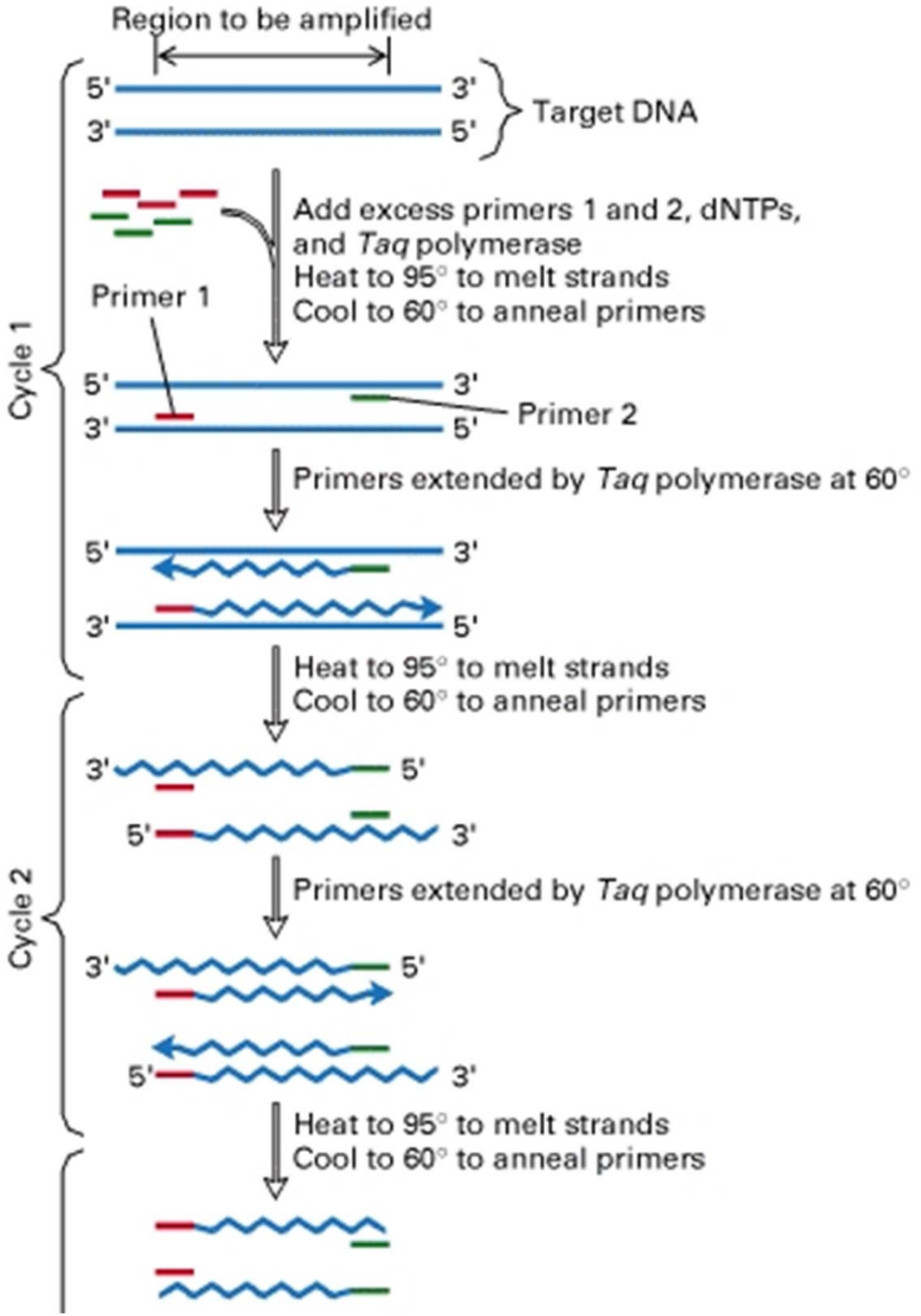
Polymerase Chain Reaction

- Developed in the 1980s by Kary Mullis
- Technique for making copies, or amplifying, a specific sequence of DNA in a short period of time
- An alternative to cloning can be used to directly amplify rare specific DNA sequences in a complex mixture when the ends of the sequence are known.
- Process
  - **Target DNA** to be amplified is added to a tube, mixed with **nucleotides** (dATP, dCTP, dGTP, dTTP), buffer, and DNA polymerase.
  - **Primers** are added – short single-stranded DNA oligonucleotides (20–30bp long)
  - Reaction tube is placed in an instrument called a **thermocycler**

