

Human Mitochondrial DNA Kit AT

- 21-1236 & 21-1236A Mitochondrial DNA Extraction and Amplification Kit
- 21-1237 & 21-1237A Mitochondrial DNA Extraction, Amplification and Electrophoresis Kit with Ethidium Bromide Stain
- 21-1238 & 21-1238A Mitochondrial DNA Extraction, Amplification and Electrophoresis Kit with *Carolina* BLU™ Stain

FOR TEACHING PURPOSES ONLY

Instructor's Manual

(For automatic or manual thermal cycling)

For technical assistance call 800-227-1150 x4381

Upon receipt of the kit, store proteinase K, mtDNA Primer/Loading Dye Mix, and pBR322/*Bst*N I markers in freezer (approximately -20°C). Other materials may be stored at room temperature (approximately 25°C).

This kit was developed in cooperation with the Dolan DNA Learning Center of Cold Spring Harbor Laboratory. The experiment was adapted with permission from *Laboratory DNA Science: An Introduction to Recombinant DNA Technology and Methods of Genome Analysis*, by Mark V. Bloom, Greg A. Freyer, and David A. Micklos, ©1996, Benjamin/Cummings Publishing Company, Inc., Menlo Park, CA.

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Individuals should use this kit only in accordance with prudent laboratory safety precautions and under the supervision of a person familiar with such precautions. Use of this kit by unsupervised or improperly supervised individuals could result in serious injury.

Mitochondrial DNA Amplification 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 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 their 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, during which the primers form hydrogen bonds with their complementary sequences on either side of the mt control region, and
- a 30-second incubation at 72°C, during which the *Taq* polymerase makes complementary DNA strands that begin with each primer.

You may 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) of Cold Spring Harbor, 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.

Materials

The materials in the Mitochondrial DNA Amplification Kit *AT* are sufficient for 25 reactions. Prior knowledge of basic methods of gel electrophoresis and staining of DNA is presumed. The materials are supplied for use with the exercise described in this kit only. Carolina Biological Supply Company disclaims all responsibility for any other use of these materials.

Upon receipt of the kit, store proteinase K, mtDNA Primer/Loading Dye Mix, and pBR322/*Bst*N I markers in freezer (approximately -20°C). Other materials may be stored at room temperature (approximately 25°C).

Included in the kit

For extraction and amplification (all kits)

1.5 g Chelex® 100 resin	mineral oil, 5 mL
5 mL proteinase K, 100 µg/mL	Student Guides
25* Ready-to-Go PCR Beads™	Instructor's Manual
700 µL mtDNA primer/loading dye mix	
130 µL pBR322/ <i>Bst</i> N I markers, .075 µg/µL	

*Ready-to-Go PCR Beads incorporate *Taq* polymerase, dNTPs, and MgCl_2 . Each bead is supplied in an individual 0.5-mL tube or a 0.2-mL tube.

For Carolina BLU™ electrophoresis (Kits 21-1238 and 21-1238A)

5 g agarose	4 latex gloves
250 mL Carolina BLU™ final stain	6 staining trays
7.0 mL Carolina BLU™ gel/buffer stain	150 mL 20× TBE

For ethidium bromide electrophoresis (Kits 21-1237 and 21-1237A)

5 g agarose	6 staining trays
250 mL ethidium bromide, 1 µg/mL	150 mL 20× TBE
4 latex gloves	

Note: For Kits 21-1236 and 21-1236A, electrophoresis agents will need to be purchased separately.

Needed but not supplied

aluminum foil
beakers containing ice
camera for photographing gels (optional)
centrifuge, clinical, for 15-mL tubes, minimum 500 × g (CC)
(optional, but useful)
centrifuge, micro- or nano-, for 1.5-mL tubes (optional, but very
useful)
DNA thermal cycler, programmable (can hand cycle, but requires
multiple water baths)
electrophoresis chambers for agarose gels
electrophoresis power supplies
forceps
laboratory markers, 1 per student
micropipets, 1–10 µL or 1–20 µL, one for instructor or several shared
by students
tips, several per student
micropipets, 100–1000 µL (or 1-mL transfer pipets)
tips, several per student
microtube racks, may be shared
microtubes, 1.5-mL polypropylene, 3 per student
paper cup, 1 per student (CC)
razor blade or scalpel, 1 per student (HS)
saline solution, 0.9% NaCl in water, 10 mL per student in 15-mL
tube (CC)
6 staining trays, 1 per agarose gel
UV transilluminator with UV-blocking screen or glasses (for
ethidium bromide staining)
vortexer (HS) (optional)
water bath, boiling, per 12 students
water baths, 37°C and 65°C (used at different times)
white light box (for *Carolina* BLU™ staining)

