Methods

Weaver 4th ed Ch 4+5 Methods etc

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Chapter 4 & 5

- Labeling: to “mark” molecules to simplify their tracking
- Electrophoresis: To separate of different sizes from each other.
- Ion-exchange chromatography: to separate molecules of different charge
- Gel filtration chromatography: to separate molecules of different size
- S1 mapping: to map the boundaries of nucleic acids, to determine their abundance
- Primer extension: to map the boundaries of nucleic acids, to determine their abundance
- Run-off transcription: to determine the level of transcription rate in vitro.

Labeling

Purpose: to make it easy to track molecules when they are in low abundance and therefore invisible.

^{32}P: Radiolabeled nucleotides such as [alpha -^{32}P]dATP can be used to label DNA molecules. When ^{32}P decays it emits high energy β particles which can be detected using film. However, these particles are so energetic that they produce big 'fuzzy' exposures and are actually somewhat hazardous to work with.
RNA polymerases will use [alpha 32P]UTP for the in vitro radiolabeling of RNA. 31P is the non-radioactive isotope.
^{35}S: [alpha-^{35}S]dATP can be used by DNA polymerases also. It contains an ^{35}S that will be incorporated into the growing nucleic acid chain. RNA polymerases will use [α -^{35}S]UTP to make RNA radiolabeled with 35S. Major advantage is that it emits β particles which are less energetic. Therefore, they produce sharper autoradiographic images. Furthermore, they are safer to work with because their weaker β particles are unable to penetrate deeply. Disadvantage is that film requires longer periods of exposure. 32S is the non-radioactive isotope

Comparison:	
Resolution:	$^{35}S > ^{32}P$
Exposure time:	$^{35}S > ^{32}P$
Hazard:	$^{32}P > ^{35}S$

Techniques used to prepare probes:

Nick Translation

Add Pancreatic DNAase I and E. coli DNA polymerase I to the DNA in the presence of all four dNTPs, plus one radiolabeled dNTP (eg. [alpha32P]dATP).
During the reaction period the DNAase I produces random single stranded nicks in the DNA. DNA polymerase I treats these as primers and begins DNA synthesis. The polymerase's 5'-3' exonuclease activity removes unlabeled DNA 'in front' of the enzyme and the polymerase activity synthesizes radiolabeled DNA 'behind' the enzyme.

Random Primer Labeling

Uses the Klenow fragment of E. coli DNA polymerase I. The Klenow fragment has 5'-3' polymerase activity but has lost it's 5' to 3' exonuclease activity.
[Originally: The Klenow fragment is the large fragment produced when DNA polymerase I is digested with a protease (subtilisin). Now a cloned fragment.]
Denatured DNA, random primers, dNTPs and a radiolabeled dNTP (eg. [α 32P]dATP) are mixed together. The Klenow fragment used the DNA as template, random primers as primers and synthesizes radiolabeled DNA.

End-labeling of oligomers

Oligomers: T4 polynucleotide kinase will accept gamma-32P-ATP and transfer the 32P to the 5’ end of the oligomer.
DNA: First treat with Calf Intestinal Alkaline Phosphatase, then treat as above OR just add ADP to the reaction above.

PCR labeling of DNA fragment

If the PCR reaction is run in the presence of a radiolabeled nucleotide then the amplification product produced will be radiolabeled.

In vitro transcription

This technique allows one to make radiolabeled RNA complementary to only one strand of the DNA template. This are sometimes called strand specific probes. Bacteriophage RNA polymerases are used in this procedure.
SP6 polymerase is purified from Salmonella typhimurium (a gram negative bacteria) which is infected with the bacteriophage SP6. The polymerase is very specific for its own promoter. Plasmids which contain this promoter can be used to produce in vitro transcribed transcripts. In the plasmid below the promoter directs the transcription of RNA from the cloned insert. Prior to addition of the polymerase the plasmid is digested with a restriction enzyme which linearizes the plasmid in a fashion that prevents the polymerase from making RNA complementary to the plasmid.

Agarose DNA gel electrophoresis

Purpose: to separate DNA or RNA molecules based on differences in their size. Molten agarose is poured into a mold. The mold includes a comb which forms the wells. The gel is submerged in a buffer. DNA is loaded into the wells. Sucrose or Ficoll makes the DNA solution dense enough to sink to the bottom of the wells.

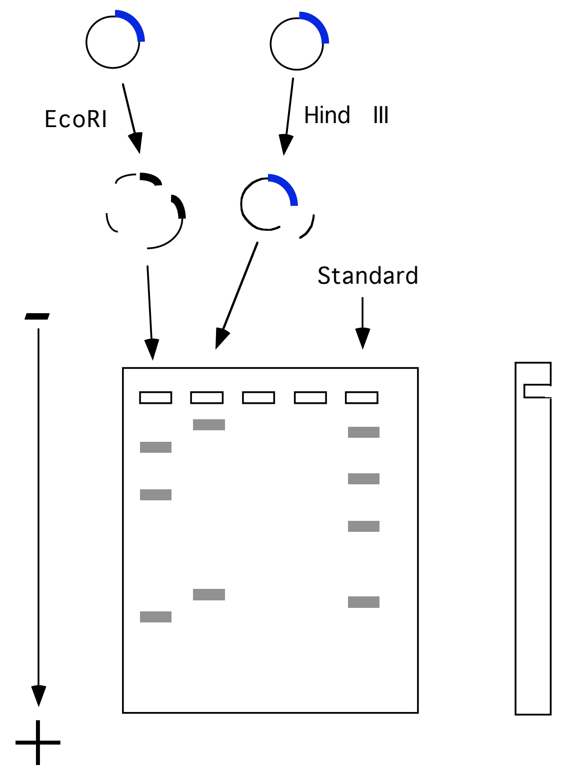
A current is run through the gel (~5V/cm length). The DNA moves in the field. The DNA behaves for the like a piece of string and the gel behaves like a sponge with large channels through it. Longer DNA molecules move more slowly because they are bigger and experience more friction with the gel.

