Sengamala Thayaar Educational Trust Women's College
(Affiliated to Bharathidasan University)
(Accredited with 'A' Grade {3.45/4.00} By NAAC)
(An ISO 9001: 2015 Certified Institution)
Sundarakkottai, Mannargudi-614 016.
Thiruvarur (Dt.), Tamil Nadu, India.

III B.SC MICROBIOLOGY
SEMESTER VI
RECOMBINANT DNA TECHNOLOGY

Dr.M.Kannahi,
Assistant Professor ,
P.G Research Department of Microbiology

Recombinant DNA

- **Recombinant DNA (rDNA) molecules are DNA molecules formed by laboratory methods of genetic recombination (such as molecular cloning) to bring together genetic material from multiple sources, creating sequences that would not otherwise be found in the genome.**

- **Recombinant DNA is possible because DNA molecules from all organisms share the same chemical structure. They differ only in the nucleotide sequence within that identical overall structure.**

- **Recombinant DNA is the general name for a piece of DNA that has been created by the combination of at least two strands.**

- **Recombinant DNA molecules are sometimes called chimeric DNA, because they can be made of material from two different species, like the mythical chimera.**

- **R-DNA technology uses palindromic sequences and leads to the production of sticky and blunt ends.**

Enzymes involved in rDNA Technology

1. **Restriction endonuclease, RE**
2. **DNA ligase**
3. **Reverse transcriptase**
4. **DNA polymerase, DNA pol**
5. **Nuclease**
6. **Terminal transferase**

Restriction Enzymes and DNA Ligases Allow Insertion of DNA Fragments into Cloning Vectors

- **The DNA sequences used in the construction of recombinant DNA molecules can originate from any species.**
- **For example, plant DNA may be joined to bacterial DNA, or human DNA may be joined with fungal DNA.**

Stanley N. Cohen , who received the Nobel Prize in Medicine in 1986 for his work on discoveries of growth factors.

Stanley N. Cohen (1935–) (top) and Herbert Boyer (1936–) (bottom), who constructed the first recombinant DNA using bacterial DNA and plasmids.

Six steps of Recombinant DNA

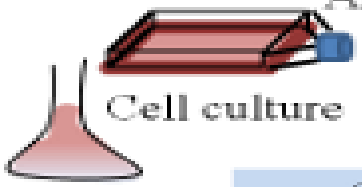
1. **Preparation of desired gene (vector and target gene)**
2. **Isolation of DNA vector (Cleavage)**
3. **Construction of rDNA (Ligation)**
4. **Introduction of rDNA in to host cell (Transforming)**
5. **Selection and multiplication of recombinant host cell (Cloning)**
6. **Expression of cloned gene (Screening)**

Preparation of desired gene (vector and target gene)

To construct rDNA, the desired gene can be isolated from

1. Genomic DNA
2. mRNA
3. Free nucleotides by chemical synthesis

DNA extraction



Any source

Cell culture

Tissue extract

Cell Lysis
Physical/Chemical

Wash steps

Detergents/ Separation of DNA by specific adsorption to membranes

Removal of membrane lipids

Wash steps

Protease treatment

Protein denaturation and removal

Wash steps

Removal of other cellular contaminants

RNase treatment

RNA removal

Elute in slightly alkaline buffer/ double distilled water

Purified DNA

Desired gene from mRNA

Isolation of mRNA

↓

Fractionation of mRNA by Gel Electrophoresis

↓

Treatment of mRNA with reverse trancriptase with deoxy ribonucleotide

↓

Synthesis of cDNA

↓

Separation of cDNA from mRNA by alkali treatment

↓

Treatment of cDNA with DNA Polymerase and a primer

Formation of cDNA clone

↓

cDNA used to construct rDNA

Free nucleotides by chemical synthesis

- **Small oligonucleotides can be synthesized from free nucleotides using gene machine**
- **The oligonucleotides are bound together to get a complete gene**

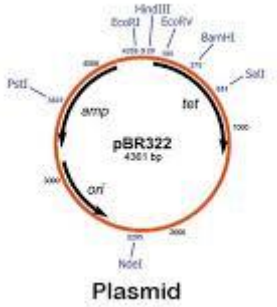
Vectors- Cloning Vehicles

A cloning vector is a small piece of DNA, taken from a virus, a plasmid, or the cell of a higher organism, that can be stably maintained in an organism, and into which a foreign DNA fragment can be inserted for cloning purposes.

- **All cloning vectors have in common at least one unique cloning site, a sequence that can be cut by a restriction endonuclease to allow site-specific insertion of foreign DNA.**
- **The most useful vectors have several restriction sites grouped together in a multiple cloning site (MCS) called a polylinker.**

Types of vector

1. **Plasmid Vectors**
2. **Bacteriophage Vectors**
3. **Virus vectors**
4. **Shuttle Vectors--can replicate in either prokaryotic or eukaryotic cells.**
5. **Yeast Artificial Chromosomes as Vectors**



Plasmid

Isolation of DNA vector (Cleavage)

The cell wall of bacteria is ruptured by treating with lysozyme and EDTA

↓

The cells are treated with SLS

↓

Centrifugation of cell wall debris and proteins

↓

Fractionation of supernatant using Cesium Chloride density gradient centrifugation

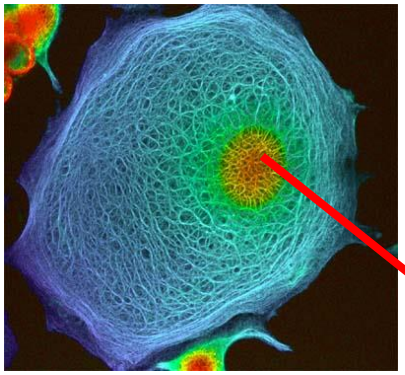
↓

Collection of plasmid from the lower layer of tube

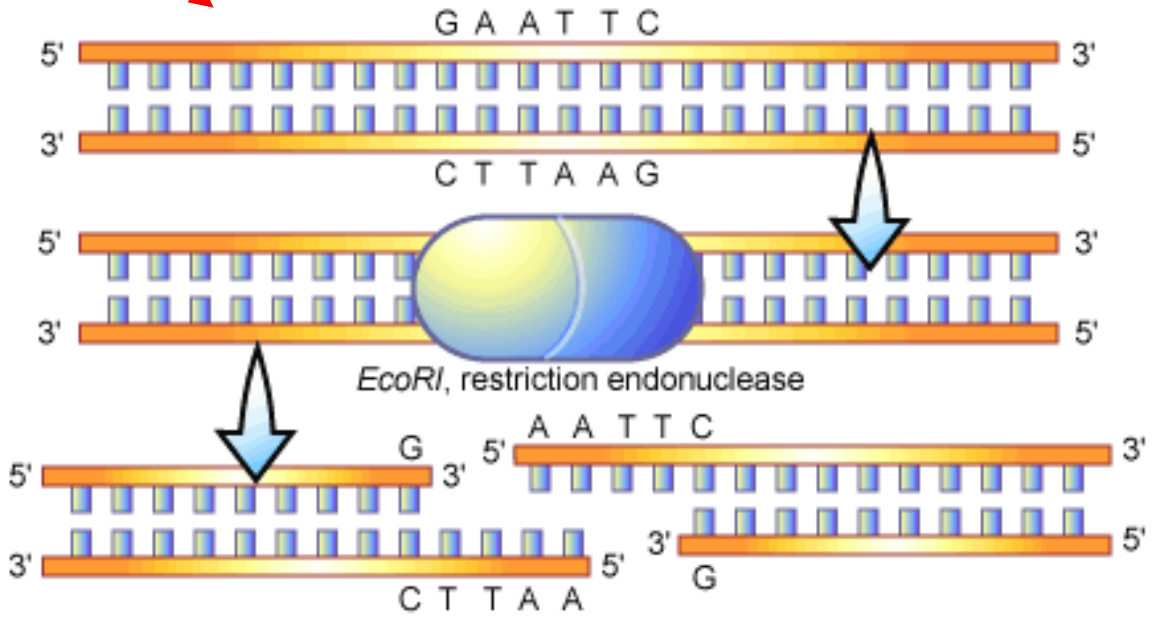
↓

Plasmid used to construct rDNA

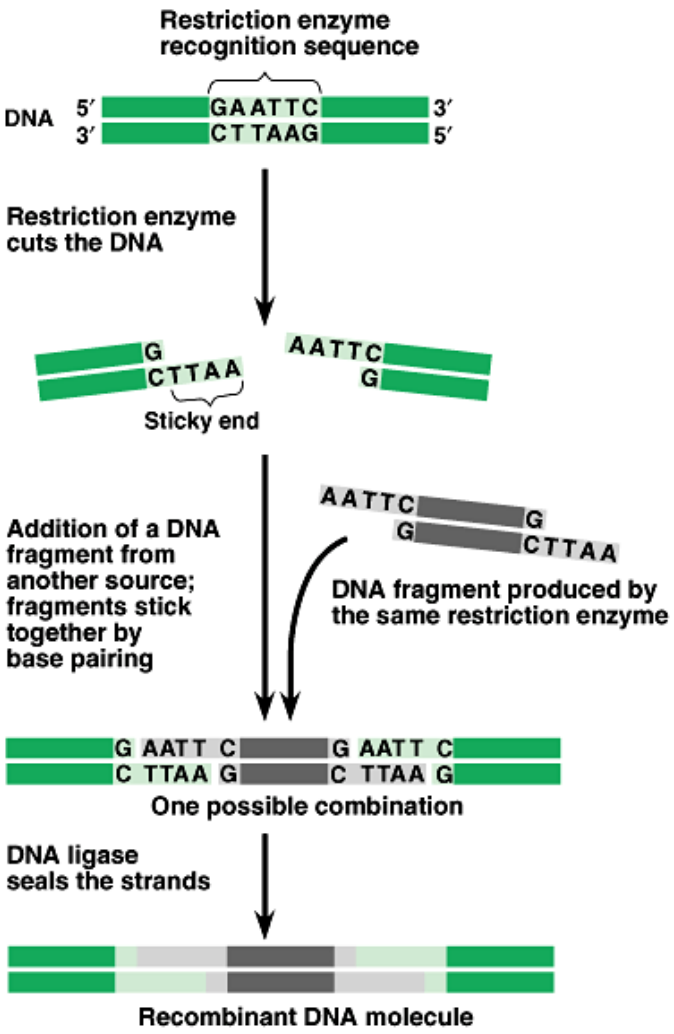
Cut out the gene



Restriction enzymes



Restriction enzymes cleave DNA



- The same sequence of bases is found on both DNA strands, but in opposite orders. GAATTC / CTTAAG
- This arrangement is called a palindrome. ***Palindromes are words or sentences that read the same forward and backward***.
- form **sticky ends**: single stranded ends that have a tendency to join with each other (the key to **recombinant DNA**)

