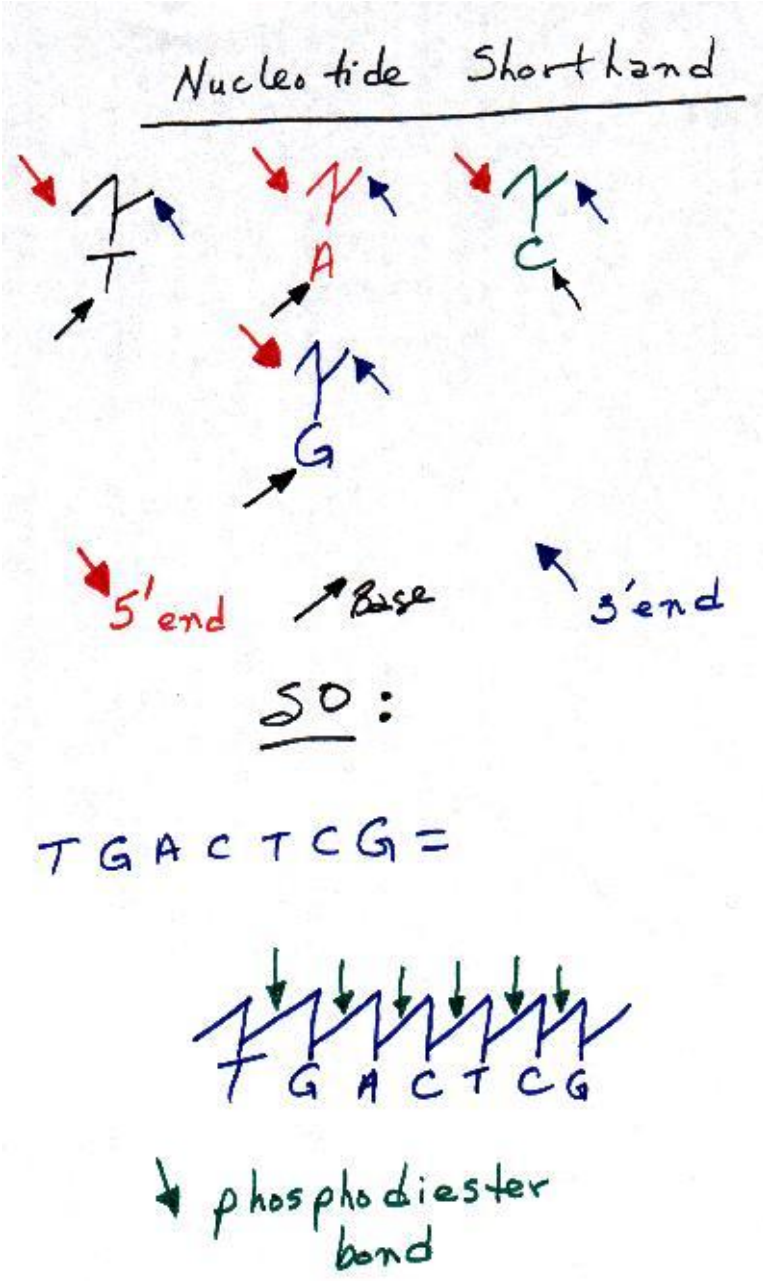
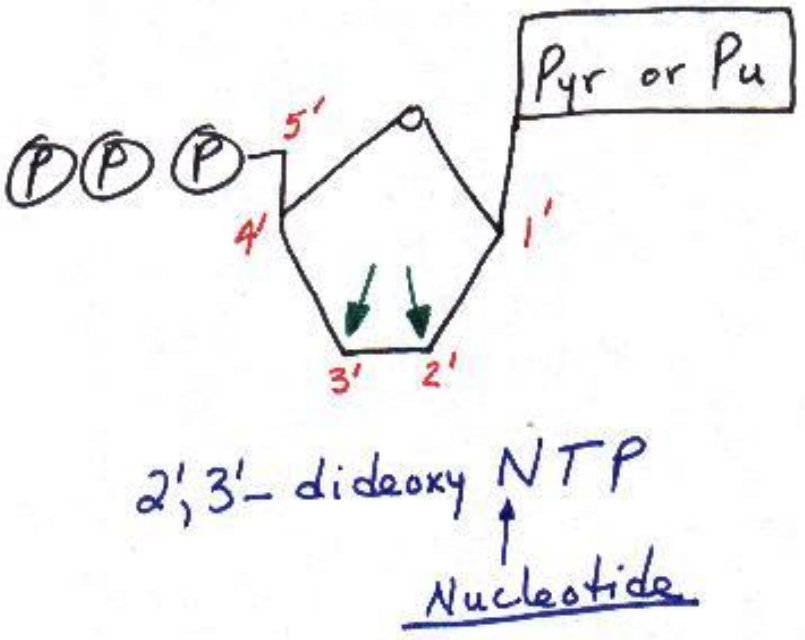
Techniques for Isolating and Identifying Nucleic Acids

- Several techniques have been developed to determine the sequence of DNA: the Maxam-Gilbert and Sanger methods. In order to follow how the techniques work, it is necessary to understand a little nucleotide shorthand, image at right:
- Note: Maxam-Gilbert will not be discussed as the Sanger method has been digitized due to its more simple and rapid approach in determining DNA sequences.