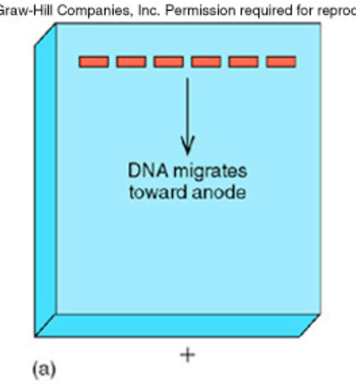
Shorter molecules travel faster, longer molecules travel more slowly.

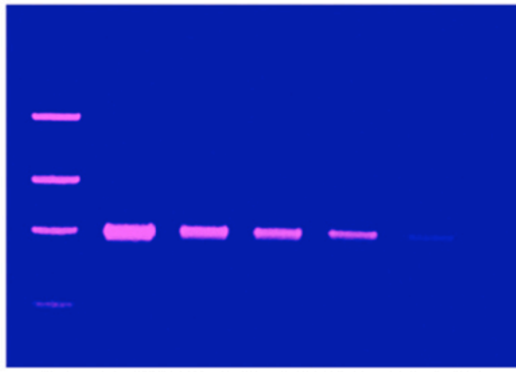
A standard is a solution containing DNA fragments of known size. One uses these to determine the size of other molecules on the gel.

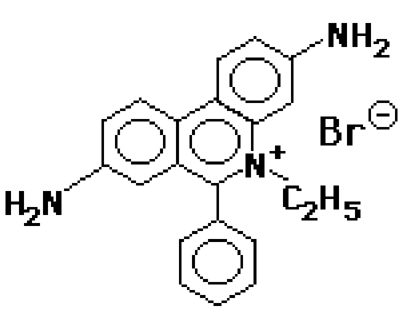
Ethidium bromide has long been used to stain these gels.

Fig 5.1 Weaver 4th ed.









Ethidium Bromide

SDS gel electrophoresis

Purpose: to separate protein molecules based on differences in their size.

- Protein is boiled in a SDS solution. SDS is a detergent with a negative charge. The amt of SDS bound to a protein is proportional to the length of the protein.
- SDS gives each part of the protein an approx uniform negative charge. Without SDS each protein would have a different charge distribution. Boiling in SDS also causes the proteins to denature and behave more like linear molecules. This gel can separate linear molecules of uniform charge according to their size.
- The acrylamide gel is matrix. The electric field pulls the protein through the gel. The longer the protein the more friction with the gel and the slower the migration.
- The rate of migration through the gel is proportional to the length of the protein.