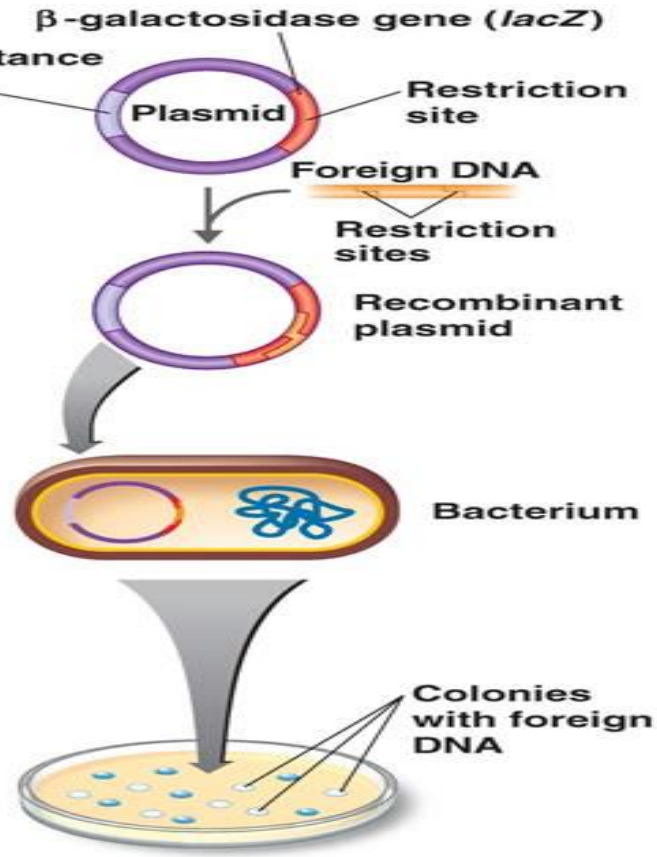
Ligation of DNA fragments.

A recombinant DNA molecule is usually formed by cleaving the DNA of interest to yield insert DNA and then ligating the insert DNA to vector DNA (*recombinant DNA* or *chimeric DNA*). DNA fragments are typically joined using *DNA ligase* (also commercially produced).

- **T4 DNA Ligase**

Construction and Introduction of rDNA in to host cell (Transforming)

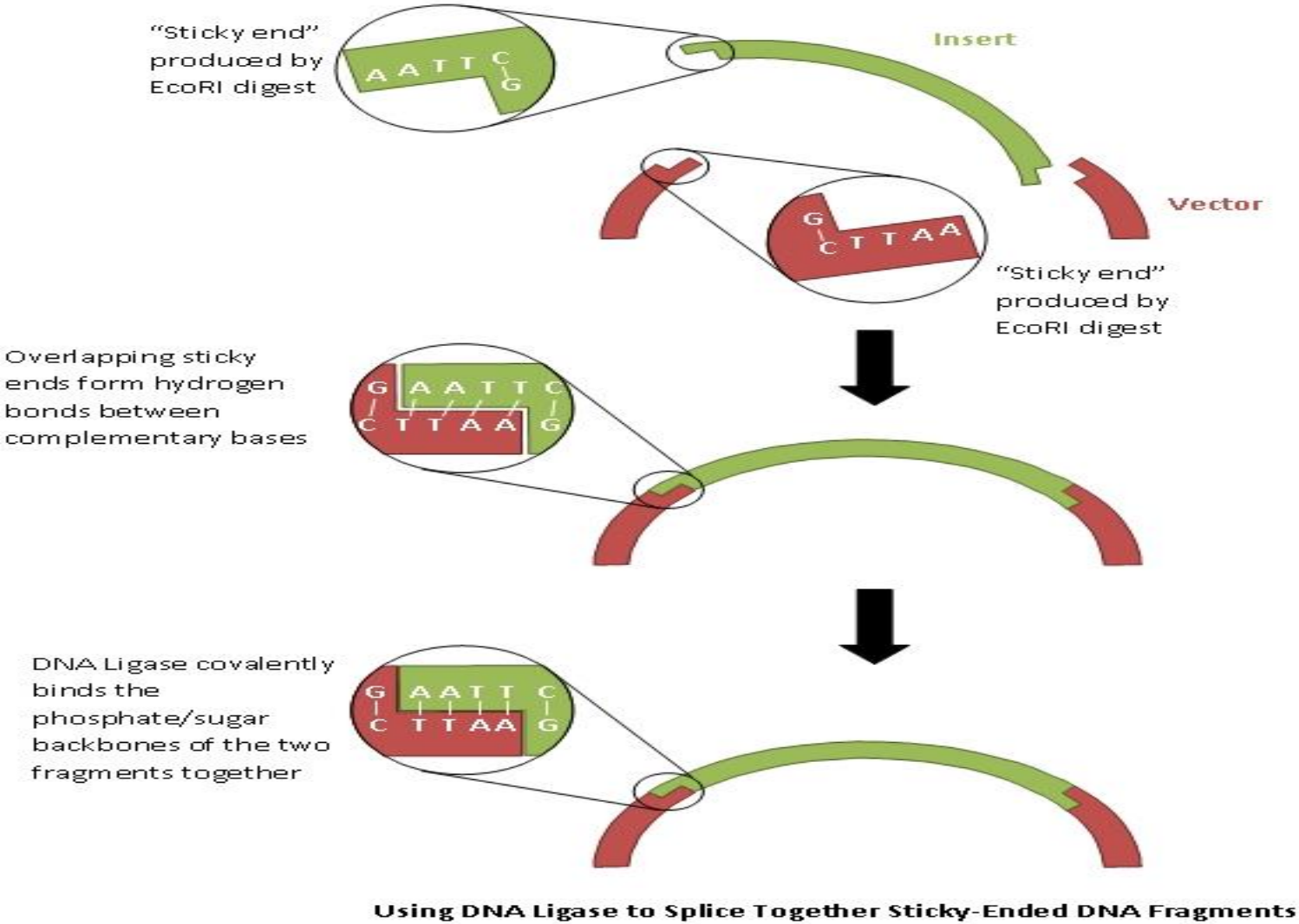
1. Plasmid DNA and foreign DNA are both cut with the same restriction enzyme. The plasmid has the genes for lactose hydrolysis (the *lacZ* gene encodes the enzyme β-galactosidase) and ampicillin resistance.
2. Foreign DNA will insert into the *lacZ* gene. The bacterium receiving the plasmid vector will not produce the enzyme β-galactosidase if foreign DNA has been inserted into the plasmid.
3. The recombinant plasmid is introduced into a bacterium, which becomes ampicillin resistant.
4. All treated bacteria are spread on a nutrient agar plate containing ampicillin and a β-galactosidase substrate and incubated. The β-galactosidase substrate is called X-gal.
5. Only bacteria that picked up the plasmid will grow in the presence of ampicillin. Bacteria that hydrolyze X-gal produce galactose and an indigo compound. The indigo turns the colonies blue. Bacteria that cannot hydrolyze X-gal produce white colonies.



Methods to construct rDNA

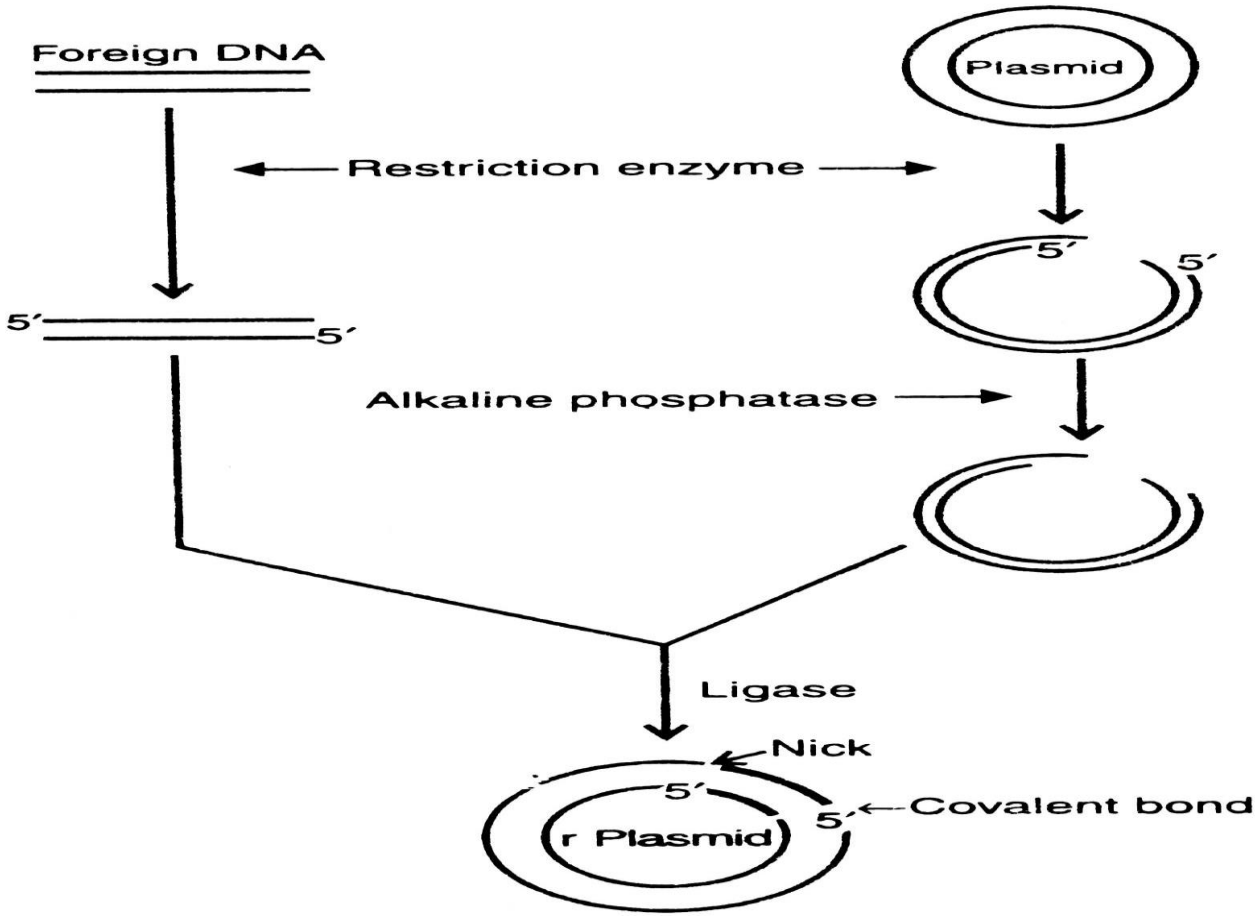
- Cohesive end ligation
- Cohesive end ligation using alkaline phosphatase
- Blunt end ligation
- Blunt end ligation using linkers
- Blunt end ligation using adaptors
- Homopolymer tailing

