# CYCLE SEQUENCING

**Introduction:**

The process of sequencing of a gene of interest is to generate fragments that are one nucleotide different in size and can be distinguished from each other when viewed on an electrophoresed gel. DNA is sequenced using a replicative (amplification) process that uses one strand of the PCR fragment as the template, and includes deoxynucleotides and dideoxynucleotides in the reaction. A DNA polymerase uses deoxyribonucleotides (dNTPs) to generate new strands of DNA from the DNA template provided. To generate different sized fragments, dideoxyribonucleotides (ddNTPs = missing -OH group at 3’ carbon of sugar) are incorporated by the DNA polymerase and act as terminator molecules by not allowing polymerization to continue. The random process of adding both dNTPs and ddNTPs on newly made strands results in many copies of different sized fragments of the target DNA. Although DNA sequencing is the ultimate in determining differences among individuals, it is not typically used in forensic cases. The reason is that only a very small portion of the genome is compared. There are many portions of the genome that are identical from person to person. Therefore, it is important to find a polymorphic region of the genome to do comparisons. A polymorphic region refers to a DNA region that has some sequence variation (nucleotide differences) from individual to individual.

 **Sanger Sequencing:** The DNA sequencing technique that uses radioactivity is called the Sanger method (named after Frederick Sanger). The dCTP nucleotides are tagged with 35S. Since there is no distinction between the four ddNTPs, the whole sample cannot be run in the same lane. Four separate sequencing reactions have to be performed, with each having only one type of dideoxyterminator. For example, reactions 1 = ddGTP, 2 = ddATP, 3 = ddTTP, and 4 = ddCTP. These samples are then loaded in four different lanes on the polyacrylamide gel. After electrophoresis, the gel is transferred and dried onto a long filter paper, and is placed in a cassette containing X-ray film. After exposure to the 35S-tagged DNA fragments, the film is developed and a black & white image containing dark bands appears. This is called an autoradiogram (or autorad). Since the shortest fragments migrate the fastest and furthest on the gel, the beginning of the sequence is read starting at the bottom. Although many researchers manually read the results off of the autorad, there is now computer software that can read an autorad using a gel scanner. Using radioactivity requires more safety precautions and is much more time consuming, taking 5-8 days to generate results. However, it is less costly than automated sequencing.

**Automated Sequencing:** The most current way of distinguishing among DNA fragments is by tagging the 4 types of ddNTPs with 4 different color dyes. For example, thymine = red, adenine = green, cytosine = blue, guanine = yellow. The common automated sequencing method requires samples to be electrophoresed on a long vertical polyacrylamide gel. The sample is loaded in one lane, and each base type can be distinguished from each other by the difference in its color that it fluoresces. As the DNA fragments reach 3/4 of the way down the gel, the dyes become fluoresced as a laser beam scans the gel. The fluorescent light images are then converted to computerized images. The results come in both text and graphical form, showing the intensities of the bands as different colored peaks. This method is safer to use than those using radioactivity, it can run up to 36 samples concurrently, and it only takes 36 hours to generate results. Since, this method is relatively quicker than the standard Sanger sequencing method, it is more commonly used today, especially for the human genome project. However, an automated sequencer is quite expensive to purchase. In *Investigation 14*, you will perform automated sequencing using the newest technology in sequencing, that is, using a thin capillary system instead of a long vertical gel.

In this procedure, dye-labeled dideoxyterminators will be used. That is, each terminator base has a different color dye attached it. Each fragment size generated will contain a specific dye, depending on what the last base is in the DNA fragment generated. Once cycle-sequencing is performed, samples will be purified and electrophoresed in an Applied Biosystems 310 capillary automated sequencer. The sequencer contains a laser beam that excites each dye as the fragments migrate through a polymer within a thin capillary tube. The fluorescent image is eventually converted to a graphical image on a computer screen, as shown below. Data is saved as a computer file.