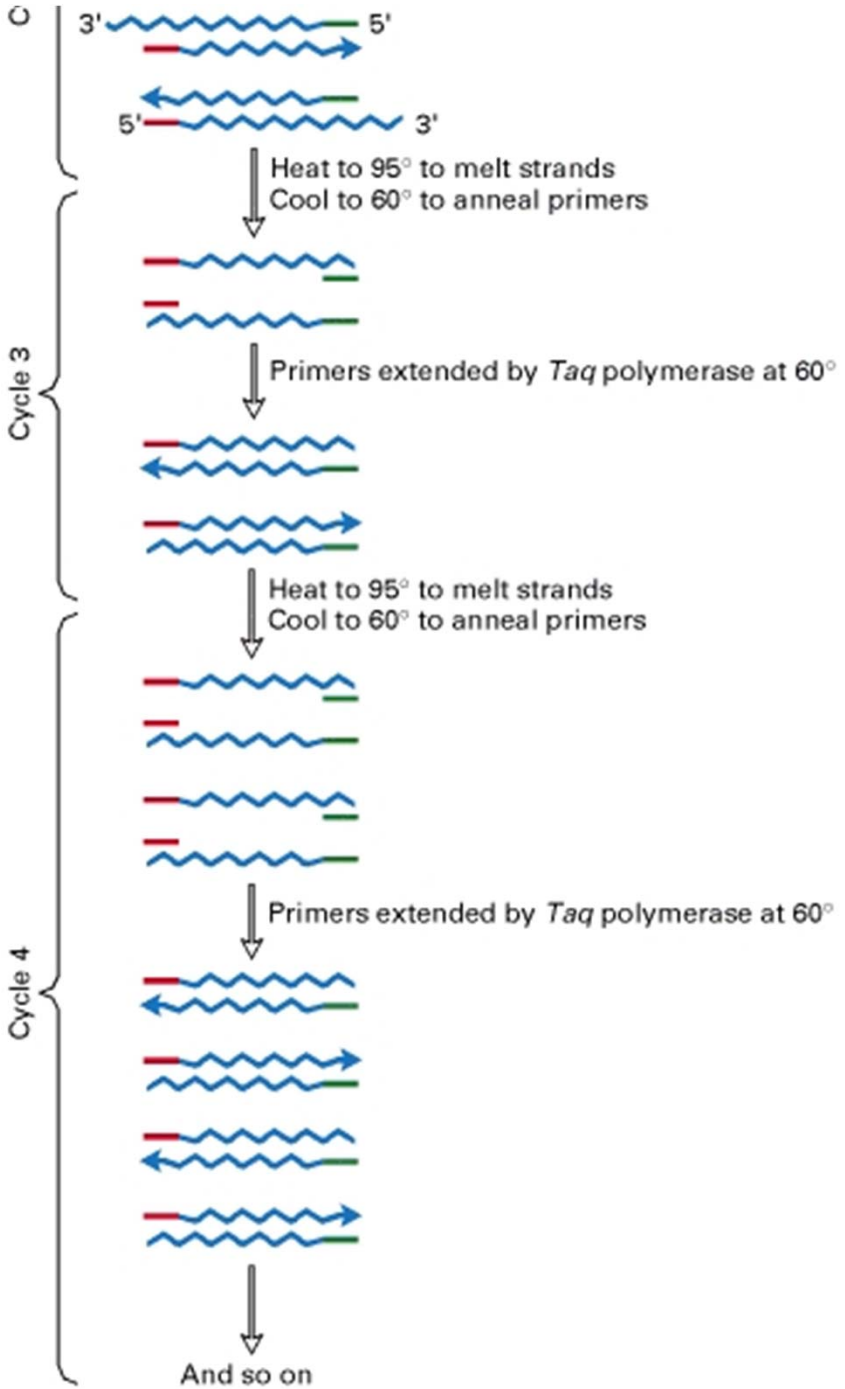
Process

- Thermocycler will take DNA through a series of reactions called a **PCR cycle**
- Each cycle consists of three stages
  - Denaturation
  - Annealing (hybridization)
  - Extension (elongation)
- At the end of one cycle, the amount of DNA has doubled
- Cycles are repeated 20–30 times

Polymerase chain reaction



Polymerase chain reaction



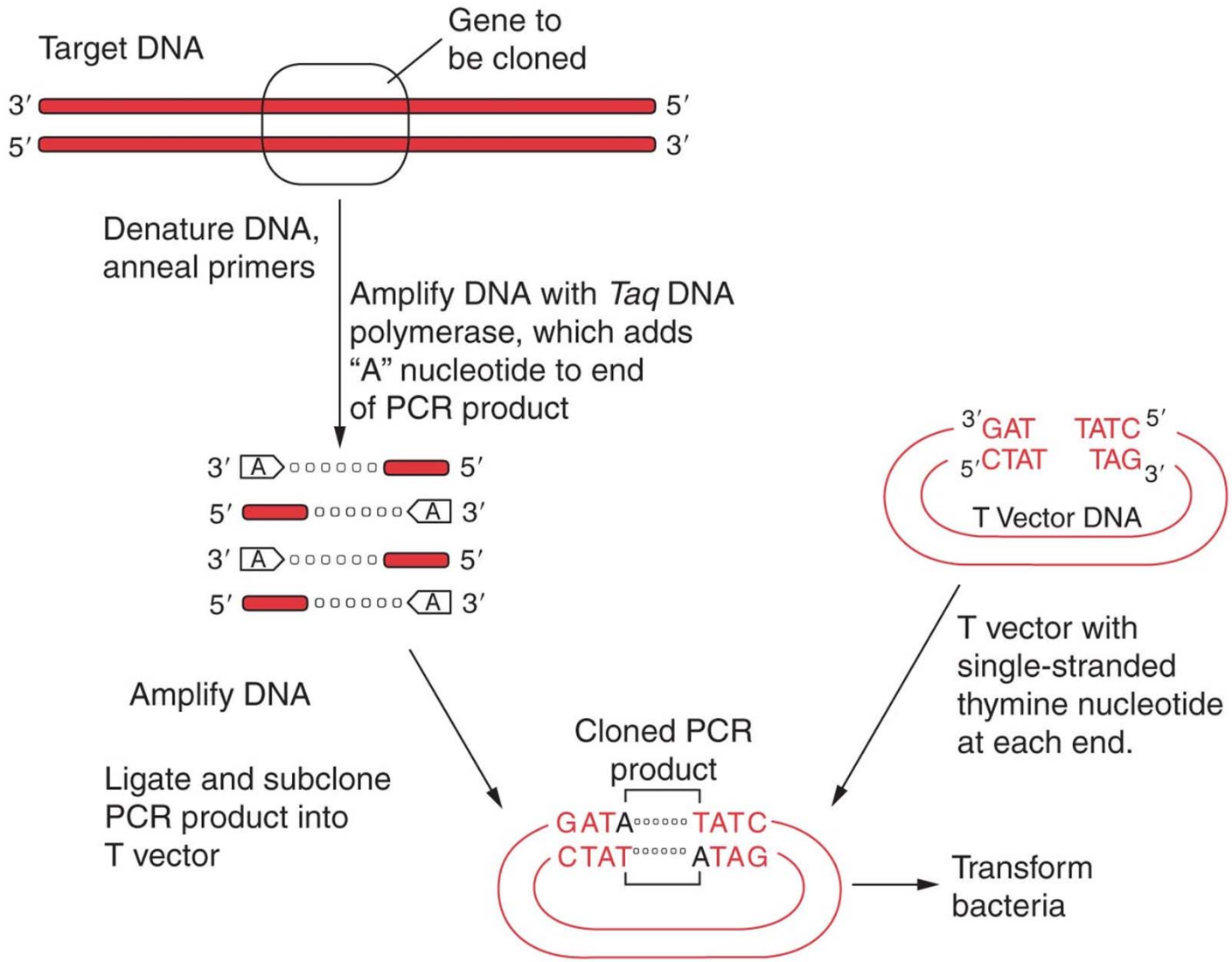
PCR

- The type of DNA polymerase used is very important
  - *Taq* DNA polymerase – isolated from a species known as *Thermus aquaticus* that thrives in hot springs, can work at 60^{o}C
- Advantage of PCR
  - Ability to amplify millions of copies of target DNA from a very small amount of starting material in a short period of time
- Applications
  - Making DNA probes
  - Studying gene expression
  - Detection of viral and bacterial infections
  - Diagnosis of genetic conditions
  - Detection of trace amounts of DNA from tissue found at crime scene

