

DNA SEQUENCING (using an ABI automated sequencer)

OBJECTIVE:

To label and separate DNA fragments varying by single nucleotides, in order to determine the sequence of nucleotides.

INTRODUCTION:

Determination of a DNA sequence is accomplished using one of two basic methods, and their derivations. Both methods were first described in 1977. The first method (Maxam and Gilbert 1977) is based on specific chemical degradation of the DNA. The DNA is first end-labeled using ^{35}S or ^{33}P , followed by separation of the two strands on a gel. Four aliquots of the desired strand are then subjected to a set of four DNA degradation reactions. One cuts the DNA at A residues, one at G residues, one at C residues and one at pyrimidine residues. Each of the four reactions is loaded into individual lanes of a thin (0.4 or 0.6 mm) polyacrylamide gel (5-15%). Electrophoresis is then performed at high voltage (usually 2,000 to 2,500 V, approximately 50-60 mA), for several hours, depending on the total length of the DNA fragment, and the portion of the sequence desired. If the fragment is short, and the sequence desired is close to the 5' end of that fragment, then the run time will be short (1-2 h). If the fragment is long, and the desired sequence is closer to the 3' end, then the run time will be longer (2-5 h).

The other method of sequencing DNA (Sanger 1977) uses a polymerization reaction (using DNA polymerase) in conjunction with a mix of deoxynucleotide triphosphates (dNTPs) and dideoxynucleotide triphosphates (ddNTPs). Since dideoxynucleotides terminate the growth of the DNA polymer once they are incorporated (since the hydroxyl at the 3' position is absent), a series of fragments is produced dependent on the dideoxynucleotide used and the DNA sequence of the template. Since this is a polymerization reaction, the DNA is labeled during the reaction by the addition of an alpha-labeled nucleotide. Each of four reactions contains one of the four dideoxynucleotides, and is loaded into a separate well of a polyacrylamide gel (as above). This method has also been adapted for use with PCR. In this case, a small amount of DNA is used in conjunction with ddNTPs, dNTPs, and a labeled dNTP. This adaptation has also been used in automated sequencing. In this case, each of the ddNTPs is also labeled with a fluorescent dye (one color for each dNTP). Only one reaction mix is needed in this case. The single lane is run on a polyacrylamide gel which is connected to a laser light source and a light sensor and computer. The sensor and computer determine which color of light is being emitted, and record the corresponding nucleotide at that position. [Note: Other labeling and detection methods using infrared light sources and a single dye are also available commercially.]

DNA sequencing has been a molecular biology method that has developed from a technically difficult procedure for the elite labs, to a method that is indispensable for all molecular biology labs, as well as many other kinds of labs and classes. While automated DNA sequencing has become an increasingly popular choice for many labs, radio-labeled methods are still used in many cases where automated sequencing fails (for a variety of reasons).

