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Topic 15:Recombinant DNA Technology

Recombinant DNA Technology

- Recombinant DNA Technology is the use of laboratory molecular techniques to isolate and manipulate fragments of DNA (recombinant DNA molecules).

- The first successes in making recombinant DNA molecules were made in the early 1970s by two research groups at Stanford University.

Uses and Applications of recombinant DNA Technology

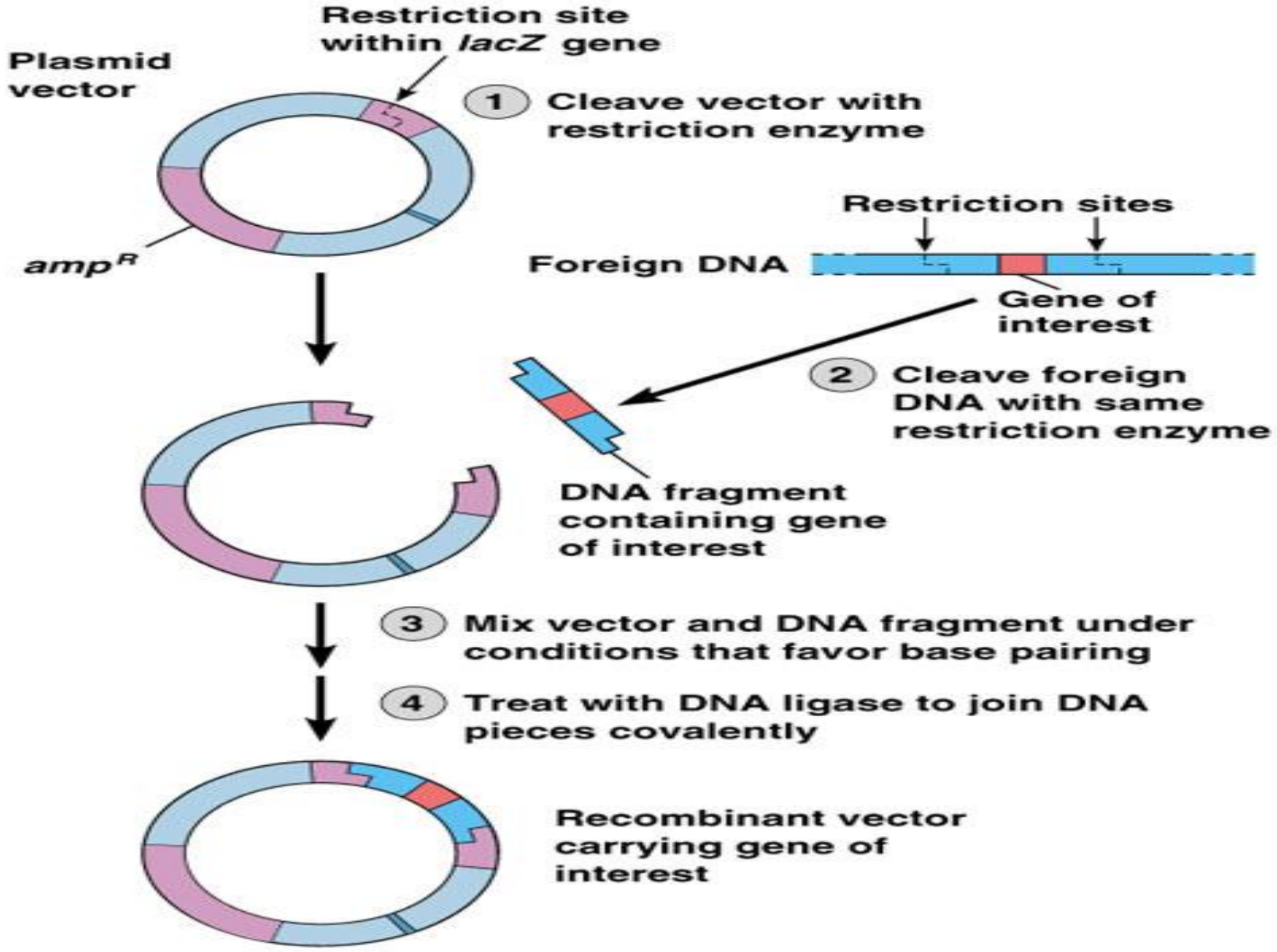
1. Improve our understanding of gene structure and function.
2. Gene therapy
3. Production of transgenic plants and animals in agriculture: the cloned gene from one species is transferred to other species.

How Do Researchers Make Multiple Copies of Recombinant DNA?

1. Cloning
2. PCR
3. Making cDNA from mRNA by reverse transcriptase.

Gene Cloning

- Refers to isolating and making many copies of a gene. Involves the introduction of a single recombinant DNA (gene and plasmid) molecule into a bacterial host cell.
- A gene is removed from its native site within a chromosome and inserted into a smaller segment of DNA called "**vector**".
- The purpose of the vector is to act as the carrier of the gene to be cloned.
- When the vector is introduced into a living cell (called host cell) it can replicate itself in the cell and produce many identical copies of the gene.