(HS) = Needed for DNA isolation from hair sheaths (procedure A1)

(CC) = Needed for DNA isolation from cheek cells (procedure A2)

Scheduling

The Human Mitochondrial DNA Kit *AT* includes several different activities. Plan your time as follows:

Day	Time	Activity
1 or more days before lab	30 min	Pre-lab: Mix and aliquot Chelex/proteinase K (HS) Mix and aliquot saline solution (CC) Aliquot Chelex (CC) Aliquot mtDNA Primer/Loading Dye Mix
Lab period 1	30 min 30 min 10 min 60-180 min	Pre-lab: Set up workstations Isolate Squamous Cell DNA Set up PCR reactions Post-lab: Amplify DNA in thermal cyclers
Lab period 2	40 min 15 min 20+ min 20 min 30-45 min 10 min	Pre-lab: Prepare gel solution and cast gels Load DNA samples into gels Electrophoresis Post-lab: Stain gels Post-lab: De-stain gels Post-lab: Photograph gels
Lab period 3	40 min	Results and Discussion

Pre-Lab Preparation

Laboratory Period 1

Make up a 10% Chelex solution: 1.5 g Chelex + 15 mL distilled or deionized water.

For DNA Isolation from Hair Sheaths (A1):

1. On the day of the lab, prepare a 1/1 mixture Chelex solution and proteinase K, by adding 5 mL of each component into a 15-mL tube. Be sure to shake the Chelex stock tube to re-suspend the Chelex

beads before adding to the mixture. Store refrigerated (4°C) until use.

2. For each student, aliquot 300 μL of Chelex/proteinase K in a 1.5-mL tube. Be sure to shake the stock tube (or draw liquid in and out of pipet tip several times) to re-suspend the Chelex beads each time before pipetting a student aliquot.

For DNA Isolation from Cheek Cells (A2)

1. For each student, prepare and aliquot 10 mL 0.9% saline solution into a 15-mL polypropylene tube. The formula is 0.9 g NaCl per 100 mL distilled or deionized water.
2. For each student experiment, aliquot 500 μL 10% Chelex suspension into a 1.5-mL polypropylene tube. Be sure to shake the stock tube (or draw liquid in and out of pipet tip several times) to re-suspend the Chelex beads each time before pipetting a student aliquot.

Remaining Preparation Is Common to Isolation Methods A1 and A2

3. Some of the mtDNA Primer/loading dye mix may collect in tube cap during shipping. To have full volume available for student use, pool reagents by spinning tubes briefly in a microcentrifuge or tapping tube end on desktop.
4. Set up one boiling water bath per 12 students. This water bath should consist of a beaker and test tube rack to allow 1.5-mL centrifuge tubes to be suspended with the reaction in the boiling water. The entire tube should not be submerged. Alternatively, affix a double layer of aluminum foil over a beaker, and use a pencil or other object to punch holes for the 1.5-mL tubes.

5. Prepare student stations, each with the following materials:

Chelex solution, 500 μL (CC)	razor blade or scalpel (HS)
Chelex/proteinase K, 300 μL (HS)	Ready-to-Go PCR Bead™ and tube
Student Guide	
laboratory marker	saline solution, 0.9% (CC)
micropipet tips	microtube rack
micropipet, 1–10 μL or 1–20 μL	microtubes, 1.5 mL
micropipet, 100–1000 μL (or 1-mL transfer pipet)	
paper cup (CC)	

6. Students will share the following materials:

centrifuge, micro- or nano-	mineral oil
centrifuge, clinical (CC)	mtDNA primer/loading dye mix
beakers containing ice	water bath, boiling
DNA thermal cycler	vortexer (HS) (optional)
forceps	

7. Program thermal cycler with a step file:

94°C – 30 sec

58°C – 30 sec

72°C – 30 sec

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

Laboratory Period 2

1. Dilute the 20× TBE to 1× by adding 150 mL 20× TBE to 2850 mL of distilled or deionized water. Prepare 1.5% agarose solution sufficient for the number of gels needed to hold all student samples, by adding 5 g agarose to 333 mL of 1× TBE. Each gel will need to be approximately 8 mm in depth to accommodate samples of up to 25 μL. Cover agarose solution with aluminum foil and keep warm in a 65°C water bath until ready for use.

