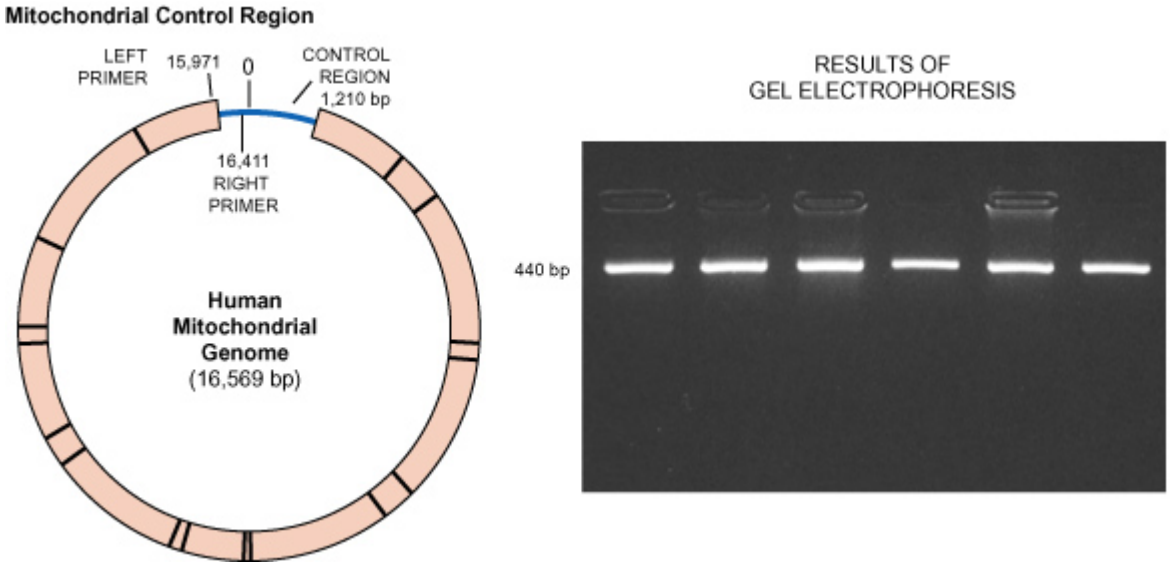
Laboratory Protocol

INTRODUCTION

Every human cell has a "second" genome, found in the cell's energy-generating organelle, the mitochondrion. In fact, each mitochondrion has several copies of its own genome, and there are several hundred to several thousand mitochondria per cell. This means that the mitochondrial (mt) genome is highly amplified. While each cell contains only two copies of a given nuclear gene (one on each of the paired chromosomes), there are thousands of copies of a given mt gene per cell. Because of this high copy number, it is possible to obtain a mt DNA type from the equivalent of a single cell's worth of mt DNA. Thus, mt DNA is the genetic system of choice in cases where tissue samples are very old, very small, or badly degraded by heat and humidity.

Under good circumstances — working from fresh cell samples — mt DNA is the easiest human DNA to amplify by PCR. This experiment examines a 440-nucleotide sequence from the noncoding region of mt genome. Hand cycling is a realistic alternative to automated thermal cyclers, and the high yield of amplified product can be visualized in an agarose gel with a variety of stains.

Because each student is amplifying the same region, the gel electrophoresis results will also be the same for each. However, amplified student samples may be submitted to our Sequencing Service (http://www.geneticorigins.org/geneticorigins/mito/mitoframeset.htm), which will generate student mt DNA sequences and post the results on our Sequence Server. Comparison of control region sequences reveals that most people have a unique pattern of single nucleotide polymorphisms (SNPs). These sequence differences, in turn, are the basis for far-ranging investigations on human DNA diversity and the evolution of hominids.



PROCEDURE

Part I: DNA Isolation by Saline Mouthwash

1. Pour 10 ml of the saline solution (0.9% NaCl) into mouth and vigorously swish for 30 seconds.
2. Expel saline solution into a paper cup.
3. Swirl to mix cells in the cup and use a micropipet to transfer 1 ml (1000 µl) of the liquid to 1.5 ml tube.
4. Place your sample tube, together with other student samples, in a balanced configuration in a microcentrifuge, and spin for 1 minute.
5. Carefully pour off supernatant into paper cup or sink. Be careful not to disturb the cell pellet at the bottom of the test tube. A small amount of saline will remain in the tube.
6. Resuspend cells in remaining saline by pipetting in and out. (If needed, 30 µl of saline solution may be added to facilitate ressupension.)
7. Use a micropipet to withdraw 30 µl of cell suspension, and add to tube containing 100 µl of Chelex. Shake well to mix.
8. Boil cell sample for 10 minutes. Use boiling water bath, heat block, or program thermal cycler for 10 minutes at 99°C. Then, cool tube briefly on ice (optional).
9. After boiling, shake tube. Place in a balanced configuration in a microcentrifuge, and spin for 1 minute.
10. Use a micropipet to transfer 30 µl of supernatant (containing the DNA) to clean 1.5 ml tube. Avoid cell debris and Chelex beads. This sample will be used for setting up one or more PCR reactions.
11. Store your sample on ice or in the freezer until ready to begin Part II.