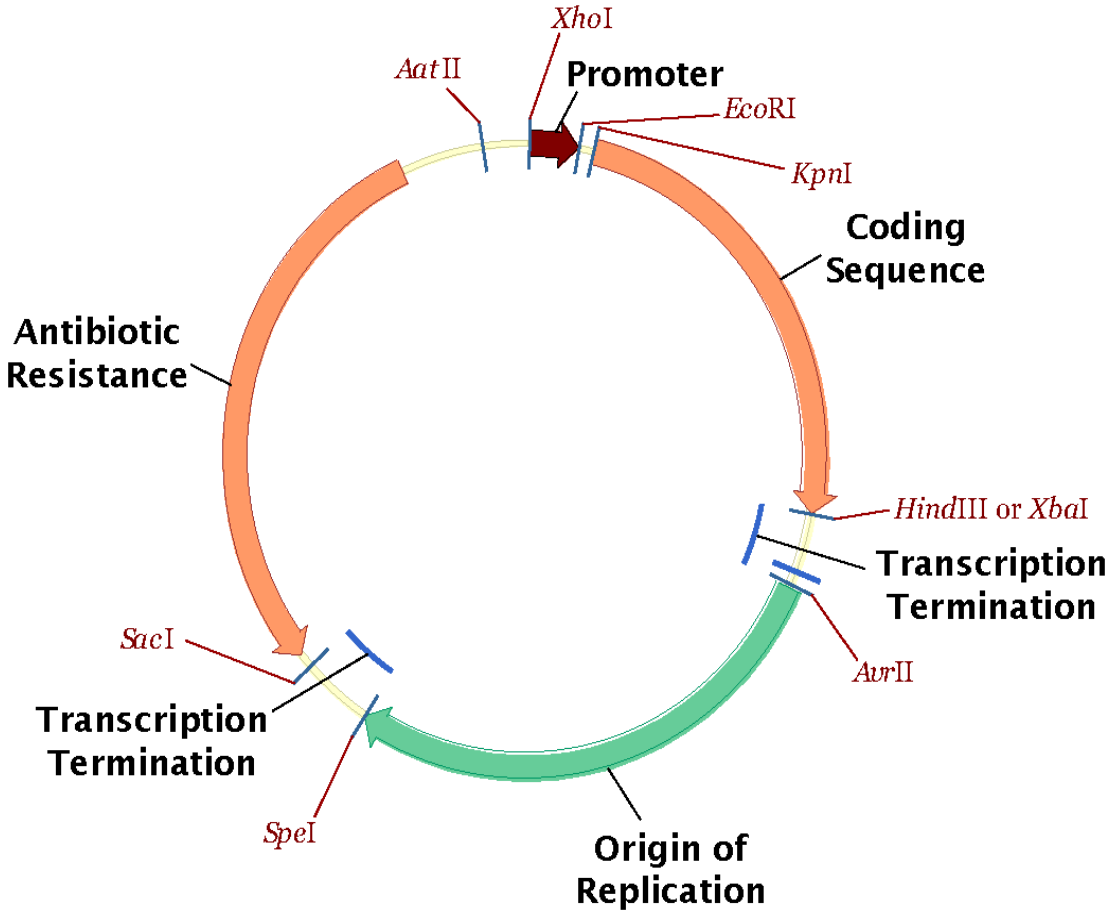


(b) Preparation of recombinant plasmid vector

Types of Vectors

1. **Plasmids:** small circular pieces of DNA. They are found naturally in many strains of bacteria.

- Many naturally occurring plasmids carry genes that confer resistance to antibiotics and other toxic substances. These plasmids are called **R factors**.
- Plasmids also contain a DNA sequence known as the **origin of replication** that is recognized by the replication enzymes of the host cell. This is the sequence which specifies the host cell specificity (determines the type of bacteria which can host the plasmid)


XhoI
AatII
Promoter
EcoRI
KpnI
Coding Sequence
Antibiotic Resistance
HindIII or XbaI
Transcription Termination
AvrII
SacI
Transcription Termination
SpeI
Origin of Replication

2. **Viral vectors:** viruses can infect living cells and propagate themselves. Therefore they can be used as vectors to carry genes and replicate them in host cells.

   Plasmids and viruses can accept small inserts (only few thousands of nucleotides in length).

3. **Cosmid:** is a hybrid between a plasmid and phage λ. Its DNA can replicate in a cell like a plasmid or be packaged into a protein coat like a phage. Can accept DNA inserts that are tens of thousands of bp.

4. **YAC** (Yeast Artificial Chromosome) and BAC (Bacterial Artificial Chromosome). These vectors can carry much larger segments of DNA than plasmids and viruses (several hundred thousand to 2 million nucleotides in length).

