# Mitochondrial DNA
# Cycle Sequencing Supplement

**Some classes may have the opportunity to prepare and run their own DNA sequencing reactions. If you have access to a DNA sequencer, you may not need to send your samples to Cal State East Bay, If you would like to run your own sequencing reactions, please follow the instructions provided in this supplement.**

## Inventory Sheet

Listed below are the reagents and consumables provided in the Cycle Sequencing kit from BABEC. If you need access to some of these additional materials, please contact your local biotech education partnership coordinator. Following the aliquoting procedures outlined in this teacher packet, 1 kit contains reagents for approximately 50 reactions. In a class of 35–40 students, 40 students will have enough reagents for each student, a positive control, a negative control and 15% overage for transferring reagents. In the table below, the volume of each reagent is listed per student. If aliquoting for groups, use the following equation:

$$\frac{\text{x µL}}{\text{student}} \times \frac{\text{\# of students}}{\text{team}} \times 1.1 \text{ (overage factor)} = \text{amount per team}$$

**Cycle Sequencing Reagents Provided in BABEC/Applied Biosystems Kit:**

| √ | Item | Storage | Volume Per Kit | Volume Per Student (see directions above) |
|---|---|---|---|---|
| | Big Dye Terminator RR Mix | Freezer (-20 ˚C) | 400 µL per tube | 8 µL/student |
| | mtDNA cycle sequencing primer | Freezer (-20 ˚C) | 100 µL per tube | 2 µL/student |
| | pGEM 3Zf: positive control DNA | Freezer (-20 ˚C) | ~ 50 µL | 2 µL/reaction |
| | -21 M13: positive control primer | Freezer (-20 ˚C) | ~ 50 µL | 4 µL/reaction |

**NOTE:** Thawed reagents can be stored in the refrigerator for up to one month. **If you are not going to use the reagents within a month, you should store in the freezer.** If you do not use the frozen reagents by the end of the school year, please contact the BABEC education manager or your partnership coordinator to determine if they should be discarded.

**Cycle Sequencing Reagents/Consumables NOT Provided in BABEC/Life Technologies Kit:**

| Item | Comments |
|---|---|
| Centri-Sep Columns (ABI PN401763) | After the sequencing reaction, it is important to remove unincorporated dye terminators and salts that may compete for capillary electrophoretic injection. Unincorporated terminators can co-migrate with the sequencing template, resulting in basecalling errors, and excess salt translates to poor signal-to-noise ratios. |
| Performance Optimized Polymer (POP) | After performing the post-sequencing reaction purification, samples are ready for analysis on an Applied Biosystems capillary electrophoresis-based genetic analyzer. During capillary electrophoresis, the products of the cycle sequencing reaction migrate through capillaries filled with polymer. The negatively charged DNA fragments are size separated as they move through the polymer in the capillaries toward the positive electrode. |

**Additional Equipment Required for Lab:**

- P-20 Micropipettes and tips
- PCR tube racks and PCR tubes
- Permanent markers
- Waste containers
- Microcentrifuges
- Ice containers
- Applied Biosystems Thermal Cycler
- Applied Biosystems DNA Sequencer (Genetic Analyzer, models 310, 3130, 3500, or 3730)
- Appropriate Performance Optimized Polymer (POP) for capillary electrophoresis

For further information about mtDNA sequencing and working with ABI Genetic Analyzers, please see the ABI website on the topic:
http://www.appliedbiosystems.com/absite/us/en/home/applications-technologies/dna-sequencing-fragment-analysis/mitochondrial-sequencing.html