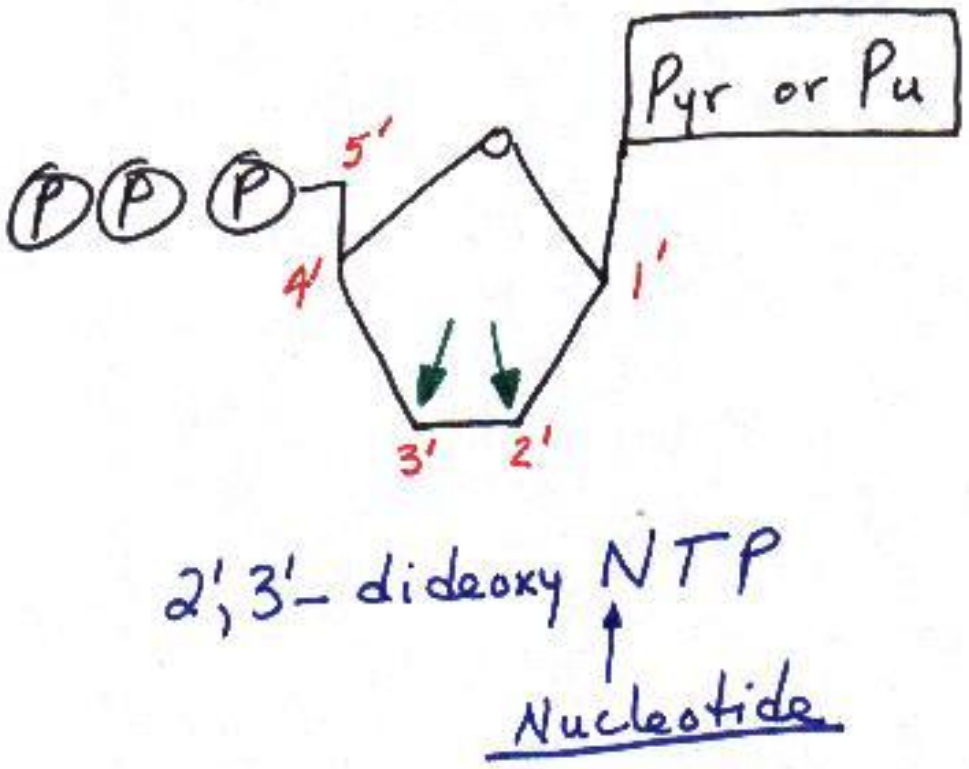


Sanger Method

- It is also known as the chain termination, controlled interruption of enzymatic replication and dideoxy methods of DNA sequencing.
- The key to this method is the utilization of a 2',3'-dideoxy compound, Figure, right, green arrows.



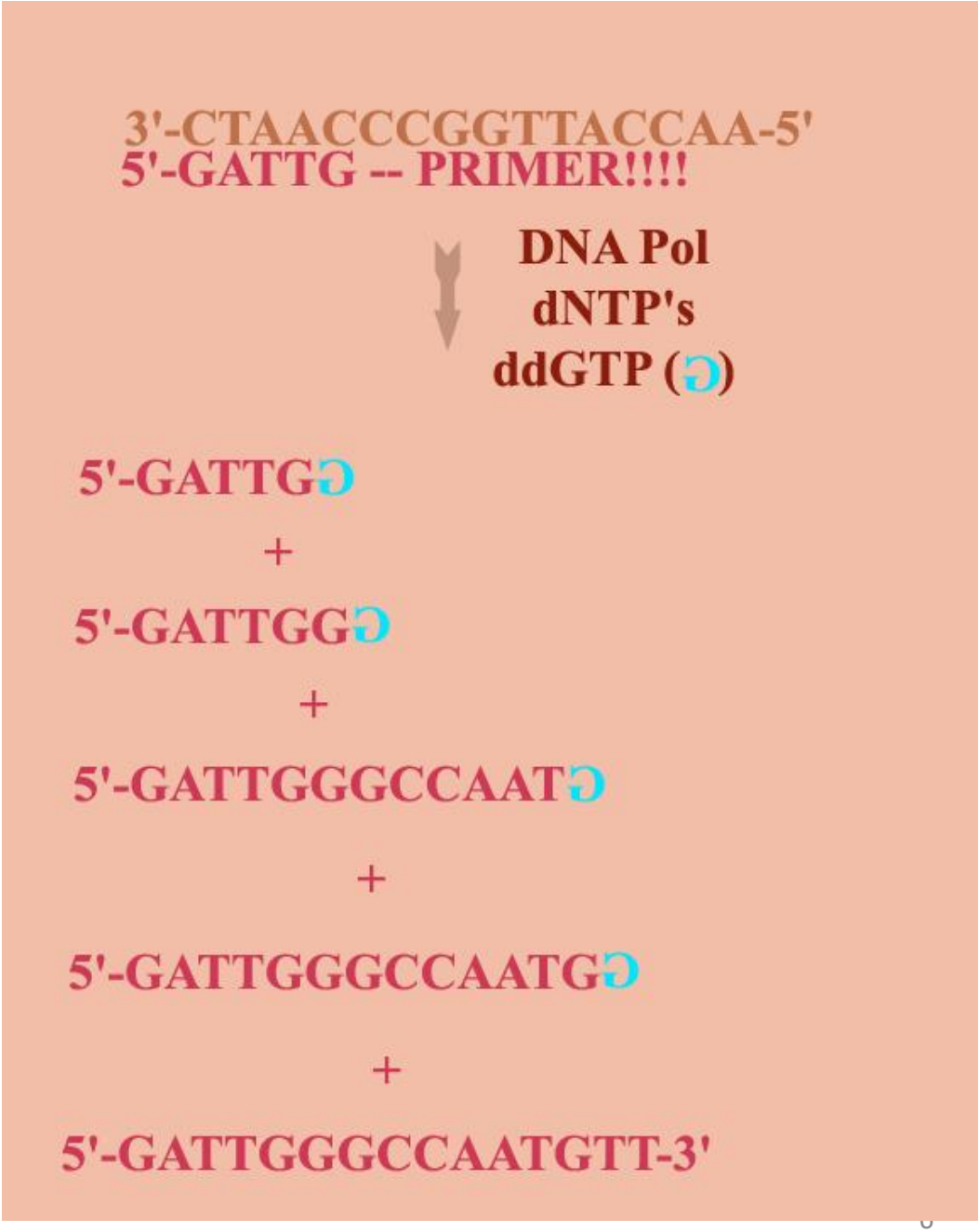
- In this compound, besides having no 2' -OH, there is also no 3' -OH.
- Without this -OH, it is impossible to make 3',5'-phosphodiester bonds and the DNA chain will not continue to elongate.