Table 5.2 Examples of vectors generally available for cloning DNA fragments

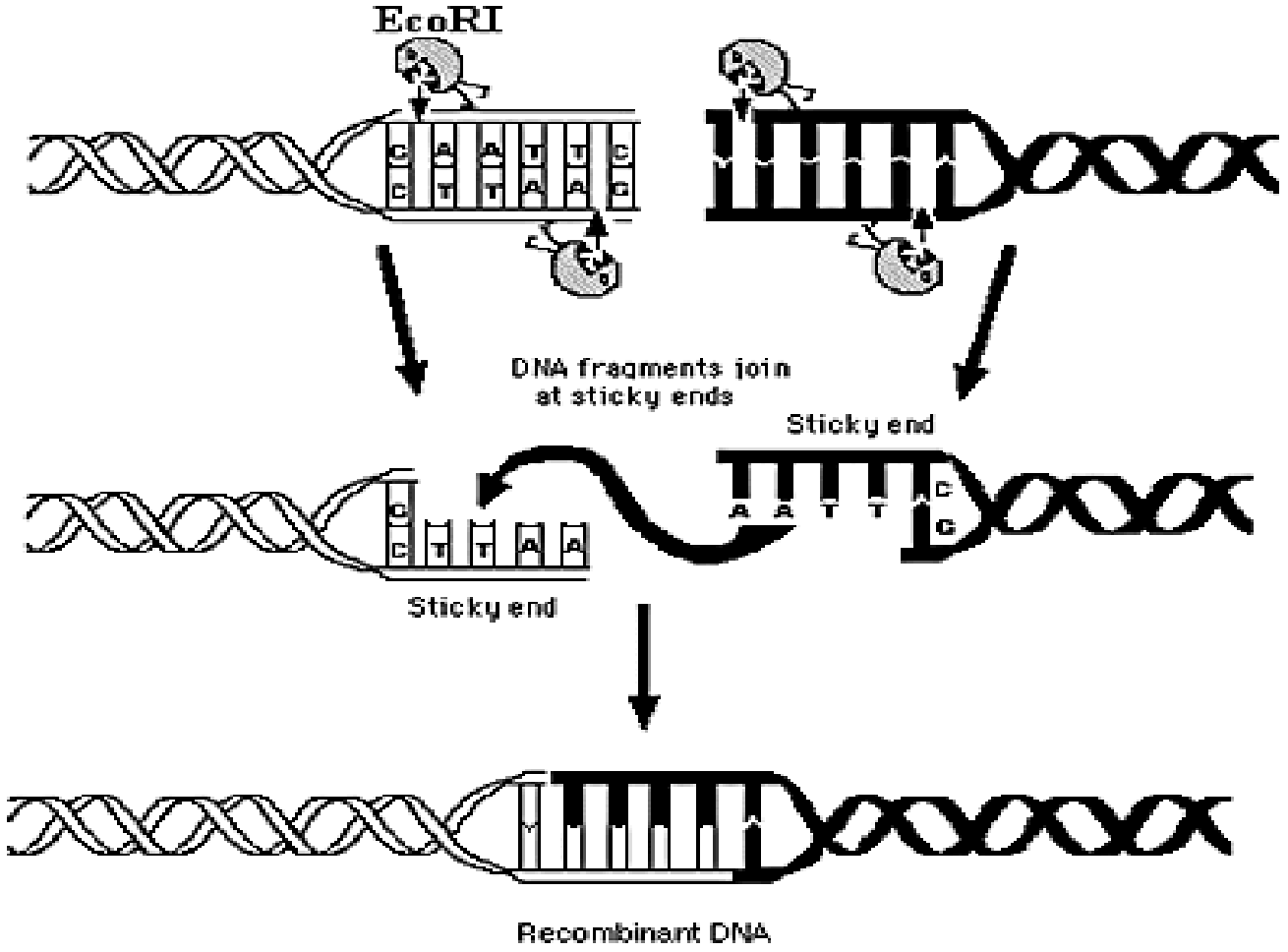
Vector	*Host Cell*	*Vector Structure*	*Insert Range (kb)*
M13	*E. coli*	Circular virus	1–4
Plasmid	*E. coli*	Circular plasmid	1–5
Phage λ	*E. coli*	Linear virus	2–25
Cosmids	*E. coli*	Circular plasmid	5–45
BACs[a]	*E. coli*	Circular plasmid	50–500
YACs[b]	*S. cerevisiae*	Linear chromosome	100–2000

[a]BAC: bacterial artificial chromosome
[b]YAC: yeast artificial chromosome

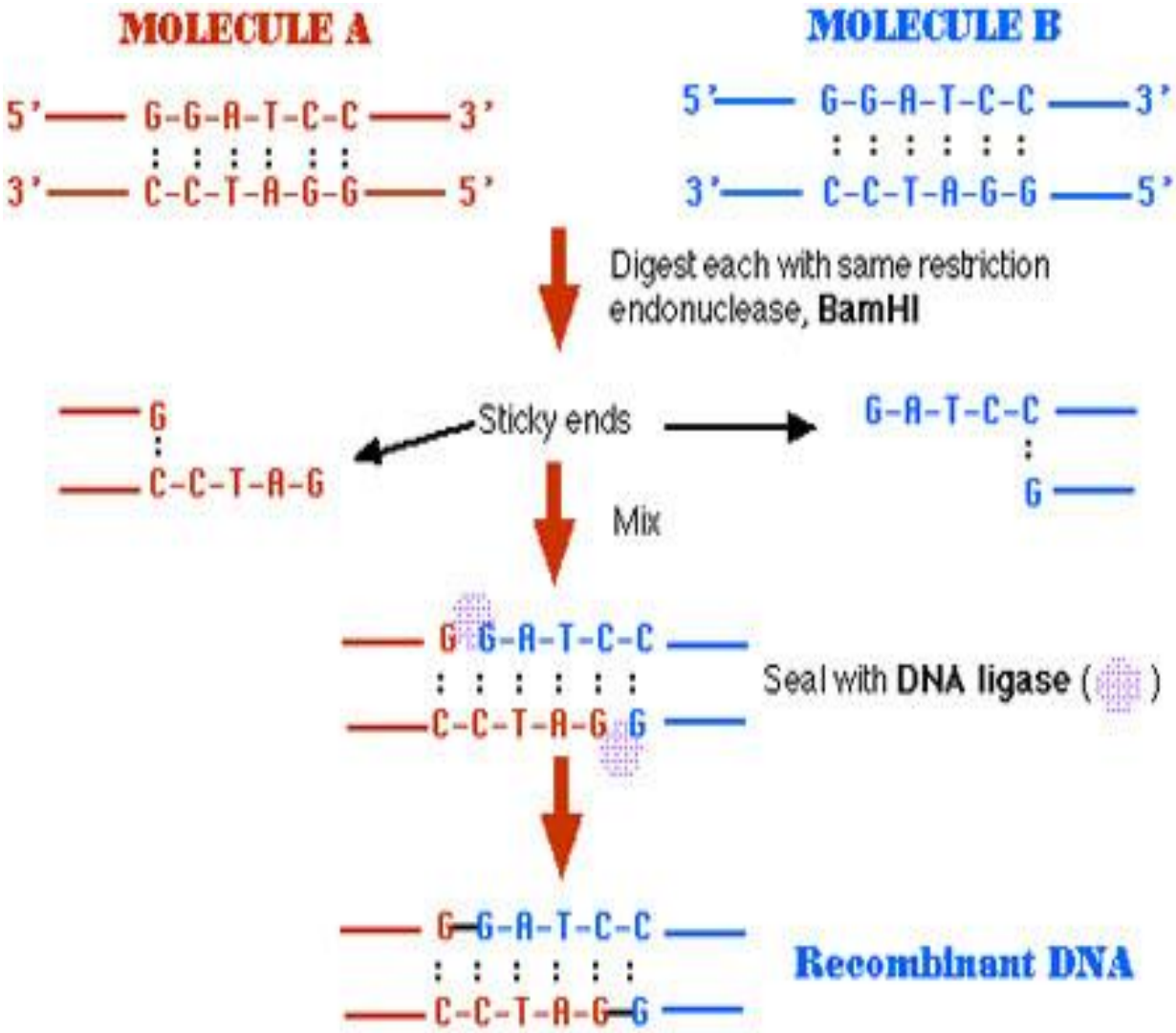
- Insertion of the chromosomal DNA fragment into the vector requires the cutting and pasting of DNA fragments. To cut DNA, researchers use enzymes known as "**Endonucleases**" or "**restriction enzymes**".
- They have been called **Restriction Enzymes** because they restrict viral replication.
- The restriction enzymes used in cloning experiments bind to a specific base sequence and then cleave the foreign DNA backbone at two defined locations, one on each strand.
- They are made naturally by many different species of bacteria.