Cloning PCR Products

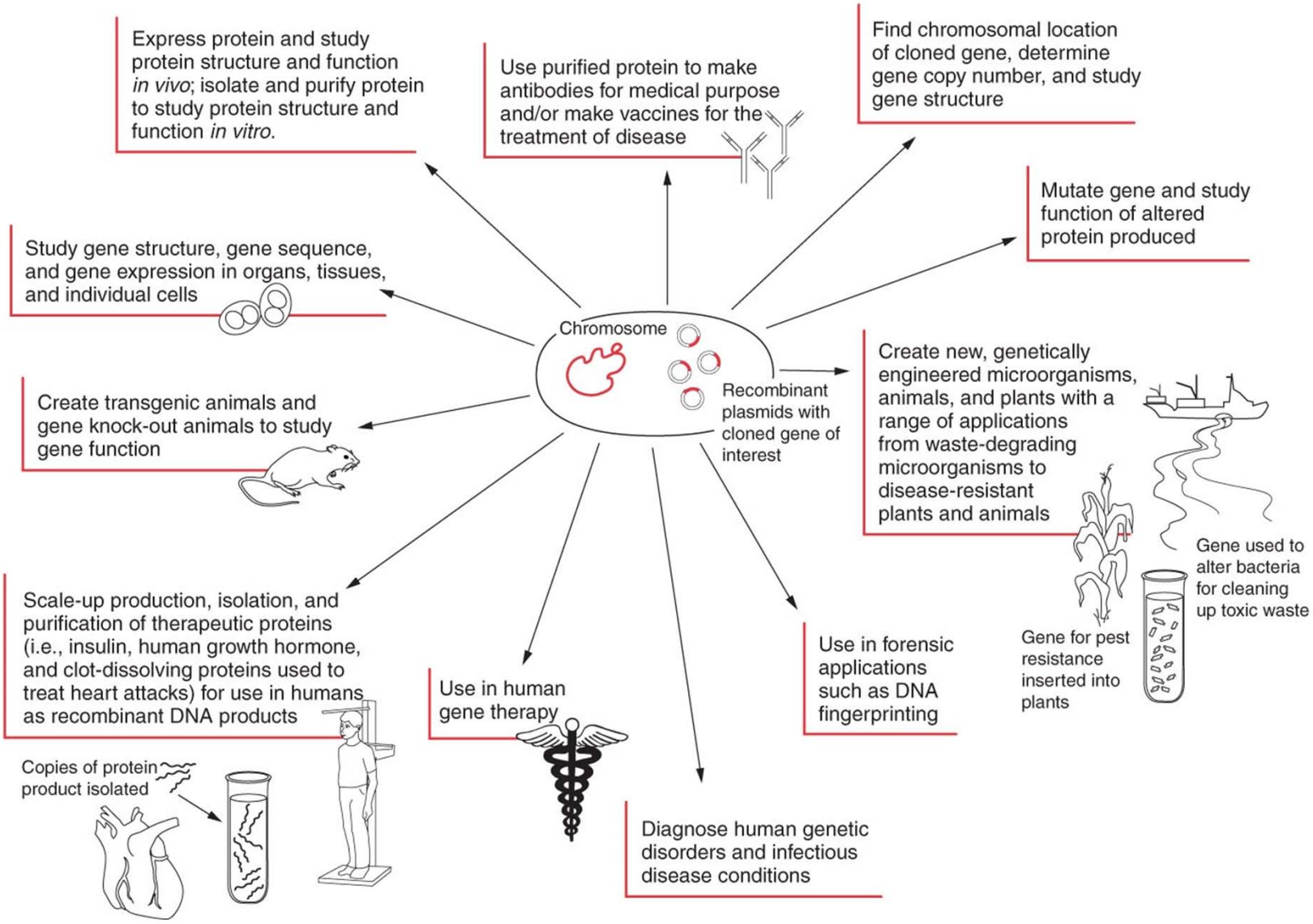
- Is rapid and effective
- Disadvantage
  - Need to know something about the DNA sequence that flanks the gene of interest to design primers
- Includes restriction enzyme recognition sequences in the primers
- Uses T vector
  - *Taq* polymerase puts a single adenine nucleotide on the 3’ end of all PCR products

Cloning PCR Products



What Can You Do with a Cloned Gene?

Applications of Recombinant DNA Technology

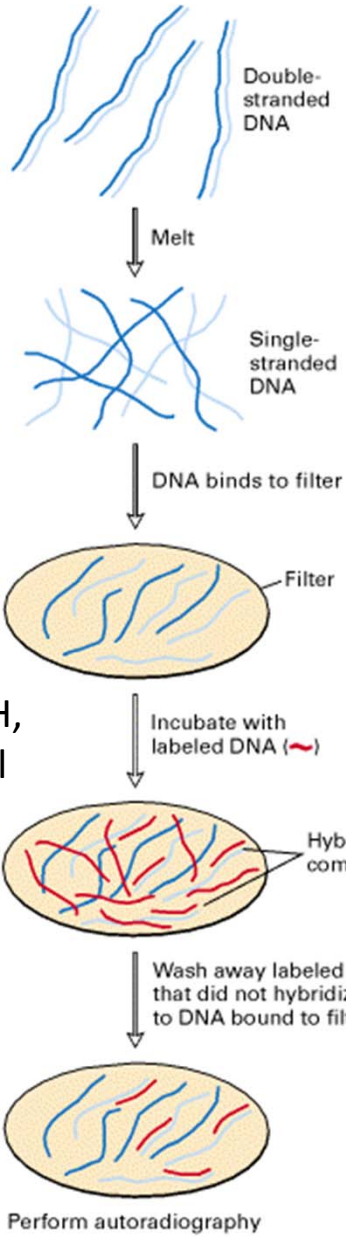


What after cloning?

