DNA detection and hybridization

DNA detection is an important analytical tool in molecular biology. We will discuss ways of detecting DNA quantitatively in solution and in gels, and also ways of detecting specific nucleotide sequences in a mixed collection of DNA fragments.

Detection of nucleic acids

When we handle nucleic acids in a laboratory, we are often concerned with the concentration of DNA in our sample. Of course in many cases our sample could be quite filthy! It might have both DNA and RNA nucleic acids, as well as proteins and polysaccharide contaminants. The ideal assay for DNA concentration would be specific, not combining the levels of RNA and DNA in a single quantitative figure. Such an ideal assay would also be linear across a broad range, meaning that the numerical data (i.e. the raw data), when plotted as a function of DNA concentration, would yield a straight line. The ideal assay would also be safe, fast, and non-destructive of the sample.

Here are two old standard approaches for detection:

1. UV spectroscopy at 260 nm. This method works quite well if you have a pure sample of nucleic acids not badly contaminated by proteins (which have an absorbency maximum at 280 nm). An absorbency of one A260 unit per ml = 50 micrograms/ml DNA, so you can establish a quantitative measure of your sample. The disadvantages of this method are that RNA and DNA both absorb, as does protein to some extent; you can't tell how large your DNA fragments are (bacterial chromosome and plasmid both being detected); you can't tell what sequences they may contain; and you need a solution of 500 ng/ml (and perhaps 0.2 to 1 ml of that, depending on your cuvette) to reach an absorbency of 0.01, the usual limit of detection. Generally speaking, you cannot make use of the portion of the sample you put in a cuvette, because there is too much risk of cross-contamination from other samples.

2.Ethidium bromide fluorescence. RNA and DNA can both be stained by ethidium bromide (in a solution of 1 microgram ethidium bromide/ml). If the ethidium bromide is used in conjunction with gel electrophoresis, the two nucleic acids are not easily confused, and 10 to 50 ng of DNA can be readily detected in a single band. This method has an advantage in that you can measure the size (mobility on a gel) of your DNA, which provides you with information on the identity of the DNA. For gel electrophoresis, your DNA must be concentrated in bands to achieve the most sensitive levels of detection -- if your sample is a random collection of sizes, your fluorescence is spread out over a greater area. If you have a pure sample, you can also use ethidium bromide without resorting to a gel, by making spots of your DNA sample (mixed with ethidium bromide) on a piece of plastic wrap. By comparing the fluorescence of your DNA dilutions against a standard of known concentration, you can estimate the concentrations of samples that are too valuable to use in spectroscopy.

Quite a bit has been happening in the field of nucleic acid detection in the last few years, and one of the leaders is Molecular Probes, Inc., a company based in Eugene, Oregon. Check out their searchable handbook (suggested search terms: sybr, ethidium, DAPI, Hoechst)

While you are browsing this site, note the discussions of sensitivity, selectivity, and handling characteristics. Try searching on the word "sensitivity."

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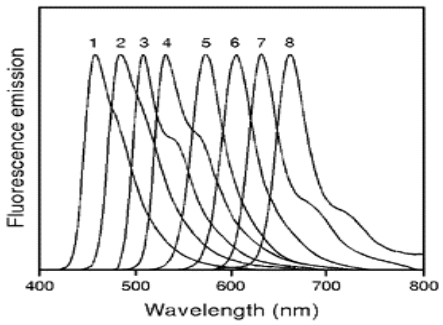
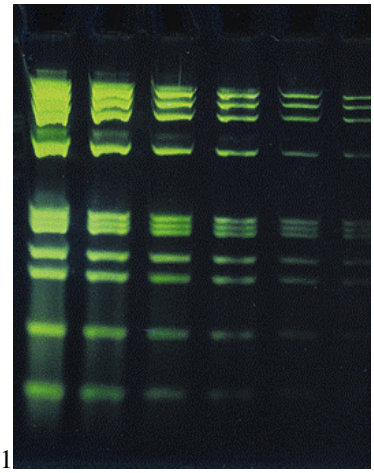
Cyanine dyes are now available with wavelengths of emission ranging from blue to near infrared, as the figure below demonstrates.





What is similar about all of the methods described so far? They all detect "bulk" DNA, and can' t report what nucleotide sequence is present. For the remainder of this lecture, we will concentrate on methods that detect DNA by its hydrogen bonding capability - we call this "probing" the DNA, and we say that the probe "hybridizes" to the target sequence.

