# CC9TH 4TH SEMESTER
# MOLECULAR BIOLOGY

# RECOMBINANT DNA TECHNOLOGY IN BRIEF AND ITS APPLICATIONS

# Introduction

- **restriction endonuclease –** An enzyme that recognizes specific short sequences of DNA and cleaves the duplex (sometimes at the target site, sometimes elsewhere, depending on type).

# Introduction

- **cloning vector –** DNA (often derived from a plasmid or a bacteriophage genome) that can be used to propagate an incorporated DNA sequence in a host cell.
  - Vectors contain selectable markers and replication origins to allow identification and maintenance of the vector in the host.

# Nucleases

- **Nucleases** hydrolyze an ester bond within a phosphodiester bond.
- **Phosphatases** hydrolyze the ester bond in a phosphomonoester bond.

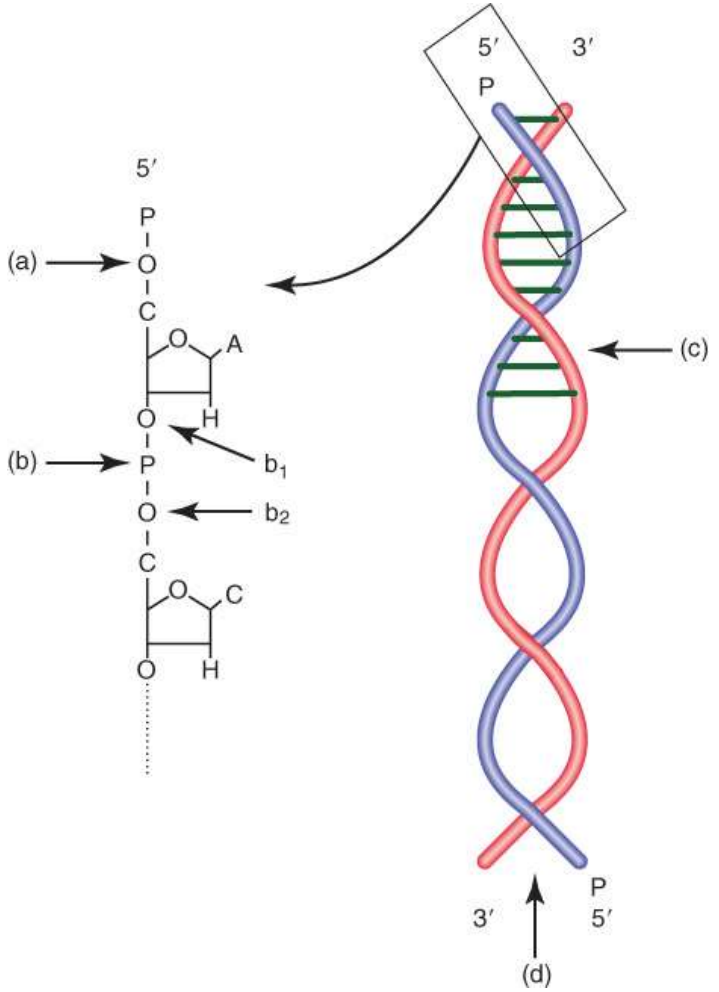
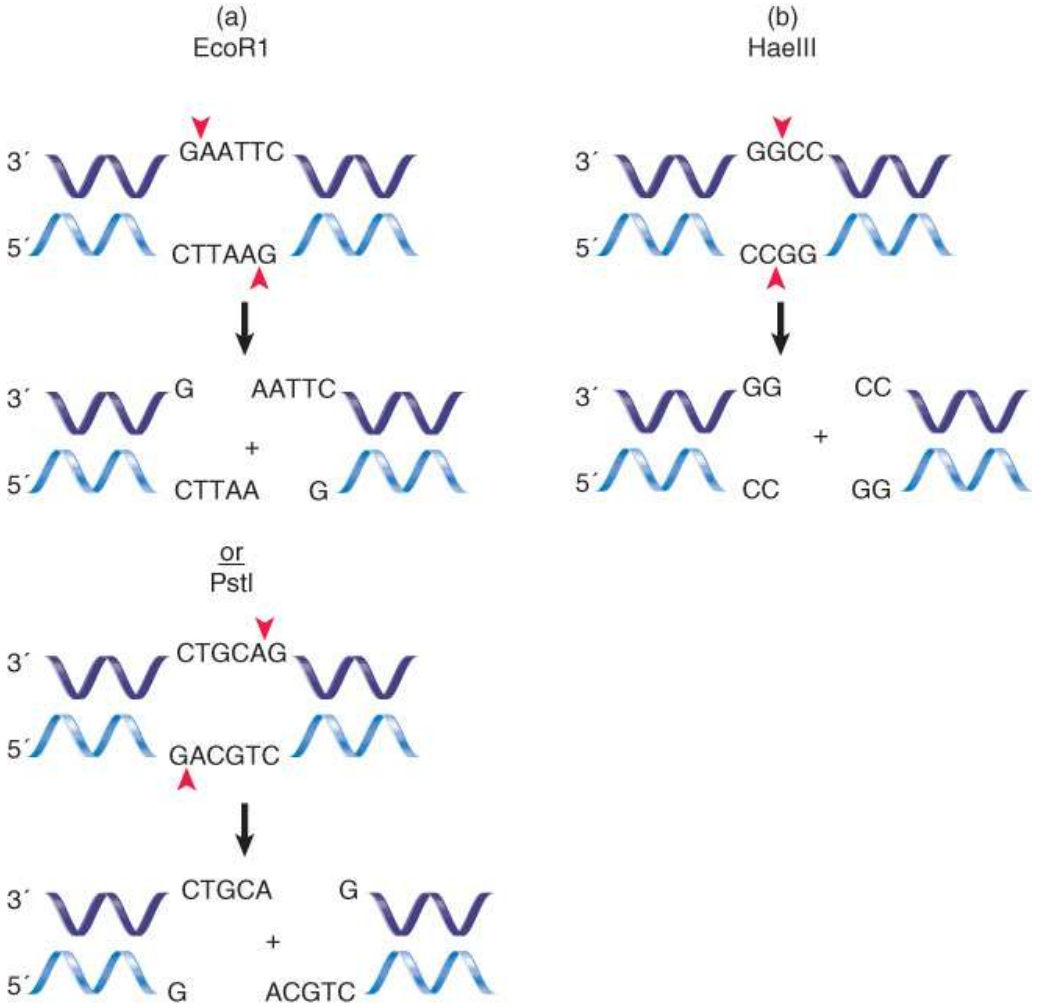



