

DETERMINATION OF RIBOSOMAL RNA GENE COPY NUMBER

PART 1: DNA DILUTION

OBJECTIVE:

The accurate measurement of DNA in solution is difficult, especially when only small amounts of DNA are present. The purpose of this exercise is to accurately determine the concentrations of DNA to be used to determine gene copy number (or for other uses). Part 1 describes the process of physically dilute the DNA into capillaries. Part 2 details the densitometric methods to quantify the DNA by measuring the density of silver grains on the photograph of the capillaries. Part 3 introduces the first part of slot-blotting of the samples prepared in parts 1 and 2. The second part, hybridization of the blot, will be performed later.

INTRODUCTION:

It is often necessary to determine the copy number of a particular gene. This can be done by analysis of the Southern hybridization pattern from a digest with a restriction enzyme, but there are limitations to this method. First, the gel must not be overloaded or underloaded, since this can cause problems with the quantitation of the DNA loaded onto the gel. Second, the transfer of the DNA might not be uniform throughout the blot, making accurate comparison to the standards impossible. An alternate method, that has been used successfully is the dot-blot (more recently, the slot-blot) method. For this technique, a known amount of DNA is loaded directly to a spot on the blotting membrane, followed by hybridization to a labeled DNA probe.

Several steps are involved in this process. First, the DNA must be accurately measured. This can be accomplished by using spectrophotometry, on a gel or by the capillary method of Moore and Sutherland (1985). We will use the latter method, since this is very accurate and requires very little DNA for the assay. Second, homologous probe DNA standards must be loaded onto each blot for comparison to the unknowns. Third, a negative control must be loaded onto each blot. This will indicate the amount of nonspecific hybridization on the blot and may indicate the lower limit of the assay. Fourth, densitometry must be performed for the initial DNA quantitation and for the hybridization signal quantitation. This assures that the readings obtained are within the linear range of the film and maintains consistency in the analysis.

MICROCAPILLARY QUANTITATION METHOD:

This method (Moore and Sutherland, 1985) uses desitometric analysis of a photograph (actually, a fluorograph) of the UV illuminated DNA stained with ethidium bromide.

The amount of ethidium bromide used and the concentration of DNA are crucial to obtaining accurate results. Since RNA will also add to the fluorescence, any RNA in the sample must be digested with RNase before performing the assay. Even then, the oligoribonucleotides do cause some added fluorescence. Typically, in your diluted samples, the amount of fluorescence added by the RNA oligonucleotides should cause no more than a slight change in the apparent concentration of DNA. The final DNA concentration in the capillaries (after addition of the ethidium bromide and water) must be between 5 ng/ μ l and about 50 ng/ μ l. Below 5 ng/ μ l, the fluorescence becomes inconsistent and above 70 ng/ μ l, the fluorescence levels off to a fairly constant value, no matter how much more DNA is added.

STEPS IN THE PROCEDURE:

1. Make serial dilutions of lambda DNA and of the probe DNA. The plasmids pBD4, pSR118 or pSR125 (containing the ribosomal RNA genes of the yeast, *Saccharomyces cerevisiae*) will be used. They must first be linearized by digesting with *EcoRI* (which cuts several times in each plasmid, generating several fragments). Treat with RNase solution. Both phage and plasmid DNAs should also be heated to 65 °C for a few minutes, then put on ice prior to dilution.

a. Make the following solutions of lambda DNA (in nanograms, ng/ μ l): 2, 5, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 110, 120, 130, 140, 150 and 200. It is absolutely necessary that these be SERIAL DILUTIONS. In other words, the solution of the highest concentration is used to make the solution of the next lower concentration. That solution is used to make the next solution, and on down the line. In this way, each solution (except the last one) is used only once to make the solution of the next lower concentration, and is not used to make any other dilution. This is the best way to achieve a smooth dilution series.