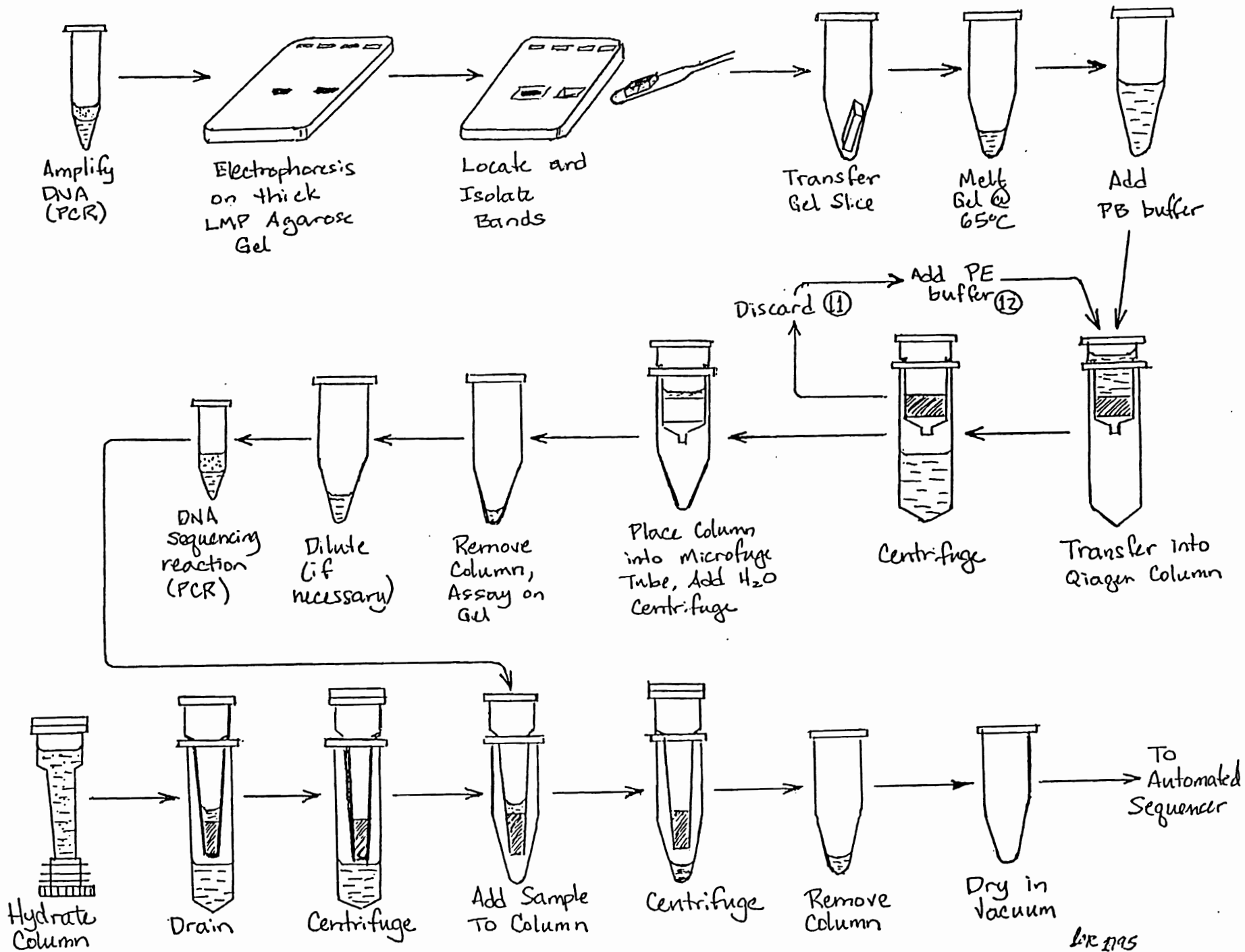
STEPS IN THE PROCEDURE:

General Steps (explained in detail below):

- A. Prepare or purify a suitable PCR product.**
- B. Perform Cycle Sequencing on the PCR product.**
- C. Dry samples.**
- D. Initialize automated DNA sequencer and computer.**
- E. Prepare samples for loading into DNA automated sequencer.**
- F. Prepare, load and begin sequencer run.**

A. Prepare or purify a suitable PCR product:

1. Use primers prITTS4 and prITS5 in a 100 μ l PCR reaction to amplify the desired DNA (starting with 5-10 ng).
2. Run 2 μ l of this DNA on a 1.5% agarose gel to determine the result of the PCR reaction. If a single heavy band is seen, proceed to step 3. If not, go back to step 1. [Separation of the PCR product from the gel can be accomplished in several ways, including melting the band out of a low melting point gel and various column purification methods. In steps 3 - 14 below, a column purification method is described. On pages 86 - 91, a gel elution method is described using low melting point agarose gels. Both methods generally yield good results.]
3. Prepare a thick (double the standard volume) 1.0% low melting-point agarose gel (molecular biology grade). Be sure to use a comb that will allow for a marker lane, plus all of your samples with every other lane empty (e.g., three samples plus a marker lane can be run on an 8 well gel).
4. Add 12 μ l of RS to 40 μ l of the PCR reaction (avoiding the oil layer) for each well to be loaded.
5. Load the gel and start as usual, but the running voltage should be 75 V rather than 100 V (at 100 V the gel will heat up and melt).
6. Take a picture of the gel as usual. Using a fresh razor blade, turn on the UV source and cut around each band. Turn off the UV source, turn on the room lights, and transfer each of the bands into separate microfuge tubes using a clean spatula.
7. Place the microfuge tubes into a 65°C water bath until the gel slices melt. Add approximately 5 volumes of the PB buffer (Qiagen DNA purification kit) to each tube. In most cases this will be approximately 500 μ l. Mix thoroughly and return to the water bath.
8. Place one Qiagen spin column into the Collection tube.