Part II: DNA Amplification by PCR

1. Use a micropipet with a fresh tip to add 22.5 µl of the appropriate primer/loading buffer mix to a PCR tube containing a Ready-To-Go PCR Bead. Tap tube with finger to dissolve bead.
2. Use a micropipet with a fresh tip to add 2.5 µl of human DNA (from Part I) to reaction tube, and tap to mix. Pool reagents by pulsing in a microcentrifuge or by sharply tapping tube bottom on lab bench.
3. Label the tube cap with a number, as assigned by your teacher. This way, your results will be anonymous.
4. If necessary, 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 until ready to amplify according to the following profile. Program thermal cycler for 30 cycles according to the following cycle profiles. Each program may be linked to a 4°C cycle to hold samples after completing the cycle profile, but amplified DNAs also hold well at room temperature.

Denaturing time and temperature	30 sec - 94°C
Annealing time and temperature	30 sec - 58°C
Extending time and temperature	30 sec - 72°C

Part III: DNA Analysis by Gel Electrophoresis

1. Use a micropipet with a fresh tip to add 15µl PCR sample/loading dye mixture into your assigned well of a 2% agarose gel. (This will leave enough product if you intend to sequence the mt control region.) 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.
2. Load 5 µl of the pBR322-BstNI size markers into one lane of 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. Gel may be stained either with 1 µg/ml ethidium bromide for 10 minutes or 0.05% methylene blue (or proprietary) stain for 30 minutes, followed by 20-30 minutes destaining with water.

Part IV: DNA Labeling by Dideoxy Chain Termination

1. Add 2 µl of mt amplicon (product) to 13 µl of master mix.
2. Store all samples on ice or in the freezer until ready to amplify. Program thermal cycler for 25 cycles according to the following cycle profile. The program may be linked to a 4°C cycle to hold samples after completing the cycle profile, but amplified DNAs also hold well at room temperature.

Denaturing time and temperature 10 sec - 96°C
Annealing time and temperature 5 sec - 50°C
Extending time and temperature 4 min - 60°C

Part V: DNA Purification and Automated Sequencing

1. Add to a 1.5 ml tube: 2 µl of 3 M sodium acetate, pH 4.6 and 50 µl of 95% ethanol.
2. Transfer the entire 15 µl contents of the mt DNA sequencing reaction to the 1.5 ml tube containing the ethanol solution.
3. Vortex and place tube on ice for 20 minutes.
4. Microfuge for 30 minutes.
5. Use a micropipet to carefully remove as much of the ethanol solution as possible.
6. Rinse pellet by adding 250 µl 70% ethanol. Microfuge for 15 minutes.

7. Carefully aspirate all the alcohol solution with a micropipet. Use a Kimwipe or paper towel to remove any alcohol from the sides of the tube. Be careful not to disturb the pellet, which may or may not be visible.

8. If possible, let the pellet air dry overnight.

9. After the pellet is thoroughly dry, resuspend in 2.5 µl of loading dye.

10. Incubate at 95° C for 5 minutes to denature DNA.

11. Load 2 µl on sequencing gel (ABI 373 Sequencer).

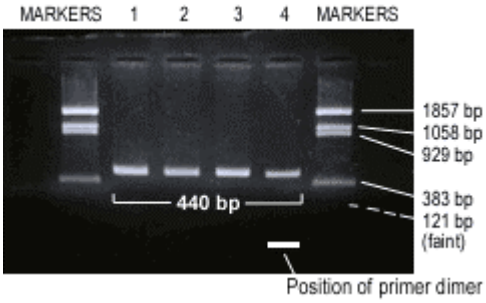
RESULTS AND DISCUSSION

Interpreting Your Gel

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/BstN I markers. Working from the well, locate the bands corresponding to each restriction fragment: 1,857 bp, 1,058 bp, 929 bp, 383 bp, and 121 bp (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 ten times the rate of nuclear DNA. Propose a biological reason for the high mutation rate of mt DNA.

4. The high mutability of the mt 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 mt DNA 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. 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?

FURTHER SUGGESTIONS for exercises can be found at the Dolan DNA Learning Center's Internet site Genetic Origins (http://www.geneticorigins.org/geneticorigins/mito/mitoframeset.htm).