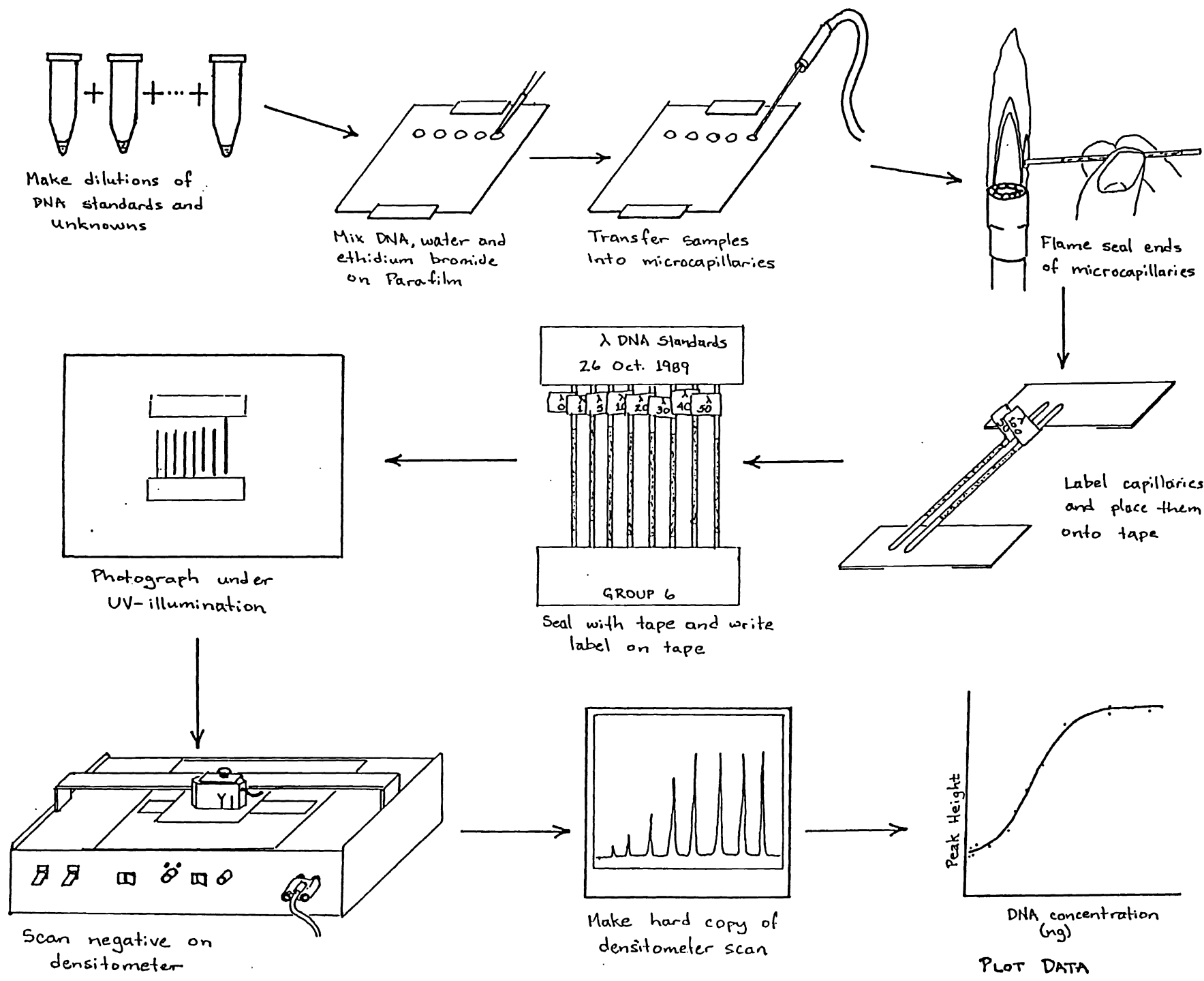
b. You will also be provided the following solutions of a ribosomal DNA-containing plasmid to be used for DNA quantitation: (ng/ μ l) 2, 5, 10, 20. A second set of solutions of the same plasmid will be supplied (in picograms, pg/ μ l) to be used as standards later on the slot-blot: 1, 2, 5, 10, 15, 20, 25, 50, 75, 100, 250, 500 and 1000.

2. Tape a piece of Parafilm onto the benchtop and mix the following (for each sample in part 1a, above) on the Parafilm:

- 1.5 μ l sterile dH₂O
- 1.0 μ l ethidium bromide (50 μ g/ml)
- 2.5 μ l DNA solution

3. Transfer the solution into a 10 μ l microcapillary (which has been broken in half). Draw the solution to within about 1-1.5 cm from one end. Flame-seal that end, then flame-seal the other end.

4. Place the sealed capillary onto two pieces of tape (stuck to the bench), one at each end of the capillary. Place the capillaries at least 4-5 mm from each other.



5. When all have been prepared, place another piece of tape over the top of each end of the capillaries and label the tape for later reference.

