AGAROSE GEL ELECTROPHORESIS AND RESTRICTION ENDONUCLEASE DIGESTIONS

INTRODUCTION:

Two very powerful techniques of molecular biology are those of restriction endonuclease cleavage of DNA and gel electrophoresis for separation of DNA on the basis of size (and sometimes on the basis of other characteristics).

Restriction endonucleases:

Restriction enzymes cut double stranded DNA at specific sites. Most recognize four, five or six base pair "pseudo-palindromic" sequences (i.e., the sequence of one strand is the same as the sequence of the other strand read in the opposite direction). Some recognize longer sequences, and some recognize non-palindromic sequences. Nearly six hundred have been identified, and over one hundred are commercially available. The names are almost always based on the genus and species names of the bacteria from which they were isolated. For example, *Eco*RI, was the first restriction enzyme (RI) isolated from *E. coli*. Some make staggered cuts that leave the 5' ends of the strands exposed (e.g., *Eco*RI, *Bgl*II, *Bam*HI), some leave the 3' end exposed (e.g., *Hha*I, *Kpn*I, *Pst*I) and some make blunt end cuts (e.g., *Alu*I, *Eco*RV, *Hae*III). Many are affected by modified (methylated) bases. Some will cut only if the bases are methylated, but more commonly, some enzymes will not recognize the proper sequence if the bases are methylated. Most require magnesium ions, specific salt concentration ranges and physiological pH ranges to function properly. Most are also inhibited by certain polysaccharides (found in plants and fungi). If the proper environment is not provided for the enzymes, they will either cut inefficiently (causing only partial digestion of the DNA), not function at all, or their base recognition may be altered (so-called "star" activity). Since the enzymes cut at specific sites and much of the DNA changes little, these enzymes can cut the DNA into fragments of reproducible lengths. When run on gels and hybridized to gene (or other) probes, maps of the DNA regions of interest can be produced. In this exercise, the DNA will be digested using restriction enzymes, then the resulting fragments will be separated by agarose gel electrophoresis.

Gel Electrophoresis:

Linear DNA fragments can be separated on the basis of size using agarose gels. The practical range for good separation on standard gels (0.4-2.0%) is from about 100 base pairs up to about 30-40 kilobase pairs (kb). [As you will see in later exercises, molecules up to 12 Mb (megabases) can be separated on agarose gels by altering the angle and timing of the electrical field.] Agarose is a mixture of carbohydrate polymers. It is made mainly of 1,3-linked β-D-galactopyranose and 1,4-linked 3,6-anhydro-α-L-galactopyranose. Depending on the source of the agarose, varying degrees of substituted groups (half-ester sulfate groups, pyruvic acid ketals and methyl ethers) on the sugars can be present. These substituted groups can have large effects on the properties of the agarose and can have detrimental effects on some molecular biology methods. For this reason, the type of agarose to be used should be chosen very carefully. For general purposes, agarose from BioRad, Fisher, Sigma, and FMC are good. For special purposes, the FMC agaroses are excellent.