9. Load entire sample into the column.
10. Spin in a microfuge for 60 s.
11. Remove the Collection tube and discard the liquid.
12. Add 750 μl of PE buffer to the spin column. Spin in microfuge for 60 s.
13. Discard the liquid in the collection tube.
14. Replace the Collection tube with a clean 1.5 ml microfuge tube labeled accordingly. Add 15 μl of sterile distilled water to the column and centrifuge again for 60 s. The tube will now contain the eluted DNA. Keep this on ice or store at -20°C .
15. Assay the DNA by running 2 μl of this in a single lane of a 1.0% standard agarose gel.

B. Perform Cycle Sequencing on the PCR product.

1. From the gel picture of the PCR amplifications, dilute the amplification products to 5-10 ng/ μl .
2. Set up Cycle Sequencing reactions. Full reactions consist of the following:

- 8.0 μl Terminator Ready Reaction Mix (Perkin Elmer)
- 1 - 2 μl PCR product
- 0.5 - 1 μl primer (10 mM stock solution)
- 9.0 - 10.5 μl H_2O (to reach a final volume of 20.0 μl)

[NOTE: Half reactions (i.e. 4.0 μl of Reaction Mix and correspondingly lower amounts of all components) and quarter reactions (i.e., 2.0 μl , etc.) have been used successfully.]

3. Perform PCR using the following program:

- 94 $^{\circ}\text{C}$ (1 min)
- 25 - 30 cycles of:
 - 94 $^{\circ}\text{C}$ (10 s)
 - 50 $^{\circ}\text{C}$ (30 s)
 - 60 $^{\circ}\text{C}$ (4 min)
- Hold @ 4 $^{\circ}\text{C}$ until removed to freezer or column (steps 4 - 11 below)

4. Add enough sterile distilled water to each reaction to bring the volume to about 20 μl .
5. Hydrate a Centri-Sep column for at least 30 min in 800 μl of sterile distilled water. This can be done the day before, followed by storage at 4 $^{\circ}\text{C}$. Be sure that all of the gel matrix material is in the water.
6. When ready for use, remove all air bubbles by tapping on the column. Remove the stopper from the bottom end and allow the column to drain completely by gravity into the Wash tube.

Discard the water.

7. Mark the tube at the top on one side. This will be the side on the outside of the microfuge during each centrifugation.
8. Place the column into the Wash tube and then place into the centrifuge. Centrifuge on low speed (2,500 RPM for most microfuges) for 2 minutes.
9. Remove and discard the Wash tube. Place the column into a clean Sample Collection tube.
10. Carefully remove the DNA sequencing reaction mixture (avoiding ALL oil) and load it onto the top of the column (in the center).
11. Centrifuge at low speed for 2 minutes.