Cohesive end ligation

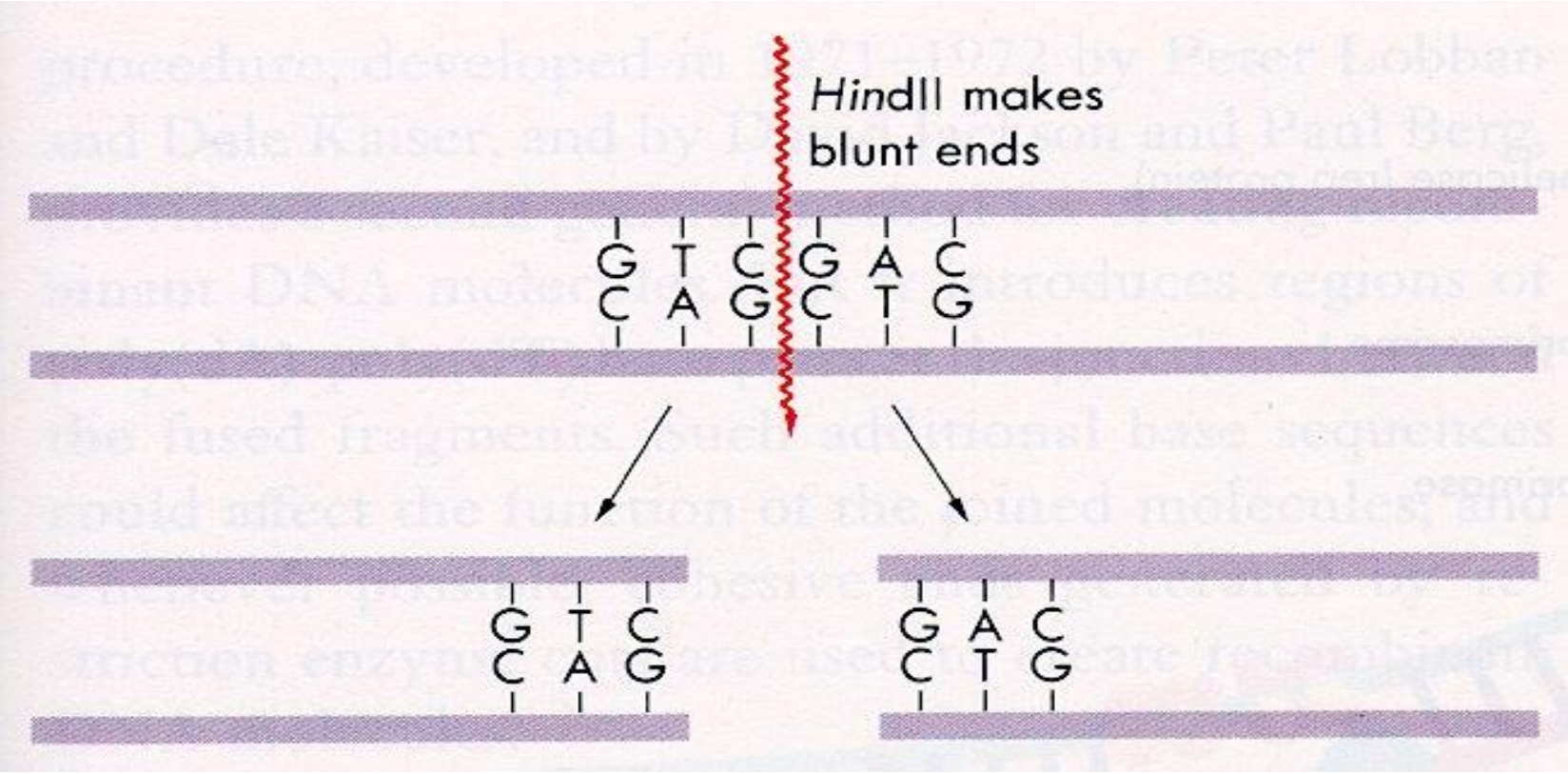


Using DNA Ligase to Splice Together Sticky-Ended DNA Fragments

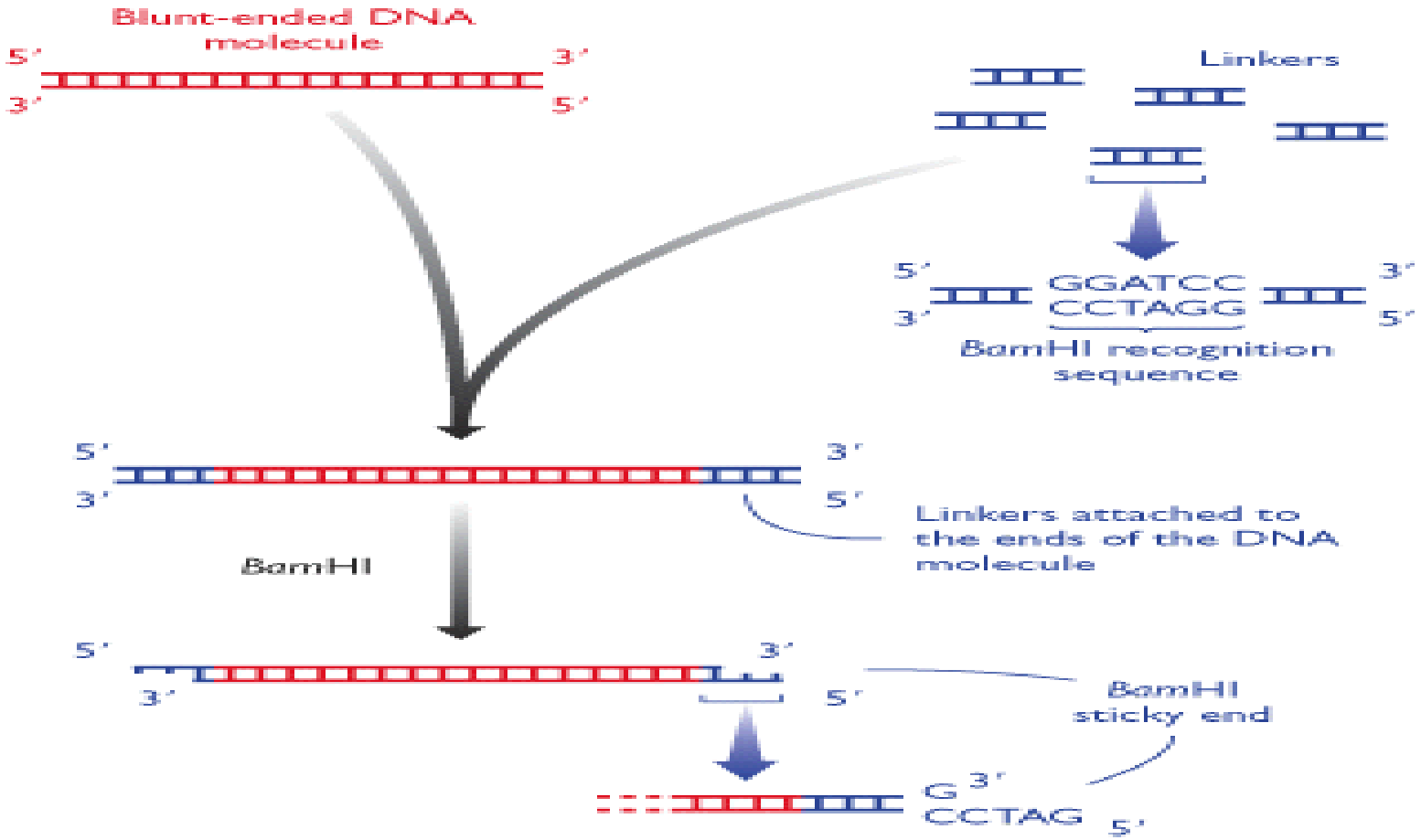
Cohesive end ligation using alkaline phosphatase