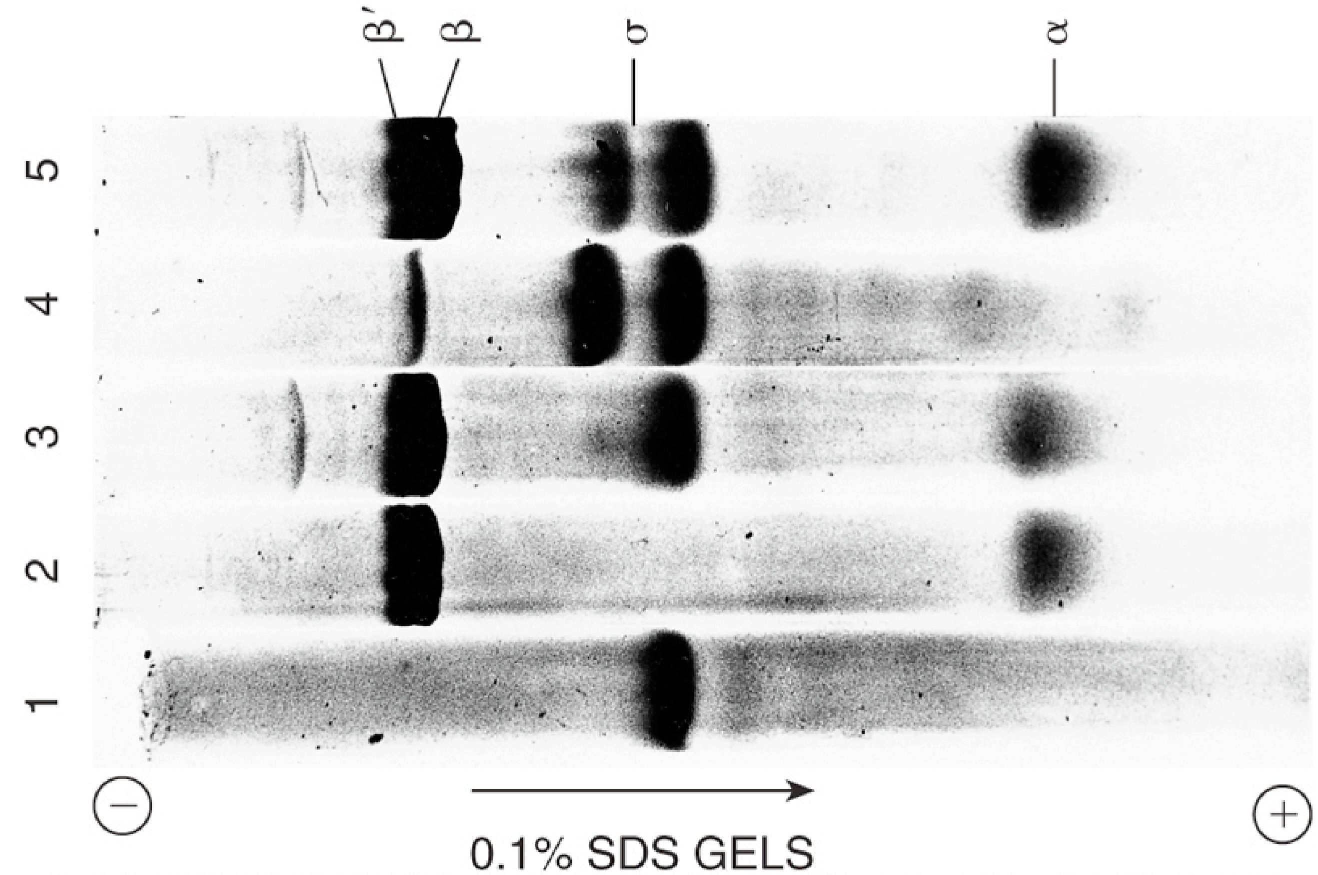


Fig 6.1 Weaver 4th ed

Molecular exclusion Chromatography

Figure5.7