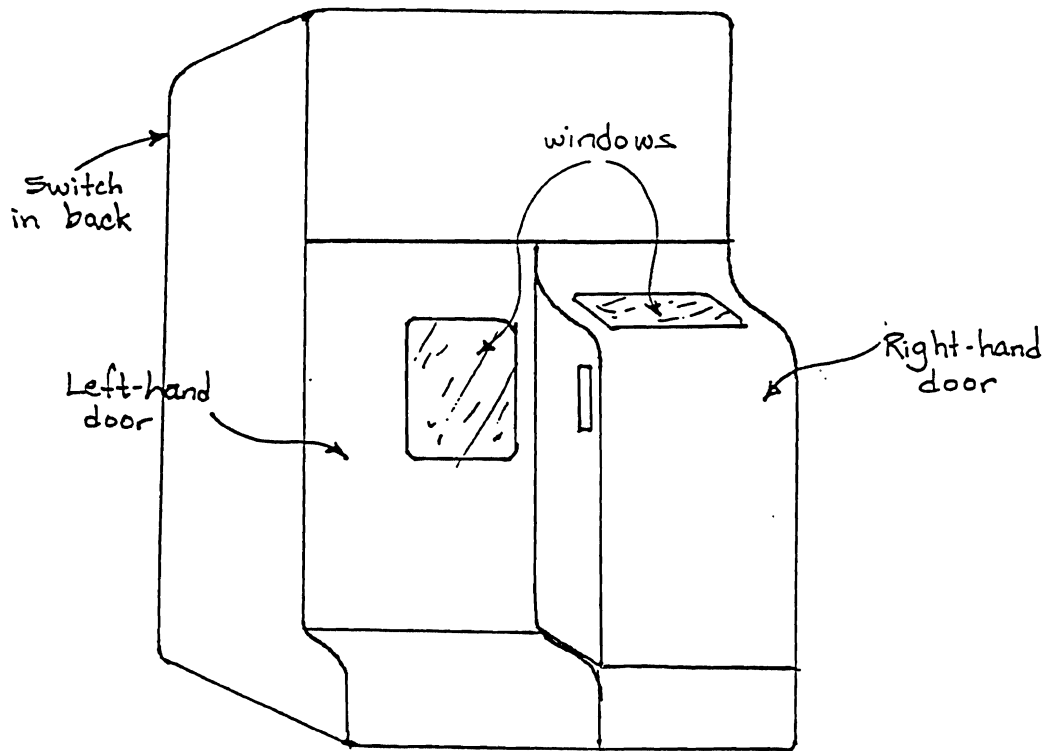
[Note: An alternative to steps 5 - 11, above, is to perform an ethanol precipitation to purify the labeled DNA from the unincorporated dye terminator nucleotides. First, mix 2.0 μ l 3 M sodium acetate and 50 μ l 95% ethanol in a 1.5 ml microfuge tube. Add water to the Sequencing Reaction to bring the volume to 20 μ l, mix and add this to the tube containing the ethanol and sodium acetate. Place in freezer (-20°C or -80°C) for at least 30 min (and up to overnight). Centrifuge at full speed for 15-30 min. Pour off supernatant and add 250 μ l 70% ethanol and centrifuge again. Remove all supernatant and dry pellet. Although this method works in some cases, it is not as acceptable as column purification. Usually, some high background is present near the beginning of the sequence, so that this portion of the region is obscured and cannot be reliably read.]

C. Dry samples.

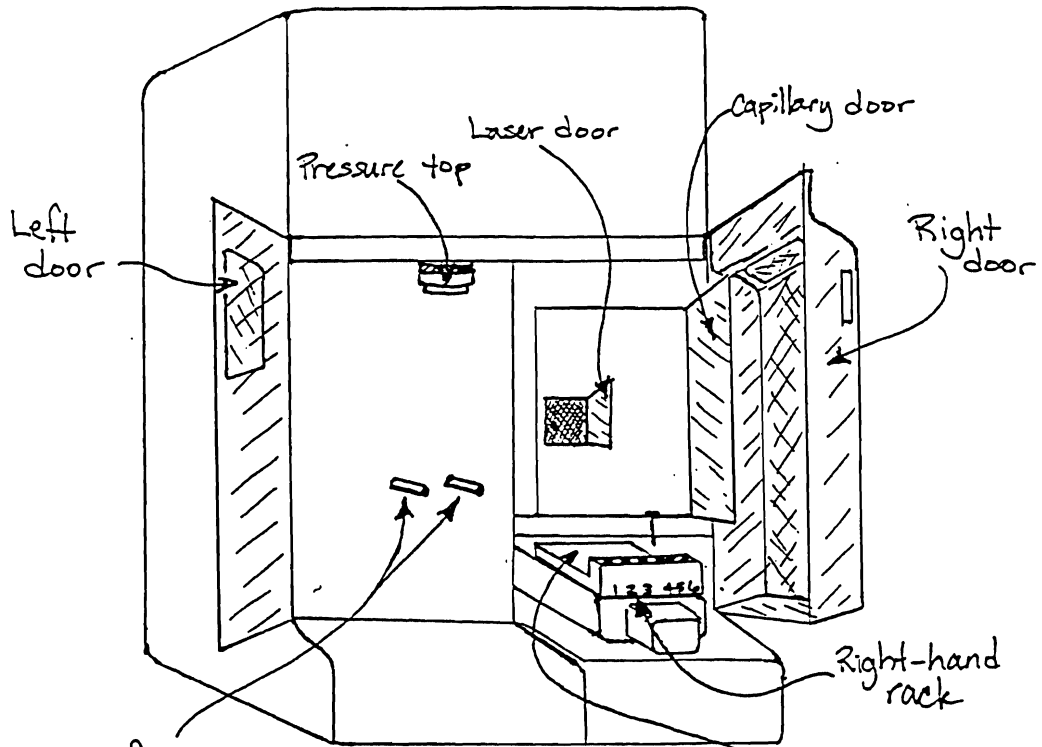
1. Dry the liquid in the Sample Collection tube under vacuum. This can be done using a Speed-Vac (or similar vacuum centrifuge). A mechanical pump vacuum is best, since this will take about 1 hour or less to dry the 20 - 50 μ l volumes. Less efficient vacuum systems may take overnight, thus compromising the DNA quality.