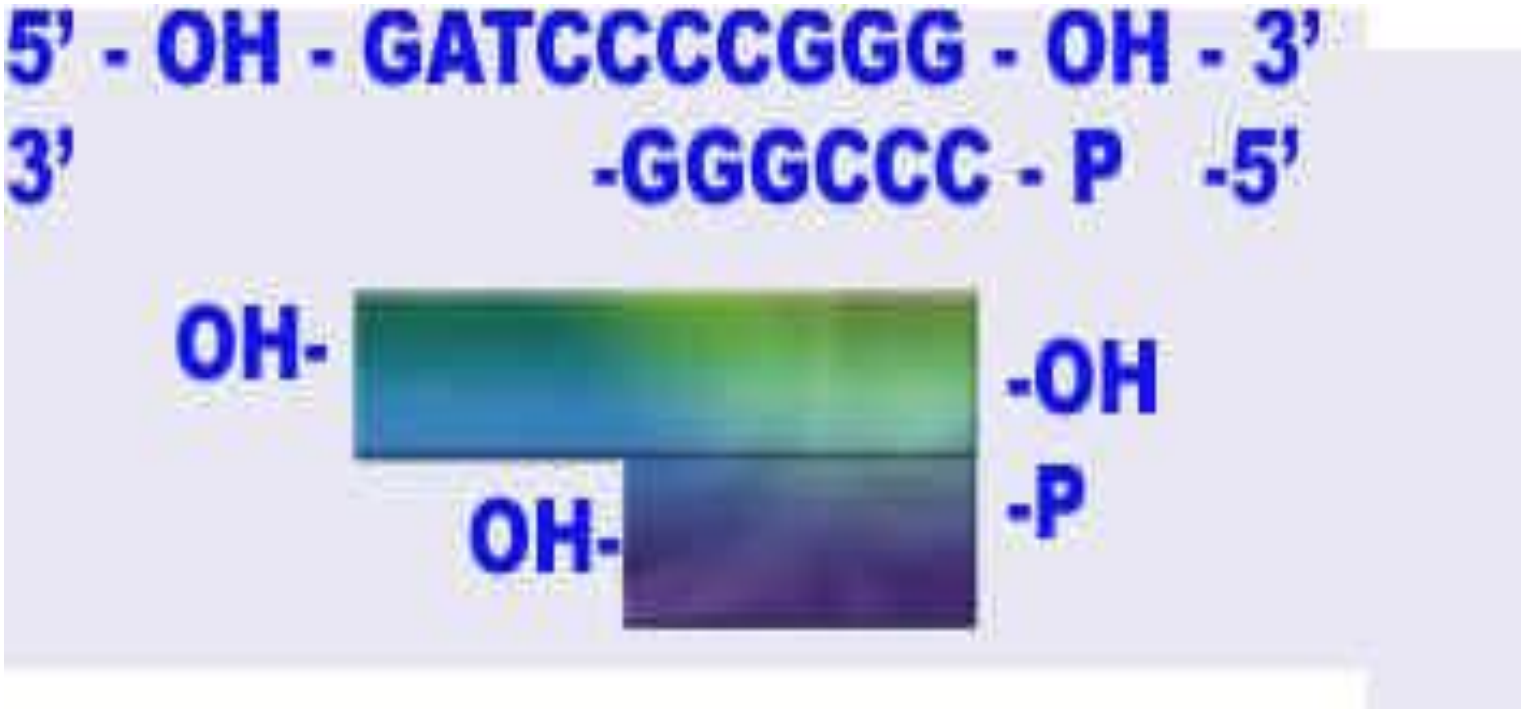
Blunt end ligation



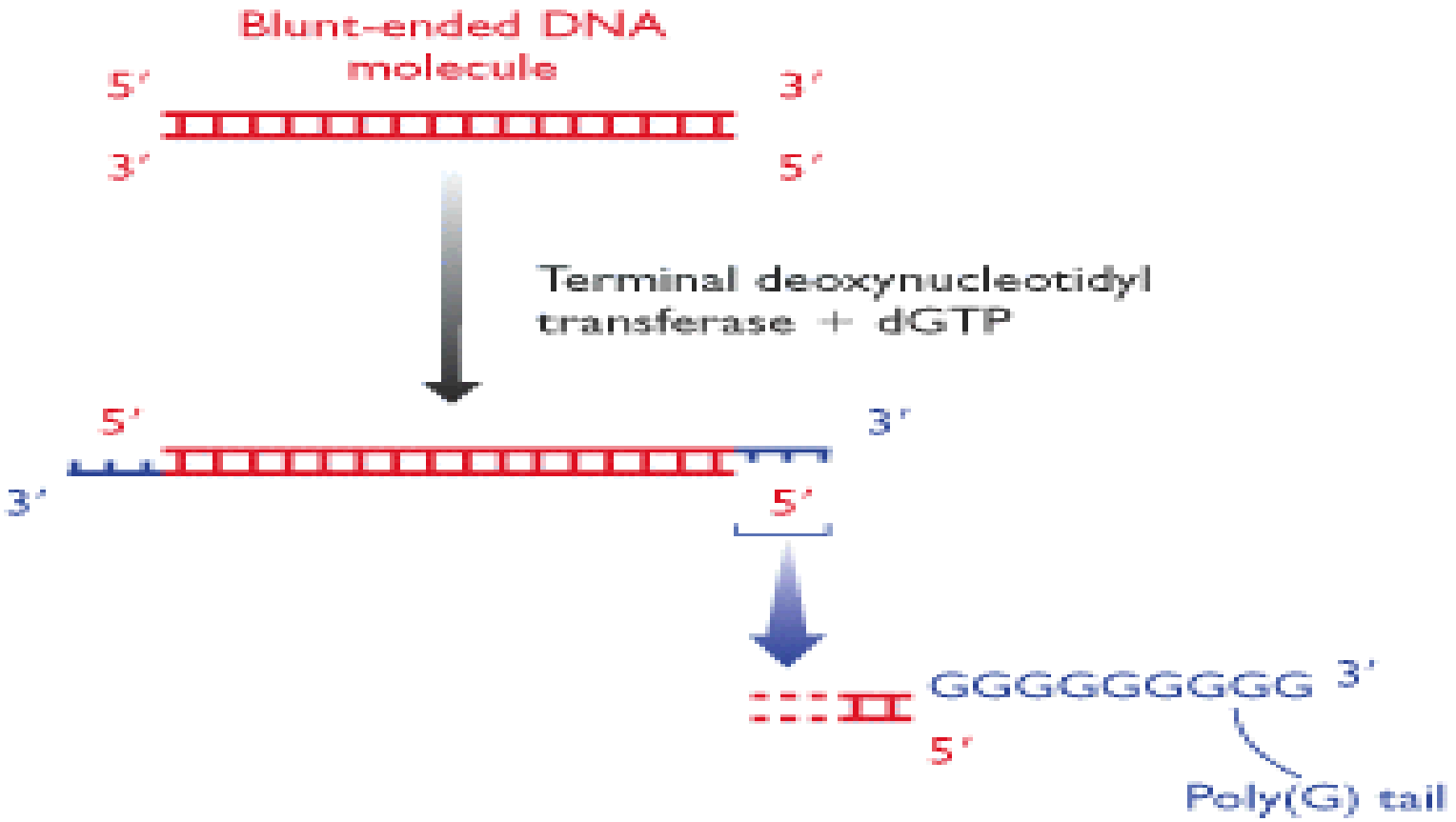
Blunt end ligation using linkers



Blunt end ligation using adaptors



Homopolymer tailing



Inserting DNA Fragments into Vectors

- **DNA fragments with either sticky ends or blunt ends can be inserted into vector DNA with the aid of DNA ligases.**
- **For purposes of DNA cloning, purified DNA ligase is used to covalently join the ends of a restriction fragment and vector DNA that have complementary ends . The vector DNA and restriction fragment are covalently ligated together through the standard 3 → 5 phosphodiester bonds of DNA.**
- **DNA ligase "pastes" the DNA fragments together**

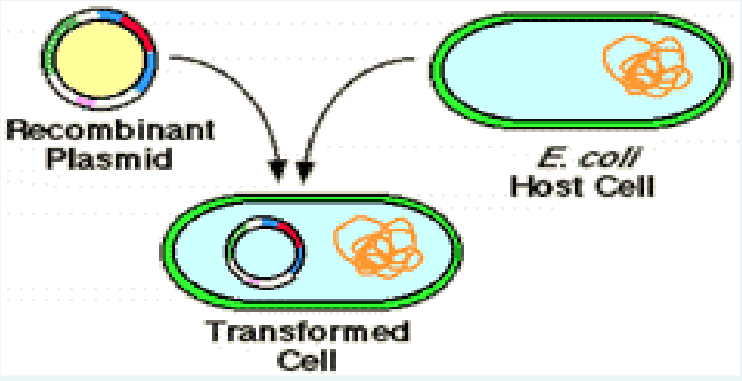
4. *Introduction of recombinant DNA into compatible host cells.*

In order to be propagated, the recombinant DNA molecule (insert DNA joined to vector DNA) must be introduced into a compatible host cell where it can replicate.

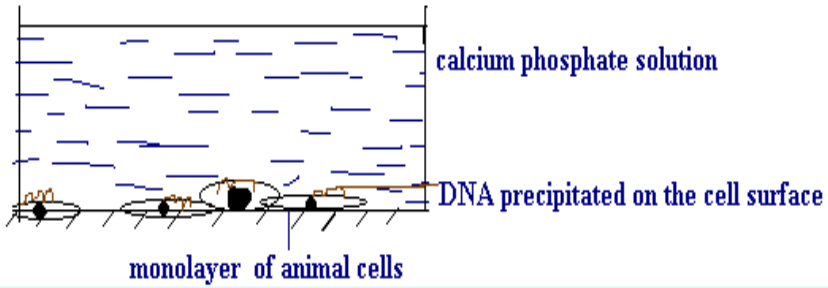
The direct uptake of foreign DNA by a host cell is called genetic transformation (or transformation). Recombinant DNA can also be packaged into virus particles and transferred to host cells by transfection.