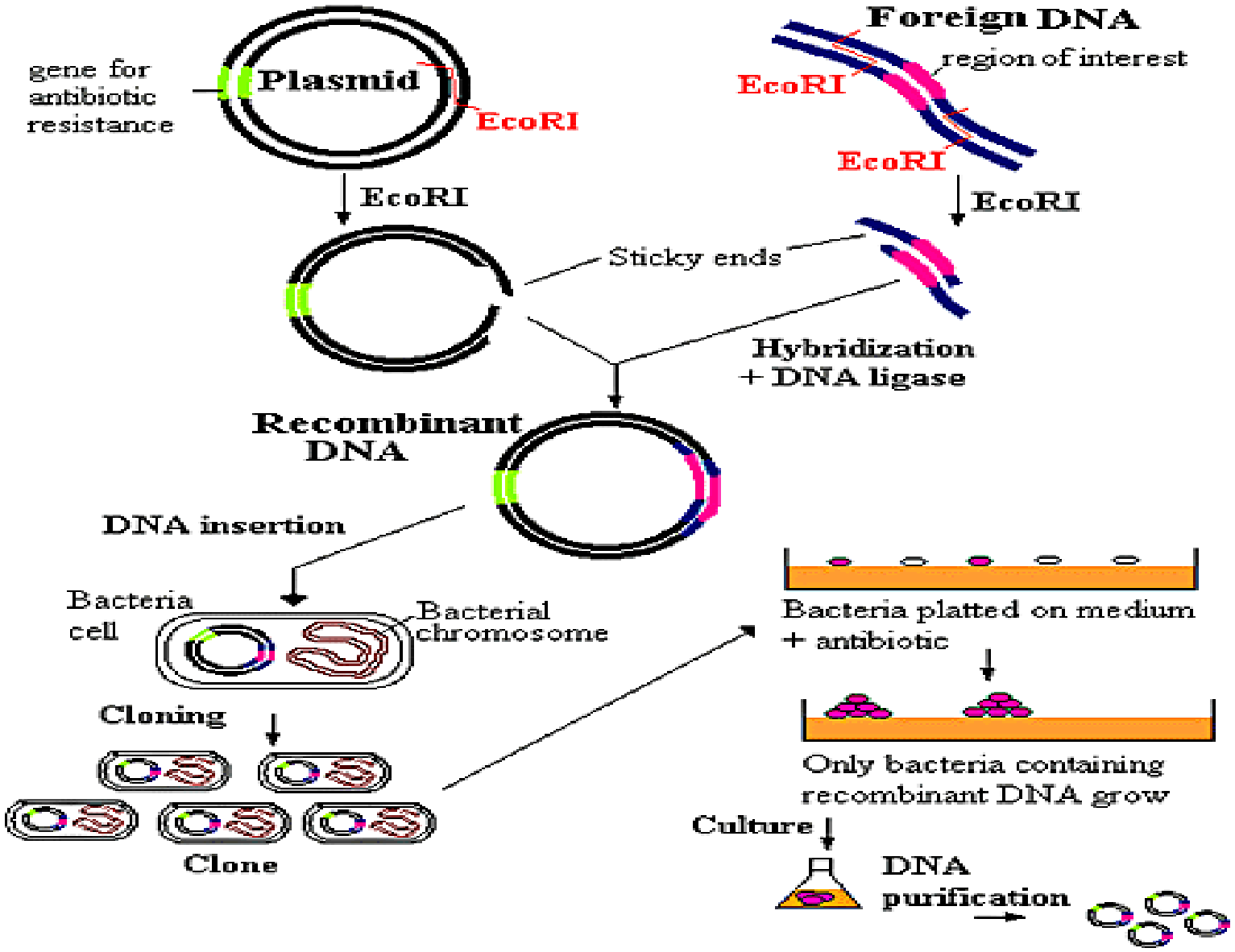
- Restriction enzymes usually recognize sequences that are **palindromic** (the sequence is identical when read in the opposite direction in the complementary strand).
- Example: the sequence recognized by the enzyme ***EcoR1 is:*** ***5` -GAATTC- 3`***

  3` -CTTAAG- 5`
- *Restriction enzymes usually cut DNA into fragments with* ***"sticky ends".*** *These DNA fragments can hydrogen bond to each other due to their complementary sequences.*
- *The fragments are called* ***"restriction fragments"***
- Restriction fragments are pieces of DNA that begin and end with a restriction site.