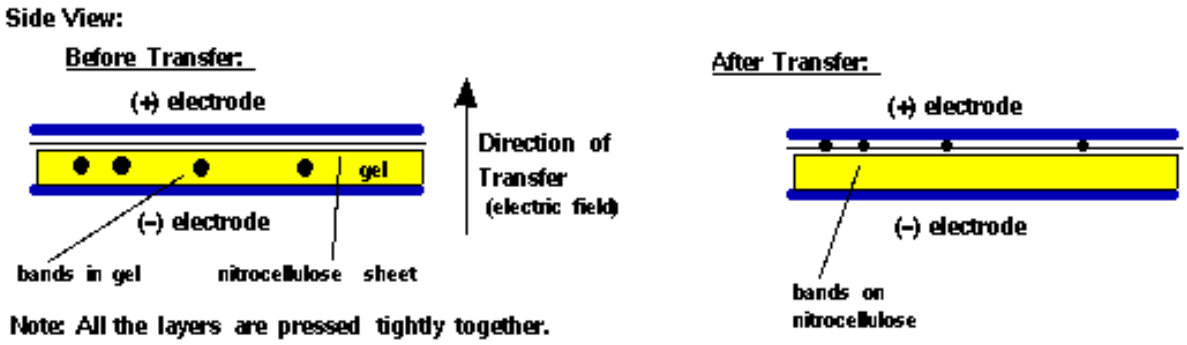
Southern blotting

By hybridization of labeled nucleic acid probe, DNA can be detected by blotting methods (called Southern blots, after the inventor Dr. Southern). After a nucleic acid sample is run on a gel, and a picture is taken to establish the mobilities of known molecular weight standards, the DNA in the gel can be "blotted" onto a special type of membrane (nitrocellulose or nylon-based membranes) so that an imprint of the separated nucleic acids is made.

Then, a labeled nucleic acid (marked with either radioisotope or chemical label) is allowed to anneal or hybridize to the DNA on the filter -- this "**probe**" bonds only to the DNA to which it is complementary, by base-pairing rules. Once the excess probe is washed away, and the hydrogen bonded probe is detected, an image of the gel can be created that shows where sequences complementary to the probe happened to be when you halted the electrophoresis. The great advantage of this method is that you can detect small quantities (1 to 10 pg per band) of a specific nucleic acid, so it is at least 1000 times more sensitive than ethidium bromide.

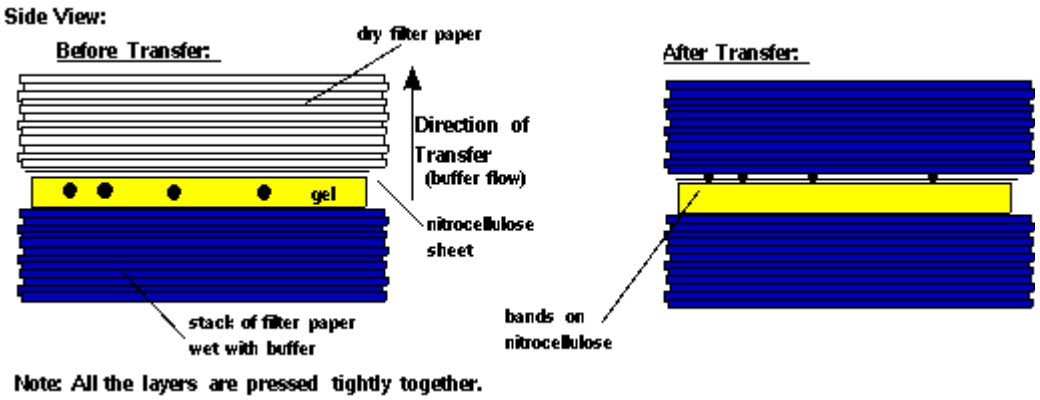
There are two common ways of transferring nucleic acids from a gel to a membrane.

- One is to use an electrophoresis tank to apply an electric field across the face of the gel, causing the molecules to leave the gel (in the direction of the membrane).



Here is an **example of a commercially available electrotransfer unit**.