Introduction of rDNA into host cell

Transformation

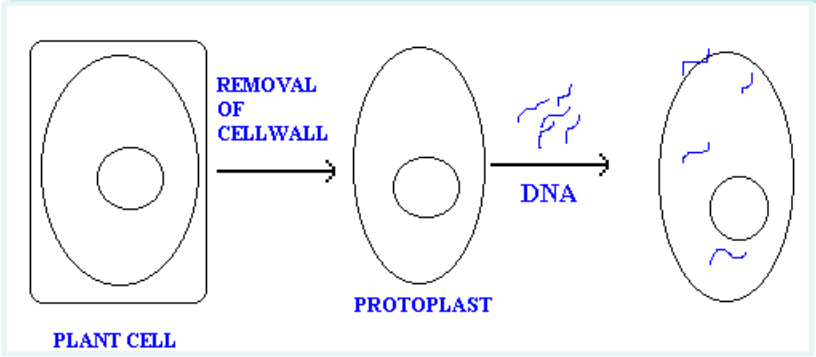


Precipitation

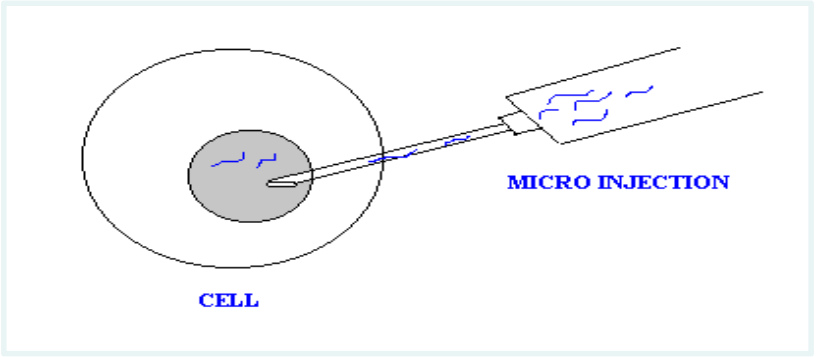


Introduction of rDNA into host cell

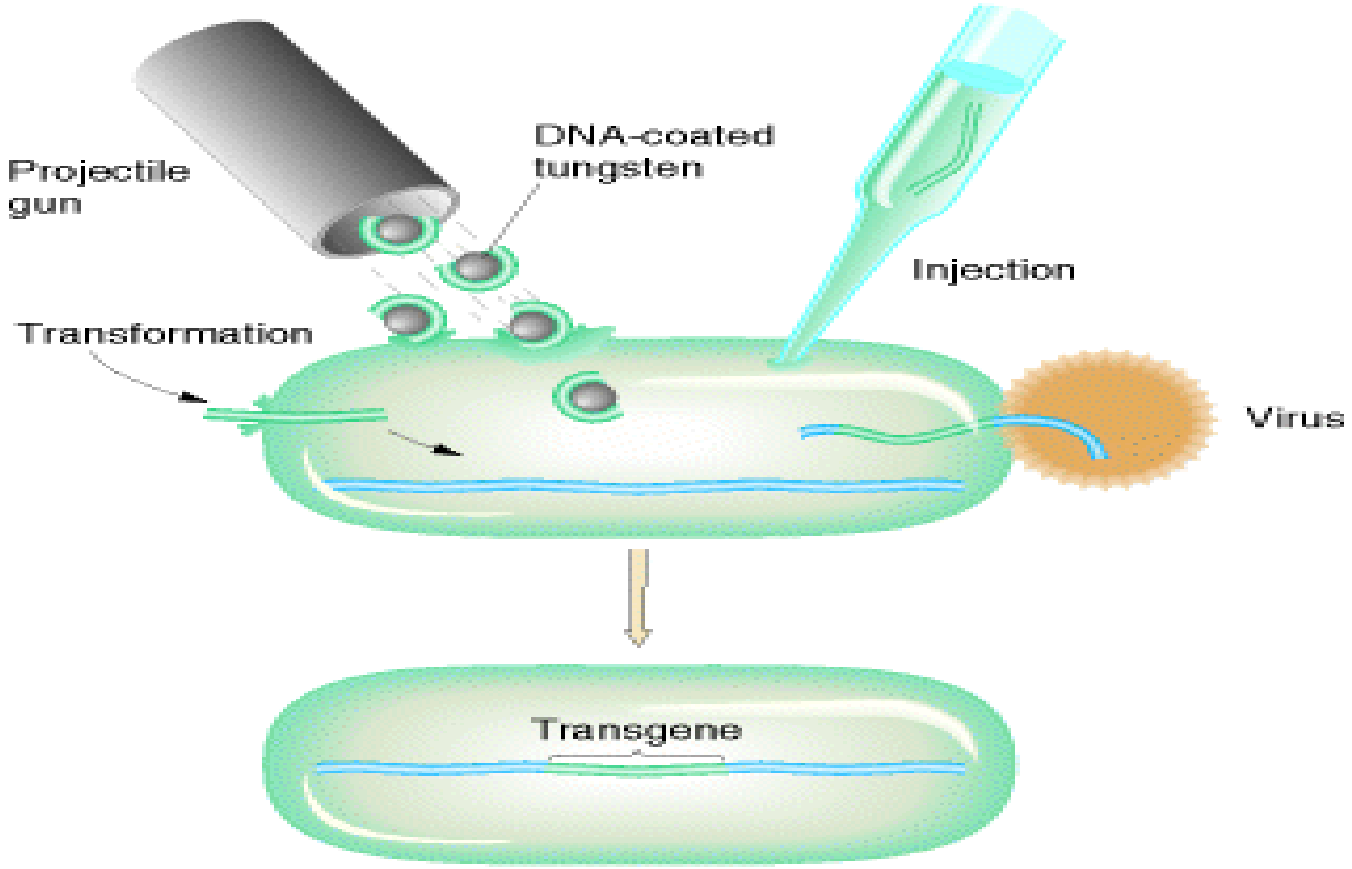
Protoplast fusion



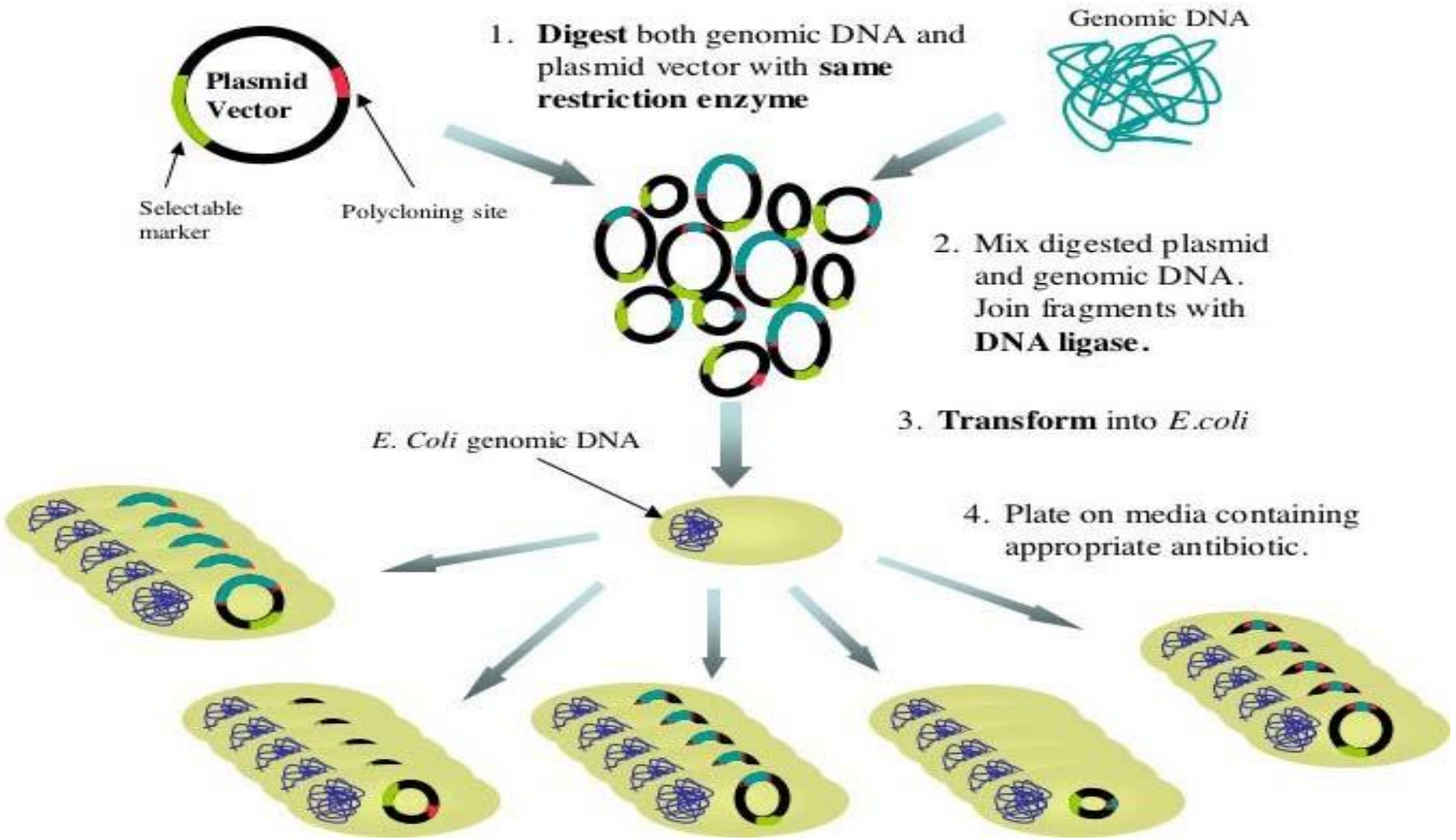
Microinjection



Microprojectiles (Biolistics or gene gun)



Cloning a DNA library in a plasmid vector



Identification of Host Cells Containing Recombinant DNA

- **Once a cloning vector and insert DNA have been joined *in vitro*, the recombinant DNA molecule can be introduced into a host cell, most often a bacterial cell such as *E. coli*.**
- **In general, transformation is not a very efficient way of getting DNA into a cell because only a very small percentage of cells take up recombinant DNA. Consequently, those cells that have been successfully transformed must be distinguished from the vast majority of untransformed cells.**

- **Identification of host cells containing recombinant DNA requires genetic selection or screening or both.**
- **In a *selection*, cells are grown under conditions in which only transformed cells can survive; all the other cells die.**
- **In contrast, in a *screen*, transformed cells have to be individually tested for the presence of the desired recombinant DNA.**
- **Normally, a number of colonies of cells are first selected and then screened for colonies carrying the desired insert.**

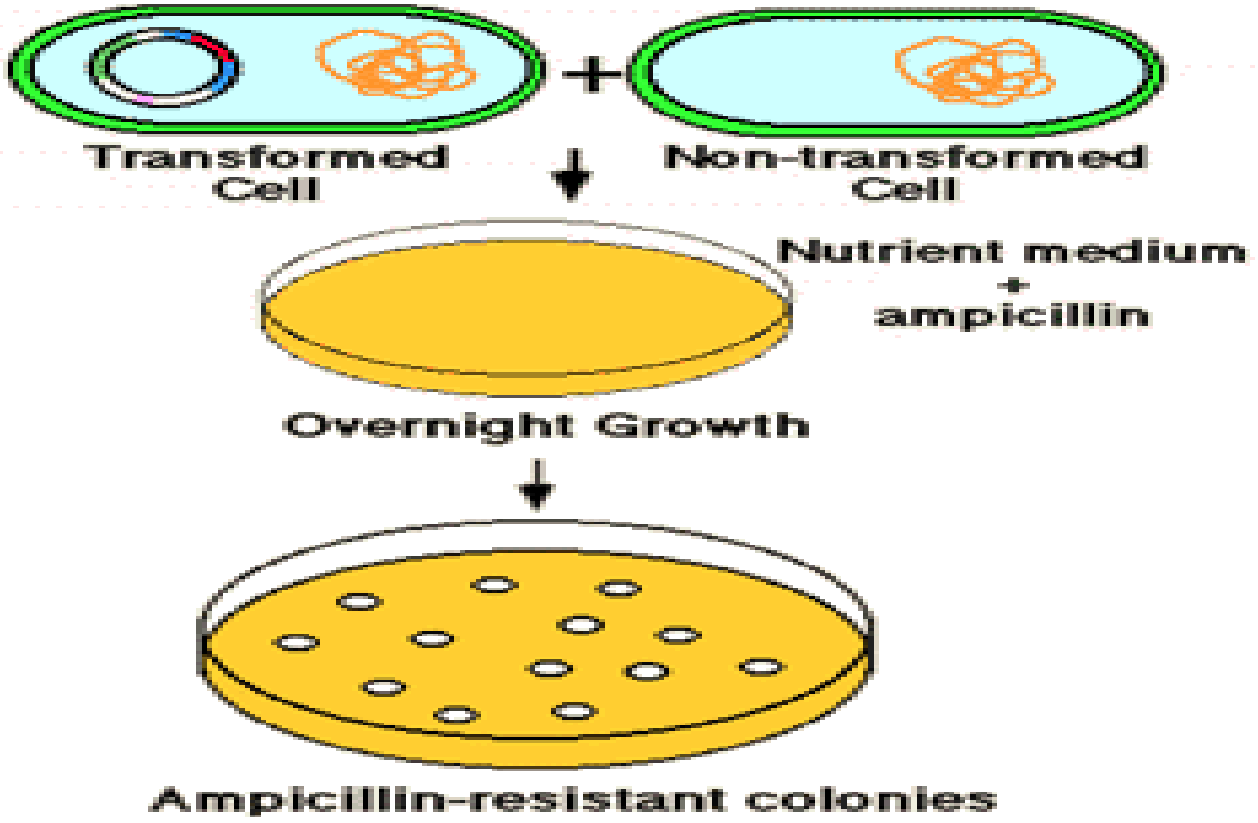
Selection Strategies Use Marker Genes (Primary screening)