6. Repeat steps 2-5 with your unknown samples.

7. Photograph the microcapillaries the same way you would a gel, with the following differences: a. the lens should not be completely open (@ F4.5), use F8 instead (the reason is that the lenses tend to distort the images at the edges, so that the final intensities as recorded on the film are not accurate representations of the actual intensities); b. the frame should not be filled with the microcapillaries, a generous border should be maintained (again, because of lens distortion), c. Open the shutter for 1.5 minutes. RECORD ALL SETTINGS ON THE CAMERA, including the F-stop, the height of the lens, etc. The next time you photograph the microcapillaries, the settings must be EXACTLY the same. If they are not, comparisons between negatives is impossible.

8. Take two exposures, one long one (1.5 minutes) and one shorter one (1 minute) so the linear reactivity of the film is achieved.

9. Scan the negative on a densitometer and construct a standard curve.

SOLUTIONS/SUPPLIES:

10 μ l glass capillaries:

(These must be made of soft glass so that they can be melted in the bunsen burner flame)

Ethidium Bromide:

50 μ g/ml in sterile dH₂O

REFERENCES:

Moore, S.P. and Sutherland, B.M. 1985. Anal. Biochem. 144:15.

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PART 2: DENSITOMETRY AND QUANTITATION

INTRODUCTION:

Densitometry, in this case, is simply determining the density of silver grains at a location on a photographic negative. Films differ in their reactivities to light (or radioactive decay). High contrast films respond over a shorter range of light flux than do lower contrast films. Because of this, high contrast films are not as useful for densitometry, since an area of the film can change from no exposed silver grains to a high density of exposed silver grains with only a slight change in light flux. Lower contrast films, on the other hand, undergo more gradual changes in the number of exposed silver grains in a particular area of the film. Each film also has a light flux range where the number of silver grains reacting to the light is directly proportional to the amount of light hitting the silver grains. In other words, the film is reacting in a linear way to the amount of light it is exposed to. Below this linear range, the film does not respond to the light. Above this range, all of the silver grains have reacted. In other words, the film cannot respond to any more light. As long as you are working within the linear range of the film, the measurements are accurate. Outside of this range, and the measurements cannot be trusted.