- Identification of specific clones of interest in a large collection of clones
- Isolation of the clone of interest
- Separation of cloned DNA from the vector DNA and its analysis
- Characterization of cloned DNA by determining its nucleotide sequence

Membrane hybridization assay

Identification & Isolation of specific clones of interest

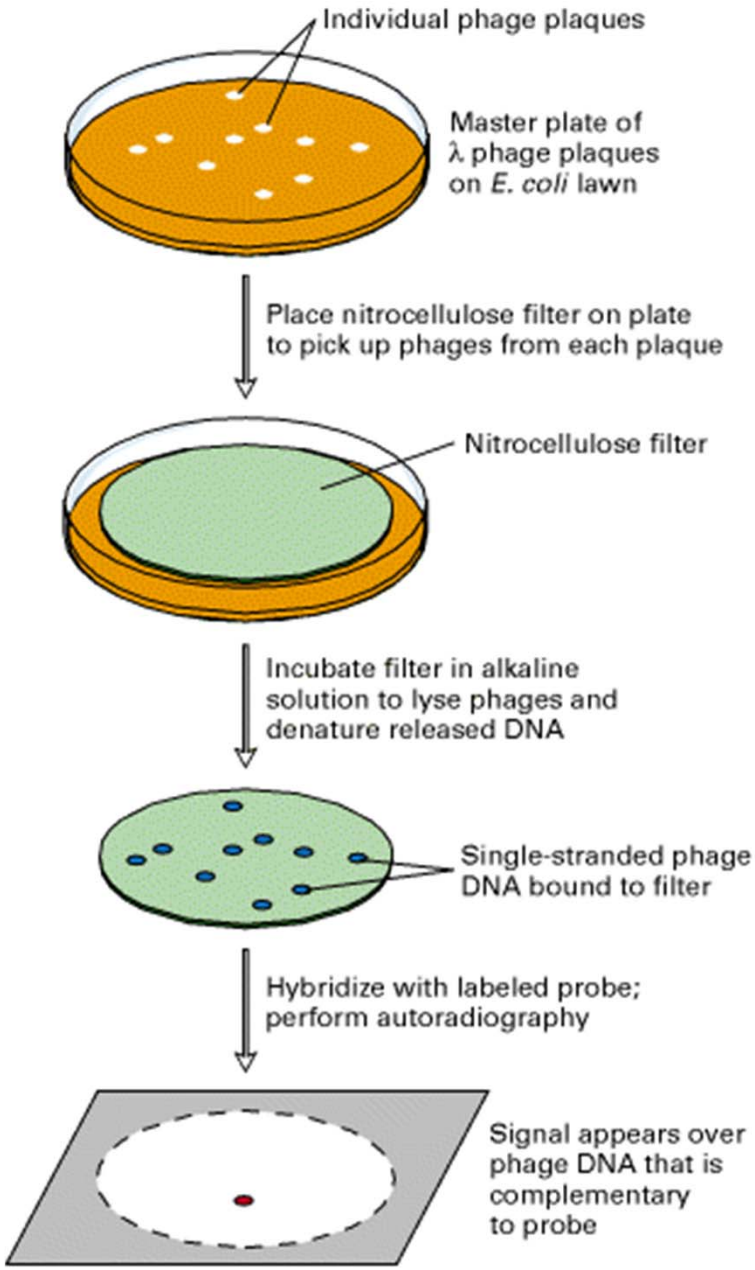


Nitrocellulose filter / treated nylon membrane

Hybridize at near neutral pH, 40 – 65 °C, 0.3 – 0.6 M NaCl

Radioactively labeled single stranded DNA or RNA complementary to some of the nucleic acid bound to the membrane