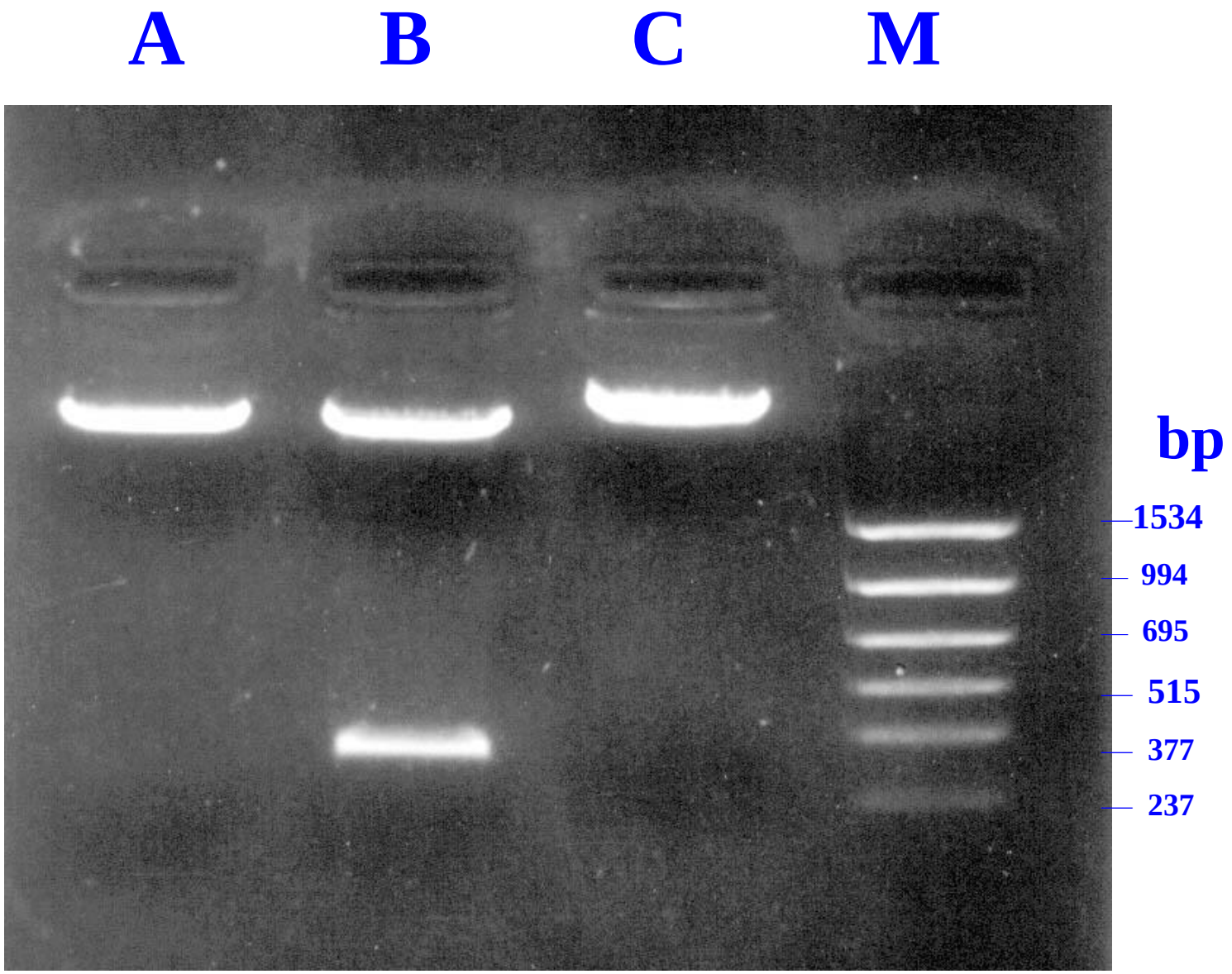
- **Many selection strategies involve selectable marker genes— genes whose presence can easily be detected or demonstrated. *amp*^{R}**
- **Selection or screening can also be achieved using insertional inactivation.**

Screening (Strategies)

1. **Gel Electrophoresis Allows Separation of Vector DNA from Cloned Fragments**
2. **Cloned DNA Molecules Are Sequenced Rapidly by the Dideoxy Chain-Termination Method**
3. **The Polymerase Chain Reaction Amplifies a Specific DNA Sequence from a Complex Mixture**
4. **Blotting Techniques Permit Detection of Specific DNA Fragments and mRNAs with DNA Probes**
5. **Antibiotic resistance**

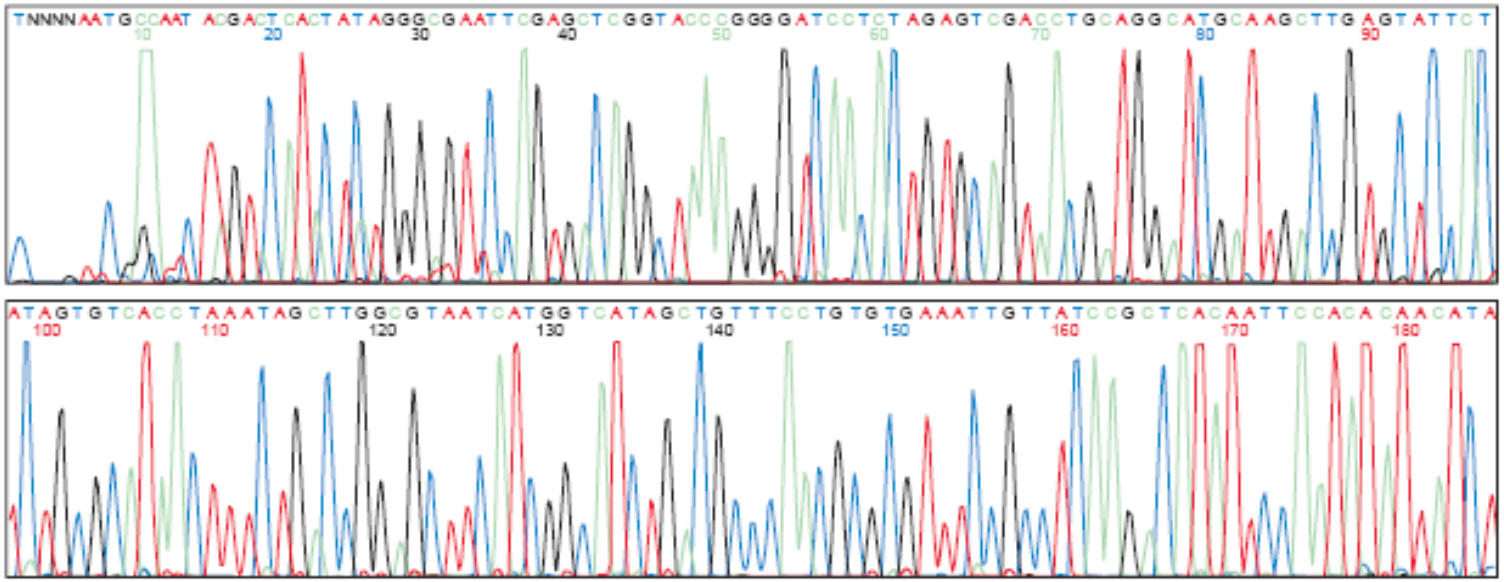
Antibiotic resistance





Gel Electrophoresis

negative charged DNA run to the anode



Sequencing results

Southern blot technique can detect a specific DNA fragment in a complex mixture of restriction fragments.

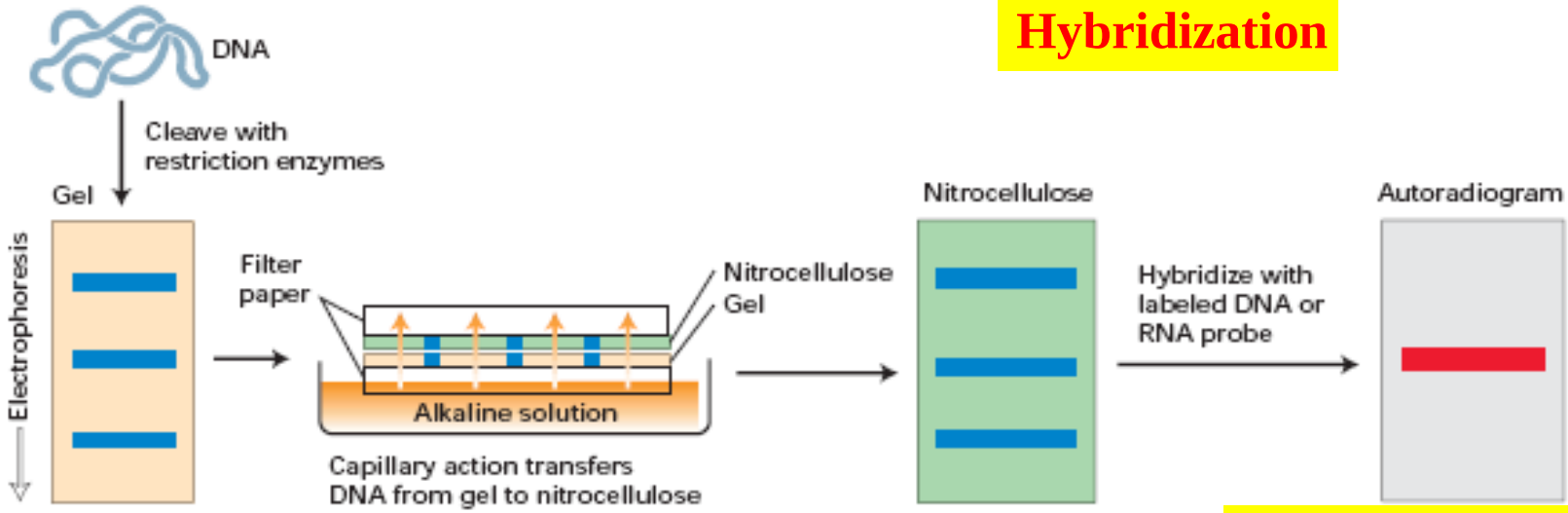


Table 7.2. Blotting Techniques

	What Is Probed	Nature of the Probe
Southern blot	DNA	DNA
Northern blot	RNA	cDNA or RNA
Western blot	Protein	Antibody