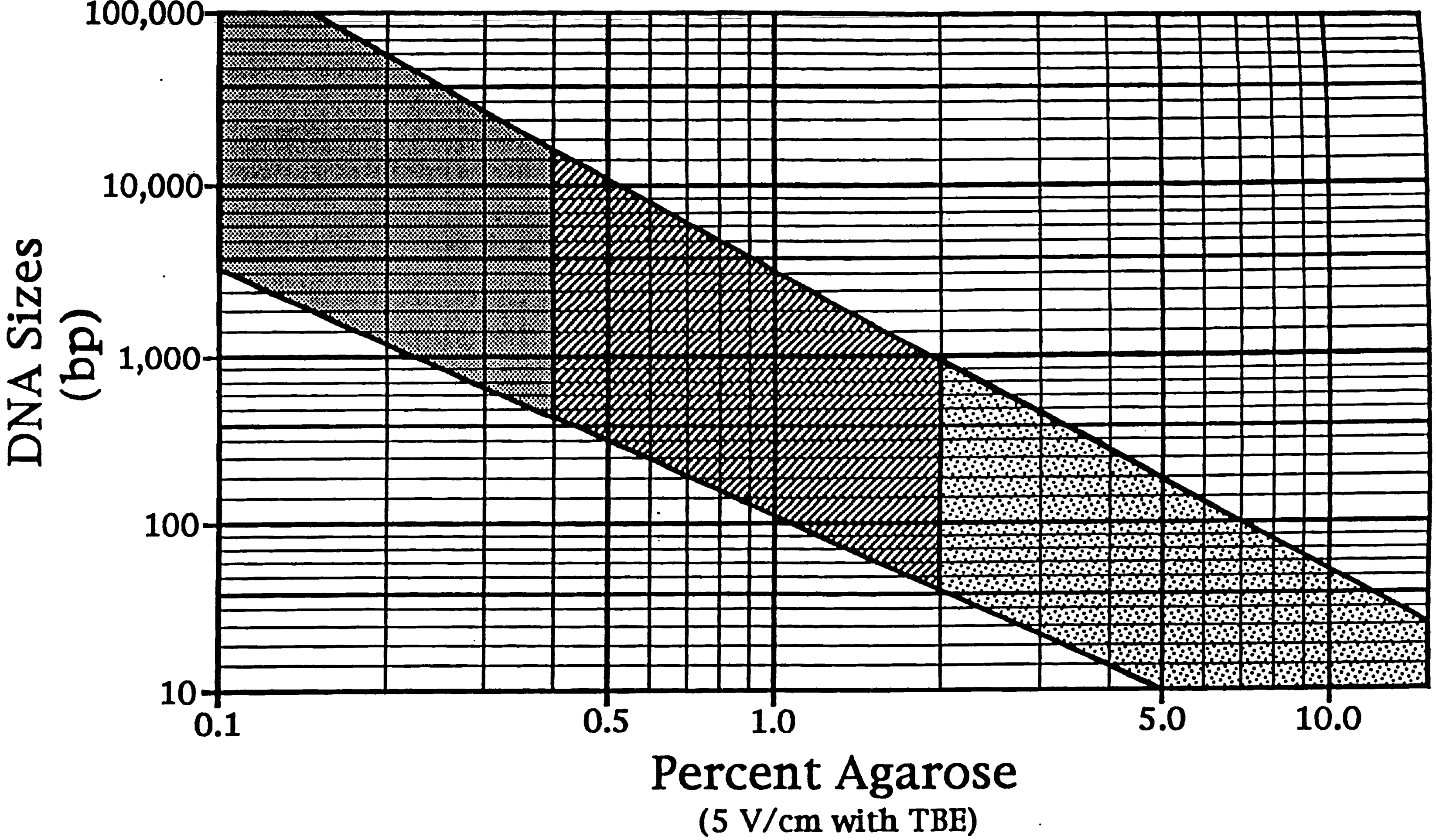
Agarose solutions do not react like most other solutions. At specific temperatures, hydrogen bonding between polymers causes a gel to form. This is a matrix of the carbohydrate polymers, filled with water. Because of a phenomenon known as hysteresis, the melting temperature of the gel is higher than the gelling temperature. General purpose agarose gels gel around 35-40 °C but melt at about 95 °C. Low melting point agaroses gel at 25-30 °C and melt at around 65 °C. Concentration can affect the gelling and melting temperatures.

Agarose goes through several stages during dissolution. First the powder disperses in the water (at cooler temperatures), then it becomes hydrated (at moderate temperatures) and finally melts or dissolves (at high temperatures). If the temperature is too high before it becomes dispersed in the water, a shell of hydrated agarose can prevent rapid penetration of more water and can slow dissolution greatly. Sometimes, this is not easy to see, and the agarose may appear to have dissolved, when it has not. If cast at this point the gel will be inconsistent and will be virtually useless for good separation of the DNA. Therefore, start with cold water.

When voltage is applied through the gel, the DNA migrates toward the positive (red wire) pole (since DNA has a net negative charge). When a voltage of between 1 V/cm and 5 V/cm is applied, linear DNA molecules migrate as the log of their molecular weights. The linear range of this separation depends on the agarose concentration and on the voltage (and current) applied through the gel. Lower percentage gels (0.4%-0.6%) generally give a broader linear range than do higher percentage gels. However, they are harder to work with because they are fragile and below 0.4% they tend to float in the buffer. Below 0.3%, the wells of the gel tend to collapse and the gel must be additionally supported by a higher percent agarose gel surrounding the low percentage agarose gel (see figure on next page to determine percentage of agarose for your specific application).