Screening λ library with membrane-hybridization technique

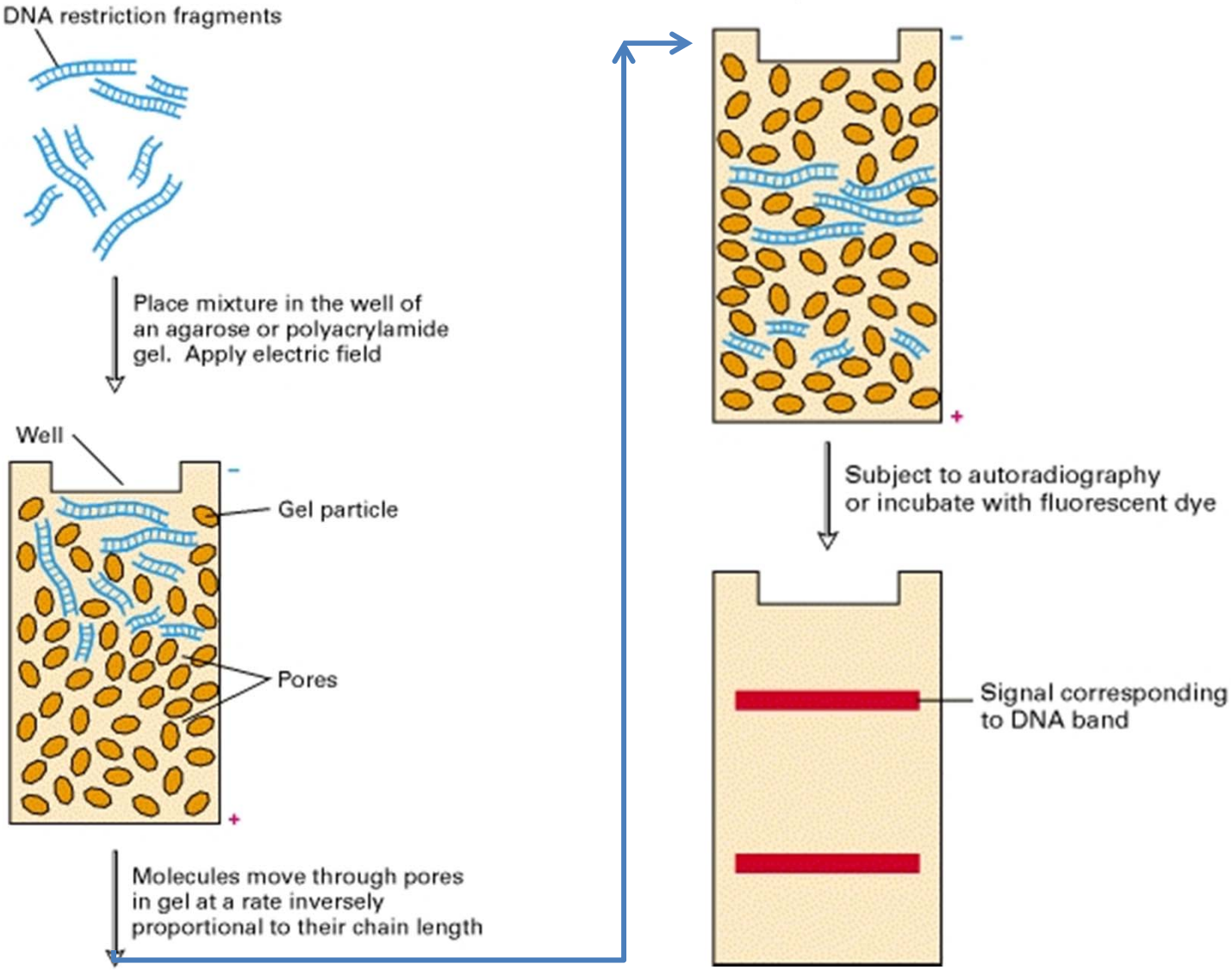


Align the autoradiogram and the petri dish to remove viral particles from the clone corresponding to the spot

Isolated cloned DNA is treated with the restriction enzyme used to form the recombinant plasmid to separate cloned DNA and vector DNA

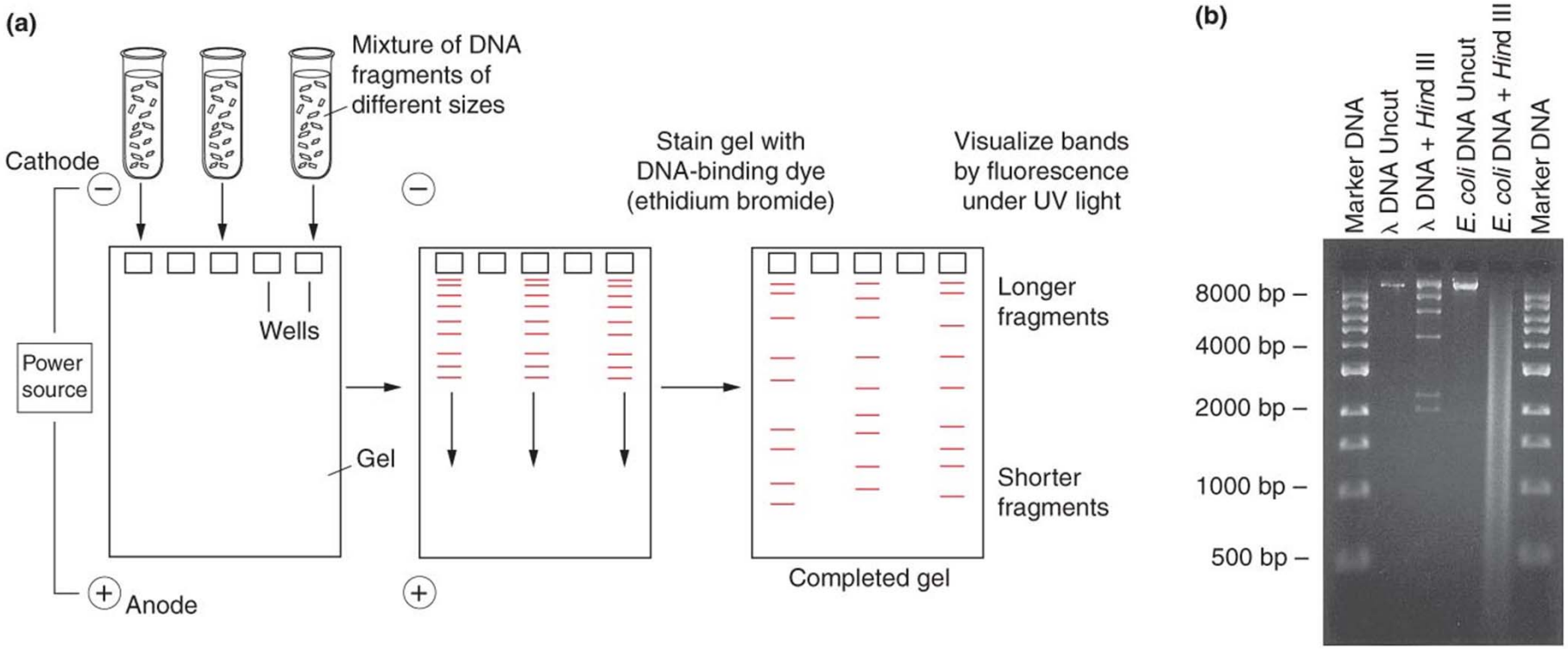
Separation of DNA fragments of different lengths by gel electrophoresis

Separation of cloned DNA from the vector DNA



- Agarose Gel Electrophoresis
  - Agarose melted in a buffer and poured into a horizontal tray
  - When solidified, the gel contains small holes or pores through which the DNA fragments will travel
  - The percentage of agarose used to make the gel determines the ability of the gel to separate DNA fragments of different sizes
    - Most common is 0.5–2% agarose
    - Higher percentage will separate small DNA fragments better
    - Lower percentage is better for separating large fragments

- Agarose Gel Electrophoresis
  - To run a gel, it is submerged in a buffer solution that conducts electricity
  - DNA is loaded into wells at the top of the gel
  - Electric current is applied to the ends of the gel
    - DNA migrates according to its charge and size
    - Rate of migration through the gel depends on the size of the DNA
      - Large fragments migrate slowly; smaller fragments migrate faster
  - Tracking dye is added to the samples to monitor DNA migration during electrophoresis
  - DNA can be visualized after electrophoresis by the addition of DNA staining dyes


(a)
Mixture of DNA fragments of different sizes
Cathode
Stain gel with DNA-binding dye (ethidium bromide)
Visualize bands by fluorescence under UV light
Wells
Power source
Gel
Longer fragments
Shorter fragments
Anode
Completed gel
(b)
Marker DNA
λ DNA Uncut
λ DNA + Hind III
E. coli DNA Uncut
E. coli DNA + Hind III
Marker DNA
8000 bp –
4000 bp –
2000 bp –
1000 bp –
500 bp –