2. Students will share the following materials:

agarose or polyacrylamide gels	loading dye
camera system	micropipet, 1–10 μL or 1–20 μL
centrifuge, micro- or nano-	staining trays
pBR322/ <i>Bst</i> NI size markers	microtube rack
electrophoresis apparatus	UV transilluminator with safety screen
electrophoresis buffer	
ethidium bromide staining solution (Kits 21-1237 and 21-1237A)	<i>Carolina</i> BLU™ staining solution (Kits 21-1238 and 21-1238A)

For Kits 21-1238 and 21-1238A:

Add Carolina BLU™ Stain to Agarose and Buffer

The concentration of stain added to the agarose/buffer is dependent on the voltage used for electrophoresis. If electrophoresing at voltages less than 50 volts, a slightly lower concentration is used than if running at voltages greater than 50 volts. The stain may be added to the entire volume of agarose and distributed, or the agarose may be distributed to each lab station and the stain added by the students in the amounts listed in the following table:

Voltage	Agarose Volume	Stain Volume
<50 volts	30 mL	40 µL (1 drop)
	60 mL	80 µL (2 drops)
	400 mL	520 µL (13 drops)
>50 volts	50 mL	80 µL (2 drops)
	400 mL	640 µL (16 drops)

After addition of the stain to the agarose, swirl to mix and immediately pour the gel. Gels may be prepared one day ahead of the lab day, if necessary. Gels stored longer tend to fade and the stain to lose its ability to stain bands during electrophoresis. Store gels covered with a small amount of buffer (leaving masking tape in place), or store covered with buffer in the gel box. Don't try using more stain than recommended in your gel. This leads to precipitation of the DNA in the wells and can create artifactual aggregated DNA bands in the agarose gel.

Use the chart below for addition of the stain to 1× TBE electrophoresis buffer:

Voltage	Buffer Volume	Stain Volume
<50 volts	500 mL	480 µL (12 drops)
	2.6 L	2.6 mL (65 drops)
>50 volts	500 mL	960 µL (24 drops)
	2.6 L	5 mL (125 drops)

The dropper bottle provided delivers 40 μL /drop. If a calibrated pipet is available, the dropper tip can be removed for quicker addition of larger volumes of stain. The volume of buffer and agarose required for some gel box options are listed below:

Type Gel Box Required	Volume Buffer Required	Volume Agarose Required
Mini Gel System Box	200 mL	30 mL/casting tray
Carolina Gel Box, 1 tray	250 mL	50 mL
Carolina Gel Box, 2 trays	450 mL	100 mL

While *Carolina* BLU™ is not toxic, we recommend that the students wear gloves to prevent staining their skin. TBE buffer containing *Carolina* BLU™ stain may be reused if the reuse occurs within a day or so. If left longer, the dye loses its ability to stain DNA during electrophoresis. If reusing the buffer for several days is important, we recommend using *Carolina* BLU™ in the gel and as a final stain only.

Student Lab Briefing

Students should be familiar with the polymerase chain reaction and electrophoresis. The introductory material assumes students are familiar with basic Mendelian genetics.

It is highly recommended that students be given a chance to view their own cells under a light microscope—preferably before beginning the experiment. **For cheek cells**, use a toothpick to gently scrape inside of mouth or to pick up some of the pellet formed in Step A2-6. Smear cell debris on a microscope slide, add a drop of 1% methylene blue (or other stain), and add a coverslip. **For hair sheath**, cut hair just above sheath material and place on microscope slide. Add a drop of methylene blue, a drop of proteinase K (100 $\mu\text{g}/\text{ml}$), and a coverslip. Observe at several time points, to see the effect of enzyme digestion. Gently smashing the hair under the coverslip will aid in disrupting the sheath membrane.

Additional Background Resources

Kreuzer, H., and A. Massey. 2007. *Molecular Biology and Biotechnology: A Guide for Teachers*. American Society for Microbiology (Carolina catalog #21-2240), Washington, DC.

Bloom, M., G. Freyer, and D. Micklos. 1996. *Laboratory DNA Science: An Introduction to Recombinant DNA Technology and Methods of Genome Analysis*. Benjamin/Cummings Publishing Company, Inc., Menlo Park, CA.

An animation of the PCR process can be viewed at the Dolan DNA Learning Center Web site. Search for PCR animation at <http://www.dnalc.org>.