Types of blotting techniques

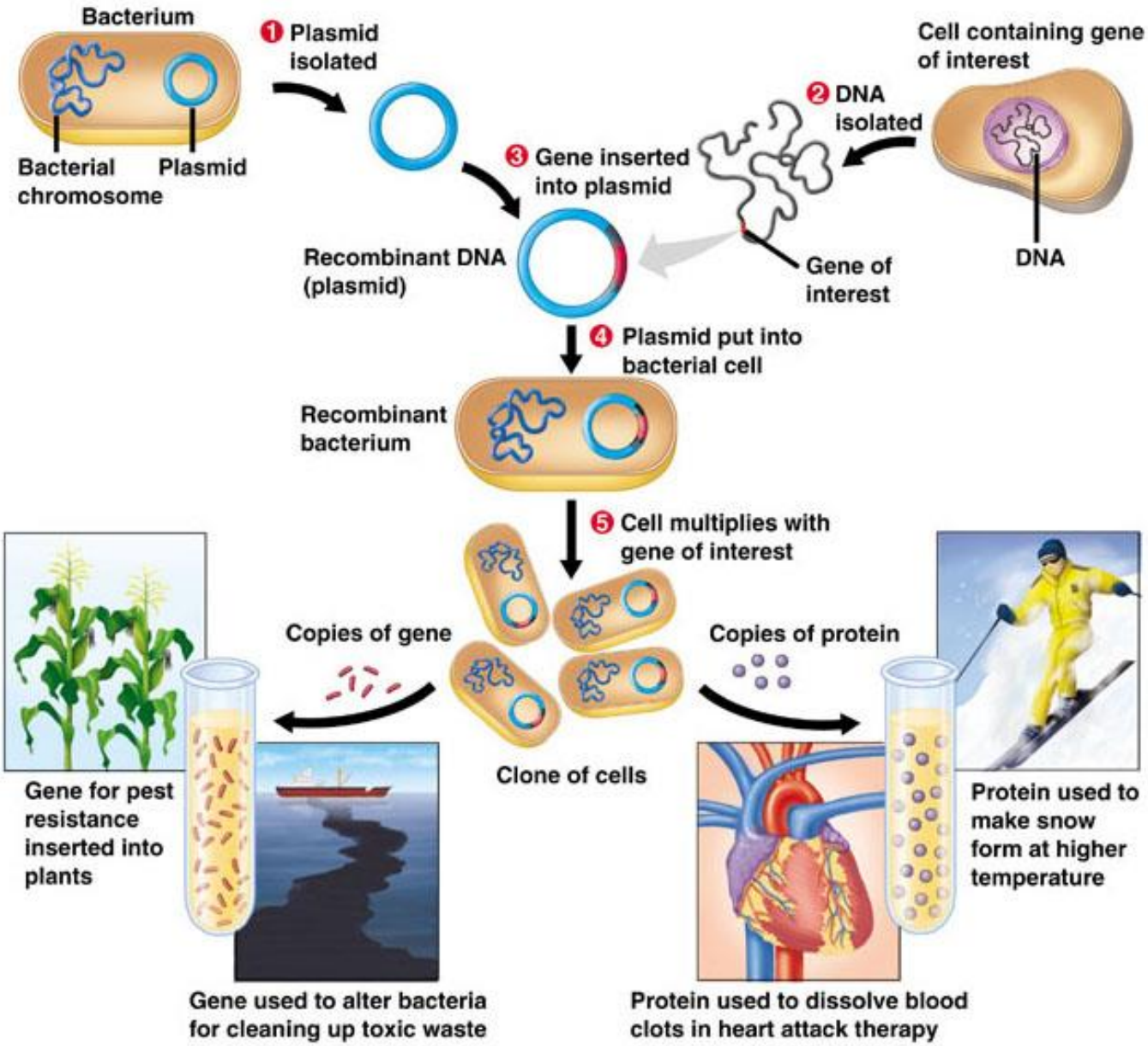
- **Southern blotting**
  - **Southern blotting techniques is the first nucleic acid blotting procedure developed in 1975 by Southern.**
  - **Southern blotting is the techniques for the specific identification of DNA molecules.**
- **Northern blotting**
  - **Northern blotting is the techniques for the specific identification of RNA molecules.**
- **Western blotting**
  - **Western blotting involves the identification of proteins.**
  - **Antigen + antibody**

Expression of Proteins Using Recombinant DNA Technology

- **Cloned or amplified DNA can be purified and sequenced, used to produce RNA and protein, or introduced into organisms with the goal of changing their phenotype.**
- **One of the reasons recombinant DNA technology has had such a large impact on biochemistry is that it has overcome many of the difficulties inherent in purifying low-abundance proteins and determining their amino acid sequences.**

- **Recombinant DNA technology allows the protein to be purified without further characterization. Purification begins with overproduction of the protein in a cell containing an expression vector.**
  - **Prokaryotic Expression Vectors**
  - **Eukaryotic Expression Vectors**

Brief Introduction



Applications of Recombinant DNA Technology

- **Pharmaceutical Products**

- **Some pharmaceutical applications of DNA technology:**
  - **Large-scale production of human hormones and other proteins with therapeutic uses**
  - **Production of safer vaccines**
- **A number of therapeutic gene products —*insulin, the interleukins, interferons, growth hormones, erythropoietin, and coagulation factor VIII*—are now produced commercially from cloned genes**

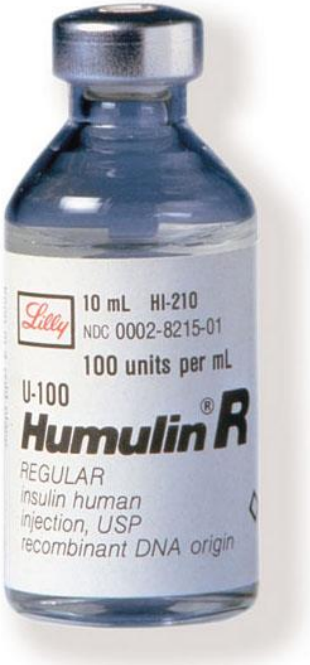
- **Pharmaceutical companies already are producing molecules made by recombinant DNA to treat human diseases.**
- **Recombinant bacteria are used in the production of human growth hormone and human insulin**

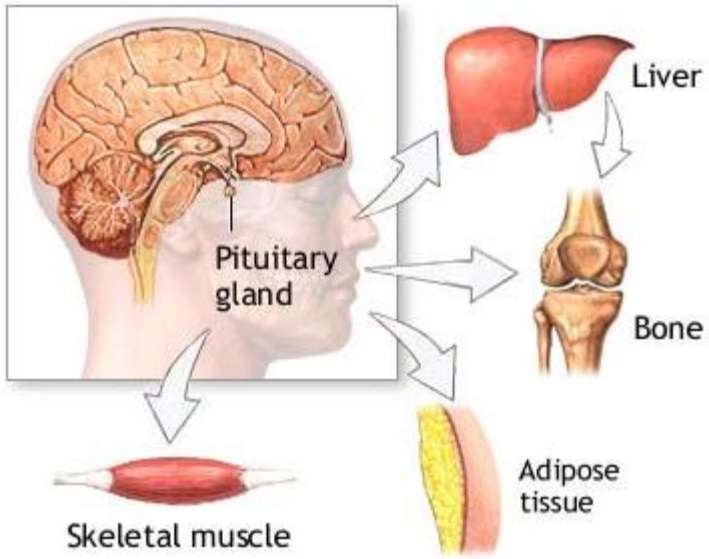
SOME PROTEIN PRODUCTS OF RECOMBINANT DNA TECHNOLOGY

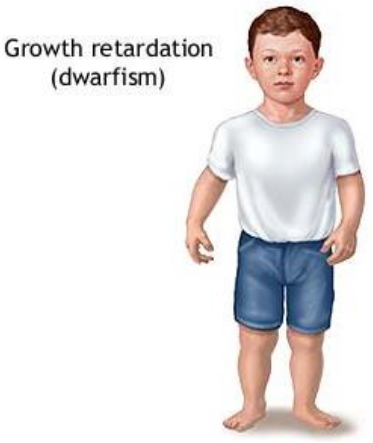
Product	Made In	Use
Human insulin	*E. coli*	Treatment for diabetes
Human growth hormone (GH)	*E. coli*	Treatment for growth defects
Epidermal growth factor (EGF)	*E. coli*	Treatment for burns, ulcers
Interleukin-2 (IL-2)	*E. coli*	Possible treatment for cancer
Bovine growth hormone (BGH)	*E. coli*	Improving weight gain in cattle
Cellulase	*E. coli*	Breaking down cellulose for animal feeds
Taxol	*E. coli*	Treatment for ovarian cancer
Interferons (alpha and gamma)	*S. cerevisiae; E. coli*	Possible treatment for cancer and viral infections
Hepatitis B vaccine	*S. cerevisiae*	Prevention of viral hepatitis
Erythropoietin (EPO)	Mammalian cells	Treatment for anemia
Factor VIII	Mammalian cells	Treatment for hemophilia
Tissue plasminogen activator (TPA)	Mammalian cells	Treatment for heart attacks

Use recombinant cells to mass produce proteins

- **Bacteria**
- **Yeast**
- **Mammalian**



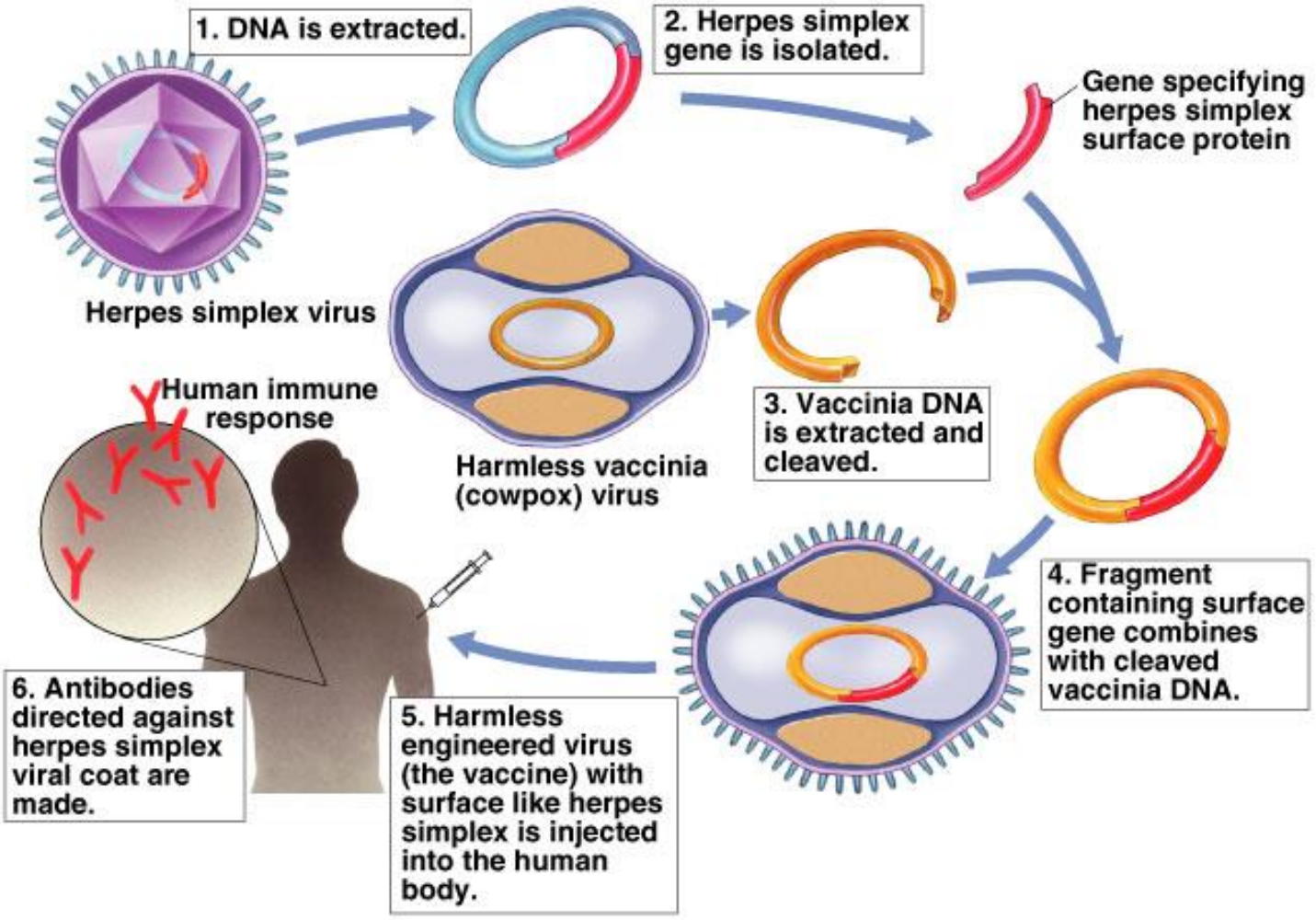




- **Insulin**
  - Hormone required to properly process sugars and fats
  - Treat diabetes
  - Now easily produced by bacteria