Another is to use the capillary action of a solution to wash the nucleic acids through the gel and onto the membrane. We do this by placing the gel on a paper wick, over a reservoir of liquid, and placing the nitrocellulose or nylon membrane on top of the gel, then placing paper towels on the membrane so that it soaks up the liquid (that' s why we call it a "blot"). As the liquid is sucked through the membrane, more is drawn up into the gel through the paper wick. The net result is a rush of liquid passing through the gel and membrane, washing the DNA onto the membrane where it will stick. Some people skip using a reservoir of liquid, and just use the liquid in the gel to transfer the nucleic acids during blotting



For the capillary blotting method, the gel must be treated in certain ways before the DNA can be eluted. In general, you follow the recommendations of the manufacturer of the membrane, but there are usually two important chemical treatment steps, and each require equilibration of the gel for about 10 to 30 minutes.

- First, the DNA is partially depurinated with weak hydrochloric acid so that it can be fragmented into small pieces for transfer. If you skip this step, the DNA molecules smaller than 5-10 kbp will transfer well, but the molecules larger than that will not transfer. If you go off to have lunch and let this reaction go too long, your hybridization signals will be reduced.
- Second, the DNA is denatured into single strands by treatment with alkali (for example, 0.2 to 0.5 M NaOH, sometimes in the presence of 1.5 M NaCl as well). With many membranes, and in particular nitrocellulose, single stranded DNA will stick to the membrane whereas double stranded DNA will not. The DNA needs to be single stranded so that it is available for hybridization to the single stranded probe. In many methods, the gel is blotted directly in 0.4 M NaOH (however that does not work with nitrocellulose).
- In some methods of blotting (for example with nitrocellulose membranes), the DNA will only stick to the membrane if the alkali is neutralized. The typical treatment in that case is to soak the gel in 0.5 M Tris-Cl pH 7.5, 1.5 M NaCl for about 30 to 60 minutes, then equilibrate in 10x SSC buffer (1 M NaCl, 0.54 M sodium citrate) before blotting in the same buffer.

Here are additional tips on how to blot well:

- To obtain the best-looking blots, it is important to handle the membrane very gently, wearing gloves to avoid transfer of skin oils.
- The wicks should be full wetted with transfer solution, and free of air bubbles. The best way to get rid of air bubbles is to roll a glass pipet over the wick (it helps if you put a layer of plastic wrap over the wick to protect it from the pipet).

We generally put the gel on the wick "upside down" because the DNA tends to run on the bottom of a gel, and so turning the gel over places the DNA in closer proximity to the membrane. The membrane should be cut to the size of the gel and pre-wetted, first in water and then in the transfer solution (with nylon-based membranes the water step is less important, but with nitrocellulose it is essential!), then applied to the gel and rolled into tight contact with a pipet. A few paper wicks are cut to the size of the membrane, then pre-wetted and placed on the membrane (and rolled into tight contact), then a stack of dry paper towels cut to the right width and length, and 3 to 5 inches thick, is placed on top of the wet wicks.

Now your blot is set up, and should be left overnight to allow completion of the transfer of DNA. When the paper towels are removed, the positions of the wells can be marked onto the membrane with a soft pencil, and the DNA can be covalently bound to the filter by either heat treatment (80 C, in vacuo. The vacuum is especially important if you are using nitrocellulose, which is explosive in air at that temperature) or UV light cross-linking (nylon membranes only). If you can't get your hands on a UV light source or a vacuum oven, the nylon filters hang onto the DNA fairly well if they are simply dried thoroughly.

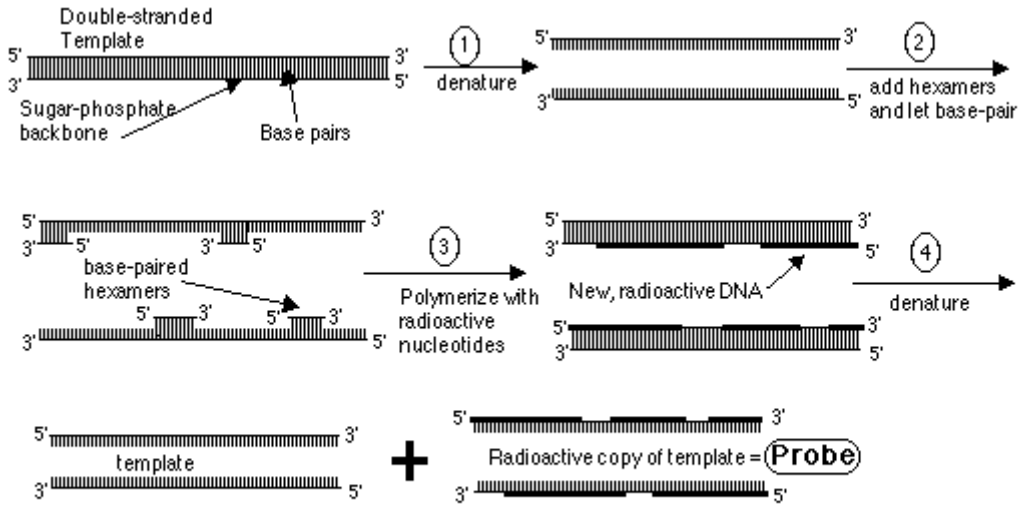
Capillary blotting methods can be accelerated by using a vacuum system...see the VacuGene!