Fine Points of Lab Procedure

Be alert to the following cautions when performing the experiment. Where appropriate, discuss fine points with students and have them make notes in their student guides.

1. Disclosure and Confidentiality

The mt control region was specifically selected for use in this laboratory because it is phenotypically neutral. The mt control region does not encode protein and has no known relationship to disease states, sex determination or any other phenotype. The amplification product of this experiment engenders no issues of disclosure or confidentiality.

However, you should take several precautions if you opt to determine the nucleotide sequences of student DNA:

- MtDNA genotypes are explicit records of maternal inheritance that are unique to each family. To avoid the possibility of discovering inconsistent mtDNA inheritance, it is best not to generate genotypes from siblings or other family members. Since mtDNA is inherited exclusively from the mother, there is no chance of showing nonpaternity, which is the most frequent problem uncovered by DNA testing.
- The confidentiality of student mtDNA genotypes can be maintained by identifying student samples only by numbers.

In a formal sense, a single DNA sequencing experiment cannot definitively prove or disprove relatedness for several reasons:

- Student samples easily can be mixed up at any point in the multi-step procedure (e.g. during DNA isolation, PCR reaction setup, transfer of samples to the sequencing lab, sequencing reaction setup or sequencing gel loading). A forensic laboratory would use approved methods for maintaining "chain of custody" of samples and for tracking samples. In an educational setting, sloppiness provides a safety valve for inconsistent results.

- Sequence errors do occur during amplification, sequencing, and base calling (by the sequencing software). “Finished” DNA sequence submitted to DNA databanks has typically been sequenced 2–6 times.
- Heteroplasmy is known to occur in mtDNA sequences. In heteroplasmy, cells harbor two populations of mitochondria, each type with a different nucleotide at a particular position.

2. Comparison of DNA Isolation from Cheek Cells and Hair Sheaths

Saline mouthwash is the most effective method of cell collection for PCR. Cells are gently loosened from the inside of the cheek, yielding small groups of several cells each. This maximizes the surface area of cells, allowing for virtually complete lysis during boiling. Experience has shown that the mouthwash procedure produces interpretable PCR results in 85%–95% of samples. Surprisingly, food particles rinsed out with the mouthwash have little effect on PCR amplification, but may obstruct passage of fluid through pipet tips and make pipetting difficult. So, it is not advisable to eat immediately before the experiment—especially fruits.

The mouthwash method does generate liquid waste; however, the risk of spreading an infectious agent is much less likely than from natural atomizing processes, such as coughing or sneezing. Several elements further minimize any risk of spreading an infectious agent that might be present in mouthwash samples:

- Each experimenter works only with his or her sample.
- The sample is sterilized during a 10-min boiling step.
- There is no culturing of the samples that might allow growth of pathogens.

Hair sheaths and roots are the cell source of choice in mtDNA amplifications. Hair sheaths and roots are the safest source of human cells from which to extract DNA for PCR amplification. Risk of spreading an infectious agent is minimized by “dry” collection, which does not involve any body fluid or generate any supernatant. This method also stresses the power of PCR in forensic cases—even one good sheath or root can provide enough DNA for excellent amplification.

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 (.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 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 sample, and begin thermal cycling immediately. **Keep the reactions on ice until the thermal cycling begins.**

To insure maximum specificity, some experimenters employ a “hot start,” where one reagent is withheld from the reactions until the samples are cycled to the initial denaturing temperature. You can perform a hot start by adding the student samples during the first denaturation step. Either program an extended first denaturation of 10

min, or stop cycling and restart after adding the samples. **A simpler alternative is to set up reactions on ice, start the thermal cycler, and then place the tubes in the machine as the temperature approaches the denaturing set point.**

7. Hand Thermal Cycling and Time Course

Amplification of mt control region is not demanding, and good results can be obtained with hand cycling. Simply set up three constant temperature water baths (or heat blocks) at 94°C, 58°C, and 72°C. Secure student reactions in a test tube rack, and rotate the rack successively through the three baths for 30 sec each.

For a time course, have students set up three identical reactions. Segregate one of each student reactions in different racks or in different areas of a single rack. Stop (remove) one set of tubes after 22 cycles, one set after 26 cycles, and one set after 30 cycles. If you like, you can add additional cycle points to the course.

8. 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). Use 20 µL of the DNA marker per gel.

