Mitochondrial DNA Sequencing
Teacher Packet Cover Sheet

Dear Teacher,
This packet is designed to make your job easier. Please read over the **Teacher Packet** carefully before beginning this lab. Included in this packet are the following:

1. **Inventory Sheet**. Please check to see that all the materials listed on the Inventory Sheet have safely arrived to you and store each item appropriately.

2. **Laboratory Preparation Sheet**. Read the Laboratory Preparation carefully for suggestions on setting up this PCR activity in your classroom.

3. **Thermal Cycler Grid**. Schematics showing the layout of the spaces in the GeneAmp 2400 and GeneAmp 9700 thermal cyclers are provided for students to record where they put their samples.

4. **Teacher's Manual**. The teacher's version of the lab manual includes parts of the student version and important notes about the protocol where it is easy to make mistakes. You will also need a copy of the student version of this lab for copies of the reading and internet instructions. Please make sure to read the lab manual carefully and closely monitor student technique during lab steps.

5. **Sending samples for sequencing.** Please contact Chris Baysdorfer at CSUEB prior to sending your samples. Keep the samples refrigerated until you send them. Label them clearly, save a copy of the labels for yourself, and send.
Chris' email address is : chris.baysdorfer@csueastbay.edu

Mail clearly labeled samples to Chris Baysdorfer, CSUEB Department of Biology, 15800 Carlos Bee Blvd, Hayward, CA 94542.

When the sequences are complete, they will be sent to you by email. You can then upload them to the DNALC website for comparison at your discretion.

PLEASE DO NOT SEND ANY SAMPLES FOR WHICH THERE WERE NO RESULTS ON THE GEL. We have to pay for each sequence, so if the student did not have a band on the gel, there will be no sequence either, and we will have to pay for no reason.

If you have questions please contact me by email at sharyrosenbaum@hotmail.com or by phone at (925) 376-4041.

Shary Rosenbaum
EBBEP Coordinator

Inventory Sheet

Listed below are the reagents and consumables provided in the PCR kit you should receive as well as additional reagents and consumables you will need to acquire/provide yourself. Make sure to also read the list of equipment needed for this PCR activity. If any reagents are missing please contact Shary Rosenbaum ASAP. If any of the kit materials are missing, please contact your cluster leader immediately (or the teacher that you got the kit from)

1 reagent kit contains reagents for 40 reactions. A class of 38 students will have just enough reagents for each student and a positive and negative control.

PCR Reagents Provided in BABEC/Applied Biosystems/EBBEP Kit

	ITEM	**STORAGE**	**VOLUME PER KIT (tube)**	**VOLUME STUDENT**
	5 % Chelex 5% weight/volume Chelex 100 Resin Bio-Rad catalog # 143-2832	Refrigerator	**10 mL**	200 µL/student
	Master Mix** 2.5X PCR buffer II, 3.75 m*M* MgCl2, 2 m*M* dNTP blend, 0.075 Units/µL AmpliTaq Gold ® DNA polymerase	Freezer until ready to use, then Refrigerator	1000 µL (1 mL)	(20 µL/reaction)
	Primer Mix** 0.5 µM each primer 5'-TTAACTCCACCATTAGCACC–3' 5'-GAGGATGGTGGTCAAGGGAC–3'	Freezer until ready to use, then Refrigerator	1000 µL (1 mL)	(20 µL/reaction)
	+ Control DNA 1.0 ng/µL female genomic DNA 2.0 Novagen catalog # 70605	Refrigerator	50 µL	10 µL/reaction (at least 1 reaction/class)
	MW Marker 50 µg.mL100 bp ladder New England Biolabs #N3231L	Refrigerator	50 µL	5 µL/gel

****Master and primer mixes should come to you frozen. If they are defrosted, keep in refrigerator until ready for use. DO NOT REFREEZE!!!**

NOTE:

A great animation of sequencing, as well as good background info on this lab at:
http://www.geneticorigins.org/geneticorigins/mito/intro.html

An excellent animated tutorial showing the steps of PCR is available at the DNA Learning Center web site. **http://www.dnalc.org/resources/BiologyAnimationLibrary.htm**