**Materials:**

micropipettors & tips microfuge heat block

waste beaker vortexer thermocycler

dsH2O kimwipes tube of mineral oil

cycle-sequencing kit (on ice) gloves Ice bucket

0.5 ml microtubes microtube rack & marker

**Purifying the Gel Slice:**

 Prior to sequencing, the PCR fragments need to be purified and in liquid form. Agarase, an enzyme derived from *Pseudomonas atlantica*, digests the backbone of agarose. DNA can be separated from a gel slice that has been treated with this enzyme. Agarase is free of DNase, Rnase, or phosphatase activity. One unit of agarase is defined as the amount of enzyme that will completely digest 200 mg of a molten 1% low melting temperature agarose gel prepared in 40 mM Bis Tris/HCl, 40 mM NaCl, 1 mM EDTA (pH 6.0) in 1 hr at 40°C. You will be using the QIAquick Gel Extraction Kit by Qiagen to purify the PCR fragment. More than likely, it contains agarase in the first incubation step, and a spin column is used to separate out the DNA from solution. Up to 400 mg agarose can be processed per spin column.

**Procedure:**

1) **Put on gloves**. Set up tubes. Label 1.5ml tubes with sample # - primer name. Place the appropriate number of QIAquick spin columns in collecting tubes in a row. The number of columns should equal the number of PCR gel slices you have.

2) Depending on the how well you trimmed your bands, the melted volume should be about 50 µl. Add 3 volumes of buffer QG (~150 µl). Place samples in a 50°C heat block or water bath. Incubate for 10 min. Vortex samples briefly every 2-3 min during the incubation to help in the dissolving step.

3) After the gel slice has dissolved completely, check that the color of the mixture is yellow (similar to buffer QG without dissolved agarose), meaning that it is at the proper pH. If the color is orange or violet, add 10 µl of 3M sodium acetate pH 5.0 and mix. The color of the mixture should turn yellow.

4) Add 1 gel volume (~50 µl) if isopropanol to the sample and vortex.

5) Place a QIAquick spin column in a 2 ml collecting tube. Apply the DNA sample to the top of the column and centrifuge at 12,000 rpm for 1 min. This will bind the DNA to the membrane and collect the remaining liquid in the collecting tube.

6) Empty each collecting tube and place each column back in the same collecting tube.

7) Add 500 µl of buffer QG to the column and centrifuge at 12,000 rpm for 1 min. This step will remove all traces of agarose.

8) Add 750 µl of buffer PE to the column and let stand for 5 min. This step helps to wash the DNA. Then centrifuge at 12,000 rpm for 1 min.

9) Discard the flow-through (liquid in the collecting tube), place the column back in the collecting tube, and spin at 13,000 rpm for 1 min.

10) Place the column in a labeled 1.5 ml tube. Add 30 µl of dsH2O to the center of the QIAquick membrane and let it stand for 1 min. then, centrifuge the column at 13,000 rpm for 1 min.

11) Discard the QIAquick column, and place the opened 1.5ml tubes containing the DNA fragments in the SpeedVac. Dry samples for 10 min. This will concentrate the DNA.

**Cycle-Sequencing:**

 Since cycle-sequencing and data collecting together have about a 1% error rate, researchers sequence in both directions of the template to verify each base. To do this, the forward primer is used in one reaction, and the reverse primer is used in another reaction. The reverse sequence is reverse-complemented (on computer), both sequences are aligned, and each base is checked for reproducibility. The terminator Big Dye ready reaction mix contains deoxynucleotides (dNTPs), dideoxynucleotides (ddNTPs), buffer, *Taq* polymerase, and magnesium ions. The Big Dye kit and buffer can both be purchased from Applied Biosystems, Alcameda, Ca.

**Procedure:**

1) Quantify your DNA using the fluorometer. Use directions on page 79 in *Investigation 10*. You will need 50-100 ng of DNA for the cycle-sequencing reaction.