## Laboratory Preparation Sheet: DNA Sequencing

**What to do prior to Cycle Sequencing:**

| √ | Item | Preparation Instructions |
|---|---|---|
| | Ice (crushed) | Buy/prepare ice for each lab station. It is very important to keep the Big Dye Terminator Ready Reaction Mix and sequencing primer cold while students are preparing their cycle sequencing reactions. The DNA polymerase, dNTPs, and ddNTPs may degrade, resulting in no amplification if the reagents warm up. |
| | BigDye RR Mix | Aliquot 9 µL/student of the BigDye reaction mix. Keep this frozen until time of use, and then keep on ice after thawing. |
| | Sequencing primer | Aliquot 3 µL/student of the sequencing primer. Keep on ice after thawing. |
| | Sterile/distilled water | Aliquot 10 µL/student. |
| | Student samples | Retrieve students' amplified PCR samples so they can serve as templates in the cycle sequencing reactions. |
| | pGEM 3Zf(+) | Positive control DNA for the cycle sequencing reaction. Explain letters |
| | M13 (-21) primer | Primer for the positive control reaction for cycle sequencing. |
| | Thermal cycler grid | Photocopy and place grid(s) next to thermal cycler for students to record their tube locations with their own ID#. Two grids are provided, one for the GeneAmp 2400 (24 spaces) and one for the GeneAmp 9700 (96 spaces). |
| | Thermal cycler tray | Ensure you have the **correct tray** that sits in the thermal cycler (it is usually red, teal, tan, black or natural in color, and will have a notched upper right-hand corner to fit against its support in the thermal cycler). Without the tray, tubes may melt. |
| | P-20 micropipettes | Only the P-20 micropipettes are needed on this day. |
| | 0.2 mL PCR tubes | Use for setting up the PCR reactions. |
| | Tips | Yellow tips only. Use sterile tips if possible. |
| | Waste containers | For tips and tube disposal. |
| | Permanent markers | To label tubes, etc. |
| | Microcentrifuge | For spinning down reagents in PCR tubes. |
| | PCR tube racks/bases | For holding the PCR tubes while setting up the reaction mix. Make sure that the PCR rack or base is on ice during the PCR reaction set up. |
| | Ice containers or styrofoam cups | Use to store the PCR reagents and DNA on ice while setting up PCR reactions. |
| | Thermal cycler | Check the parameters for the MtDNA cycle sequencing in the PCR machine. Make appropriate changes where needed. |

**MtDNA Sequencing program**
1) 25 cycles of:
    96°C for 10 seconds
    50°C for 5 seconds
    60°C for 4 minutes
2) 4°C hold, ∞ infinity

**PCR Reagents—MtDNA Sequencing:**

**Big Dye Terminator RR Mix:** Contains buffer, $MgCl_2$, dNTPs, fluorescent ddNTPs, and DNA polymerase.
**Mt Cycle Sequencing Primer**: 1.6 µ*M* forward mt D-loop primer (5'- TTAACTCCACCATTAGCACC –3')
**pGEM 3Zf(+) :** 0.2 g/L positive control DNA (plasmid)
**M13 (-21) Primer:** control primer to use with pGEM control DNA (5' – TGTAAAACGACGGCCAGT – 3')

## Cycle Sequencing Reaction

1. Label a 0.2 mL tube with your 4 digit PIN.


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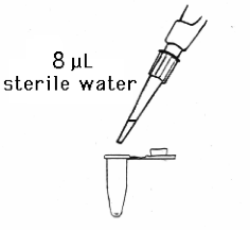
2. Set your P-20 micropipette to 8 µL and add 8 µL of sterile water to your 0.2 mL tube.

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3. Change your tip and dispense 8 µL of Big Dye Terminator RR Mix* into your 0.2 mL tube.

* RR stands for Ready Reaction. A Ready Reaction Mix contains the dNTPs, ddNTPs, buffer, enzyme, and magnesium chloride.


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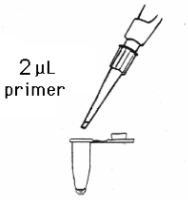
4. With a new tip, add 2 µL of mitochondrial sequencing primer into your 0.2 mL tube.