Other PCR materials provided in EBBEP PCR kit

ITEM	COMMENTS
PCR tubes (2 per student)	One tube for 200µl Chelex during DNA extraction, one tube for PCR reaction
1.0x or 0.5 X TBE buffer	Roughly 300 mL/gel for agarose electrophoresis and 45 mL per agarose gel. Store at room temperature.
Agarose	2 % agarose gel, approx. 1g agarose per gel. Store at room temperature.
Loading Dye	5 µL per student. Store at room temperature.
Ethidium Bromide	Use as provided in kit (+/- 0.5 µg/mL solution). Store at room temperature but in the plastic bag in the kit. Pour used ethidium bromide back into original bottle. DO NOT ADD ANYTHING TO ETHIDIUM BROMIDE
1.5 mL microfuge tubes 1/student	These should be clean, but don't need to be autoclaved
Sterile micropipet tips	p20s, p200s for student use and p1000s for teacher aliquotting.
Gloves	Use these when handling ethidium bromide.

EQUIPMENT YOU NEED TO GET:

1. POLAROID 667 FILM FOR PHOTOGRAPHING GELS (THIS CAN BE ORDERED FROM REED'S CAMERA IN WALNUT CREEK, 1524 Locust St., 925-938-4200)
2. Ice
3. Paper cups for DNA extraction and waste tips
4. Permanent markers
5. Disposable Staining trays (weigh boats work fine for this)
6. Goggles (use when handling ethidium bromide)
7. 0.9% saline solution (see preparation instructions below)

ALL OTHER NEEDED EQUIPMENT SHOULD BE IN THE KIT. CHECK THE KIT INVENTORY SHEET

Laboratory Preparation

Teaching Preparation:

1. Copy student guide as needed: pages 4-8 are lab instructions, pages 10-15 are internet instructions, and pages 16-19 are data collection
2. Do pop bead sequencing activity to illustrate what will be done outside of class.
3. Use animations from DNALC to review processes:
   PCR animation: **http://www.geneticorigins.org/geneticorigins/mito/intro.html**
   Sequencing animation: **http://www.dnalc.org/resources/BiologyAnimationLibrary.htm**

4 Label samples clearly, and keep a copy of the labels for yourself.

What to do prior to DNA Extraction (Lab Day 1)

1. Dispense 200 µL of 5% Chelex into 0.2 mL PCR tubes if you plan to use the thermal cycler as your heat block, one per student. Make sure to keep swirling the Chelex as you pipet so that every aliquot is actually 5% Chelex (and not more or less). Make sure to use a p1000 micropipet so the Chelex beads do not get clogged in the smaller p200 tips.
2. Make 0.9% saline. The easiest way to make this is to buy a 1 liter bottle of drinking water and add 9 grams of NON-IODIZED salt. Swirl to dissolve the salt. Use a sterile graduated culture tube or sterile pipet to transfer 10 mL into Dixie cups for students just prior to class.
3. Aliquot 150 µL of 0.9% saline for each group of 4 students for the cell pellet rehydration.
4. Only the p200 micropipets are needed on this day.
5. Set up thermocycler to heat/boil

What to do prior to PCR (Lab Day 2)

1. Buy/prepare ice for teacher lab station. It is very important to keep the master mix and primer mix cold while students are preparing their PCR reactions. The DNA polymerase and/or dNTPs may degrade, resulting in no amplification if the reagents warm up too much.
2. Photocopy and place grid(s) next to thermal cycler for students to record their ID# in the correct spot. Two grids are provided, one for the GeneAmp 2400 (24 spaces) and one for the GeneAmp 9700 (96 spaces).
3. Ensure you have the tray that sits in the thermal cycler (it is usually either red, teal, or black and has a notched upper right-hand corner). Without tray, tubes may melt.
4. Only the p20 micropipets are needed on this day.

What to do prior to Electrophoresis (Lab Day 3)

1. Make 0.5X or 1 X TBE buffer for electrophoresis and agarose gels.
2. Make 2% agarose gels (2g agarose for every 100 mL of electrophoresis buffer) for students or allow time to do this with your students. Be sure to use the same buffer as (1) above.
3. Use the p20 micropipets for gel loading.
4. Be sure to have the Polaroid 667 film on hand to photograph the gels.

Where to send the samples for sequencing:

Please contact Chris Baysdorfer at CSUEB prior to sending the samples. Keep them refrigerated until you send them. Chris' email address is : chris.baysdorfer@csueastbay.edu
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Teacher Lab Notes for DNA Extraction:

Be sure to tell students to label their mouthwash and tubes

In Step 2: Students should not discard their initial mouthwash until they have completed the whole extraction procedure just in case one of the steps fails and they need to repeat the procedure.