Restriction Mapping

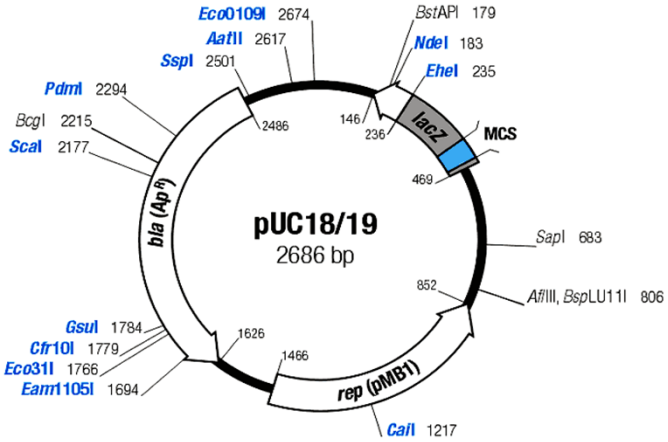
- Multiple Restriction Sites Can Be Mapped on a Cloned DNA Fragment
- Restriction mapping is a method used to map an unknown segment of DNA by breaking it into pieces and then identifying the locations of the breakpoints. This method relies upon the use of proteins called **restriction enzymes**, which can cut, or digest, DNA molecules at short, specific sequences called restriction sites.
- After a DNA segment has been digested using a restriction enzyme, the resulting fragments can be examined using a laboratory method called gel electrophoresis, which is used to separate pieces of DNA according to their size.

Method for Constructing a Restriction Map

- It involves digesting the unknown DNA sample in three ways.
- Here, two portions of the DNA sample are individually digested with different restriction enzymes, and a third portion of the DNA sample is double-digested with both restriction enzymes at the same time.
- Next, each digestion sample is separated using gel electrophoresis, and the sizes of the DNA fragments are recorded. The total length of the fragments in each digestion will be equal. However, because the length of each individual DNA fragment depends upon the positions of its restriction sites, each restriction site can be mapped according to the lengths of the fragments
- The final drawing of the DNA segment that shows the positions of the restriction sites is called a restriction map

Restriction Mapping

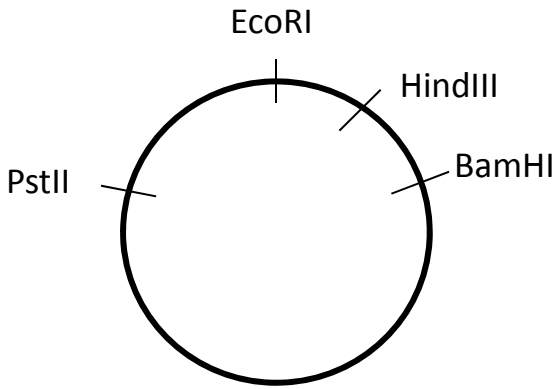
- The combination of all restriction sites in a specific DNA sequence is called *restriction mapping*. Because it indicate every single site in the sequence , that can be recognised and cut by a specific enzyme.



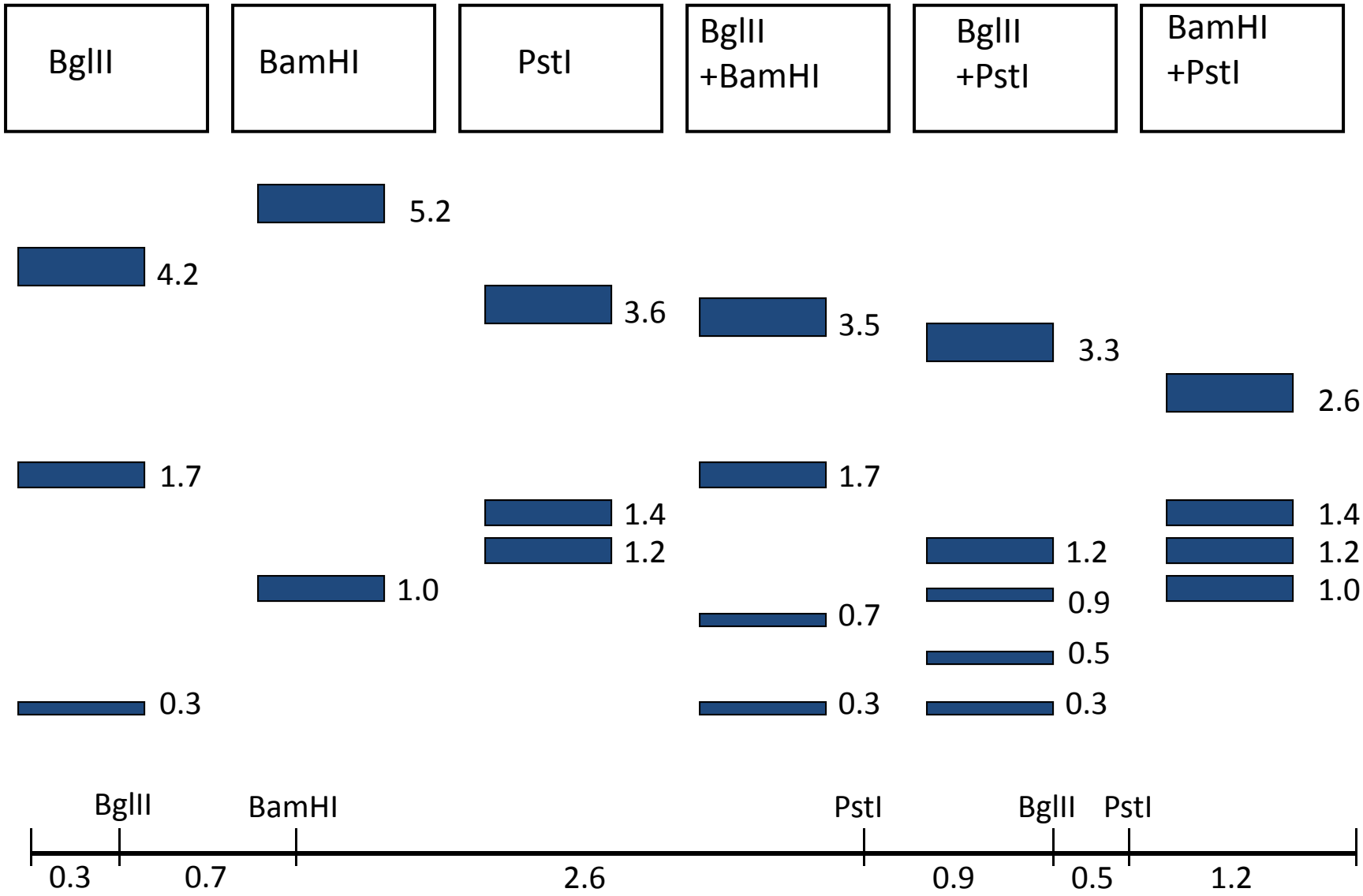
- The distance between restriction enzyme sites can be determined by the patterns of fragments that are produced by the restriction enzyme digestion
- This gives the information about the structure of unknown piece of DNA