Ethidium bromide is used in order to make the DNA visible. It intercalates between the stacked bases of the DNA and when UV-irradiation is applied (at 250-310 nm) it fluoresces in the visible range (red-orange). When a photograph is taken of this fluorescence, as little as 1 ng of DNA can be detected. The amount of DNA per band should ideally be between 10 ng and 150 ng. Above 200 ng, the bands tend to distort. At 500 ng accurate size measurements are not usually possible.

For most applications we will be dealing with linear DNA molecules, which can be compared directly on the gel to other linear DNA molecules on the basis of lengths and molecular weights. Some DNA preparations (e.g., bacterial plasmid DNAs) usually contain a mixture of linear, supercoiled (both strands being covalently bound in complete loops) and nicked circles (when one or both of the strands is broken at one or more point, the supercoiling is lost and the molecule becomes a relaxed circle). Molecules of different conformation but the same molecular weight migrate differently in the gel. Supercoiled DNAs migrate fastest, partly due to their compact size and partly to the smaller amount of ethidium bromide that intercalates into the DNA. The ethidium bromide retards the migration of the DNA, so that the less ethidium bound to the DNA, the less impeded it is in moving through the gel. Relaxed circular molecules, on the other hand, can intercalate the same amount of dye as can linear DNA.



Determination of agarose concentration given size ranges of DNAs to be separated.

- Standard agarose, but a surrounding support gel is necessary.
- Standard agarose.
- Specialty agaroses (Low melting point, wide range, and mixes).

However, circular molecules can become hooked onto spike-like obstructions in the gel, and so generally run slower through the gel than do the linear molecules. In plasmid preparations multiple interconnected DNAs, know as concatamers, are also usually obtained, and are indicated by a large diffuse band in the higher molecular weight range of the gel. Therefore, an undigested plasmid preparation may exhibit up to four bands (and sometimes more) on a gel. For most organisms, however, only one band is seen in an undigested sample.

STEPS IN THE PROCEDURE:

Restriction Endonuclease (*Eco*RI) Digestions:

1. Mix the following on ice in a 0.5 ml microfuge tube:

   1 µl DNA solution
   7 µl sterile dH_2O
   1 µl 10X *Eco*RI buffer
   0.5 µl *Eco*RI
   0.5 µl RNase solution
   total = 10 µl

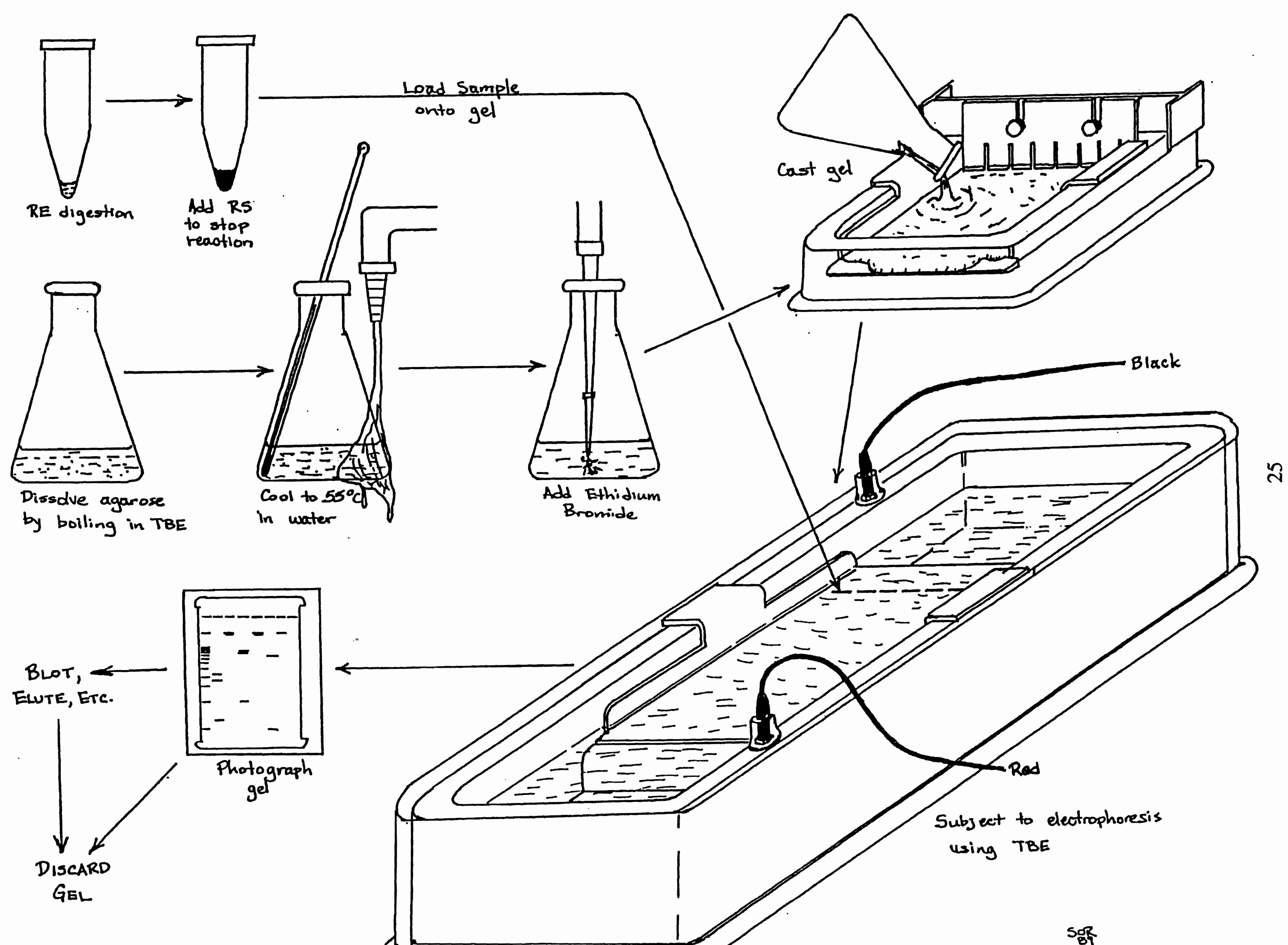
2. Make sure that the enzyme is thoroughly mixed into the solution. The enzyme comes in 50% glycerol which causes it to sink to the bottom of the tube. If left at the bottom of the tube, the layers will not mix and the DNA will probably not be digested. If some of the liquid is stuck to the upper parts of the tube, simple tap the tube on the bench top or spin the tube in the microfuge for a second.