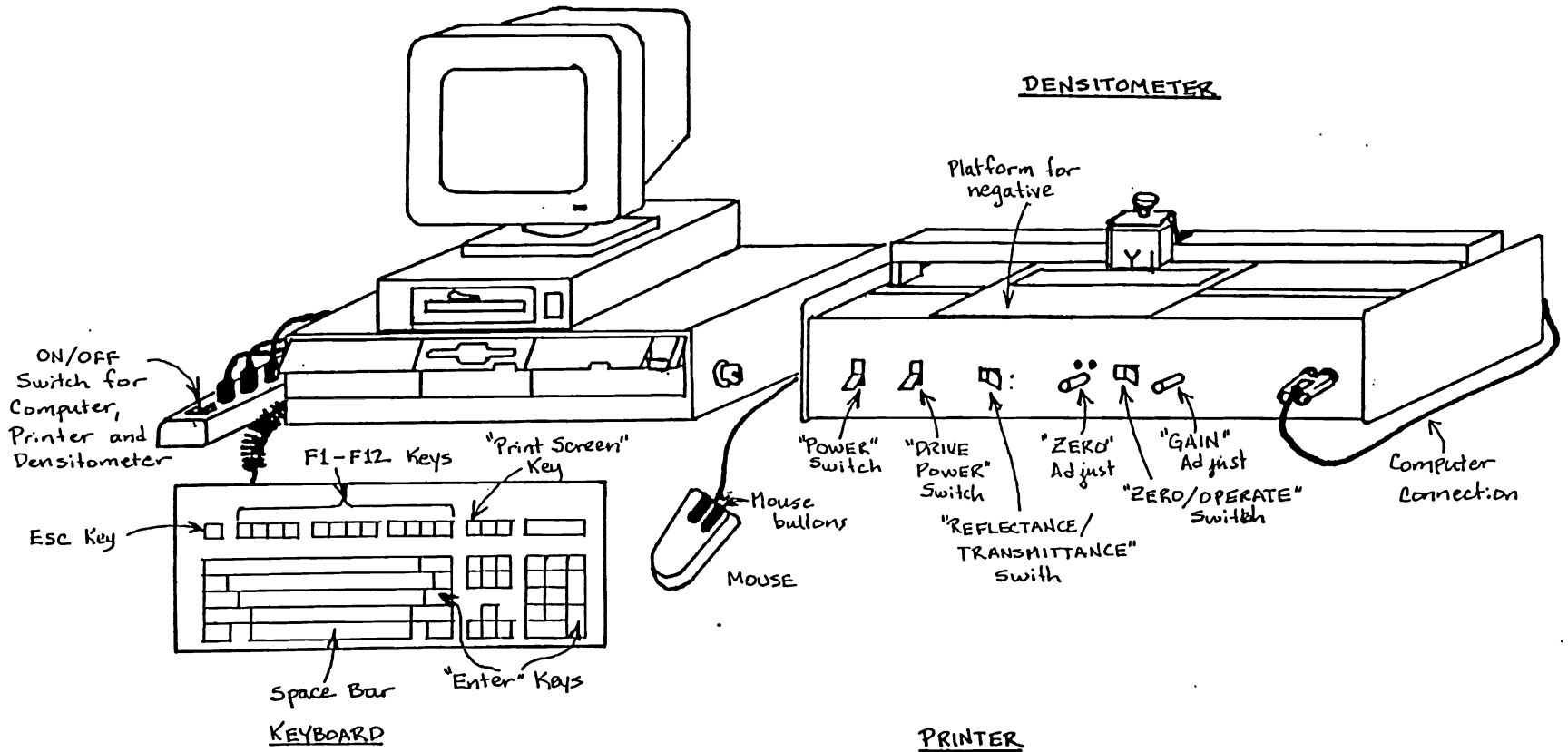
To measure the density of silver grains, we use a densitometer. This has a light that shines on one side of the photographic negative (for transmittance measurements) and a light sensor that is on the other side of the negative. After the densitometer is calibrated, it scans along the negative and simply measures the amount of light getting through the negative. In areas of high silver grain density, it records a peak. In areas of no silver grains, it records a straight baseline. Often, densitometers record on a chart recorder. Then all of the further work (calculations, integration, etc.) is done by hand. The densitometer we will use (from Hoefer Scientific) has a direct link with a computer (IBM PS/2). This allows storage of the scans on disk, and allows some of the work to be done on the computer.

STEPS IN THE PROCEDURE:

1. If the computer is not on, turn it on and wait for it to go through its "self-test" program. This takes a minute or so. If it is on, and is already in the densitometer program, then go on to step 9. Otherwise, proceed to step 2.
2. If the densitometer is not already on, then turn on the "POWER" switch. Make sure that the "DRIVE POWER" switch is off at this point.
3. The computer screen should now have "Enter new date (mm-dd-yy):" on it. Depress the "Enter" key and wait for the menu to come up on the screen.

COMPUTER

DENSITOMETER



PRINTER

4. Type: "CD\GS365PS" and depress the "Enter" key. This will change the directory of the computer to the set of densitometer programs.
5. Type: "GRAPHICS" and depress the "Enter" key. This will set the computer to use the graphics screen.
6. Type: "MOUSE" and depress the "Enter" key. This will allow use of the mouse to move around within the densitometer program.
7. Type: "METAWNDO" and depress the "Enter" key. This sets up a set of windows within the computer for use in the densitometer program.
8. Type: "GS365PS" and depress the "Enter" key. This loads and starts the densitometry program.
9. Once the densitometry screen appears, you can choose options at the bottom of the screen in two ways. First, you can use the function keys at the top of the keyboard. Second, you can move the cross (+) around on the screen using the mouse. Once the cross is over the option you want, press the button on the mouse and the computer will respond.
10. To scan a new negative, choose the F1:NEW option (at the bottom of the screen). Wait until the computer prompts you to set the "ZERO" and "GAIN" on the densitometer.
11. To begin adjustment of the densitometer, set the "MODE" selector set to "TRANSMITTANCE" and the "ZERO/OPERATE" switch set to "OPERATE."
12. Place the photographic negative onto the movable platform on the densitometer so that the area you want to scan is over the white band on the densitometer.
13. Move the platform of the densitometer to the left until an area of the negative that is of lowest silver grain density is lined up with the white line on the top of the densitometer. This is the position where the light and the light sensor are lined up.
14. Turn the "ZERO ADJUST" knob until you get a reading on the screen (above 0%). Adjust the knob until you are getting a reading of about 5%.
15. Next, move the densitometer platform until you are centered on the densest band of the area you want to scan. Adjust the "GAIN CONTROL" until the reading on the screen is about 90%. Move the platform slightly back and forth to make sure the reading isn't going above 90%.
16. Repeat steps 14 and 15 until you are certain that the lower value is around 5% and that the higher value is around 90%.
17. Move the platform so that the right edge of the negative is lined up with the white line on the top of the densitometer.