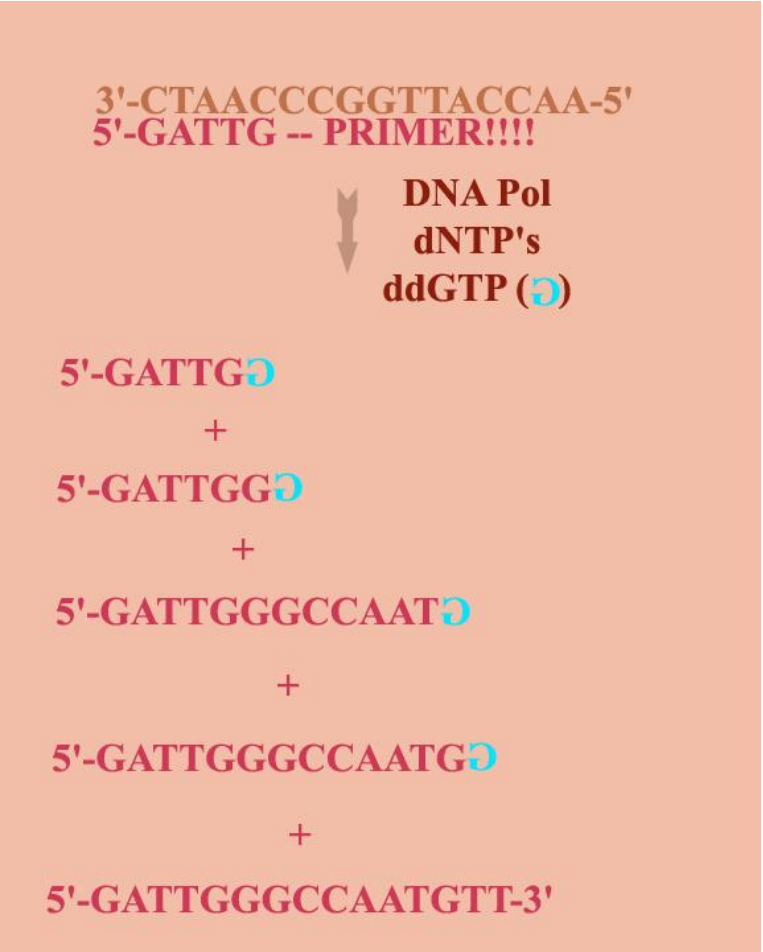
- In order to follow this method, let's consider the sequence:

- In addition, this method requires a primer that typically comes from a restriction digest. The primer for our sequence is **GATTG**, 5' to 3'.
- Note that the primer is complimentary to the first 5 nucleotides in the sequence we want to study

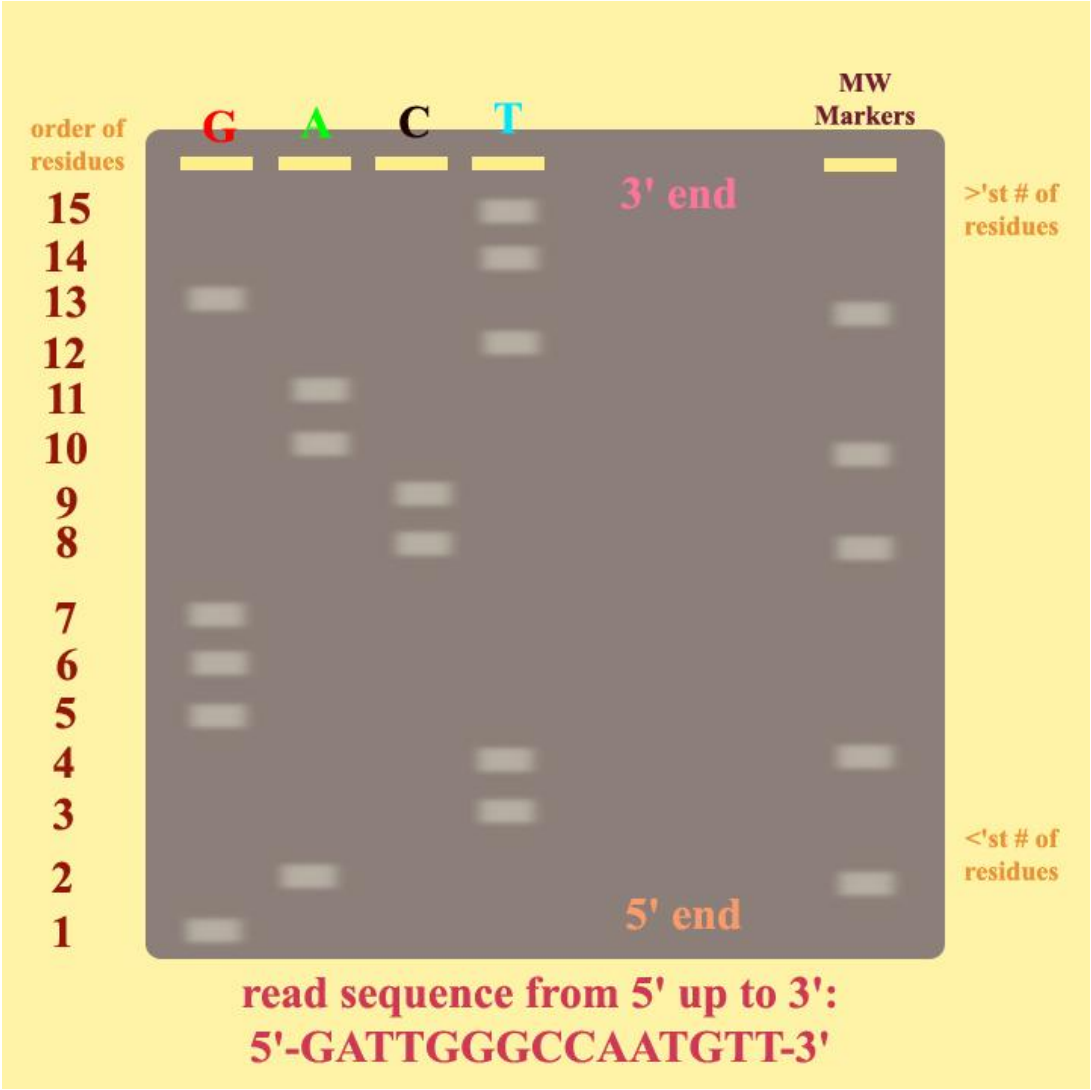
- The DNA fragment for sequencing is reacted with the complimentary primer, DNA Pol, radioactively labeled dNTP's (dNTP*) and ddGTP (dideoxy-GTP*; represented by the backwards G in the figure)