3. Place the tube into the 37 °C incubator (or water bath or heating block) and allow the reaction to proceed for at least 4 hours (better, if left overnight).

4. When the reaction is completed, add 3 µl of the RS solution and either load directly on a gel or store in a refrigerator or freezer until ready to be loaded onto the gel.

Agarose Gel Electrophoresis:

1. Mix the appropriate amount of agarose with the running buffer (in this case 1X TBE) in a flask at room temperature (do not use hot buffer or the agarose will be difficult to dissolve). The minigel boxes hold 25 ml gels (use a 50 or 125 ml flask). For a 1% agarose gel, 0.25 g of agarose is needed. For this exercise you should make a 0.6% gel. Cover the top of the flask with aluminum foil and heat the mixture to boiling in the microwave or with a Bunsen burner. BE CAREFUL!! If heated in the microwave oven, be sure that the aluminum foil does not extend out away from the flask, as this will cause arcing in the microwave, which can cause damage to the oven. Also, in the microwave the liquid can become superheated, and one bump can make it boil rapidly with an eruption of steam that can burn you! [P.S. Never handle the flask with bare hands and never get your eyes close to the open end, even if you have a cover over it!]


RE digestion
Add RS to stop reaction
Load Sample onto gel
Cast gel
Dissolve agarose by boiling in TBE
Cool to 55°C in water
Add Ethidium Bromide
Black
Red
Subject to electrophoresis using TBE
Photograph gel
BLOT, ELUTE, ETC.
DISCARD GEL
SGR 89

2. Once the agarose is completely in solution (which may take several minutes), cool the flask in a 65 °C water bath for 5-15 min.

3. Set up the casting unit. Place the gel comb into the casting tray. Be sure that the teeth are at least 1 mm above the bottom of the tray. If you have trouble determining this distance, slide a piece of white paper under the teeth. You should be able to see it better.

4. Remove the molten gel from the water bath and cool it under tap water to 55 °C, then add ethidium bromide to it. The stock solution of ethidium bromide is 10 mg/ml The final concentration in the gel should be 0.5 µg/ml. Therefore, 1.25 µl of the stock solution should be added to the molten gel. After adding the ethidium bromide to the gel, swirl the gel to mix in the ethidium bromide. REMEMBER: Ethidium bromide can be hazardous to your health and to others'. Handle it with care. If you get any on your skin, wash it off with water (no soap, this may take it through you skin). If you spill any, clean it up immediately, don't leave it for other unsuspecting people!