Figure : The targets of a phosphatase and a nuclease

# Nucleases

- **endonuclease –** Nuclease that cleaves phosphoester bonds within a nucleic acid chain.
  - It may be specific for RNA or for single-stranded or double-stranded DNA.
- **exonuclease –** Nuclease that cleaves phosphoester bonds one at a time from the end of a polynucleotide chain.
  - It may be specific for either the 5′ or 3′ end of DNA or RNA.

# Nucleases




- Restriction endonucleases can be used to cleave DNA into defined fragments.

Figure : Recognition site cleavage.

# Nucleases

- A map can be generated by using the overlaps between the fragments generated by different restriction enzymes.

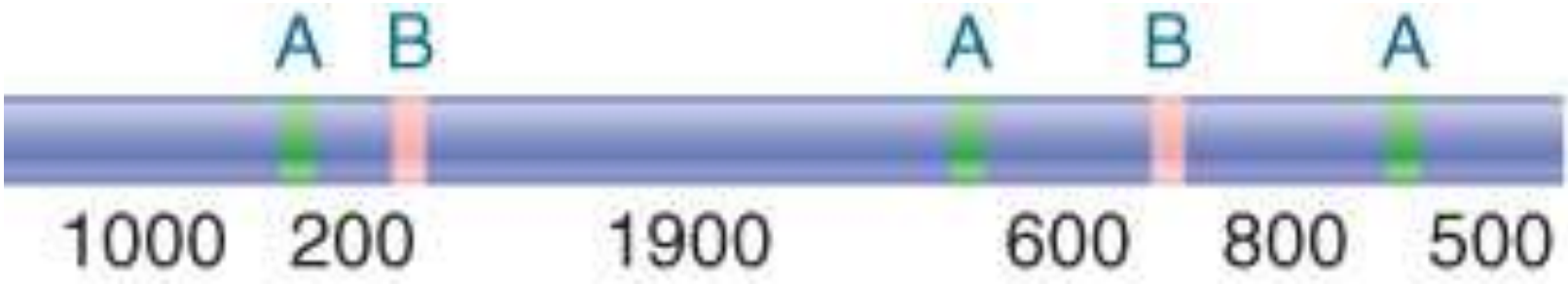



Figure : A restriction map is a linear sequence of sites separated by defined distances on DNA.

# Cloning

- **Cloning** a fragment of DNA requires a specially engineered **vector**.
- **recombinant DNA –** A DNA molecule that has been created by joining together two or more molecules from different sources.
- **subclone –** The process of breaking a cloned fragment into smaller fragments for further cloning.
- **multiple cloning site (MCS) –** A sequence of DNA containing a series of tandem restriction endonuclease sites used in cloning vectors for creating recombinant molecules.