Restriction Mapping

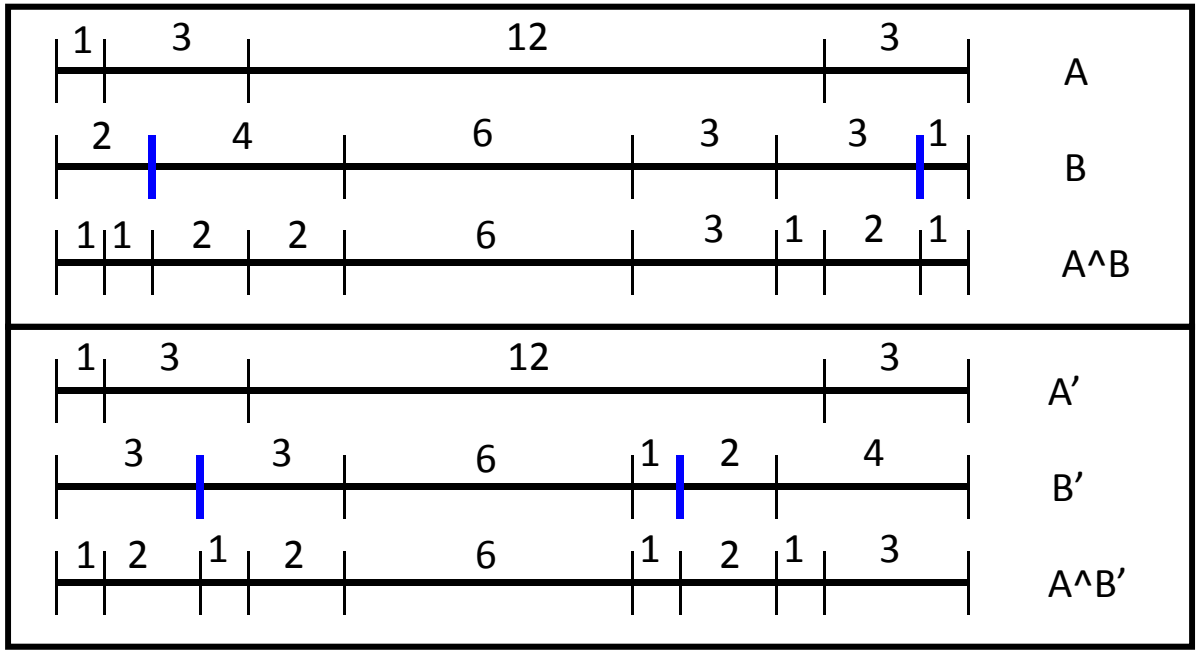
Restriction maps show the relative location of a selection of restriction sites along linear or circular DNA.



Restriction Mapping

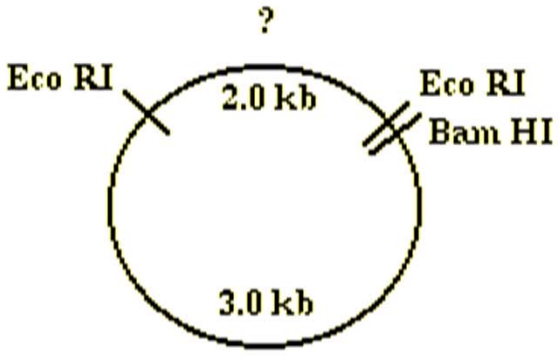


Restriction Mapping

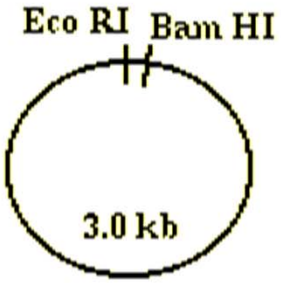


For Example

- Take a pBluescript portion of plasmid (3kb) with a known;
  - ✓ Restriction site (Eco RI & Bam HI)
  - ✓ Insert of 2 kb long



pBluescript with unknown insert in the Eco RI site



pBluescript without insert

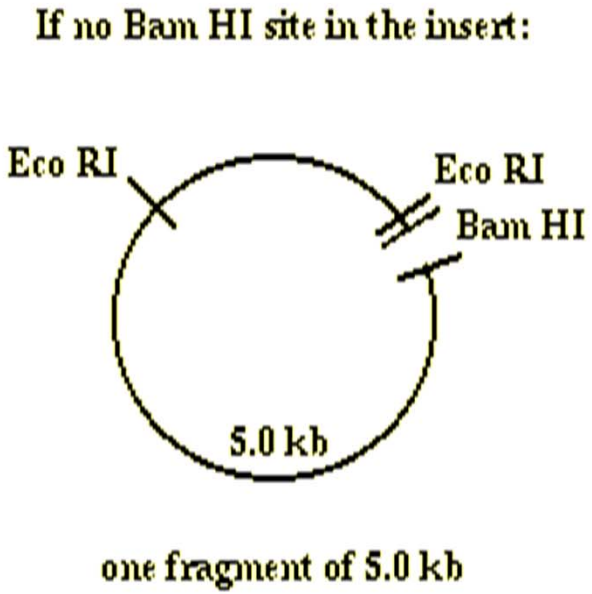
- Digest plasmid with an enzyme that you know is in the pBluescript plasmid. For example, you know that there is only one Bam HI site in pBluescript, and it is in the multiple cloning site next to the Eco RI site