5. Pour the molten gel into the casting tray. Move the flask from one side of the tray to the other while pouring so that no hot or cool spots can occur on the tray, otherwise uneven gelling may occur causing distortion of the DNA bands. Allow the gel to set 15 min or so at room temperature until the gel is firm. Then place the gel in a refrigerator for at least 1 hour to complete gelling.

6. Pour 210 ml of 1X TBE into the electrophoresis unit, then add 10.5 µl of the ethidium bromide stock solution. Tilt the unit carefully from side to side in order to mix in the ethidium bromide.

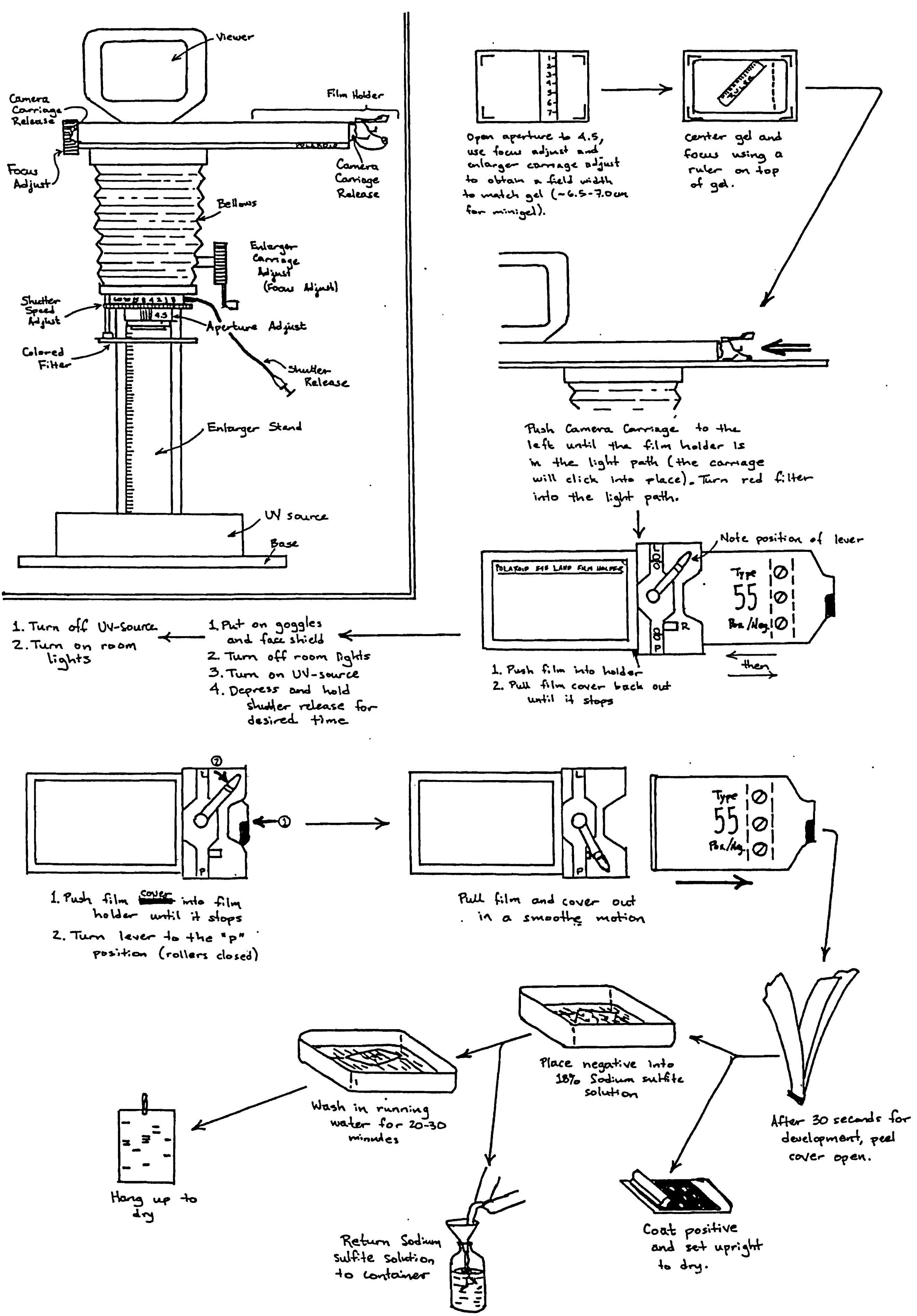
7. Pull the comb SLOWLY out of the gel. Pull one end up slightly before the other end. Once the comb is out, place the gel (with casting tray) into the electrophoresis unit.