Restriction Enzyme Action of EcoRI


MOLECULE A
5' G-G-A-T-C-C 3'
3' C-C-T-A-G-G 5'
MOLECULE B
5' G-G-A-T-C-C 3'
3' C-C-T-A-G-G 5'
Digest each with same restriction endonuclease, BamHI
G
C-C-T-A-G
Sticky ends
G-A-T-C-C
G
Mix
G G-A-T-C-C
C-C-T-A-G G
Seal with DNA ligase ( )
G G-A-T-C-C
C-C-T-A-G G
Recombinant DNA



Cloning into a plasmid

PCR (Polymerase Chain Reaction)

Allows researchers to produce multiple numbers of individual DNA sequences in a very short period of time. In PCR:

1)The selected DNA segment (template) is heated causing the two strands to separate (**denaturation step**).

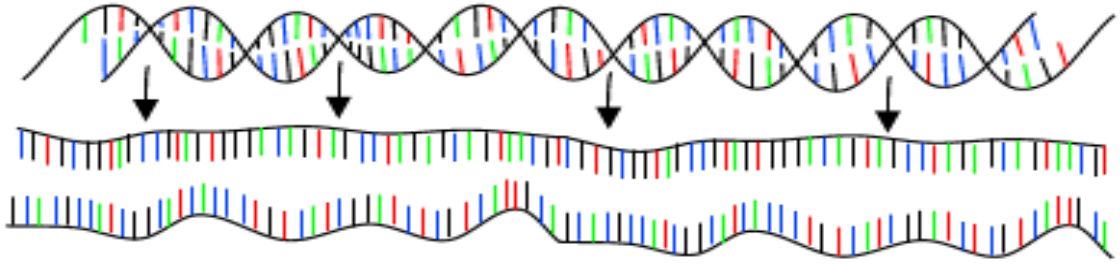
2)The DNA is cooled and two short nucleotide sequences termed primers bind to the complementary DNA strands (**annealing step)**

3)DNA polymerase (called **Taq polymerase**) then copies each strand until the researcher stops the reaction by again raising the temperature (**extension step**).

4)Increasing the temperature repeats the process.

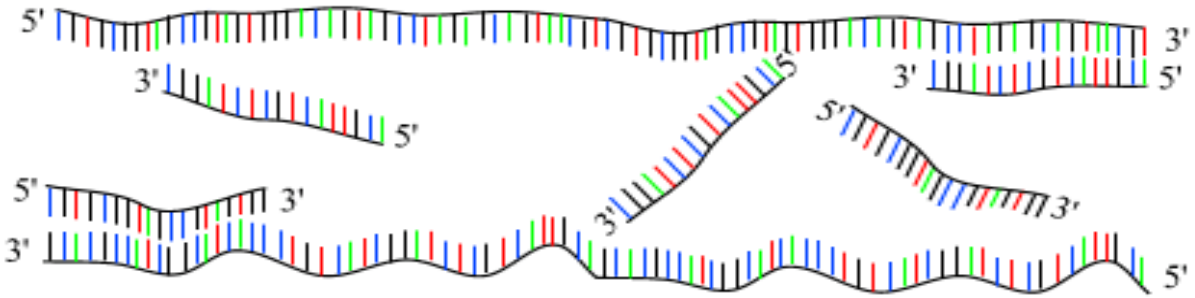
PCR : Polymerase Chain Reaction

30 - 40 cycles of 3 steps :