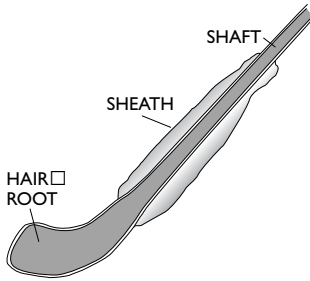
9. Viewing and Photographing Gels

View and photograph gels as soon as possible after appropriate destaining. Over time, PCR products disappear as stained bands because they slowly diffuse through the gel. If you intend to submit student samples for DNA sequencing, retain a photograph of student gels or a record of those samples that did not amplify. In general, sequence data can be obtained whenever a faint-heavy band (corresponding to 440 bp) is observed. However, it is not worth the expense or effort to attempt to sequence any samples that did not appear to amplify.

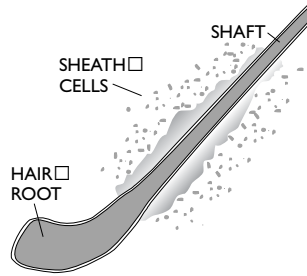
Laboratory Procedure

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.)



HUMAN HAIR



AFTER PROTEINASE K TREATMENT

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. Discard the shafts.
4. Use forceps to transfer hairs to a 1.5-mL tube containing 300 μL 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 for 30 sec (1 min in nanofuge) at full speed. Alternatively, let tube sit for 5 min to allow debris to settle.
10. Use a fresh tip to transfer 200 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 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 saline solution into the paper cup.
4. Carefully pour the saline solution from the paper cup back into the original tube and close cap tightly. Save 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 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 μL . Draw 10% Chelex suspension in and out of the pipet tip several times to suspend the resin beads. Before resin settles, rapidly transfer 500 μL of Chelex suspension to the tube containing your cell pellet.
8. Re-suspend 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 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 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 at full speed for 30 sec (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 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.
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 μL of mtDNA primer/loading dye buffer mix to a PCR tube containing a Ready-To-Go PCR Bead. Tap tube with finger to dissolve bead.
2. Use a fresh tip to add 2.5 μL of student DNA to the reaction tube, and tap to mix. Pool reagents by pulsing in a microcentrifuge or by sharply tapping the tube bottom on 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 μL) 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*N I 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, 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*N I markers. Working from the well, locate the bands corresponding to each restriction fragment: 1857 bp, 1058 bp, 929 bp, 383 bp, and 121 bp (this 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 the 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?

The presence of primer dimer confirms that the reaction contained all components necessary for amplification, but that there was insufficient template to amplify the target sequence.

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.

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.

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?

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. These make it difficult to accurately calibrate the "mutation clock."

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?

Once removed from the context of the mitochondrion, the mt insertion is subject to the lower mutation rate of nuclear DNA. The mutation clock effectively stops, preserving the insertion as a "molecular fossil" from that moment in DNA history. This means that one can study human evolution within oneself by comparing the sequence of an ancient DNA sequence (nuclear mt insertion) with the modern sequence (mt control region).

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 Web site (<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*, then on the picture labeled *Mitochondrial Control Region*, and then on *Continue on to 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 in 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 sequences 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 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 of 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.

$$4,000,000 \text{ years}/50 \text{ mutations} = 80,000 \text{ years/mutation}$$

- b. Now, use this value to calculate a divergence time for all modern humans.

$$\text{Modern human divergence} = 8 \text{ mutations} \times 80,000 \text{ years} = 640,000 \text{ years}$$

Note: The number of mutations used in the calculations for a and b may vary a small amount depending upon which sequences you have chosen to compare.

- 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?

Our direct observation of existing DNA differences underestimates the actual number of mutation events that have occurred over evolutionary time. The scientific calculation takes into account that the mt control region is so highly mutable that several mutation events have occurred at many positions:

- *In the case where an earlier mutation has back-mutated to the ancestral form found in chimps, no mutation would be observed in humans. In fact, two mutations have occurred.*

- *In the case where an earlier mutation is mutated to a second form, one mutation would be observed in humans. In fact, two mutations have occurred.*
- d. Now, calculate the divergence time for Neandertal-modern humans, using the modern human divergence estimate of 150,000 to set your mt clock.

Human divergence = 150,000 years/8 mutations = 18,750 years/mutation.

Human-Neandertal divergence = 26 mutations x 18,750 years/mutations = 487,500 years.

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

Neandertal and modern humans shared a common hominid ancestor about 500,000 years ago. However, the Neandertal and human lines had diverged for about several hundred thousand years before modern humans emerged.

Further Reading

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