In Step 3: After students have centrifuged their cheek cells, if a pellet still doesn't form, the student should dispense 1 mL of their mouthwash into a new tube and try again. A small number of people have cells that don't ever pellet. If this is the case, usually you can see the floating aggregate of cells. Assist students by trying to pipette out as much of the liquid as possible (and hopefully there will be enough cells in the tube to do the PCR)

In Step 5: After students try to dispose of the supernatant over the cell pellet, it is fine if they still have up to 60 µL of supernatant left. If there is still ~ 100µL of saline in the cell pellet tube, do not add more saline. Simply resuspend the pellet in the existing volume.

In Step 10: This step allows you to ensure that no Chelex beads have been transferred into the DNA tube. If you see beads, have the students put the DNA back into their Chelex tube, re-spin, and transfer 60 µL into a NEW tube.

Additional information:

At the Genetic Origins page of the DNALC, (http://www.geneticorigins.org/geneticorigins/) there is also a lot of information regarding the Mitochondrial Sequencing lab and background.

GeneAmp 2400 Thermal Cycler

	1	2	3	4	5	6	7	8
A								
B								
C								

GeneAmp 9700 Thermal Cycler

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D												
E												
F												
G												
H												

Staining and Photographing Agarose Gels

<u>Your teacher will stain your agarose gel and take a photograph for you so that you may analyze your results. FYI, Gel staining is done as follows.</u>

1. Place the agarose gel in a staining tray.
2. Pour enough ethidium bromide (0.5µg/ml) to cover the gel. Wait 15 minutes.
   CAUTION: Ethidium bromide is a carcinogen. Always wear gloves and safety glasses when handling.
3. Pour the ethidium bromide solution back into its storage bottle. Pour enough water into the staining tray to cover the gel. Wait 5 minutes.
4. Pour the water out of the staining tray into a hazardous waste container and place the stained gel on a UV light box.
   CAUTION: Ultraviolet light can damage your eyes and skin. Always wear protective clothing and UV safety glasses when using a UV light box.
5. Place the camera over the gel and take a photograph.

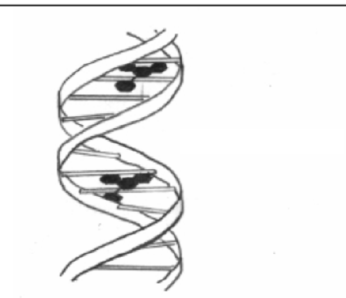

Figure 3. Ethidium bromide molecules stacked between DNA base pairs.

The PCR products run on your agarose gel are invisible to the naked eye. If you look at your gel in normal room light, you will not be able to see the amplified products of your reaction. In order to "see" them, we must stain the gel with a fluorescent dye called **ethidium bromide**. Molecules of ethidium bromide are flat and can nestle between adjacent base pairs of double stranded DNA (Figure 3). When this interaction occurs, they take on a more ordered and regular configuration causing them to fluoresce under ultraviolet light (UV). Exposing the gel to UV light after staining, allows you to see bright, pinkish-orange bands where there is DNA (Figure 4).

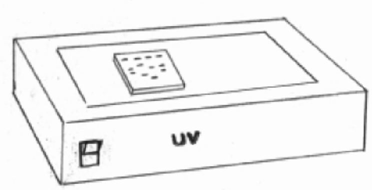

Figure 4. After staining an agarose gel with ethidium bromide, DNA bands are visible upon exposure to UV light.

<u>ALTERNATIVE FOR USING YOUR OWN DIGITAL CAMERA</u> (not provided in kit)
Use the same setup as for the Polaroid, but reach into the hood attached to the camera and gently pull the two buttons next to the lens that hold the camera attached. Remove the Polaroid camera and just use the bottom of the hood over the transilluminator. Turn off the flash on your digital camera and set it into the camera hole. Take pictures as usual. **<u>CAUTION: Use two sets of gloves so you do not contaminate your camera with ethidium bromide!!! One set for touching the gels, and a clean set for touching the camera. Cover your camera with a plastic bag to avoid EtBr contamination.</u>**

Mitochondrial D-loop PCR Amplification Results

PCR amplification of the mitochondrial D-loop region using the primers for this protocol should produce a 440 bp product as shown in the figure below.