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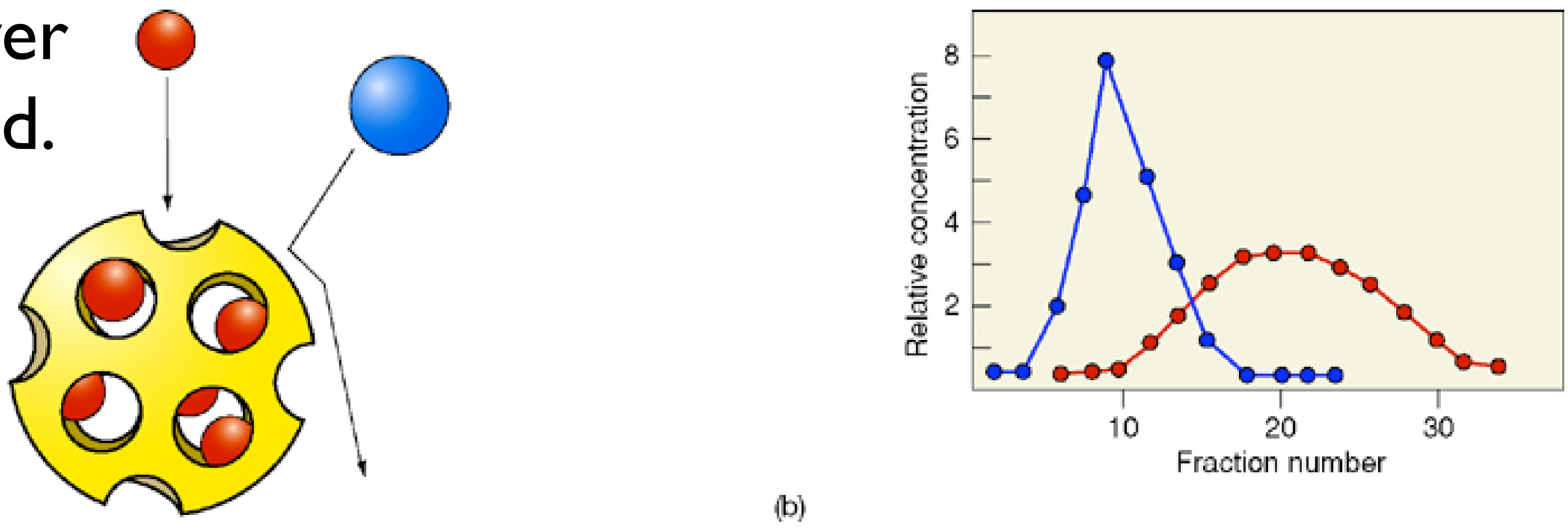
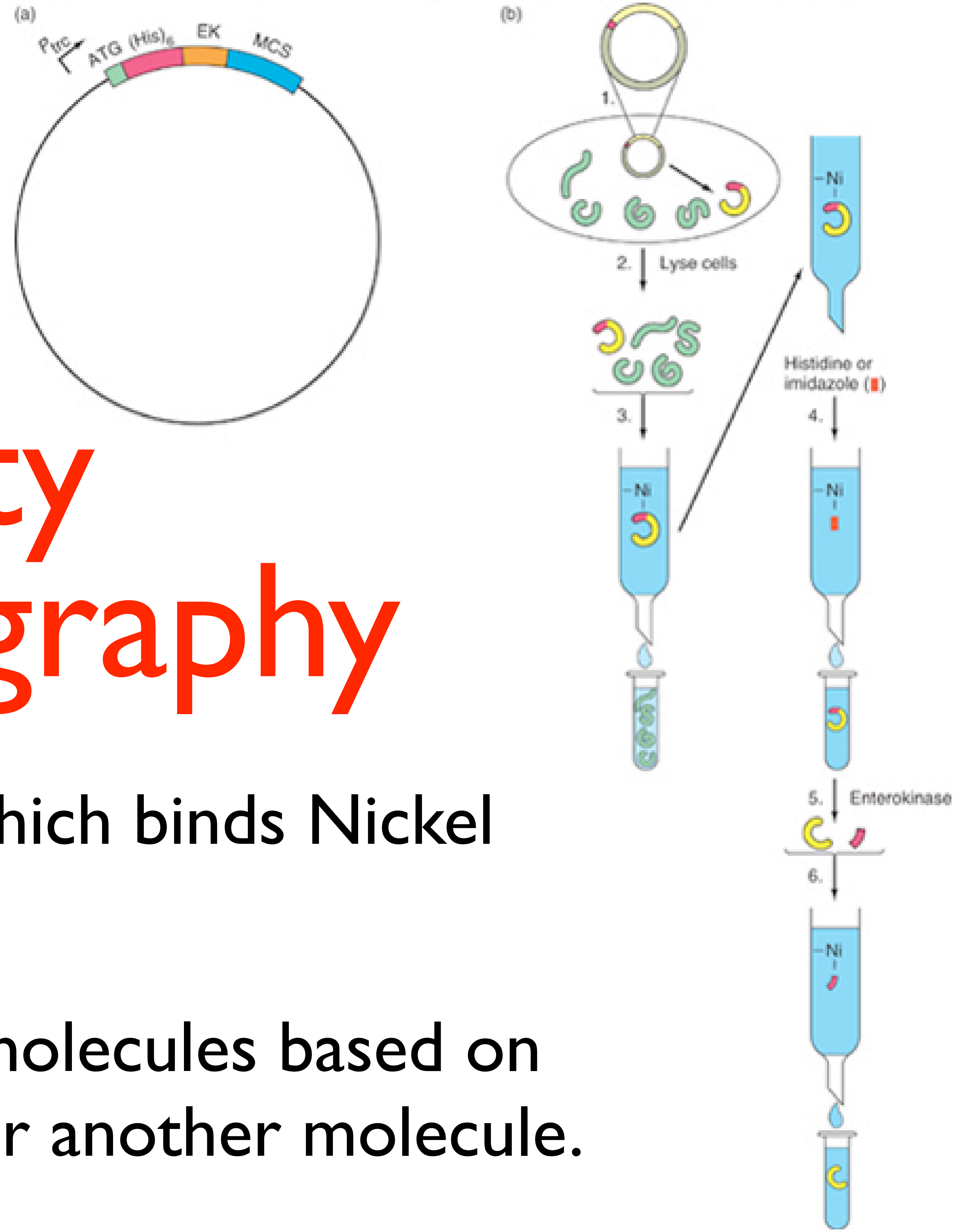