18. Select "F9:DO" on the computer.

19. The computer will "ask" you to name the scan, enter any scan notes you would like on the scan and to set the parameters for the scan. You can name your file anything you want, you can put in any scan notes you want (e.g., GROUP 1, 26 October 1989), up to 54 characters long. The parameters for the scan are as follows:

"SCAN LENGTH (CM) =" 12 (to scan the full length of a 3" x 5" Polaroid Type 55 negative)

"SCAN SPEED (CM/SEC) =" 0.22 (this matches the top speed of 13 cm/min on the Hoefer Model GS 300 scanning densitometer)

"SCAN TIME (SEC) =" (since we have entered the other two parameters, the computer will automatically set this one up for you)

20. Make sure that the densitometer is ready (POWER: on; DRIVE POWER: off; MODE: transmittance; OPERATE/ZERO: operate; right edge of negative positioned under the white line)

21. Choose the "F9:DO" option and at the SAME time turn the DRIVE POWER switch on.

22. Wait for the scan to complete. Turn the "DRIVE POWER" switch off. If you want to save this scan, choose the "F7:SAV" (save) option. To perform other manipulations on the scan you need to choose the "F2:REV" (review) option.

23. Make a hard copy (on paper) of the scan by pressing the "Print Screen" key located in the upper right hand portion of the keyboard. Printing will take a few minutes. You can either make a copy for each member of the group, or you can Xerox the printout.

24. If the scan is DNA standards, construct a standard curve for later use, by plotting the points with peak height on the Y-axis and DNA concentration on the X-axis. This can be used for later DNA concentration determinations as long as the concentration of ethidium bromide is the same and all of the camera settings are exactly the same (camera carriage height, focus adjustment, F-stop setting and the time of exposure). REMEMBER TO RECORD ALL OF THESE VALUES IN YOUR NOTEBOOK FOR LATER REFERENCE. If you don't, you'll have to reconstruct the standards each time you want to make a DNA concentration determination. Whenever you perform densitometry on the unknowns, the standards also have to be on the same negative or on a negative that has been exposed in EXACTLY the same way. The scan for the standards is set up and run first, then, WITHOUT CHANGING the "ZERO" or "GAIN," the unknowns are scanned. Only in this way can direct comparisons be made.

SOLUTIONS/SUPPLIES:

Flatbed Densitometer:

Visible light is used. Most models can be substituted for the one mentioned above.

DETERMINATION OF RIBOSOMAL RNA GENE COPY NUMBER PART 3: SLOT-BLOTTING

INTRODUCTION:

Originally, the method used was dot-blotting. The DNA samples were spotted (or dotted) onto the membrane and hybridization was then performed. The trouble with dots is that they can be difficult to read accurately using a densitometer. Densitometers have a small light that passes through the exposed film. When a dot is scanned the light passing through the middle of the dot encounters a higher density of silver grains than does the light passing through the edges of the dot. Using slots instead of dots alleviates this problem, since the spots formed are essentially rounded rectangles. The light passing through all but the ends of the spot encounters the same density of silver grains, so the accuracy and reproducibility is greater than with dot plots. The processes in slot-blotting are similar to Southern hybridization. First, the DNA should be digested with a restriction enzyme or acid nicked, so the solution is of uniform concentration. Second, the DNA is treated with NaOH to denature the DNA so that it will bind to the membrane. Third, the DNA is neutralized before placing on the blotting membrane. After the blot has been dried (as with Southern transfers), hybridization to a labeled probe is then done exactly as with a membrane from a Southern transfer.