# Cloning

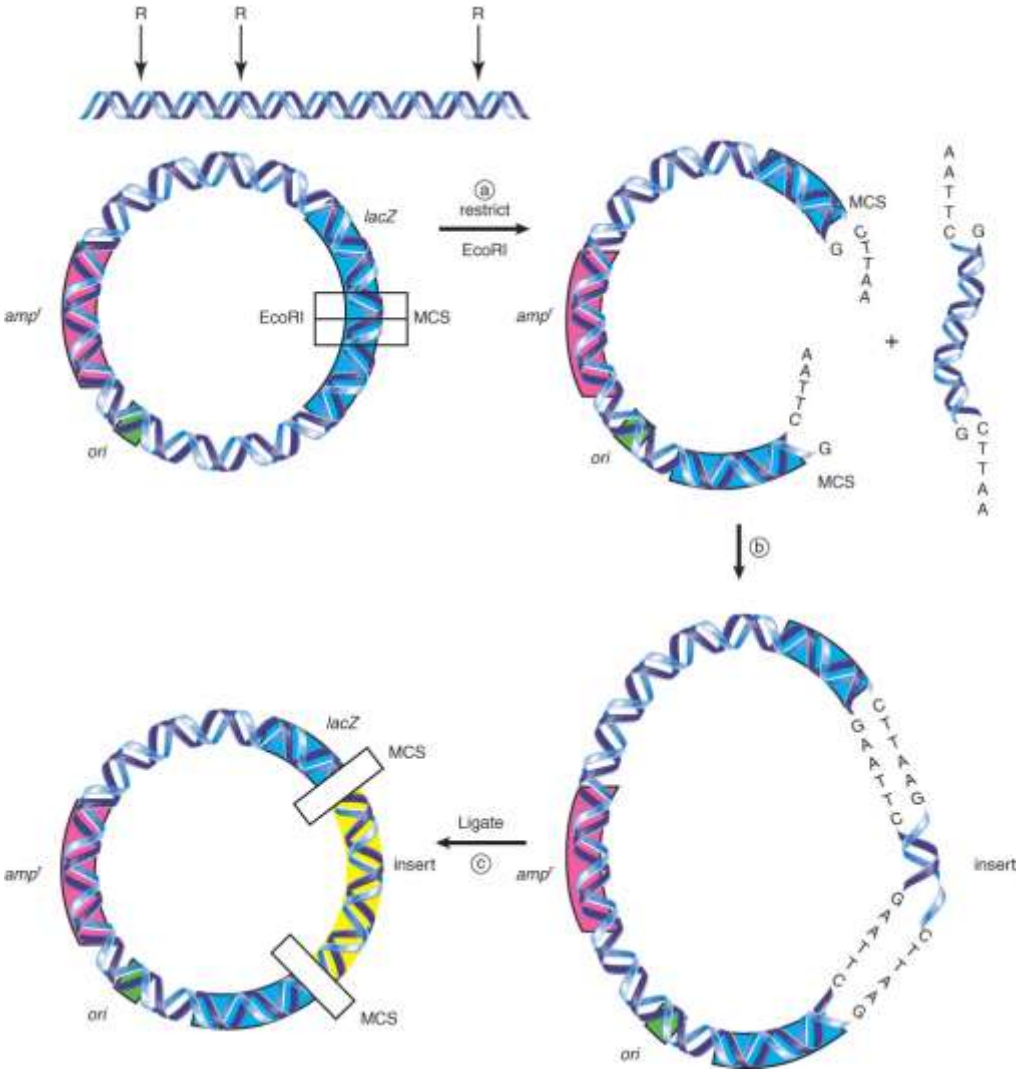



Figure : (a) A plasmid together with insert DNA (b) Restricted insert fragments and vector will be combined and (c) ligated together.

# Cloning

- **transformation** – The acquisition of new genetic material by incorporation of added exogenous, nonviral DNA.
- Blue/white selection allows the identification of bacteria that contain the vector plasmid and vector plasmids that contain an **insert**.

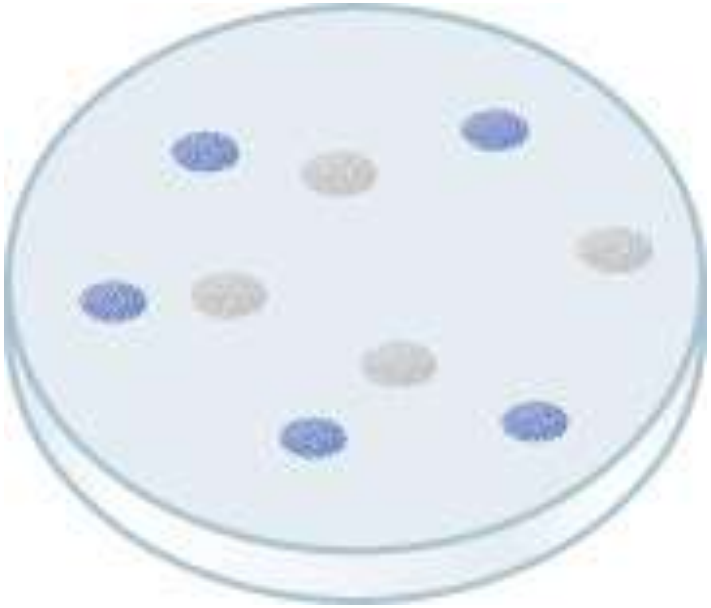


Figure : The white colonies will be used to prepare DNA for further analysis.

# Cloning Vectors Can Be Specialized for Different Purposes

**TABLE 2.1 Cloning vectors may be based on plasmids or phages or may mimic eukaryotic chromosomes.**

| Vector | Features | Isolation of DNA | DNA Limit |
|---|---|---|---|
| Plasmid | High copy number | Physical | 10 kb |
| Phage | Infects bacteria | Via phage packaging | 20 kb |
| Cosmid | High copy number | Via phage packaging | 48 kb |
| BAC | Based on F plasmid | Physical | 300 kb |
| YAC | Origin + centromere + telomere | Physical | > 1 Mb |

Table : Cloning vectors can be based on plasmids or phages or can mimic eukaryotic chromosomes.