Purpose: to separate molecules based on differences in their size.

Ion Exchange Chromatography

Purpose: to separate molecules based on differences in their charge.



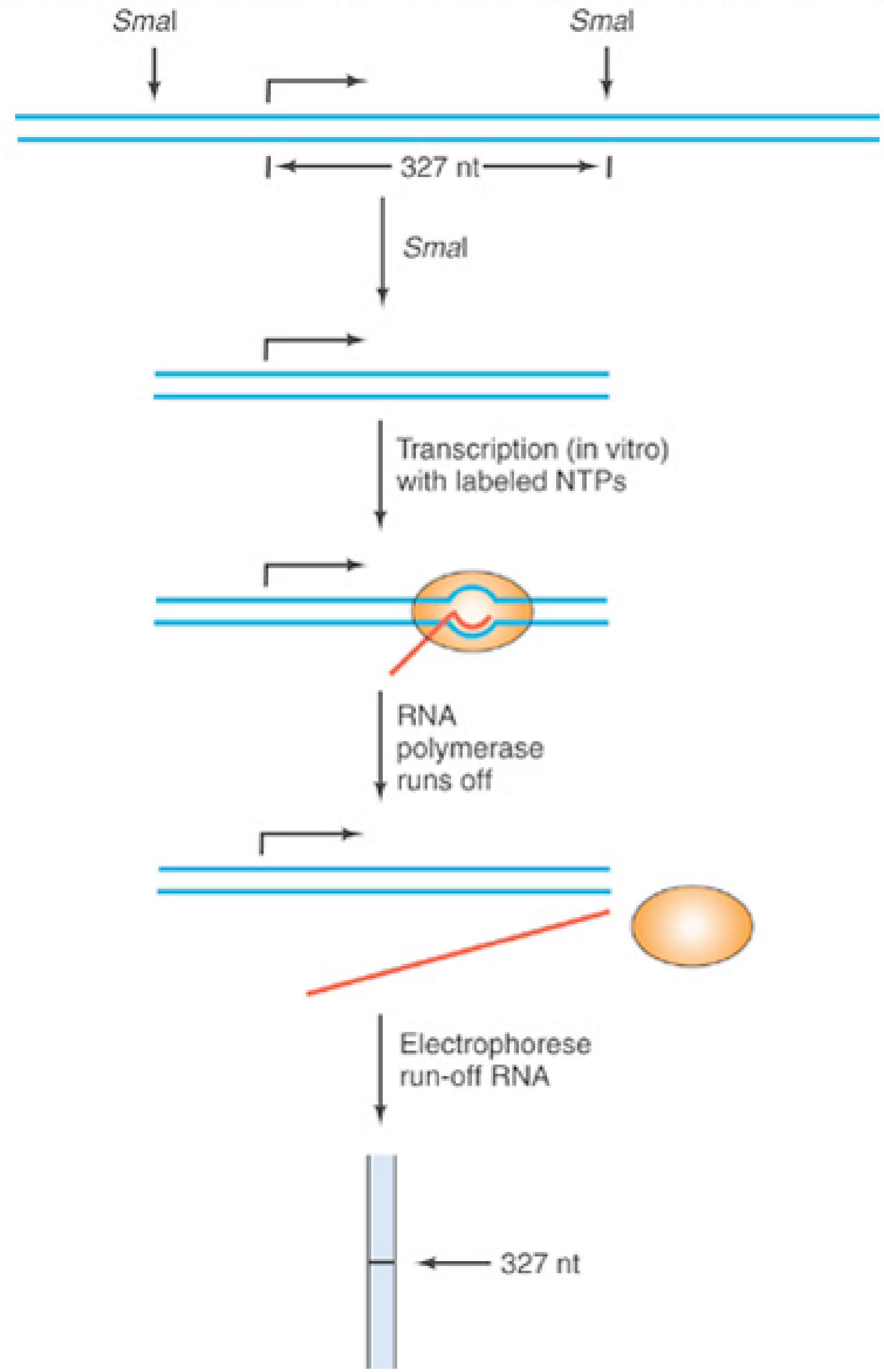
Affinity chromatography

eg. oligo histidine tag which binds Nickel
Figure 4.20 Weaver 4th

Purpose: to separate molecules based on differences in affinity for another molecule.

Run-off transcription

Purpose:
1) to determine relative level of expression
2) to determine the position where transcription begins.
3) usually limited for use in in vitro transcription studies