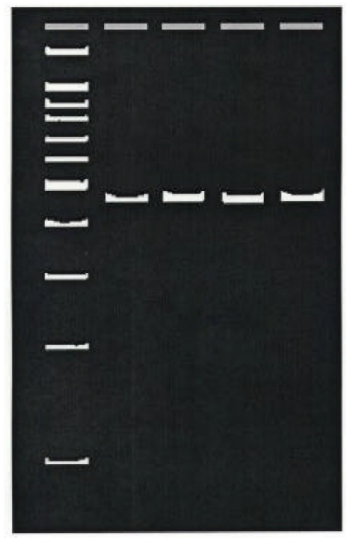

Figure 4. Representation of an agarose gel containing a 100bp ladder (leftmost lane) and lanes showing 440 bp products from D-loop PCR amplification

<u>REMEMBER DON'T SEND ANY SAMPLES FOR SEQUENCING FOR WHICH YOU DON'T SEE A BAND ON THE GEL</u>

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Student Data **NAME(S)___*Teachers guide*___**

Sequence Server Clustal W Alignments: SNPs			
Modern Human vs. Modern Human	**Number of SNPs**	**Your Average**	**Class Average**
vs.			+/- 6
----------------------- vs. --------------------------------			
vs.			
Student ______vs. Student _______			
Modern Human vs. Neanderthal			
African vs. Neanderthal #	**~18**		+/-18
Asian vs. Neanderthal#	**~18**		
European vs. Neanderthal # ---	**~19**		
Student vs. Neanderthal #____			
Neanderthal vs. Neanderthal			
Neanderthal #1 vs. Neanderthal #2	**~7**		+/- 5
Neanderthal #1 vs. Neanderthal #3	**~4**		
Neanderthal #2 vs. Neanderthal #3	**~4**		
Modern Human vs. Chimpanzee			
vs. Chimp #2			+/-42
vs. Chimp #2			
vs. Chimp #2			
Student vs. Chimp #2			

1. Calculating a Molecular Clock

Archaeologists use a number of different techniques to estimate the age of fossils. These include radiocarbon dating, measuring changes in carbonates and tooth enamel brought about by exposure to radiation over time, and determining the age of the geological strata in which the fossil was found. By dating human fossils discovered in Africa, scientists estimate that modern humans first appeared approximately 150,000 years ago. Using this value and the class average number of differences for "Modern Humans vs. Modern Humans," derive a **molecular clock,** or mutation rate, in years/mutation. Use the formula below:

_150,000 years
-------------------- = ***___25,000_____****years/mutation*
__6__*mutations*

2. Did Modern Humans Evolve from Neanderthals?

Neanderthal fossils have been discovered in Europe and the Middle East. Dating the fossils by radiocarbon decay suggests that Neanderthals inhabited the European continent as recently as 28,000 years ago. Estimates of when Neanderthal first appeared in Europe are far less precise but many scientists believe it may have been as long as 300,000 years ago. Although they are frequently depicted as stocky and brutish individuals, Neanderthals cared for their sick and injured, fashioned stone tools, used fire, lived and hunted in social units, and ritually buried their dead.

As far as we know, Neanderthals did not inhabit regions far outside the European continent. If modern Europeans descended from Neanderthals, you would expect that Neanderthals would be more closely related to modern European populations than to any other modern human population in the world. Based on your "Modern Human vs. Neanderthal" data, does it appear as though Europeans or any other modern world population descended from the Neanderthals? Explain.

No, the number of differences between Neanderthals and Modern Europeans is not significantly less than the number between Neanderthals and any other modern human population.

Note: Students may want to discuss what would be a <u>significant</u> difference.

3.Human Neanderthal Divergence

How many years ago did the common ancestor of modern humans and Neanderthals live? In the equation below, use the average number of differences (mutations) you found between modern humans and Neanderthals and your calculated mutation rate above to estimate this number.

$$\underline{\;18\;}\ \textit{mutations} \times \frac{\textit{Number of years}}{\textit{mutations}} = \underline{\;450{,}000\;}\ \textit{years}$$

4. **Did Neanderthals Contribute to the Modern Human mtDNA Gene Pool?**

A **gene pool** is the collection of all genes in a population. Members of a single gene pool would be expected to have fewer differences between them than would be expected between members of different gene pools. Did Neanderthals have a separate gene pool from that of modern humans? Could Neanderthals have contributed their mitochondrial DNA to the gene pool of modern humans? Use the comparisons below (4a through 4e) to answer this question.

a. Average difference between Neanderthals = ____***~5***___

b. Average difference between modern humans and Neanderthal = __***~18***____

c. Average difference between modern humans = ___***~6***_____

d. The *<u>closest</u>* modern human/Neanderthal alignment discovered by your class showed ___***~14***__differences

e. The two most divergent modern humans discovered by your class showed _***~11***___differences.

f. Do you think the Neanderthals used in this study are members of a single gene pool (assume that modern humans are of a single gene pool)? Explain.