Step 1 : denaturation

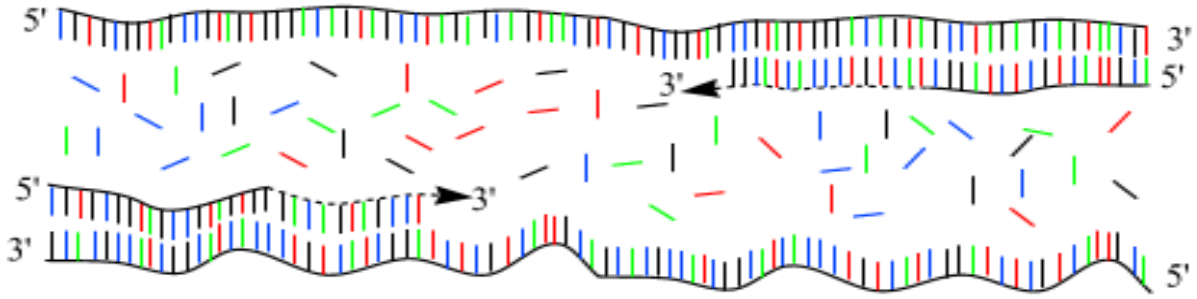
1 minut 94 °C



Step 2 : annealing

45 seconds 54 °C

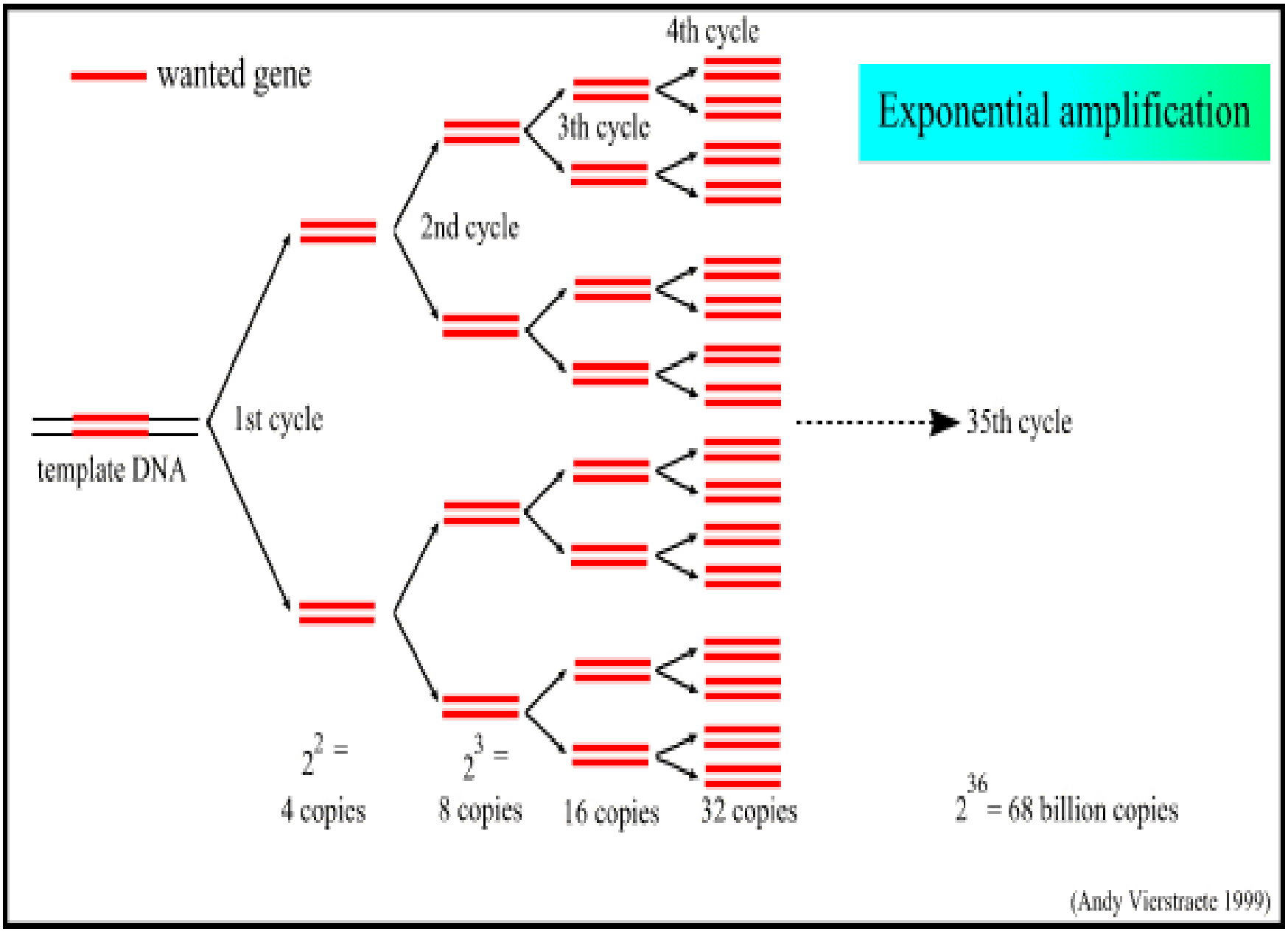
forward and reverse primers !!!



Step 3 : extension

2 minutes 72 °C
only dNTP's

(Andy Vierstraete 1999)


wanted gene
Exponential amplification
4th cycle
3th cycle
2nd cycle
1st cycle
template DNA
35th cycle
2^2 =
4 copies
2^3 =
8 copies
16 copies
32 copies
2^{36} = 68 billion copies
(Andy Vierstraete 1999)

cDNA

- RNA can be used to clone DNA.
- cDNA (complementary DNA) can be made from mRNA by the enzyme **reverse transcriptase**.
- The enzyme reverse transcriptase can use RNA as a template to make a complementary strand of DNA.
- The RNA is purified from a sample of cells and mixed with primers
- Deoxyribonucleotides and reverse transcriptase are then added to make DNA strands that are complementary to the RNA.

Gel Electrophoresis

- Electrophoresis in **agarose** or **polyacrylamide** gels is the most usual way to separate DNA molecules according to size

- The easiest and most widely applicable method is electrophoresis in horizontal agarose gels, followed by **staining** with ethidium bromide.

- This dye binds to DNA by insertion between stacked base pairs (intercalation) and it exhibits a strong **orange/red fluorescence when illuminated with ultraviolet light.**

Gel Electrophoresis

- Shorter molecules move faster and migrate farther than longer ones because shorter molecules migrate more easily through the pores of the gel. This phenomenon is called sieving.
- Most agarose gels are made with 0.7% (good separation or resolution of large 5–10kb DNA fragments) to 2% agarose (good resolution for small 0.2–1kb fragments). 1% gels are common for many applications