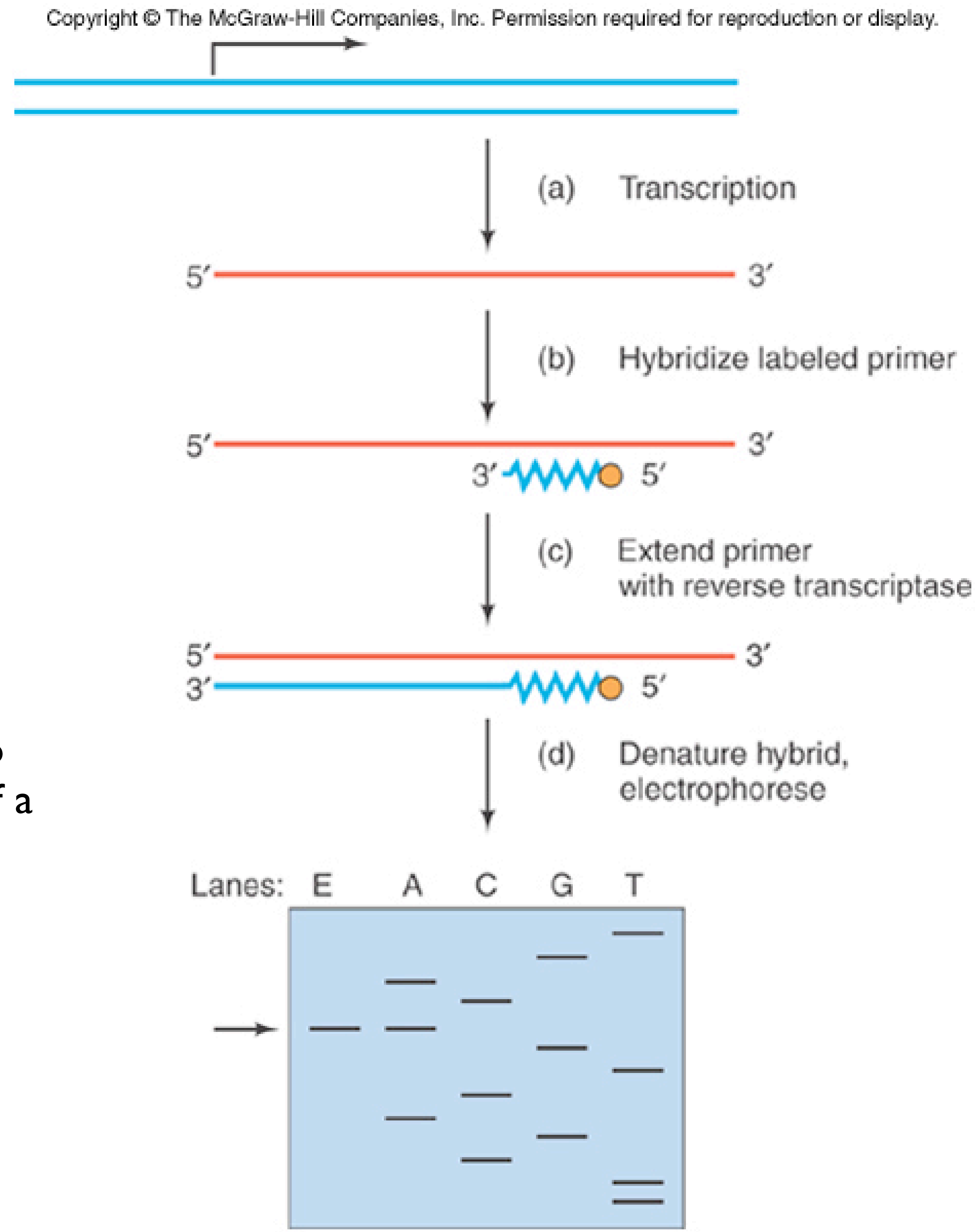


Nuclear Run-on transcription

- Purpose: to determine the level of expression of a gene in a cell.
- Purify nuclei. Cold and lack of nucleotides stop transcription elongation.
- Add radiolabeled nucleotide to the purified nuclei. Elongation of previously initiated transcriptions resumes to produce hot mRNA. New initiation does not occur because of the presence of heparin (damages free polymerase).
- Radiolabeled RNA is used as a probe.

Primer extension

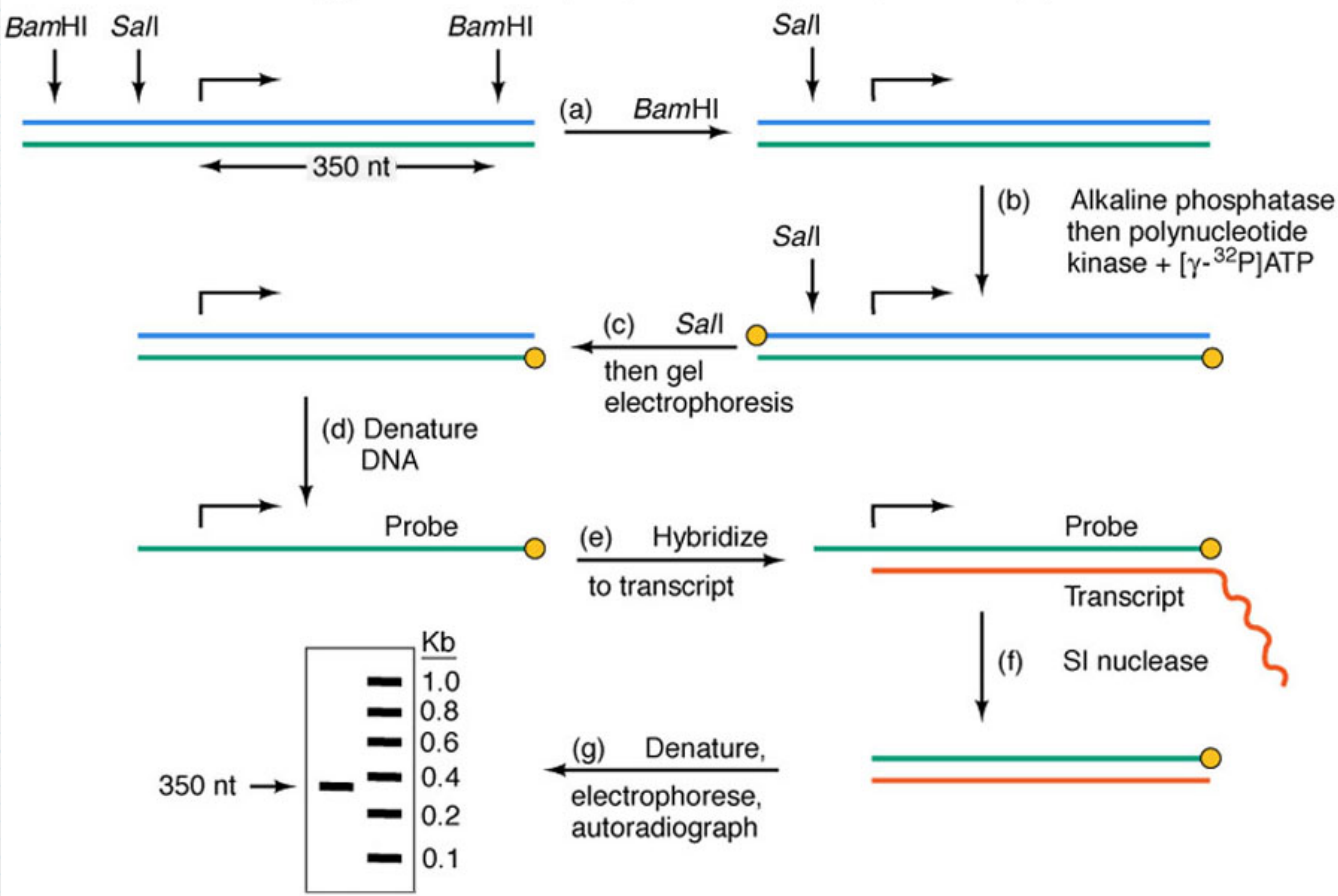
Fig 5.30 Weaver 4th edition



Purpose: to determine the relative concentration of a nucleic acid. It is also often used to determine the position of a transcription start site.