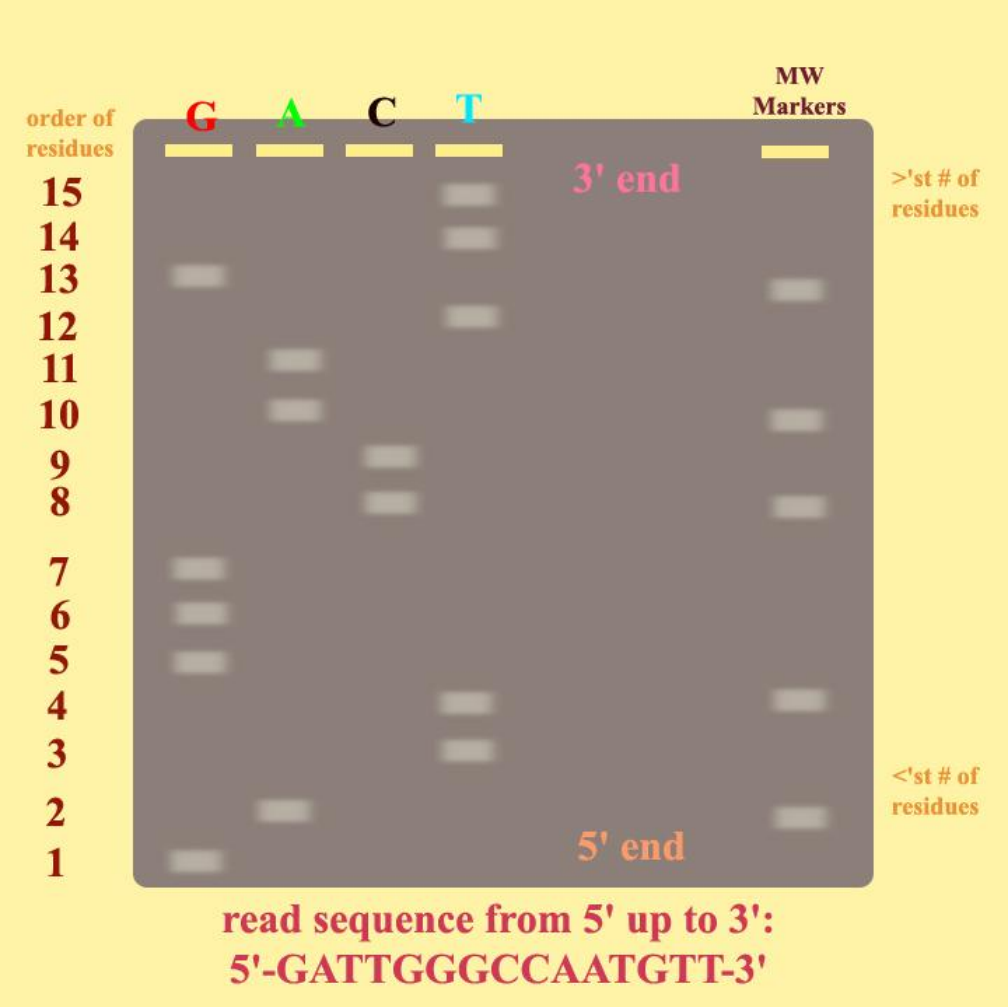
- Note that the sequence being synthesized is complimentary to the original sample.
- Whenever the newly synthesizing sequence is determined to need a "G" added to its sequence, either dGTP* or ddGTP will add on.
- If ddGTP adds on, that fragment will no longer elongate, i.e., the chain terminates -- the last residue of that fragment is a ddGTP.
- This cycle continues as desired, *ad nauseum*.