D. Initialize automated DNA sequencer and computer.

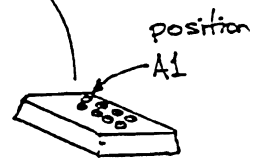
1. Turn on automated DNA sequencer (in this case an ABI 310 - details for each machine may vary slightly). Switch is on the back of the machine.
2. Turn on the computer (in this case a Power Macintosh). Wait for computer to boot up.
3. Select "310 Collection" on computer desktop.



ABI 310 (Closed)



ABI 310 (doors open)



4. Select "FILE", then "New." Select "Sample Sheet 48 Tubes." [NOTE: From 1 to 48 samples can be used. Alternative racks are also available.] Enter samples names in the desired order. Be sure to use names that will be understandable even if the samples of this run or another run are mixed up. When finished, select "Save" and name the file.

5. Select "File" and "New" and then "Injection List - Sequence." Change "length to detector" to 36 cm. Select "Sample Sheet" and choose the one with the name given in step 4.

6. Select "Print" from the "File" menu to print a copy of the list so that it can be used in part D (below).

E. Prepare samples for loading into DNA automated sequencer.

1. Add 12.5 ml of Template Suppression Reagent (kept in 4 °C refrigerator) to each tube, close tops. Heat at 95 °C for 2 - 4 min.

2. Prepare ice tray and when samples have been denatured, place immediately on ice and leave for at least 2 min. Then, place at room temperature.

3. Centrifuge briefly to move all condensed water to the bottom of the tube.

4. Transfer all of the sample into 0.5 ml ABI sequencing tubes (kept in drawer below sequencer) with sampler tops. Avoid air bubbles by tapping tubes on bench. Place each tube into ABI tube rack corresponding to the injection list (step C - 6).

F. Prepare, load and begin sequencer run.

1. Take polymer syringe, injection syringe (Hamilton) and the 10 x Genetic Analyzer Buffer out of refrigerator. Also, if a used capillary will not be used, take out a new one (with silver marking for sequencing).