The appropriate amount of the DNA sample must be loaded onto the membrane. The smallest amount of probe that can be detected on a blot is between 0.1 and 10 pg (depending on the method of detection used). Most often, you would like to have the signal much higher than this minimum value. A hybridization signal of around 100 pg is adequate for most purposes. The genome size of the organism that you are dealing with must be known or approximated. [Genome sizes are from 10^3 to 10^4 kb for bacteria, from 10^4 to 10^5 for fungi, from 10^5 to 10^8 for plants, from 10^4 to 10^7 for mollusks and insects, from 10^5 to 10^8 for amphibians and fish, and from 7×10^5 to 3×10^6 for reptiles, birds and mammals.] The final piece of information that you need before blotting the DNA is the approximate number of gene copies you expect to find with the probe. If you are attempting to detect a single copy gene in a higher plant, as much as 20 to 100 μg of DNA must be loaded onto the membrane. This amount of DNA in a small area of the membrane is pushing the binding capacity of the membrane. We will be using a ribosomal DNA (rDNA) probe. Ribosomal DNA exists in hundreds to thousands of copies per genome, so that it can be easily detected. As little as 100 ng of DNA can be loaded onto the membrane (in some cases). We will attempt to load around 0.5 - 1.0 μg per slot for this exercise. The number of rDNA copies varies from species to species and also can vary from individual to individual and from cell to cell within an individual. In this exercise we will load the DNA and later probe the blot to view this variation among the ribosomal DNA genes.

STEPS IN THE PROCEDURE:

1. For each DNA sample to be used in blotting, remove from 0.1 to 1.0 μg of each DNA sample (determined from Part 2) and place each into separate 0.5 ml microfuge tubes. [Note: Since we will be blotting with a ribosomal DNA probe and rDNA comprises an average of about 1 % of most genomes, this would yield about 1 to 10 ng of rDNA for the DNA amounts suggested here. The amount of DNA loaded depends on the proportion of the genome made up of the probed

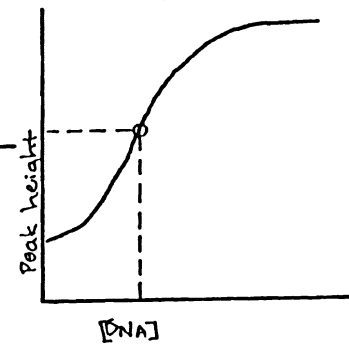
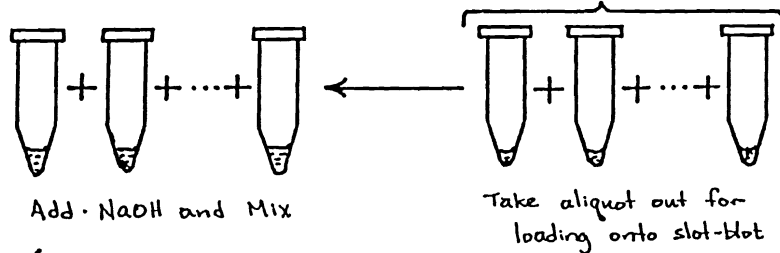
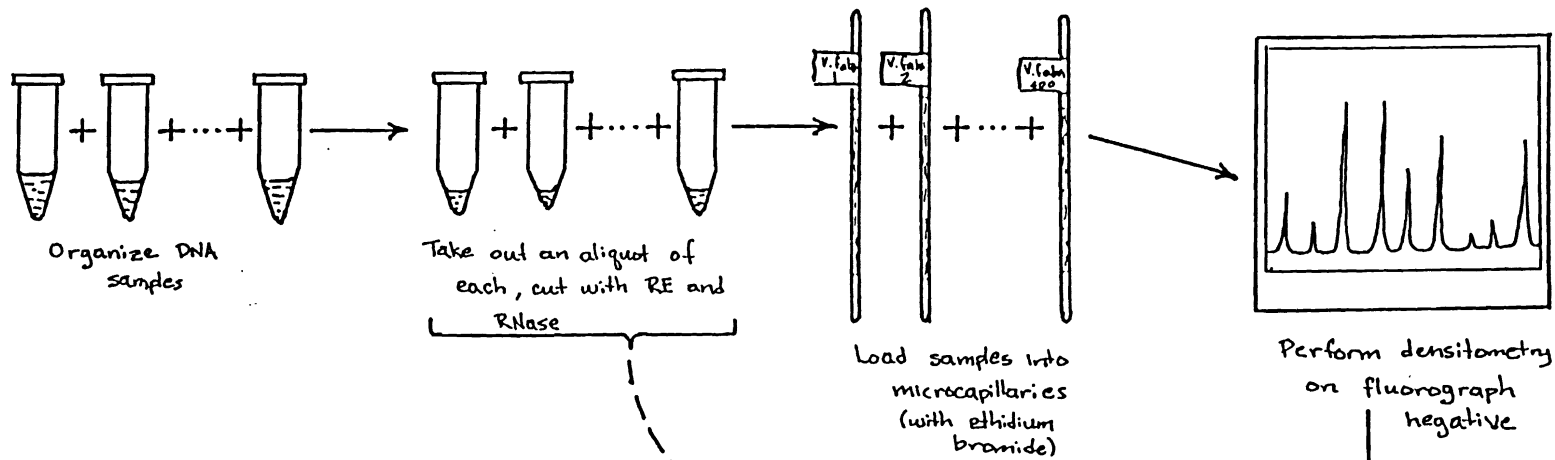
sequence, and on the sensitivity of the assay. With radioactive probes, sensitivities to about 1 - 10 pg can generally be achieved. With non-radioactive probes, sensitivities of 1 - 50 ng are common.]