- Each ddNTP will be utilized in the Sanger method so that, essentially, 4 samples will be obtained, all of which are dideoxy-derivatives of G, A, C, T.
- These samples will be electrophoresed in the much the same manner as with the Maxam-Gilbert method



- In the Sanger method, the first residue is the first nucleotide from the original nucleotide's newly synthesized complimentary DNA after the primer.

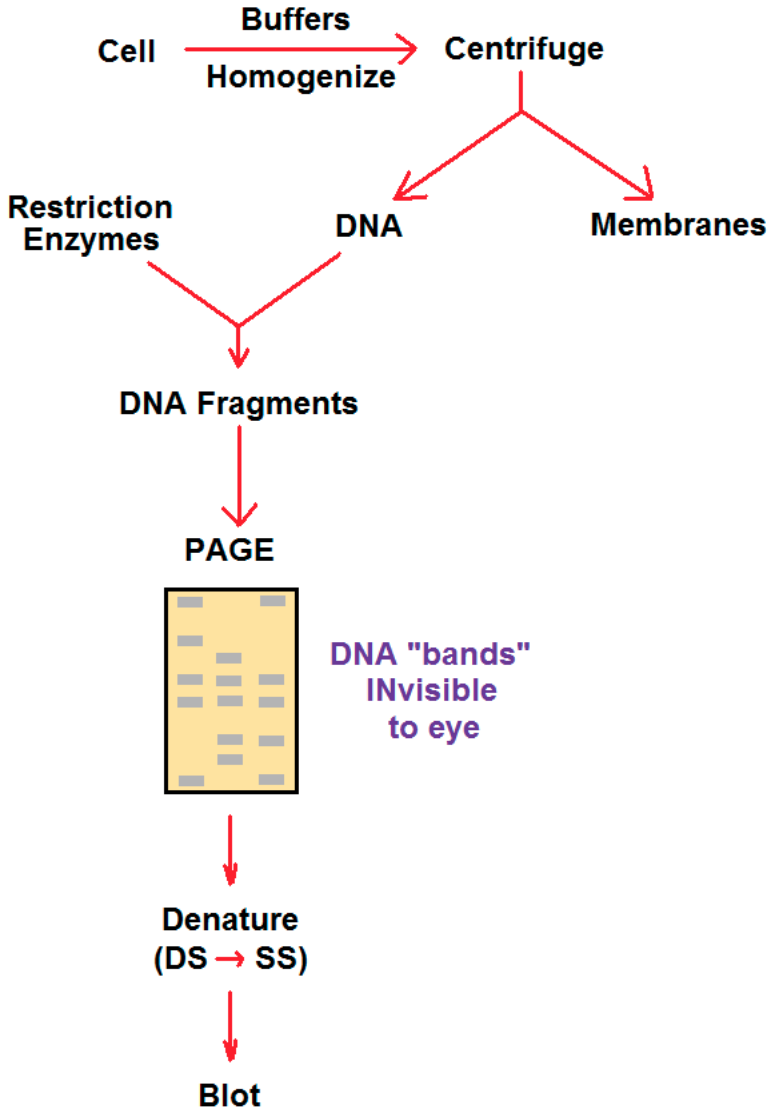


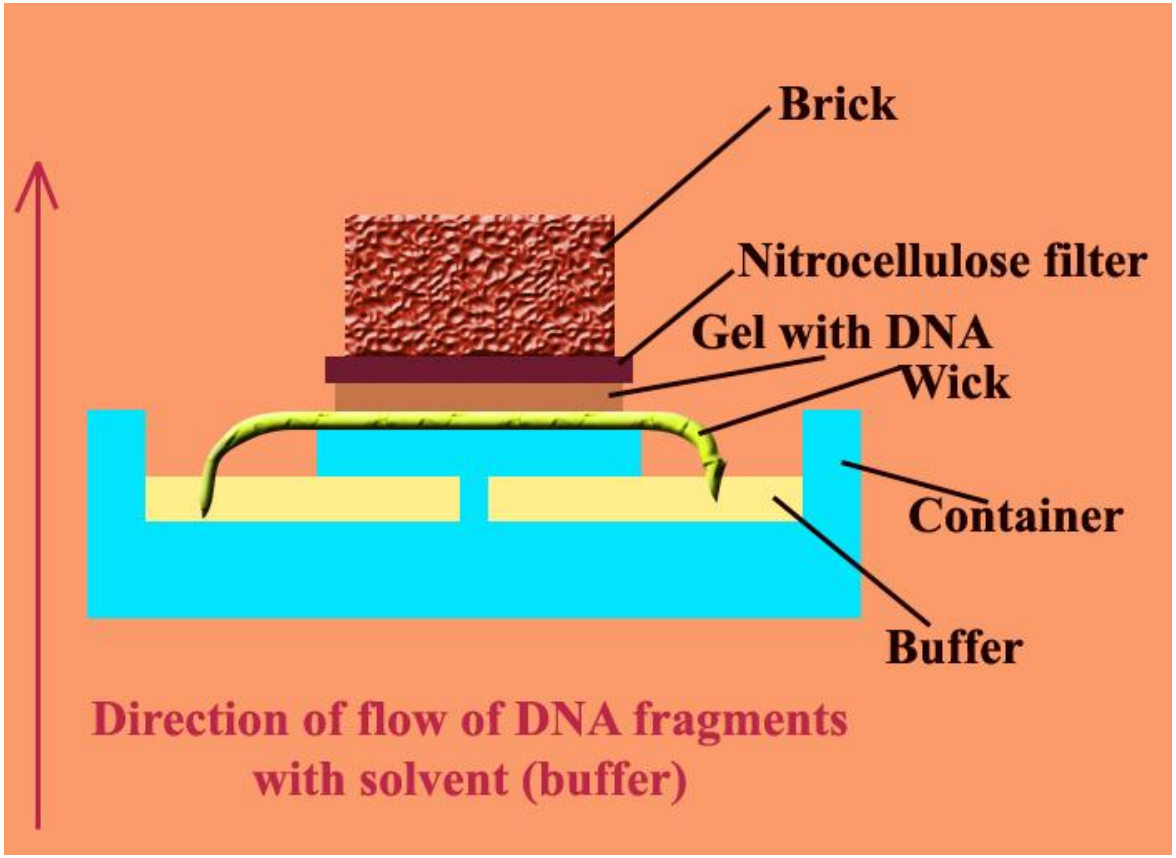
- The sequence is read directly from the autoradiograph and the **original sequence deduced from its complimentary strand sequence**.
- Although both methods are used, the Sanger method has been automated and is much faster and useful in DNA sequencing in the lab.