# Cloning Vectors Can Be Specialized for Different Purposes

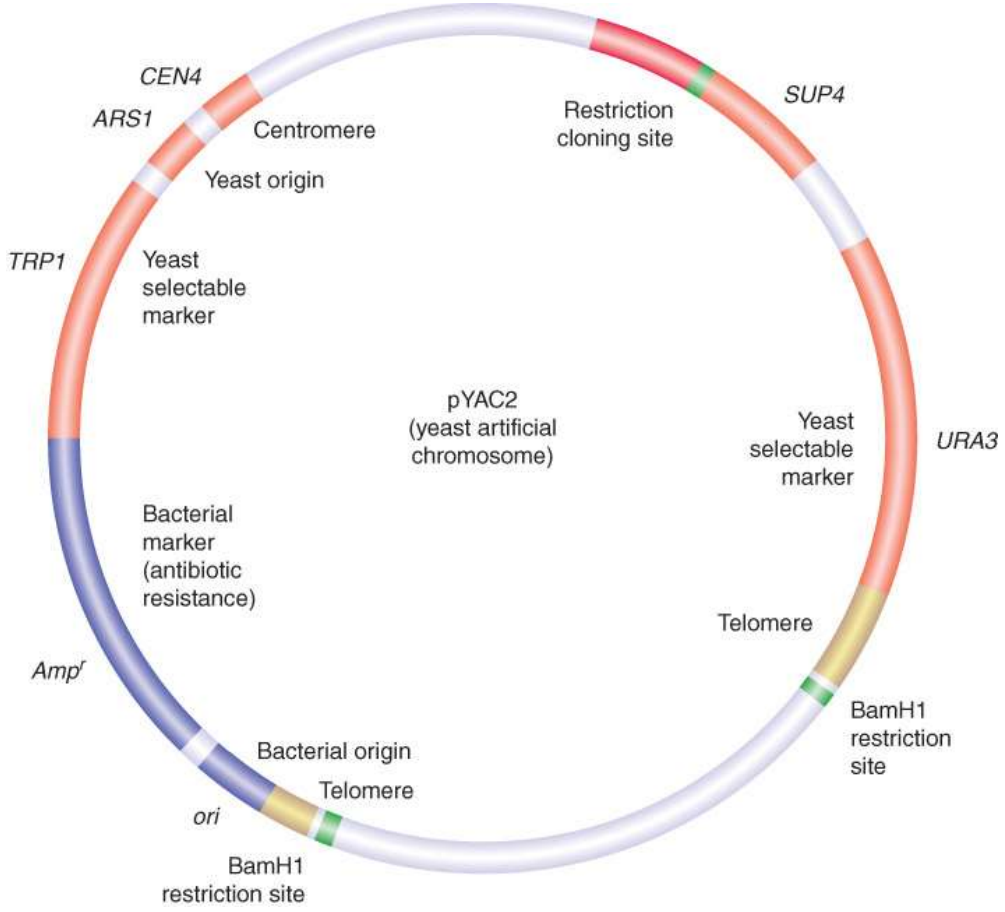



Figure : pYac2 is a shuttle vector

# Cloning Vectors Can Be Specialized for Different Purposes

- Cloning vectors may be bacterial plasmids, phages, **cosmids**, or **yeast artificial chromosomes**.
- **Shuttle vectors** can be propagated in more than one type of host cell.
- **Expression vectors** contain promoters that allow transcription of any cloned gene.

# Cloning Vectors Can Be Specialized for Different Purposes

- **Reporter genes** can be used to measure promoter activity or tissue-specific expression.

Photo courtesy of Robb Krumlauf, Stowers Institute for Medical Research

Figure : Expression of a lacZ gene can be followed in the mouse by staining for β-gal (in blue).

Courtesy of Joachim Goedhart, Molecular Cytology, SILS, University of Amsterdam.