Preparation of a labeled probe

A probe sequence can be labeled by several methods:

- "**Nick translation**" in which a double stranded DNA sequence is nicked with a limiting amount of a nonspecific nuclease such as DNase I. These breaks in the phosphodiester backbone are points of entry for the enzyme DNA Polymerase I (holoenzyme, which has 5' to 3' and 3' to 5' exonuclease activities, in addition to a 5' to 3' synthetic activity). The nicks in the DNA are "translated," or moved along the backbone, by incorporation of new nucleotides. If one of the nucleotide substrates (say dCTP) is **labeled** with radioisotope (for example the alpha phosphate can be P-32), or a chemical group (**digoxygenin** or biotin), the labeled groups will be incorporated into short stretches of DNA. This process is called "nick translation" to describe the action of the polymerase, but since it doesn't always work that well we used to call it "nicht translation" in grad school!

Random oligo primed synthesis" in which a DNA is denatured into single strands and annealed to random hexamer oligonucleotides. These random primers can then be extended using Klenow enzyme, incorporating labeled nucleotides (as above).



End labeling" in which the end of a DNA (or RNA) molecule is specifically labeled. There are methods for labeling either the 5' end or 3' end specifically. What is most common is 5' end labeling with P32-gamma- ATP, and the enzyme polynucleotide kinase. The terminal phosphate (the "hot" one) is transferred to the 5' end of the molecule. Note that only one marked residue is incorporated by this method, so the specific activity of the label (radioactive counts per minute per microgram of DNA) is lower than in the aforementioned two methods.

DNA hybridization

Single stranded nucleic acids hydrogen bond to each other efficiently, following Watson-Crick base pairing rules, at approximately 20-25 degrees centigrade below their melting point. To say it a different way, when hybridizing a probe to the DNA or RNA on a membrane, we adjust the solution conditions (for example, the salt concentration) so that the melting point of the nucleic acids is approximately 20-25 degrees higher than the incubation temperature. Lowering the salt concentration lowers the melting point, as does the addition of formamide. A typical condition for hybridization is:

6x SSC
0.2% SDS
1x Denhardt's blocking solution, or 1% w/v milk
10-50 ng/ml probe (denatured first!)

65 ^{o}C incubation, with agitation, for 18-24 hours.

Following a period of hybridization, it is necessary to wash off the probe that is loosely bound to the membrane (i.e. nonspecifically bound). This is typically done by washing the filter several times at 65 C in decreasing salt concentrations (i.e. 3xSSC/0.2% SDS, then 1x SSC/0.2% SDS, etc...).

Detection (a couple of examples only)

- If the probe is radioactively labeled, the membrane can be placed on X-ray film, and an image of the bound probe can be established by autoradiography.

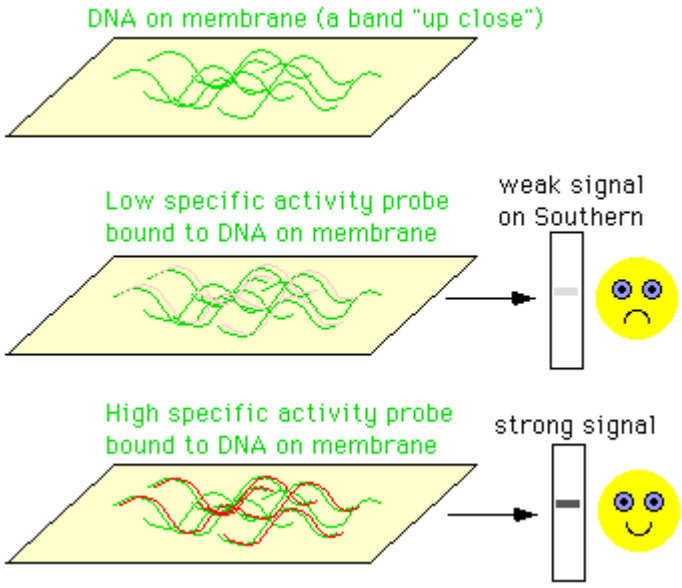
- If the probe is chemically labeled (for example, digoxygenin labeled), the filter is exposed to an anti-digoxygenin antibody conjugated to an enzyme such as alkaline phosphatase. Once the excess antibody is washed away, the substrates NBT and BCIP can be applied, which will be converted to an insoluble blue dye by the alkaline phosphatase. Therefore, the position of the bound probe is indirectly indicated by the enzymatic reaction.