- With several questions now answered about DNA and its structure, there is still a burning question regarding techniques of identifying either specific DNA regions or individuals by their DNA sequences.
- About 25% of the human genome is in many different alleles (identical genes with different sequences). These are called polymorphisms.
- Each is an inherited pattern and segregate according to Mendel's rules (coming up in the future). These **restriction fragment length polymorphisms (RFLP's)** may be used to identify diseases, criminal acts or the release of suspects for lack of correct evidence, *ad nauseum*. Only identical twins have identical RFLP's, but NOT identical fingerprints or retinal scans.
- **VNTR's (variable numbers of tandemly repeated units)** comprise very unique RFLP's. They serve as "molecular finger prints" of an individual. Both of these fragments may be studied by Southern blot and PCR.

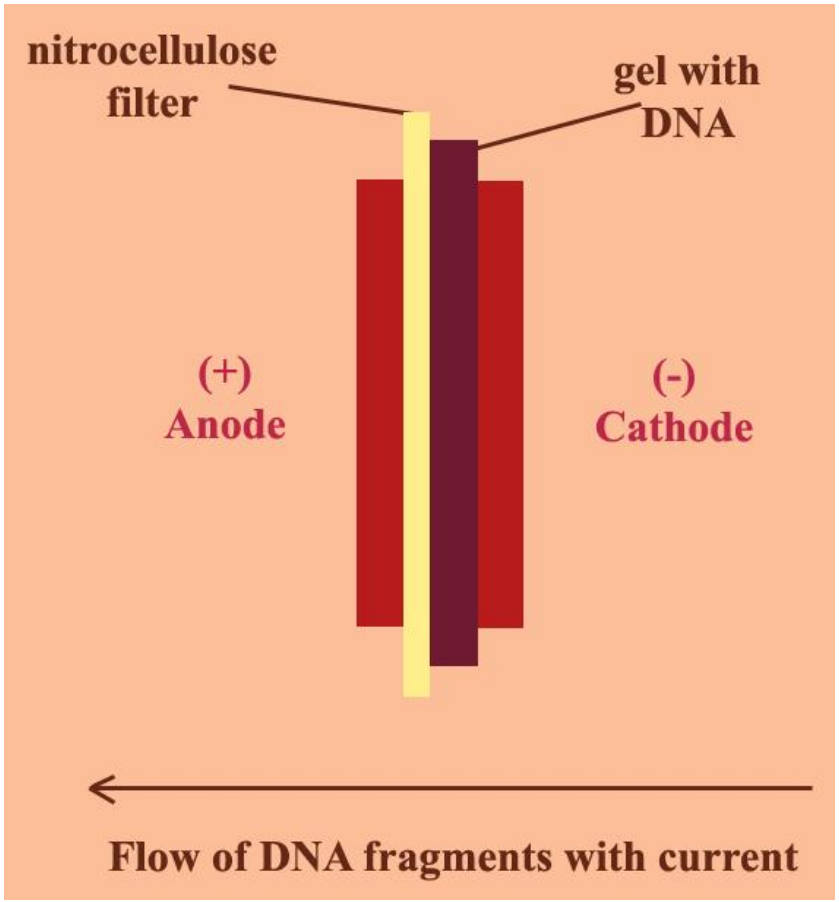
Southern Blot

- Cells are mixed with appropriate buffers and homogenized. The gimish is centrifuged to give various cell fractions, which include DNA.
- The DNA is digested by restriction enzymes to fragments and submitted to PAGE.
- There are NO fragments visible to the human eye in this gel -- yet. The gel is treated in such a manner that the DS DNA is denatured to SS DNA and then the gel is blotted onto a nitrocellulose filter.