If Neanderthals passed on their mitochondrial DNA to modern humans, you might expect that the average number of SNPs between "Modern Human vs. Modern Human" and "Modern Human vs. Neanderthal" would be similar. If Neanderthals had their own gene pool, you would expect that the average number of SNPs between "Neanderthal vs. Neanderthal" and "Modern Humans

vs. Neanderthal" would be different. Neanderthals, therefore, are probably of a single gene pool since the average difference between Neanderthals is similar to the average difference between modern humans and modern humans are considered to be of a single gene pool. However, we are only looking at a very small sample size so it is dangerous to draw a strong conclusion.
NOTE: Your students may argue the definition of a gene pool. IF there are physical barriers (lakes, mountains, etc.) between two populations, would they be in the same gene pool?

5. A Molecular Clock Based on Chimpanzee/Hominid Divergence
Based on the fossil record, scientists believe that chimpanzees and modern humans may have diverged 5,000,000 years ago.

a. Would the molecular clock be different if you used the time since chimpanzees and modern humans evolved to determine the mutation rate? Calculate a new mutation rate using the formula below and the 5 million year divergence estimate.

$$\frac{5{,}000{,}000 \textit{ years}}{\_\_\mathbf{42}\_\_ \textit{mutations}} = \_\_\mathbf{119{,}000}\_\_\_ \textit{years / mutation}$$

b. Is this value different than the one you calculated based on "Modern Human vs. Modern Human" differences? Explain.

Yes, 25,000 years/mutation vs. 119,000 years /mutation
The number of SNPs for the "Modern Human vs. Chimpanzee" comparison might be an underestimate; there may be more differences in actuality for the following reasons: back mutations would not be noticed, and multiple mutations at one base position would be counted as only one change. In addition, one of the estimates of species divergence is incorrect.

c. Using the mutation rate you calculated in 5a, when did "Mitochondrial Eve," the mitochondrial ancestor of all modern humans, live? Use the formula below for this calculation:

$$\_\_\mathbf{6}\_\_\_ \textit{mutations} \times \frac{\mathbf{119{,}000}\_ \textit{ years}}{\textit{mutations}} = \_\mathbf{714{,}000}\_\_ \textit{years}$$

How does this estimate compare with the value you used to calculate a molecular clock in Problem 1 (page 16)?

In problem 1, it was stated that modern humans arose 150,000 years ago. The value calculated here may range from 3-7 times greater than 150,000 (depending on your particular class' data)

d. Using the same molecular clock (calculated in 5a above) when did Neanderthals and modern humans diverge and how does this estimate compare with the value you calculated in Problem 3?

$$\_\_\mathbf{18}\_\_\_ \textit{mutations} \times \frac{\mathbf{119{,}000}\_ \textit{ years}}{\textit{mutations}} = \_\mathbf{2{,}142{,}{,}000}\_\_ \textit{years}$$

In problem 3, a value of 3-5 times less than this would be calculated (depending on your

particular class' data)

e. How many mutations would you need between chimpanzee and modern humans to give the mutation rate you calculated in Problem 1? Use the equation below for your calculations:

$$\frac{5{,}000{,}000 \text{ years}}{x \text{ mutations}} = \_25{,}000\_\text{years/mutation}$$

x= 200 mutations

How does this number compare with the average number of SNPs your class found for the "Modern Human vs. Chimpanzee" comparisons and how can you account for any discrepancy?

Your class probably found close to 50 SNPs when making these comparisons. The number of SNPs could be underestimated for reasons outlined in 5b above.

f. Which mutation rate might be more accurate, that derived from the modern human/modern human comparisons or that derived from the chimpanzee/modern human comparisons? Explain.

The mutation rate derived from modern human/modern human comparisons is probably closer to the actual rate. There are other lines of evidence (archeological, geological, etc.) that support the 150,000 year agod divergence. The longer ago an event occurred, the more difficult it is to estimate that point in time when it actually occurred. The small chimpanzee sample size is also a problem when trying to make an estimate of mutation rate.