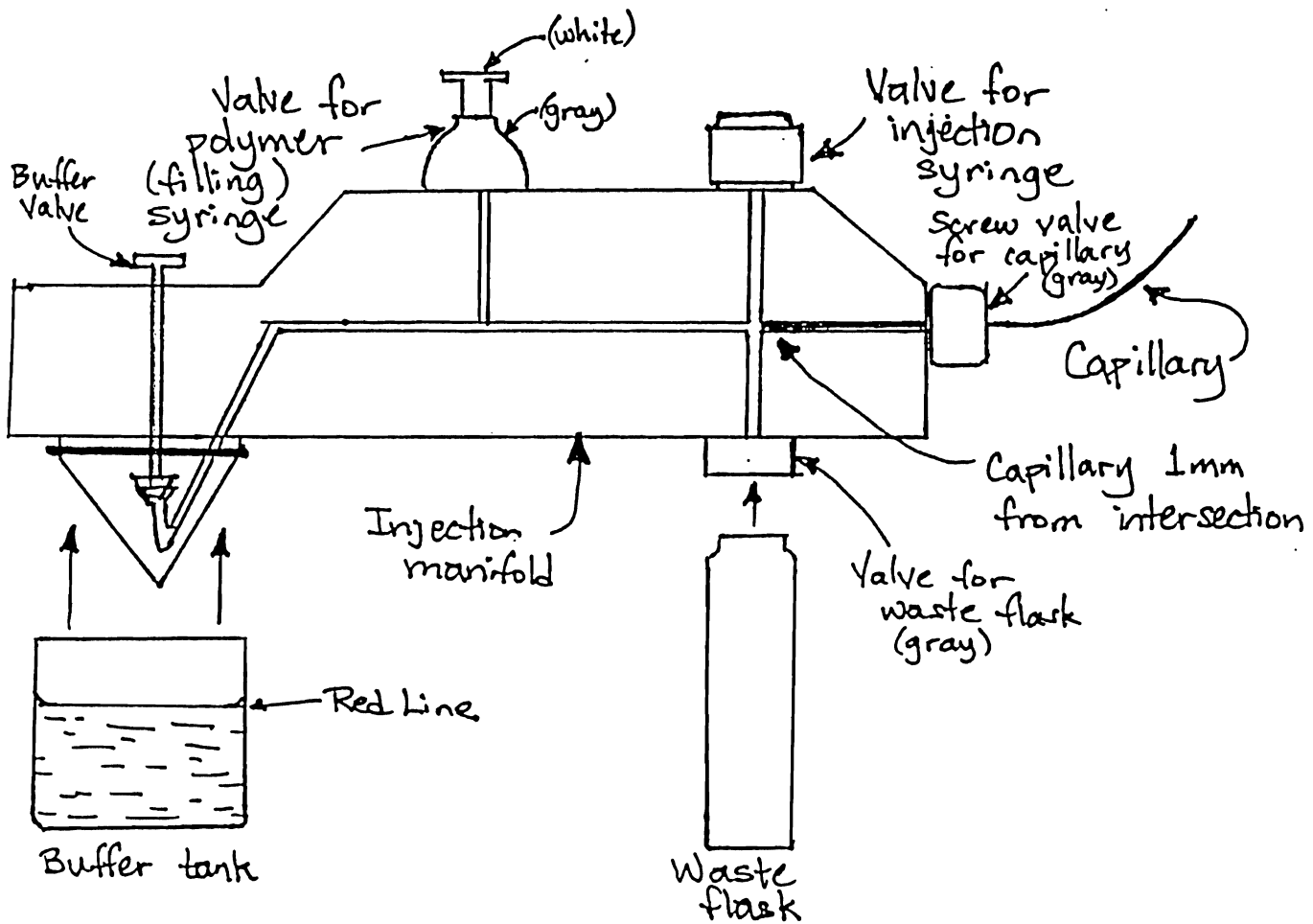
2. Prepare 15 ml of the 1 x Buffer by diluting the 10 x Buffer with sterile distilled water (or purified water purchased from chemical company).