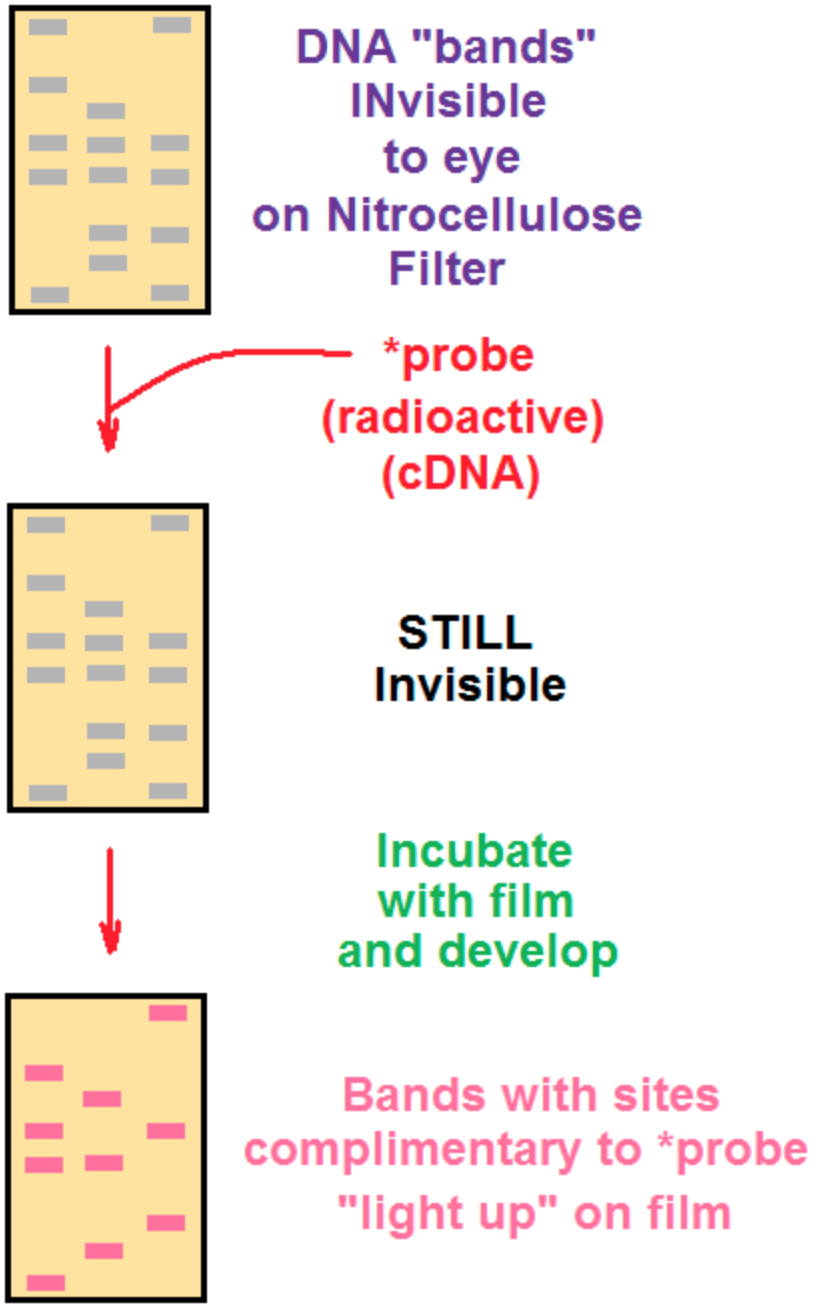


- Buffer is poured into a "pan" with a stand in its center. A wick (some just use paper towels) is laid over the stand, upon which the gel is placed. A nitrocellulose filter is placed on top of the gel and a brick (or some heavy weight) is placed on top of the filter. The solvent (buffer) flows up the wick and under the pressure of the brick, causes the fragments to be transferred to the nitrocellulose filter.



- The second way in which material is transferred from the PAGE gel to a nitrocellulose filter: by electrophoresis. The gel with the SS DNA is placed between two electrodes with a nitrocellulose filter placed on the positively charged electrode (anode) side. Electrophoresis is run as described earlier. The DNA (negatively charged) is attracted to the anode and the SS DNA is transferred to the nitrocellulose filter.

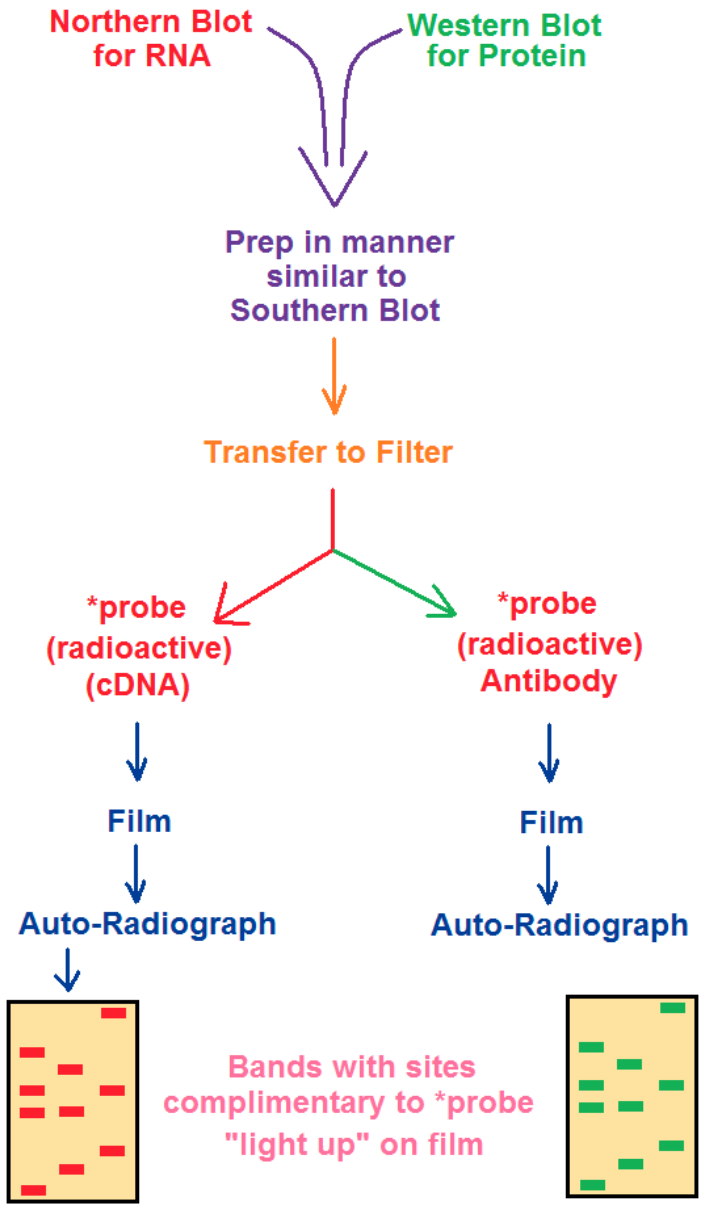
- There are STILL no bands visible on the nitrocellulose filter, yet, Figure, right.
- A radioactively (^{32}P) labeled "probe", a specific sequence of DNA complimentary to what you are looking for (cDNA -- complimentary DNA) is added to the nitrocellulose filter and incubated. Once incubation is complete, the irradiated filter is incubated with a piece of film and, then, developed. The only bands, which show up on the autoradiograph, are those bands that are complimentary to the probe and light up on the film. Those fragments may be identified on the nitrocellulose filters by matching it up with the film; the fragments may then be studied as desired.