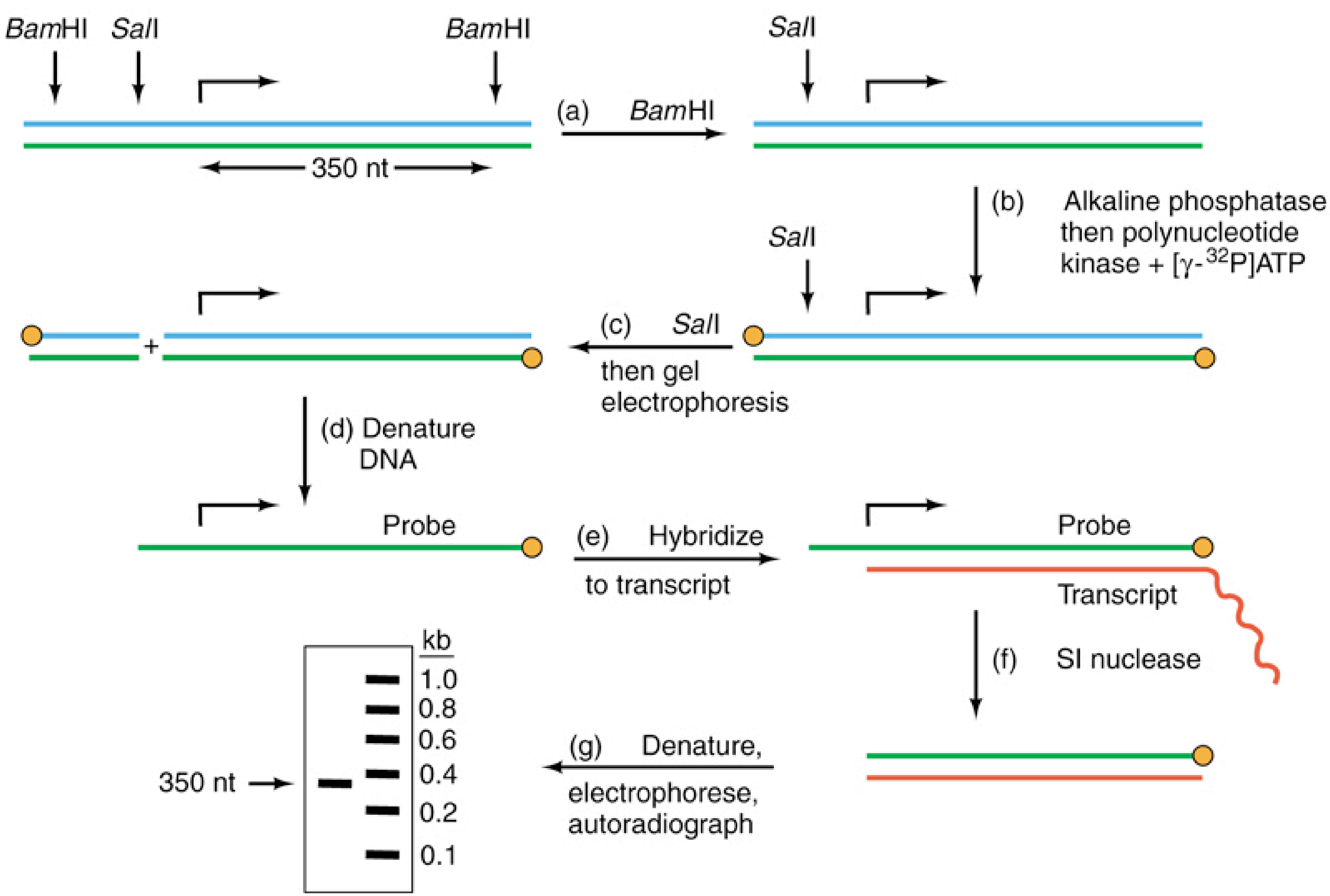
* Quantitative S1 mapping

Figure 5.27



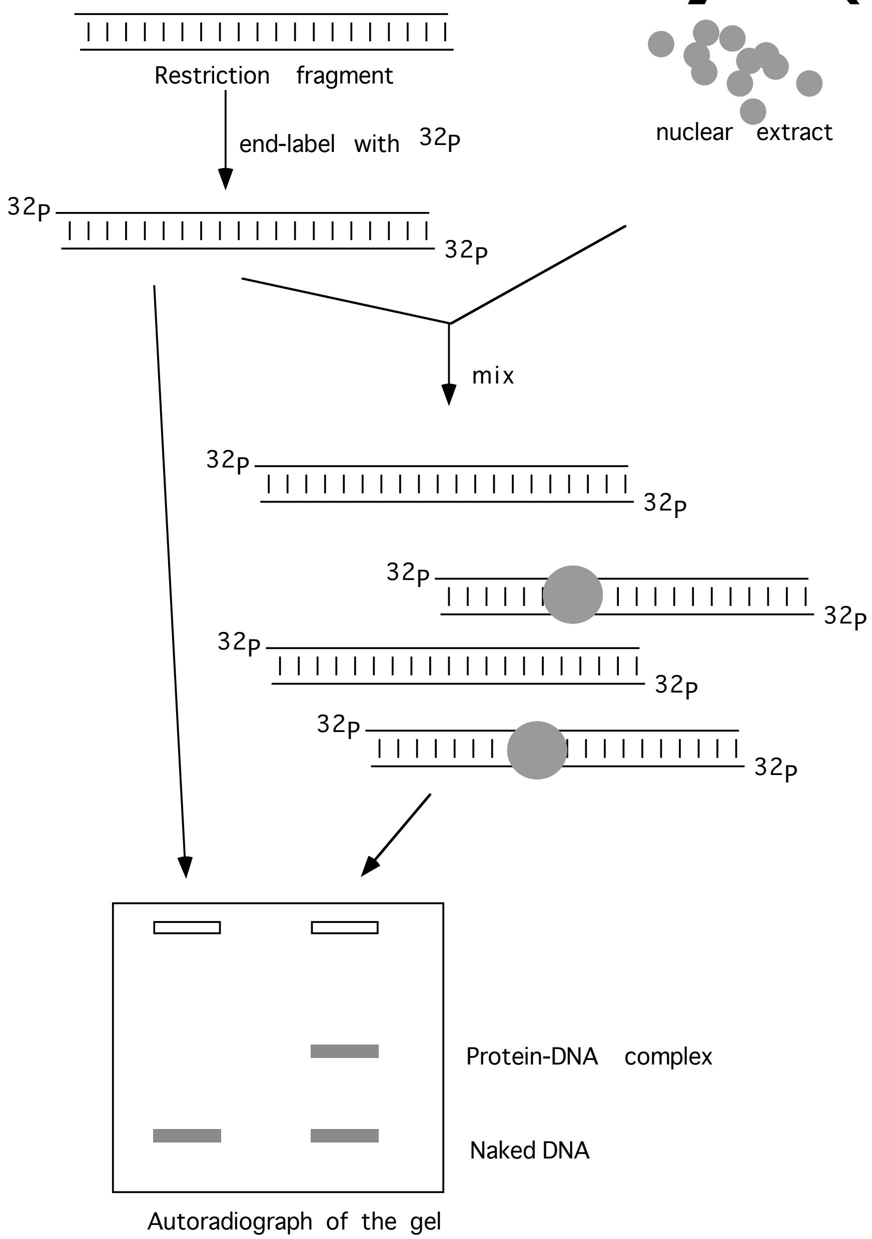
Quantitative S1

Fig. 5.27



Purpose: To determine the boundaries of exons and introns and perhaps the position of a trancription start site. One can also use it to determine the relative concentration of a nucleic acid.

Electrophoretic mobility shift assay (EMSA or gel shift)



My figure

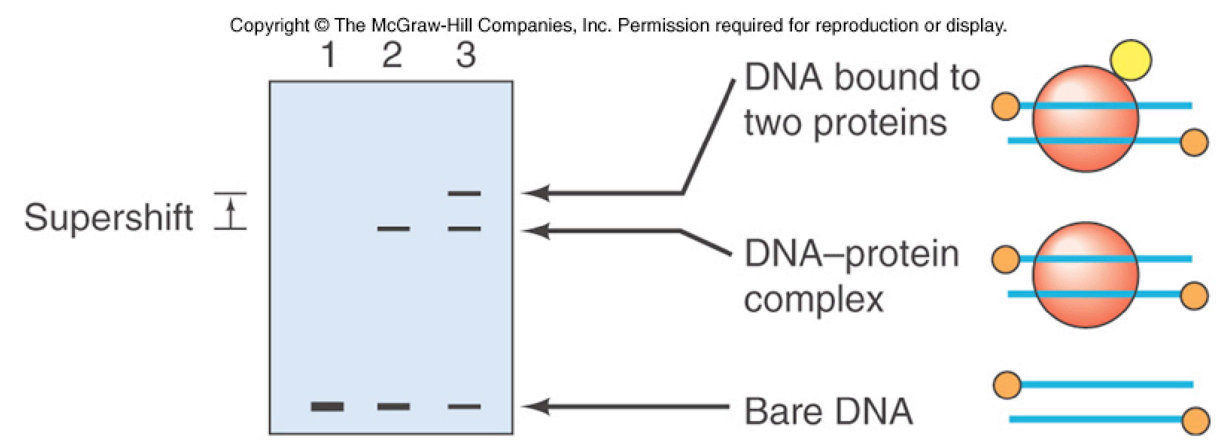


Figure 5.36 Weaver 4th ed
In supershifting the 2nd protein (yellow ball) is usually an antibody that recognizes the orange ball.

Discussed in context in Ch10

Epitope tagging

- To understand this slide you must understand what antibodies are and how they are made.
- Purpose: Antibodies are great tools for tracking and identifying a protein. A problem is that an antibody must be made against the protein that you are interested in studying. This can be pretty difficult. Furthermore, it requires a bit of luck since the antibody must be specific for your protein and not cross react with other proteins.
- If you don't have a way to obtain the protein of interest in pure form then it is just about impossible to make an antibody against it.
- A solution is epitope tagging.

- An epitope is what a single antibody recognizes.
- In proteins, epitopes are usually about 7 amino acids long.
- An antibody is made against an epitope that is not common in the organism that you are studying.
- The DNA sequence encoding the epitope is inserted into the gene that you are studying. It is inserted such that it is in the same reading frame as the gene.
- If the modified gene is inserted into the organism and expressed the antibody will now recognize the protein because it contains the epitope..
- Now your readily available and well characterized antibody will recognize the protein.