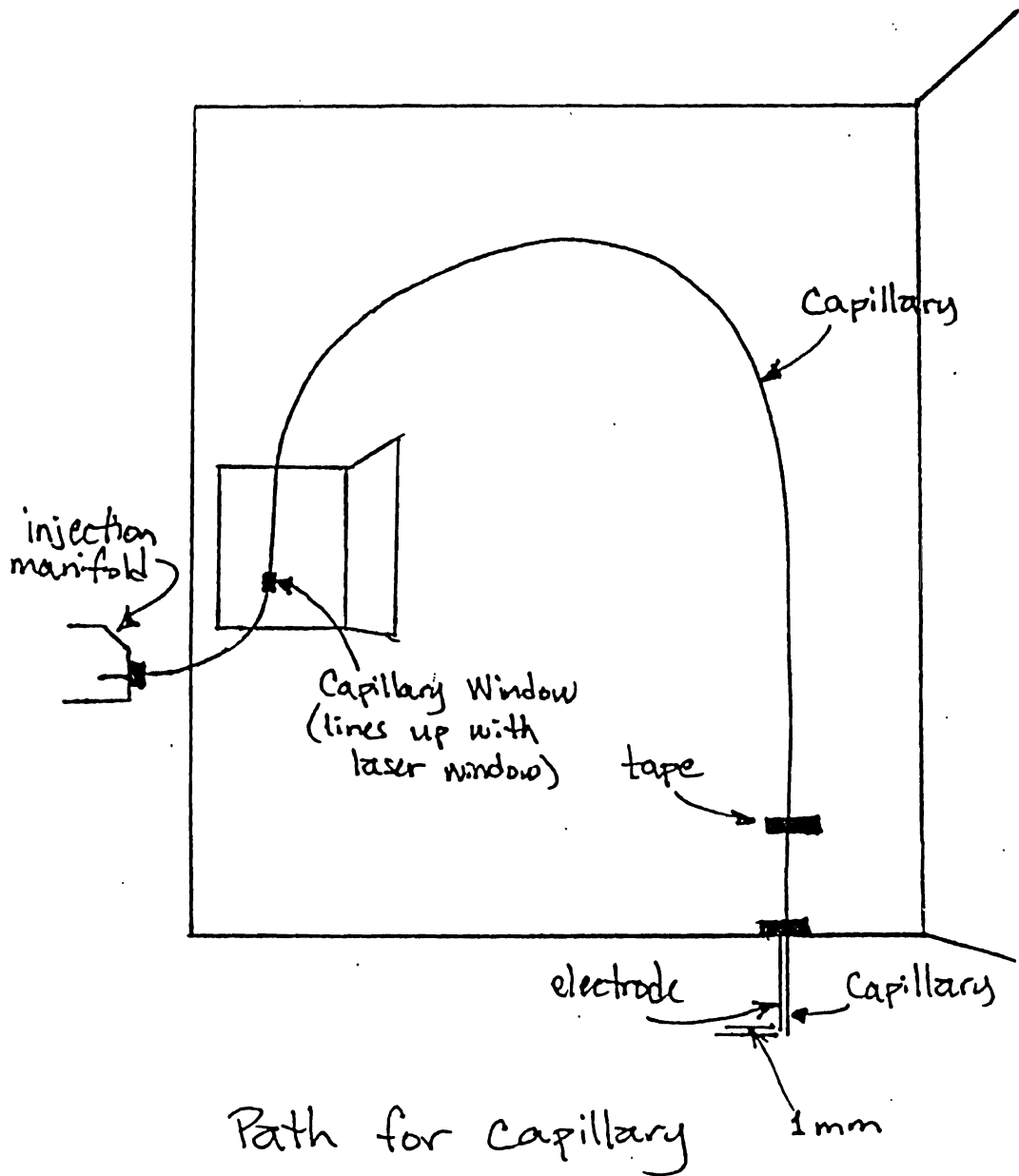
3. Open ABI 310 doors, including the right-hand door, left-hand door, inside capillary door (right side) and small laser door (see Figure).

4. Place screw valves (3) onto the plastic injection manifold (see figure) and push it onto the two pegs on the left-hand side of the ABI 310. Push waste flask into position on the lower right of the injection manifold.

5. Clean capillary window using methanol and a tissue (e.g. Kimwipe). Place the capillary into the injection manifold (as indicated in Figure). Tighten screw valve (holder) when correctly positioned.

6. Position capillary in laser window and close laser door. Position the other end so





that it is about 1 mm below the electrode (on right-hand side). Tape the capillary above to hold in position.