What is happening during hybridization of a probe?

A single stranded probe is finding DNA sequences that it can hydrogen bond to, and these hydrogen bonds cause the two molecules to stick together. In general, the DNA on the filter is denatured into single strands before it is blotted, and the probe is boiled (denatured) before it is added to the hybridization mixture. The probe is labeled on both strands (usually), so either strand can hybridize with a DNA strand on the filter. On the other hand, the labeled DNA can also re-anneal to its partner in solution -- the opposite labeled strand of DNA. What happens then is that the double stranded pair in solution does not contribute to the signal on the membrane -- they are essentially lost from the reaction.

Important concepts.

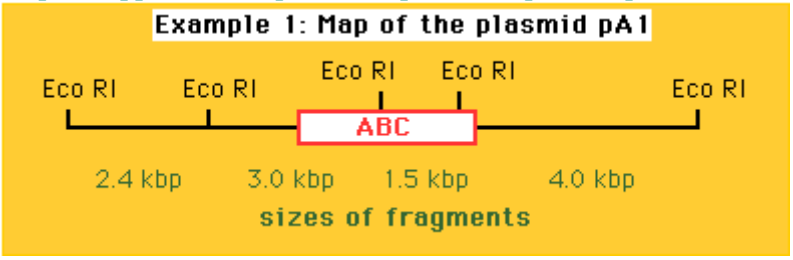
The concentration of the probe in the hybridization reaction is important, because there has to be some reasonable chance of the probe molecule "bumping into" the target molecule. If the concentration of probe is low, the time it takes to anneal a significant number of probe molecules to a target band is increased. That is, you have to hybridize your probe to your blot for a longer time - perhaps many days instead of just overnight. If you hope to use the intensity of hybridization to reflect the amount of material in your target band, the total amount of your probe should be in excess over the amount of target DNA on your blot. If you do not have excess probe, the linearity of the hybridization signals will be questionable



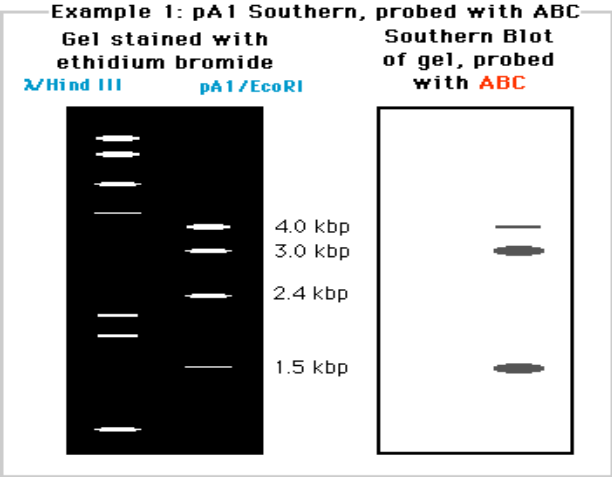
The labeled probe, once bound to a target sequence, will "broadcast" its presence by the fact that it is either radiolabeled or chemically labeled. In either case, the signal strength will depend on how much label was incorporated into the probe when it was synthesized. If only one nucleotide is labeled per probe strand, on the average, your hybridization signal will be exactly 10 times lower than if 10 nucleotides had been labeled per strand. The amount of label incorporated is called the "specific activity" of your probe. When working with radioactive materials your safety is extremely important. There are several excellent non-radioactive approaches towards probing a Southern blot, including chemiluminescence and the **BCIP/NBT Genius method we use.**

Analysis of Southern blots

Here' s a simple example. Suppose the map below represents a plasmid pA1



You perform a Southern blot, using the probe sequence marked ABC, and get the following result (compare the map printed above with the gel and Southern blot results below)



Note that there are several important concepts here:

- Not every band that is detected by ethidium bromide is detected by the ABC probe.
- There is some variation in intensity of hybridization (particularly in the case of the 4.0 kbp band, which is not heavily labeled by the hybridized probe.