Electrophoresis chamber

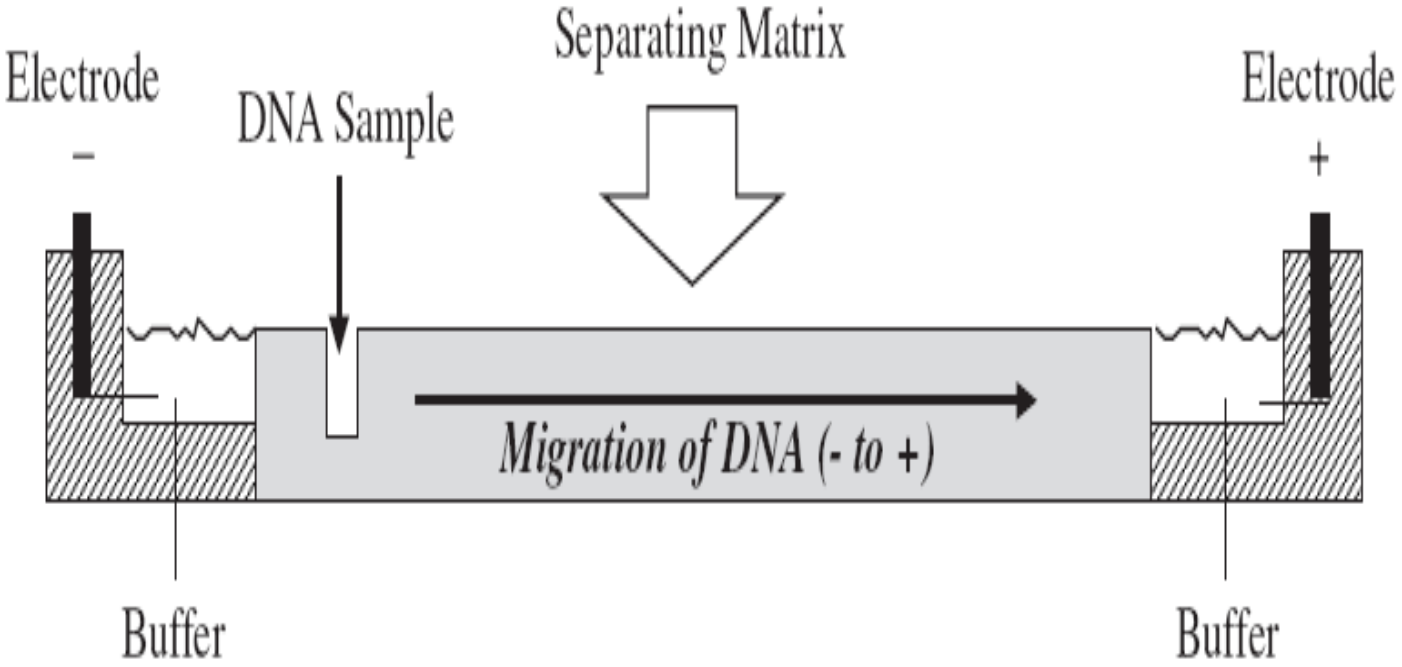
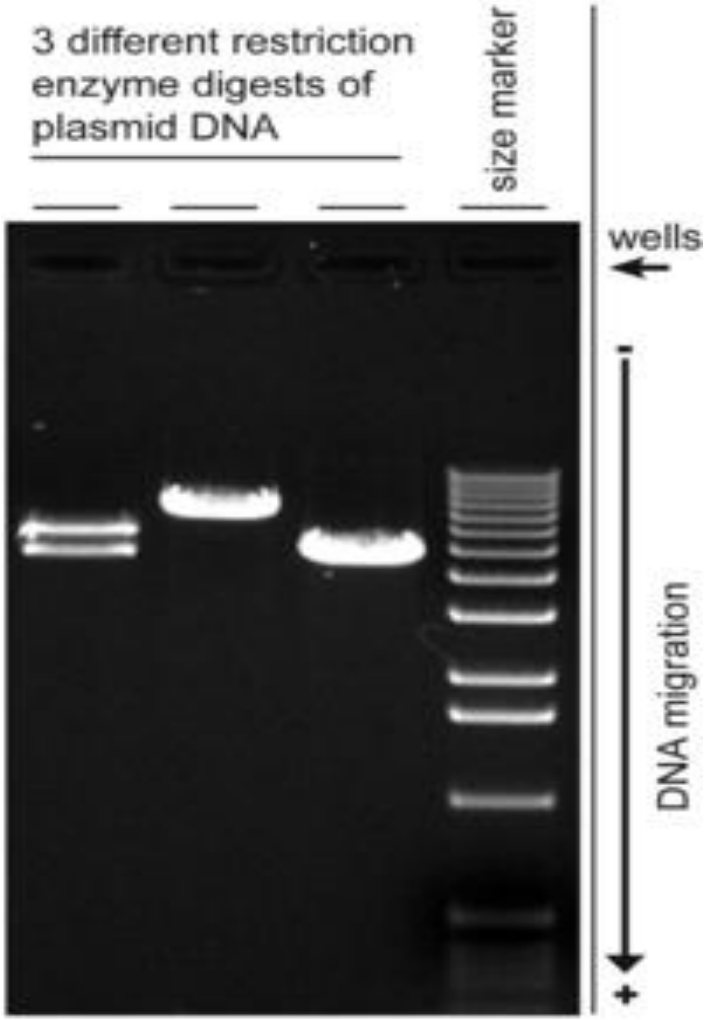


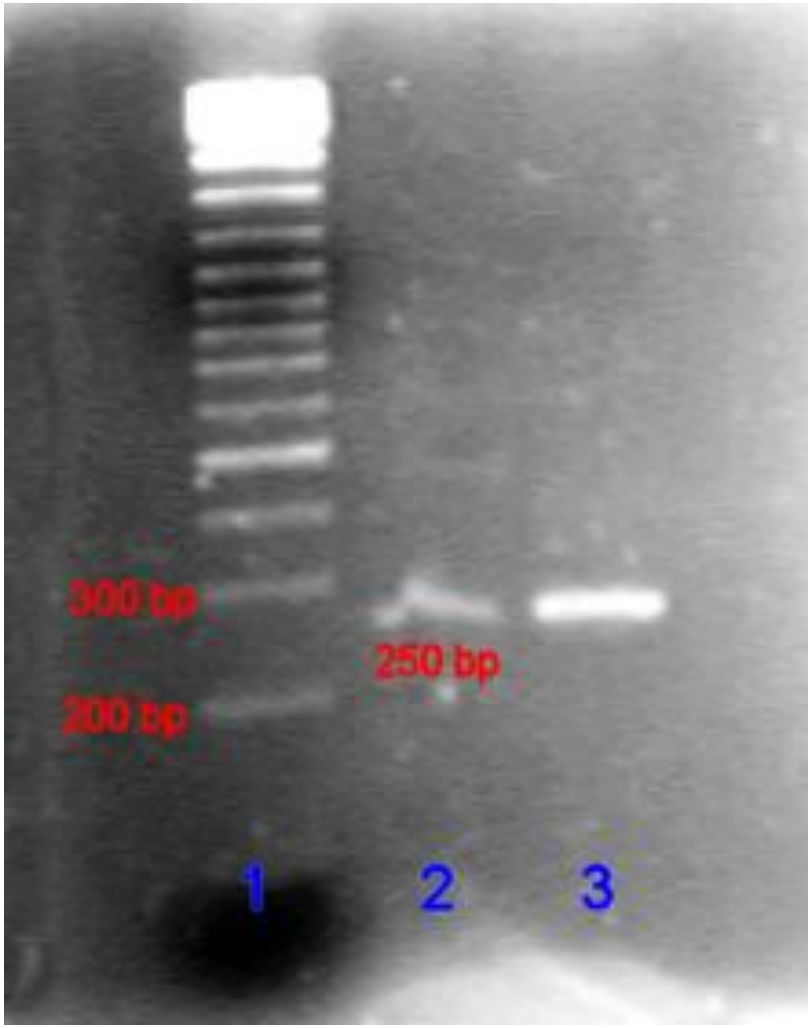
Figure 1.3 A typical setup required for agarose gel electrophoresis of DNA. The upper panel indicates a cross-section of the unit used for gel electrophoresis.