7. When capillary is in position and secure, then close the capillary door.

8. Unwrap injection syringe and place into position, on upper right of injection manifold. Screw to secure.

9. Unwrap polymer syringe and place into position, on upper left of injection manifold. Screw to secure joint.

10. Fill buffer tank to red line with 1 x Buffer and push onto bottom left position on injection manifold.

11. Go to computer and select "Window" and "Manual Control, " then "Function" and "Syringe Home." Then select "Buffer Valve Open" and "Execute."

12. On ABI 310, close waste valve by rotating (screw). Turn the polymer syringe by rotating at least half a turn to the right. Press the syringe plunger hard while watching the polymer proceed from the syringe to the buffer tank on left. Press until no bubbles can be seen in the narrow channels (including channels to waste container, capillary, and buffer reservoir). On the computer, choose "Buffer Valve Close."

13. Fill injection syringe by pressing on the polymer syringe while pulling up on the injection syringe until there is over 200 μ l of polymer in the injection syringe.

14. Close valve on filler polymer syringe and remove polymer syringe by unscrewing from mount. Cover end of polymer syringe and put it back in refrigerator.

15. Open waste valve. On computer, choose "Syringe Down." Change steps to "20" and execute until the pressure top is nearly touching the plunger on the top of the injection syringe. On the computer, change steps to "5" and continue bringing the pressure top down until it touches the injection syringe plunger.

16. Close waste valve. On computer, close "Manual Control."

17. Place tubes into front of the right-hand rack: 1 - 1 x buffer (tube with special cap, fill to line); 2 - water (fill to line); 3 - water (1.5. ml microfuge tube, without cap). Positions 4 - 6 are empty.

18. Place sample rack into position on right-hand side of machine. The tube in position A1 should be in the right-hand rear corner of the rack.

19. On computer select "Function" and "Autosampler Home X,Y Axis" and "Execute." After the rack has stopped, push "Tray" button on lower left-hand side of inside of machine. Rack should go back to home position again.

20. Close all doors on ABI 310 and again check injection list. When ready select "Run" button on computer. Select "View - Status" to monitor functioning of ABI 310. It will go

through injecting polymer into capillary and pre-electrophoresis prior to loading the first sample.

21. Once the machine has been started, it cannot be interrupted. If it is, the run must be started over again from the beginning, although the samples run through the machine to that point will have been completed. Once the run is finished, choose the second program on the computer desktop, labeled "Sequencing" to view, copy, edit, etc. the sequences.

22. Open the doors on the ABI 310, and remove the capillary. Place each end into microfuge tubes filled with water. Remove injection syringe, place Parafilm over end and place into the refrigerator for the next run.

23. Turn off computer and machine. Remove buffer tank from injection manifold and clean with water. Pull off injection manifold. Remove all valves and clean all parts and injection manifold with water. Force water through small channels in the manifold using a syringe full of water. After dry, put back in drawer under sequencer. Check inside ABI 310 to be sure all parts are clean. Clean any parts with water and Kimwipes.

24. View and analyze sequences.

SOLUTIONS:

PCR reagents:

(see previous section on PCR)

Low Melting Point Gel:

(See "Elution of DNA From Agarose Gels" section)

Qiagen Column Purification:

QIAquick PCR Purification Kit:

QIAquick Spin Columns

PB buffer

PE buffer

Collection tubes

DNA Sequencing Terminator Premix, 1000 μ l (Perkin-Elmer):

1.58 μ M A-Dye-Deoxynucleotide

94.74 μ M T-Dye-Deoxynucleotide

0.42 μ M G-Dye-Deoxynucleotide

47.37 μ M C-Dye-Deoxynucleotide
78.95 μ M dITP (inosine substitutes for guanosine)
15.79 μ M dATP
15.79 μ M dCTP
15.79 μ M dTTP
168.42 mM Tris-HCL (pH 9.0)
4.21 mM $(\text{NH}_4)_2\text{SO}_4$
42.10 mM MgCl_2
0.42 units AmpliTaq DNA polymerase

Centri-Sep Columns:

REFERENCES:

- Maxam A.M. and Gilbert W. 1977. A new method for sequencing DNA. Proc. Natl. Acad. Sci. USA 74:560.
- Sanger F., Nicklen S., and Coulson A.R. 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA 74:5463.