8. Load samples (that have added to them the RS, or running/stop solution) into the wells with a P20 Pipetman (or P200, if you have more than 20 µl to load). Be sure to load molecular weight markers (standards) into one of the lanes.

9. Once all of the samples have been loaded, hook up the electrical leads from the power unit to the electrophoresis unit and turn on the power. [Remember, DNA is negatively charged, so it will be traveling towards the positive electrode, the cathode, or the red lead. Therefore, the black lead should be on the well end of the gel and the red lead should be at the opposite end.] Begin by turning the power to 100 V for 30 seconds. Then turn the power supply off for about 10-15 seconds. Repeat this process one more time. Then turn the power on to 25 V for the first half hour, until the dye has traveled about 1 cm away from the wells. Then, turn the power up to 50-100 V.

10. Running times vary, when the dye front has gone 2/3 the length of the gel or beyond (an hour or so), this is sufficient to separate bands for most purposes.

11. Destain the gel in water for 5-60 minutes (or more).


Viewer
Film Holder
Camera Carriage Release
Focus Adjust
Camera Carriage Release
Bellows
Enlarger Carriage Adjust (Focus Adjust)
Shutter Speed Adjust
Aperture Adjust
Colored Filter
Shutter Release
Enlarger Stand
UV source
Base
Open aperture to 4.5, use focus adjust and enlarger carriage adjust to obtain a field width to match gel (~6.5-7.0cm for minigel).
center gel and focus using a ruler on top of gel.
Push Camera Carriage to the left until the film holder is in the light path (the carriage will click into place). Turn red filter into the light path.
Note position of lever
Type 55 Pos./Neg.
1. Push film into holder
2. Pull film cover back out until it stops
then
1. Put on goggles and face shield
2. Turn off room lights
3. Turn on UV-source
4. Depress and hold shutter release for desired time
1. Turn off UV-Source
2. Turn on room lights
1. Push film cover into film holder until it stops
2. Turn lever to the "p" position (rollers closed)
Pull film and cover out in a smoothe motion
After 30 seconds for development, peel cover open.
Place negative into 18% Sodium sulfite solution
Coat positive and set upright to dry.
Wash in running water for 20-30 minutes
Return Sodium sulfite solution to container
Hang up to dry

12. Photograph the gel using UV-source.

a.Slide the gel off of the gel tray onto the UV-light box.
b. Set the camera to the viewing position (top all the way to the right) and turn the red filter out of the light path (for easier focusing). Focusing is also facilitated by placing a white ruler (or other piece of plastic with letters on it) on top of the gel.
c. Raise or lower the enlarger (by rotating the large knob on the right side of the enlarger) until the image of the gel nearly fills the camera viewing frame. At the same time you need to move the focus knob (which moves the camera lens but not the camera back) to obtain a sharp image. You may need to turn both knobs repeatedly until you reach an acceptable frame and focus.
d. Push the sliding top so that the camera back is in the position to take a picture.
e. Make sure that the lever on the camera back is set so that the rollers are in the open position (lever is towards the wall).
f. Slide one of the Polaroid film holders (with film) into the slot on the camera back. Be sure that you have it right-side-up. Push it all the way into the camera back until it stops. [You may hear a click.]
g. Pull the slider back out until it stops. Do not pull it all the way out of the camera back! The film will stay in the camera back at this point, ready for the exposure.
h. Put on eye goggles and face shield.
i. Turn the shutter setting all the way to F4.5, set the shutter speed to "B" (bulb) and turn the red filter into the light path.
j. Close the door, turn off the overhead lights and turn on the UV-source.
k. Depress and hold the shutter (cable) release for desired time. Release shutter release when finished with exposure time. Depending on the sensitivity of the film and amount of DNA on the gel, the time may be from 5 to 90 (or more) seconds. For Polaroid Type 55 film, start with a 45 second exposure (time this by counting or with a stop watch - remember it will be fairly dark when you are timing this, so a watch should either have a beeper or it should have a luminescent dial). Sometimes more than one exposure may be necessary. Over time the fluorescence quenches (decreases), so the first exposure will take less time than the second (and so on). Because of this, you should attempt to make the first exposure of the gel the best (and only) one. [Remember: The film is about $1.00 to $2.00 per sheet, so don't waste it.]
l. Turn off the UV-source and turn on the room lights.
m. Push in the film slider all the way in, then turn the lever on the camera to the position that indicates that the rollers are closed (pull towards you).
n. Pull out the film slowly and evenly. Wait 20-30 seconds, then pull the outside paper pieces apart. Next, pull the negative (plastic) away from the positive (paper). Grip the metal and paper <u>firmly</u> at the end and tear out the positive by pulling at an angle. Place the positive down and next pull the negative out. You must grip firmly enough so the negative does not slide between the metal pieces at the end or this will damage the negative. Also, if you pull too hard at the wrong angle, you can tear the negative. This takes a bit of practice. Once you have the negative free from the paper/metal holder, throw the holder away. Bend the negative at the perforations, and break off and discard the small end pieces. Place the negative into a tray containing a solution 18% sodium sulfite solution (gelatin site up). Make sure it is completely covered by the solution. The gelatin goo will separate from the negative. Pull out the goo with gloved hands and discard it. Leave negative in the solution and proceed to the next steps.