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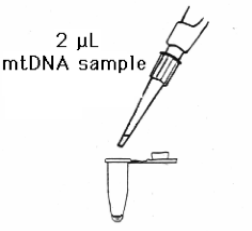
5. Using a clean tip, add 2 µL of your mtDNA PCR sample into your 0.2 mL tube. Slowly pipet up and down several times to mix.

Note: You may need to centrifuge the tubes for 5-10 seconds if the reaction components are along the walls of the tube.

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6. One student will be asked to set up the positive control reaction for the class.

**Note to teachers:**
***It is important to set up and run a positive control. Bring this with the other class samples to run on the automated sequencer.***

| Control | Ready Reaction mix | -21 M13 control primer | pGEM 3Zf(+) control DNA | Sterile water |
|---|---|---|---|---|
| + | 8 µL | 4 µL | 2 µL | 6 µL |

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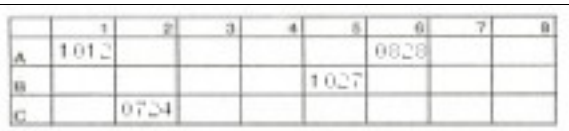
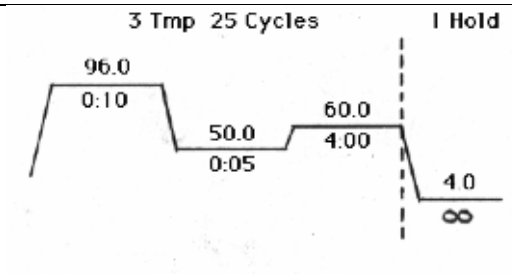
| | |
|---|---|
| 7. Place your reaction into the thermal cycler and record the location of your tube on the grid provided by your teacher. |  ☐ |
| 8. Follow the cycling parameters below for cycle sequencing of this mitochondrial control region.<br>25 cycles of:<br>96˚C, 10 seconds<br>50˚C, 5 seconds<br>60˚C, 4 minutes<br><br>4˚C hold |  ☐ |

| **Spin Column Purification and Loading the Automatic Sequencer** |
|---|
| 1. Rehydrate the Centri-Sep column in 750 µl sterile water. Remove any air bubbles from the column by tapping on the column until the bubbles float up. Allow it to sit for at least 30 minutes. |
| 2. If any bubbles appeared during rehydration, tap them out. Remove upper cap and then lower cap and place the column in the wash tube. Make sure that the column is dripping. If it is not, seal the top of the tube with Parafilm and press your finger on the Parafilm to start the flow. |
| 3. Place the wash tube with spin column in the centrifuge and spin for 2 minutes at 4,000 rpm. |
| 4. Dump the wash tube and place the column into a 1.5 mL microfuge tube, labeled with your initials. |
| 5. Apply your cycle sequencing reaction to the top center of the column matrix. Do not allow the micropipette tip to touch the matrix. |
| 6. Spin the column and microfuge tube as a unit for 2 minutes at 4,000 rpm with the column in the same orientation as in the first spin. |
| 7. After centrifugation, throw away the column and vacuum-dry the sample. |
| 8. Add 5 µL of loading buffer to resuspend the cycle sequencing sample. Pipet up and down repeatedly along the bottom sides of the tube to ensure complete resuspension of the reaction products. |
| 9. After a quick 5-second spin, transfer the sample into a 200 µL PCR tube. |
| 10. Heat samples for 2 minutes at 90˚C in the thermal cycler and then immediately place your sample in ice.<br><br>***Note to teachers:*** *Have your students take their ice containers to the thermal cycler after the 2 minute incubation so that they can place their samples into ice as quickly as possible. This will help to ensure that the strands remain denatured.* |
| 11. Load 1.5 µL into the appropriate well on the sequencing gel, following the indications on the Sample Sheet (on the computer screen). |

**You are now ready to run your samples in an ABI Genetic Analyzer. Please follow instructions for the specific model instrument that you will be using.**