2) Add the following to a 0.5 ml microtube (a 20 µl reaction mix):

 4.0 µl **Big Dye ready reaction mix**

4.0 µl **2.5X Big Dye buffer**

 1.0 µl **forward primer**

**~50 ng** **DNA template** (melted gel slice) + enough **dsH2O** to equal **11µl**

 **=** 20µl reaction

**\*\*\*\*\*\*\*Write in your notebook the amount of DNA, dsH2O, Big Dye, and primer for each sample.**

**Note\*\*\***If you do not know the DNA concentration, then use the starred values in the table.

|  |  |  |
| --- | --- | --- |
| PCR conc ng/µl | DNA amount (µl) | dsH2O (µl) |
| 1-5 | 11 | 0 |
| 6-8 | 8 | 3 |
| 9-11 | 6 | 5 |
| 12-17 | 4\*\*\* | 7\*\*\* |
| 18-23 | 3 | 8 |
| 24-40 | 2 | 9 |
| 41- | 1 | 10 |

2) Vortex briefly or pipette mix a few times and spin briefly in the microfuge.

3) Lay 2 drops of mineral oil on top of samples using a yellow pipette tip.

4) Place in thermocycler and use the following temperature profile:

 denature at 96°C for 30 sec

then linked

 to 4°C

 for

 25

cycles,

 anneal at 50°C for 15 sec

 extend at 60°C for 4 min

## Purification of Cycle-Sequencing Products:

 Unincorporated dye-labeled dideoxyterminators need to be taken out of each sample. If left in the tubes, when the samples are electrophoresed, the laser beam fluoresces the unincorporated dyes and creates large blotches in the gel image. To separate these molecules from the sequence fragments, the samples are spun through columns that contain Sephadex beads. The beads contain microscopic holes that trap small molecules like the dideoxynucleotides. While a sample is spinning in a microfuge, the DNA fragments pass between the beads and are collected at the bottom of the tube.

**Procedure:**

1) Hydrate **Centri-Sep columns** with 750 µl dsH2O for at least 30 min. They can be purchased from Princeton Separations. Take the cap off of the hydrated spin column first, then the bottom. Place the caps and bottoms in their recycling beakers.

2) Place each spin column inside a collecting tube. Place your thumb over the top and push down to cause a drop of water to exit the bottom of the tube and enter the collecting tube. Let the column drain for about 5 min. Doing this will save you time when drying the samples later on. If a substantial amount of water collects in the tube, you may need to decanter the water in the sink and replace it back under the column.

3) Place the tubes in the microfuge and have the bubble on each lid facing upward. Spin each tube combination in the microfuge at **4000 rpm** (a slower speed than normal) for exactly **2 min**. While it is spinning, label 1.5 ml microtubes with your initials and each sample number.

4) Replace each collecting tube with the labeled 1.5 ml microtube. Place the collecting tubes in their recycling beaker.

1. Load **20 µl** of the **sample** in the center of the tube. To do this, place the yellow pipette tip underneath the oil, and slowly collect the sample. You may have a little oil on the tip. A little bit is O.K. When dispensing the sample in the column, lean the tip on the inside edge of the column (like a pool cue) and dispense slowly in the center of the column without touching the Sephadex beads. The last drop may be difficult to get out. Just touch the last drop on the surface of the beads without disturbing it.

tip

 beads

 tube

6) Place the tubes in the microfuge with the bubble on their lids facing upward again. Spin samples in the microfuge at **4000 rpm** for exactly **2 min**.

7) Tap the remaining Sephadex beads out of each column and place the columns in their recycling beaker.

8) You should have about 20 µl of sample volume in your microtubes. The samples need to be lyophilized to keep them stable when transported in the mail. Place open tubes in the SpeedVac. Make sure the tubes are balanced with one opposite it. Turn SpeedVac on (without heat). It should take about 45 min - 1 hr to dry, depending on the volume in each tube. Then place them at –20°C. At this point, the samples are stable for a long period of time.