2. Add an equal volume of 1 N NaOH to each tube and mix completely. Allow to sit at room temperature for 5 minutes (or more).
3. Add an equal volume (equal to the final volume in step 2) of 1 M Tris (pH 7.5), 3 M NaCl and mix completely. Allow to sit at room temperature for a minute or so before loading onto the membrane. Do not allow it to remain for hours, since the highly repetitive DNA sequences will then have a chance to reanneal and this double-stranded DNA will not bind as well to the membrane.
4. Put the entire sample into one of the wells of the slot-blotter. Allow the solution to be drawn down completely by the vacuum.
5. When the samples have all been loaded into the wells and the solutions have all been completely drawn down, remove the membrane from the slot-blotter and allow it to dry.
6. Place the membrane into a Seal-A-Meal bag until ready to hybridize with the probe.

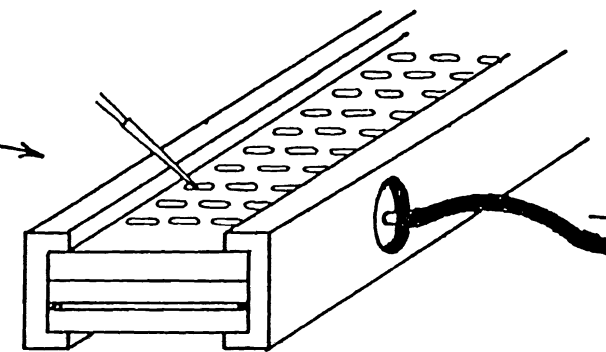
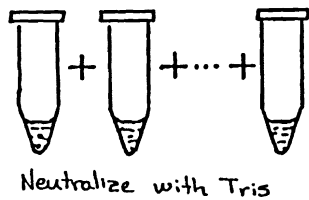
HYBRIDIZATION AND COPY NUMBER DETERMINATION: **(To be done later in the semester)**

Hybridization to the labeled probe is exactly the same as with any other blot. Copy number can be determined in the following way:

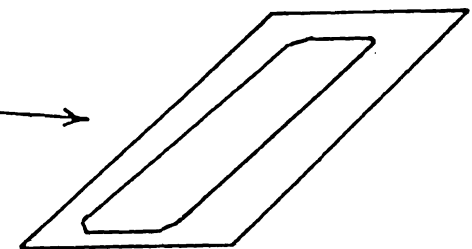
1. Calculate the proportion of hybridization signal to total DNA (i.e., picograms of hybridization signal divided by the picograms of DNA loaded onto that spot.)
2. Multiply this by the genome size of the organism. For *Vicia faba*, the value is 13.2 pg per haploid. [Some other genome sizes for other plants are listed in the Rogers and Bendich, 1985 paper that was given out in class at the beginning of the semester.]
3. Multiply this by the constant 9.11×10^5 kb/pg (which equals 6.03×10^{11} daltons/pg divided by 6.62×10^5 daltons/kb). This will give you the number of kb of DNA that hybridized. [The handout "Useful Information for DNA Handlers" that you were given earlier in the semester gives the rounded off version of the constant, $1 \text{ pg} = 10^6 \text{ kb}$, but we need to be more accurate for this exercise.]
4. Finally, divide this by the average length of the gene (actually, the length of the probe). In the case of the rDNA probe we will be using, this will be about 6.0 kb.



Determine concentration from standard curve



Load samples into slot-blotter wells (Membrane has been pre-treated and is clamped just below wells.)



After blotting is completed, dry membrane and place it into a Seal-A-Meal bag until hybridization to probe