Molecules with different sizes form distinct bands on the gel



Source: http://en.wikipedia.org/wiki/Gel_electrophoresis

An agarose gel of a PCR product compared to a DNA ladder



Source: http://en.wikipedia.org/wiki/Gel_electrophoresis

Types of buffers in Gel Electrophoresis

- There are a number of buffers used for electrophoresis.
- The most common for nucleic acids:
  - **Tris/Acetate/EDTA (TAE)**
  - **Tris/Borate/EDTA (TBE).**

Gel Electrophoresis

- **Agarose gels** can be used to separate molecules **larger than 100 bp**. For higher resolution or for the effective separation of **shorter DNA molecules, polyacrylamide gels** are the preferred method

- **Polyacrylamide** gel electrophoresis (**PAGE**) is used for separating proteins ranging in size from 5 to 2,000 kDa due to the uniform pore size provided by the polyacrylamide gel

Gel Electrophoresis

- An electric field (– to +) is applied across the gel from the top phase (stacking gel) to the bottom phase (resolving gel), causing the negatively charged "current-carrying" anions to migrate down through the gel toward the positive electrode.
- In **polyacrylamide** gels:

  - **resolving gel**: 6%, 8%, 10%, 12% or 15% polyacrylamide

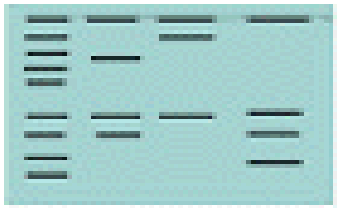
  - **stacking gel**: 5% polyacrylamide (has lower % polyacrylamide)

➔ The percentage chosen depends on the size of the protein that one wishes to identify or probe in the sample. **The smaller the known weight, the higher the percentage that should be used**

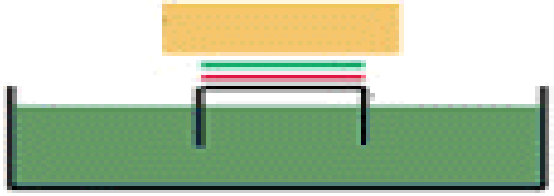
Detection of genes and gene products

- **Southern blotting:** used to detect DNA sequences. **Steps:**

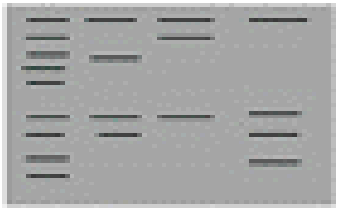
1. A sample of DNA is digested into small fragments with a restriction enzyme.
2. The fragments are separated by gel electrophoresis and then denatured by soaking in NaOH
3. The DNA bands are then transferred to a nitrocellulose filter.
4. The filter is placed in a solution containing radiolabled DNA (probe) which causes binding between the probe and the searched DNA fragments.
5. The filter is exposed to X-ray to show the radiolabeld bands.



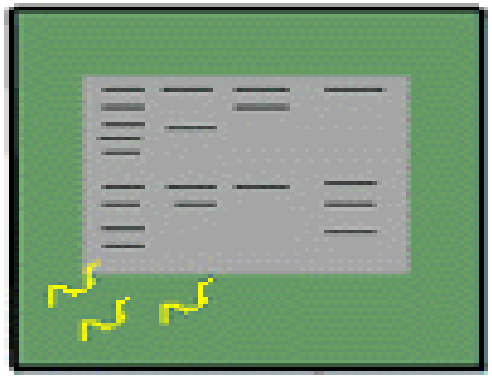
Place DNA fragments on an agarose gel and separate by electrophoresis



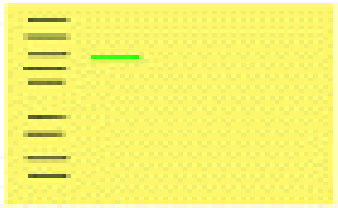
"Blot" DNA fragments from agarose gel onto membrane



Membrane imprinted with DNA bands



Add a labeled probe to the membrane (in buffer solution).



Detection (the method depends on the type of probe you use) reveals a band where your probe bound to the target sequence.

- **Northern blotting:** is used to detect RNA, this method can determine if a gene has been transcribed in a certain cell type or not.
- The technique is similar to southern plotting except that RNA fragments are used instead of DNA.
- RNAs that are complementary to the radiolabled DNA probes appear as dark bands on the X-ray film.

- **Western blotting** is used to detect proteins. The technique is similar to Southern and Western blotting but:

1. A technique called SDS-PAGE (Polyacrylamide gel electrophoresis) is used to separate the proteins on the gel.
2. Instead of using a radiolabled probe, an antibody is used as a probe.