o. Coat the positive with the coating bar provided with the film. Stand the positive up so that the coating coats it evenly. [It will take several minutes to dry completely.]
p. Pick gel up with flexible plastic ruler or similar thin piece of plastic (or with gel scoop for large gels) and place it back into the tray with water.
q. Clean up any messes you have made. In particular, clean off the plastic top of the UV-source with water and paper towels. Check for any other places on the camera, floor or tables that you might have spilled or spread ethidium bromide. DO NOT leave ethidium bromide messes for other unsuspecting people to put their unprotected hands into!
r. Take all of your materials back to the lab. If the sodium sulfite is to be reused, pour it back into the "used" container. Place the tray (with negative) into sink and run water over the negative for at least 5-10 minutes, then hang or stand it up to dry.

13. Blot gel (see Southern Blotting exercise) or discard between several layers of paper towels.

SOLUTIONS:

10 X *Eco*RI buffer

500 mM Tris (pH 7.5)
100 mM $MgCl_2$
500 mM NaCl
10 mM DTT (dithiothreitol, see below)
[Restriction enzyme buffers are now generally send along with the enzyme by the manufacturer, and this recipe is given just for information you may want to use later]

0.5 M DTT (dithiothreitol)

(usually 1 ml of this lasts a very long time)

Ethidium Bromide 10 mg/ml (stock solution)

Add 100 mg ethidium bromide to 10 ml H_20. Stir on a magnetic stirrer for several hours. Wrap in aluminum foil and keep at 4 °C

5X TBE (1 liter)

54 g Tris base
27.5 g boric acid
20 ml 0.5 M EDTA

0.5 M EDTA

18.61 g disodium EDTA
80 ml H_20
2 g NaOH pellets
Check pH (should be about 8.0, if not, then adjust)
Bring to final volume of 100 ml

Ethidium Bromide Stock Solution

10 mg/ml

RS (running/stop solution)

20% Ficoll
50 mM EDTA
0.05% bromephenol blue

Molecular weight markers (1 kb ladder)
[Use for bands of 1 kb and above]

1 µl 1 kb ladder (BRL)
1 µl lambda DNA (250 µg/ml, heat to 65 °C, then place on ice before dispensing to melt the cohesive ends apart)
10 µl 10X restriction enzyme buffer (I generally use *Eco*RI buffer, since this is the enzyme I most often use. Use of different salt concentrations will tend to alter the migration of the DNA slightly)
60 µl RS
103 µl H_20

Molecular weight markers (123 or 100 bp ladder)
[Use for bands of 3 kb and below]

5 µl 123 bp ladder (BRL) [or 100 bp ladder]
10 µl 10X restriction enzyme buffer (I generally use *Eco*RI buffer, since this is the enzyme I most often use. Use of different salt concentrations will tend to alter the migration of the DNA slightly)
60 µl RS
100 µl H_20

[Test the Molecular Weight Marker by loading 5, 10, 15, and 20 µl onto a gel. Ideally, the 5 and/or 10 µl amounts should yield bright, clear bands. If they are too faint, add more of the stock solutions of DNA markers to the mixes and test again.]