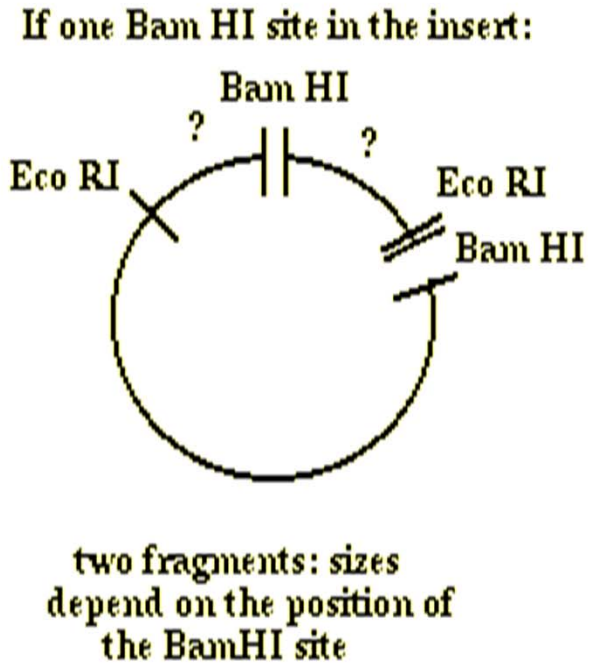
- If you digest this plasmid with Bam HI, there are two possibilities:

1) There are no Bam HI sites in the insert. If this is the case, when you run this digestion on a gel you will see only one DNA fragment, and it will be 5.0 kb long (3.0 kb of pBluescript DNA and 2.0 kb of insert DNA).

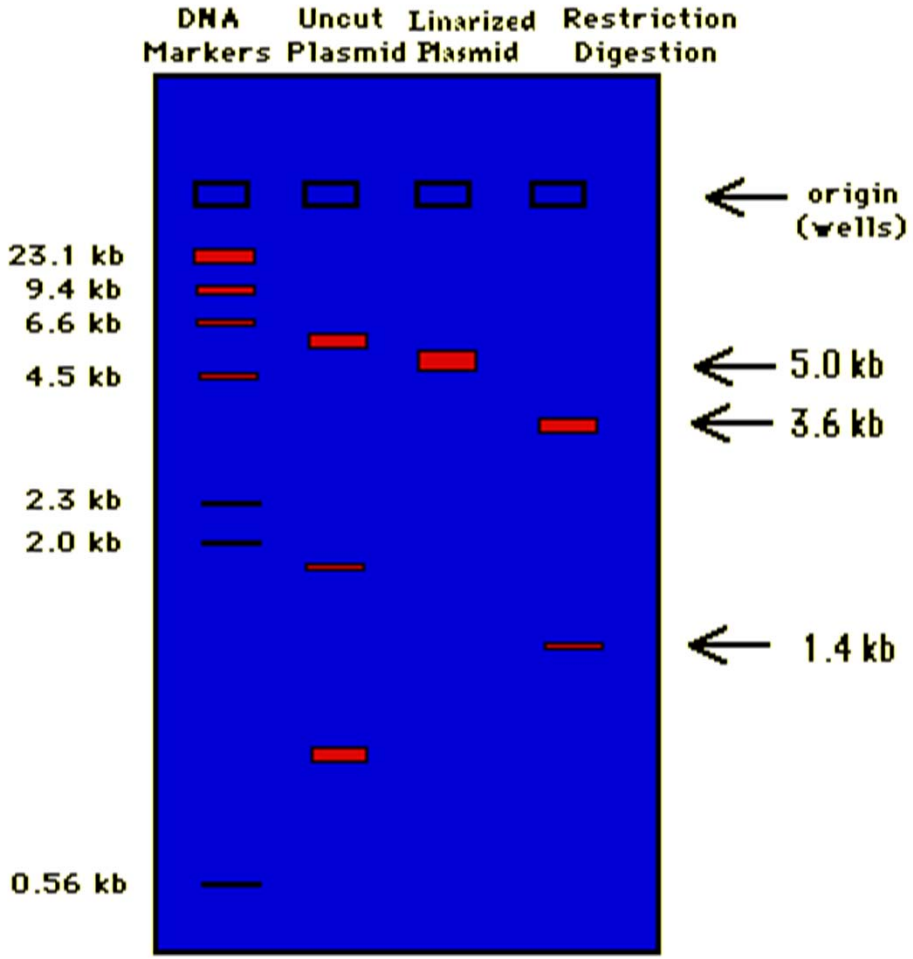
2) There is a Bam HI site in the insert. If this is the case, then the enzyme will cut the circular plasmid in two places, in the pBluescript part of the plasmid and in the insert.

- In 2nd case, you will end up with two fragments of DNA. One will be pBluescript with some of the insert still attached and the other will be just insert. The sizes of the two fragments (determined by electrophoresis) will tell you where the site is. These two possibilities are shown below





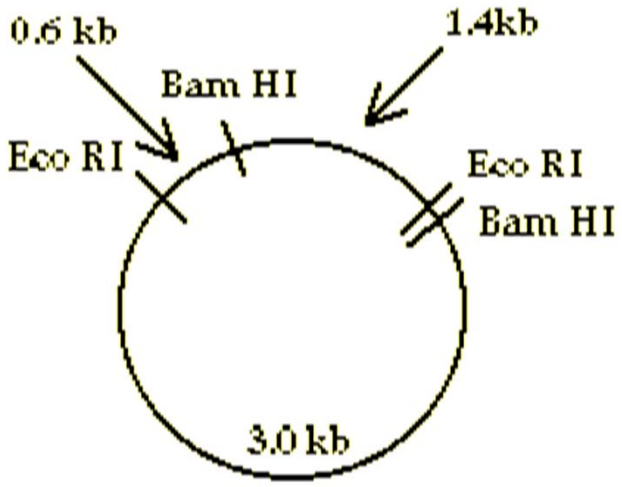
- In the second case, where there is a site in the insert, the gel might look like this:



- This case,show two pieces of information:
  - ✓ That there is a Bam HI site in the insert,
  - ✓ where the site is in relation to the one end of the insert.

- When the Bam HI digestion is separated on an agarose gel, the sizes of the two fragments can be determined. In the above gel, the fragments are 3.6 kb and 1.4 kb. Therefore, we know that the Bam HI site is 1.4 kb away from the right hand side of the insert . In this way, you have "mapped" the Bam HI site:

- By testing the insert for the presence and location of sites of many different restriction enzymes,a "restriction map" of the clone is made.



pBluescript + insert