Northern and Western Blots

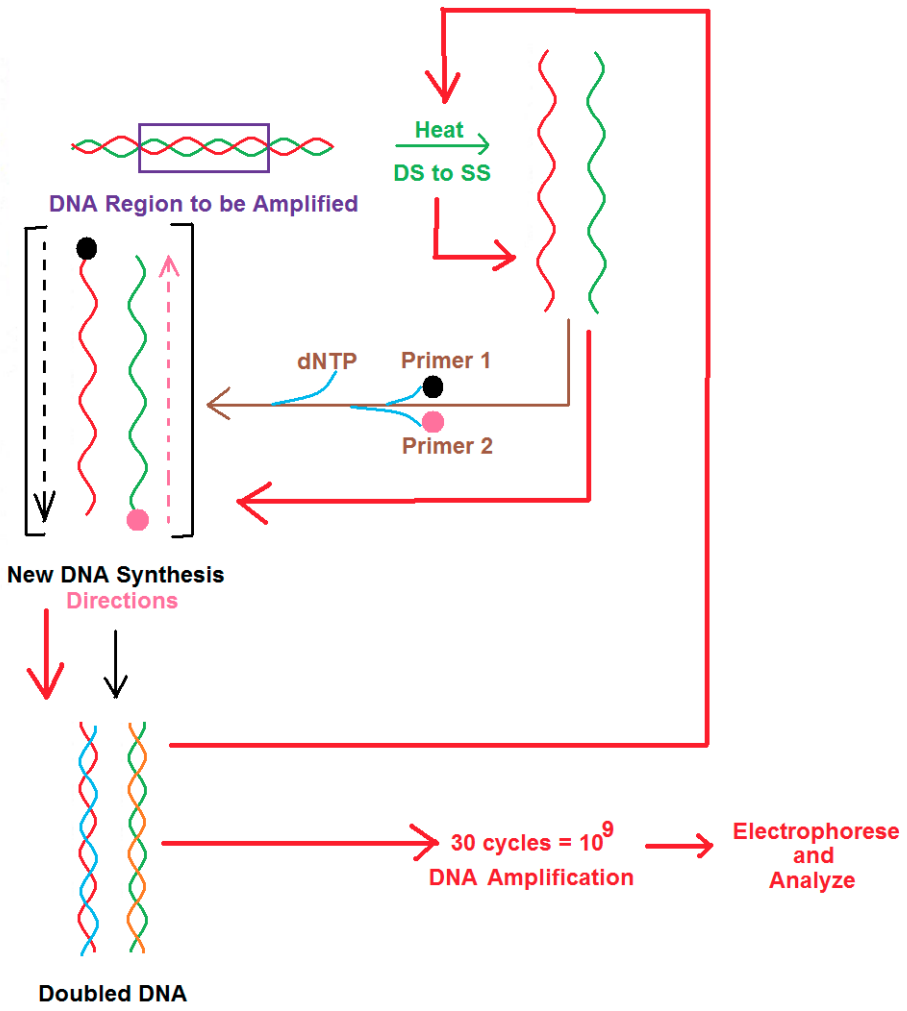
- Two other blots are of significance, as well:
  - the Northern and
  - Western blots.
- While the techniques to do these blots are similar, they examine DIFFERENT macromolecules.

- The Northern blot (named for geography rather than someone's name) examines RNA fragments.
- The probe for the Northern blot is radioactively labeled cDNA.
- The Western blot (more warped geneticist's humor) **looks for proteins**, Figure, right.
- The probe for the Western blot is radioactively labeled antibodies (more on this in the future).
- The Western blot has gained notoriety as it is the confirming test to positively diagnose people who are infected with HIV.



Polymerase Chain Reaction

- What happens, though, if there isn't enough DNA in a sample to be studied adequately? A technique called the PCR (polymerase chain reaction) was developed just for this purpose.
- In brief, a fragment of DS DNA is heated to denature it.
- To the two strands of SS DNA, two primers are added, as well as DNA Pol and dNTP's.
- As this chain reaction gets going, after 20 cycles, this small amount of, previously unstudyable, DNA is amplified one million fold! After 30 cycles, this DNA is amplified a billion-fold!



- This technique has proven useful in numerous trials.
- The PCR is also used
  - 1) to detect viruses of a "sneaky" nature (e.g., HIV),
  - 2) prenatally, e.g., to identify genetic defects,
  - 3) to detect polymorphisms,
  - 4) in tissue typing to reduce tissue rejections,
  - 5) in old DNA samples, e.g., bacteria from pyramids in amber to track evolutionary changes and
  - 6) in forensics, e.g., to identify perpetrators and separate them from innocent suspects.