- **Growth hormone deficiency**
  - Faulty pituitary and regulation
  - Had to rely on cadaver source
  - Now easily produced by bacteria

Subunit Herpes Vaccine



Genetically modified organisms (GMO)

Use of recombinant plasmids in agriculture

- plants with genetically desirable traits
  - herbicide or pesticide resistant corn & soybean
    - Decreases chemical insecticide use
    - Increases production
  - “Golden rice” with beta-carotene
    - Required to make vitamin A, which in deficiency causes blindness

- **Crops have been developed that are better tasting, stay fresh longer, and are protected from disease and insect infestations.**

"Golden rice" has been genetically modified to contain beta-carotene

Genetic Engineering of Plants

- **Plants have been bred for millennia to enhance certain desirable characteristics in important food crops.**
- **Transgenic plants.**

Table 19.4

Genetically Modified Crops

Altered Plant	Effect
Rice with beta carotene and extra iron	Added nutritional value
Canola with high-laurate oil	Can be grown domestically; less costly than importing palm and coconut oils
Delayed ripening tomato	Extended shelf life
Herbicide-resistant cotton	Herbicide kills weeds without harming crop
Minipeppers	Improved flavor, fewer seeds
Bananas resistant to fungal infection	Extended shelf life
Delayed-ripening bananas and pineapples	Extended shelf life
Elongated sweet pepper	Improved flavor, easier to slice
Altered cotton fiber	"Plasticized" fabric
Altered paper pulp trees	Paper component (lignin) easier to process
High-starch potatoes	Absorb less oil when fried
Pest-resistant corn	Can resist European corn borer
Seedless minimelons	Single serving size
Sweet peas and peppers	Retain sweetness longer
Sugarcane with corn gene	Resists bacterial and fungal toxins

The luciferase gene from a firefly is transformed into tobacco plant using the Ti plasmid. Watering the plant with a solution of luciferin (the substrate for firefly luciferase) results in the generation of light by all plant tissues.

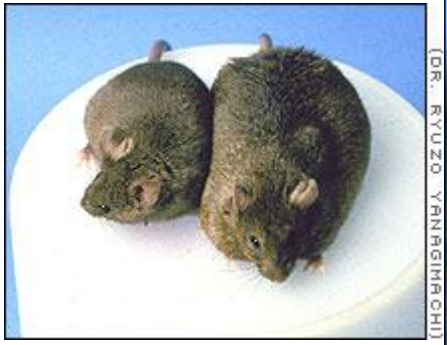
Insect-resistant tomato plants

The plant on the left contains a gene that encodes a bacterial protein that is toxic to certain insects that feed on tomato plants. The plant on the right is a wild-type plant. Only the plant on the left is able to grow when exposed to the insects.

Transgenic animals

Green fluorescence

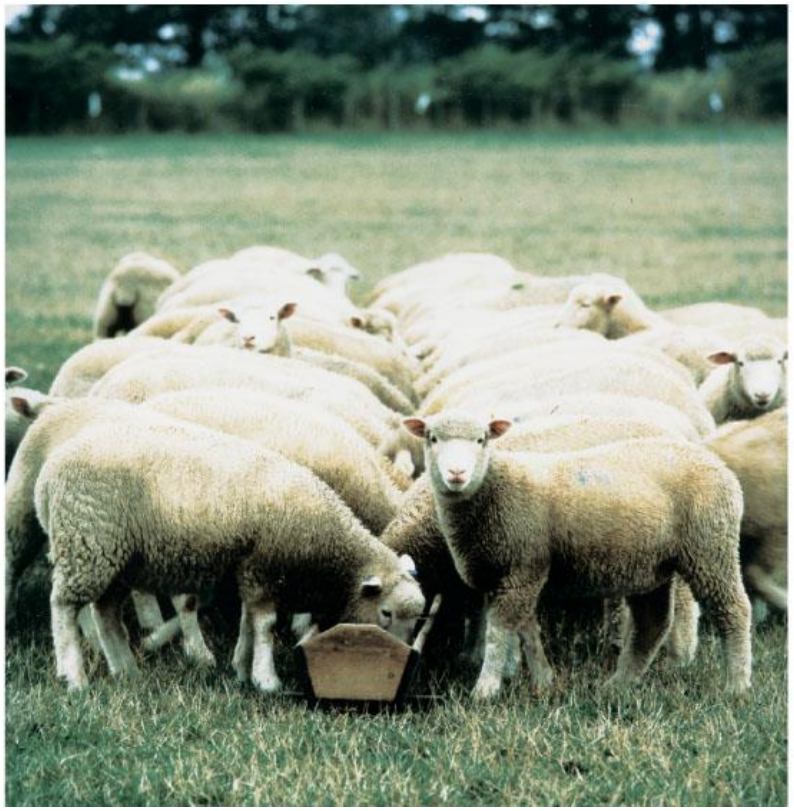
Red fluorescence



A transgenic mouse

Mouse on right is normal; mouse on left is transgenic animal expressing rat growth hormone

Farm Animals and "Pharm" Animals



- **Trangenic plants and animals have genes from other organisms.**
- **These transgenic sheep carry a gene for a human blood protein**
  - **This protein may help in the treatment of cystic fibrosis**

Other benefits of GMOs

- **Disease resistance**
  - **There are many viruses, fungi, bacteria that cause plant diseases**
  - **“Super-shrimp”**
- **Cold tolerance**
  - **Antifreeze gene from cold water fish introduced to tobacco and potato plants**
- **Drought tolerance & Salinity tolerance**
  - **As populations expand, potential to grow crops in otherwise inhospitable environments**

Application in medicine

Human Gene Therapy

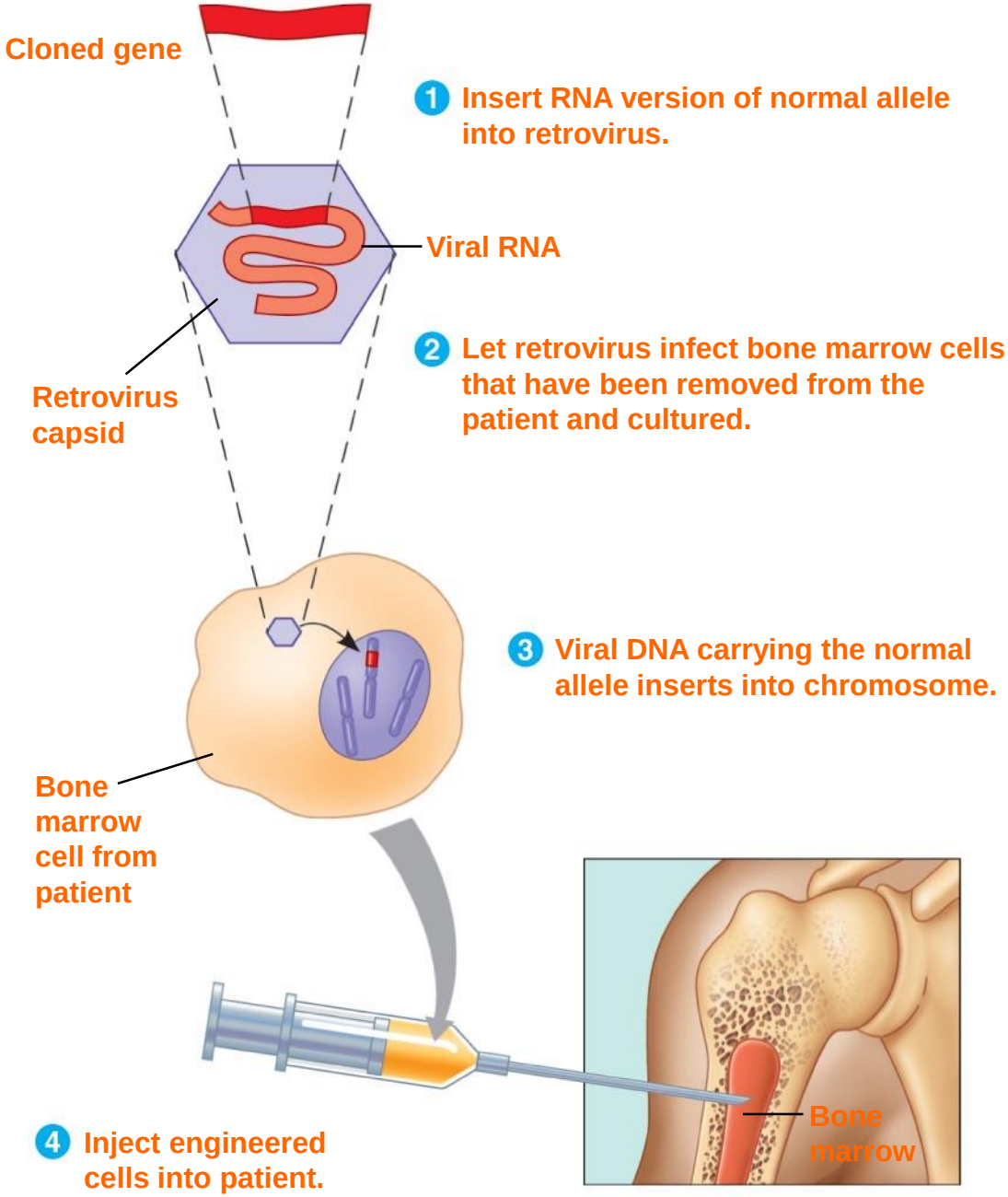
Diagnosis of genetic disorders

Forensic Evidence

Human Gene Therapy

Human gene therapy **seeks to repair the damage caused by a genetic deficiency through introduction of a functional version of the defective gene. To achieve this end, a cloned variant of the gene must be incorporated into the organism in such a manner that it is expressed only at the proper time *and* only in appropriate cell types. At this time, these conditions impose serious technical and clinical difficulties.**

- **Gene therapy is the alteration of an afflicted individual's genes**
- **Gene therapy holds great potential for treating disorders traceable to a single defective gene**
- **Vectors are used for delivery of genes into cells**
- **Gene therapy raises ethical questions, such as whether human germ-line cells should be treated to correct the defect in future generations**
- **Many gene therapies have received approval from the National Institutes of Health for trials in human patients, including the introduction of gene constructs into patients. Among these are constructs designed to cure *ADA- SCID* (severe combined immunodeficiency due to adenosine deaminase [ADA] deficiency), neuroblastoma, or cystic fibrosis, or to treat cancer through expression of the E1A and p53 tumor suppressor genes.**



Somatic cells Only!

Not for reproductive cells !!

Thank you