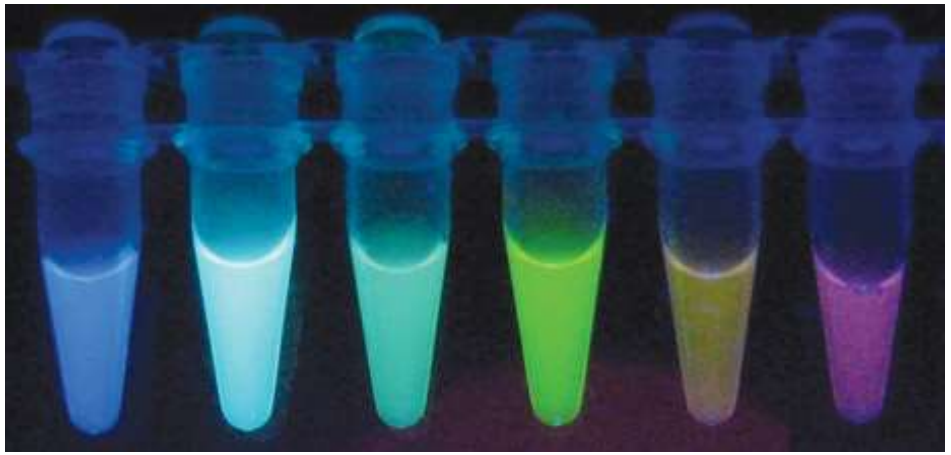


Figure : (a) Since the discovery of GFP, derivatives that fluoresce in different colors have been engineered.

# Cloning Vectors Can Be Specialized for Different Purposes

- Numerous methods exist to introduce DNA into different target cells.

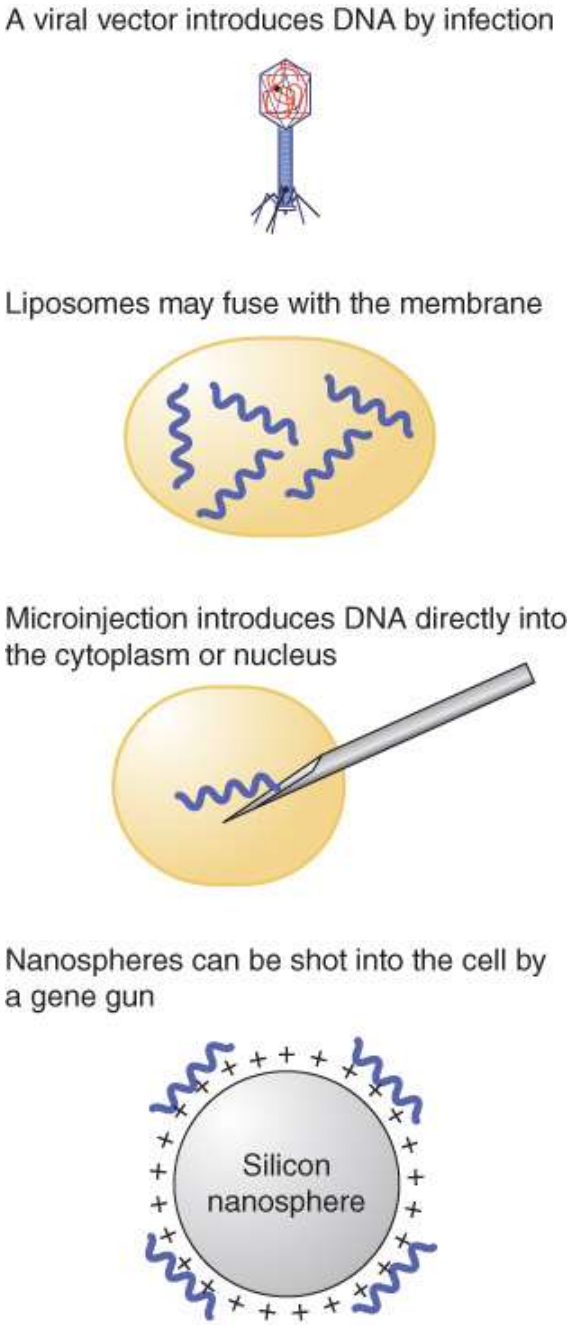



Figure : DNA can be released into target cells by several methods.

# Nucleic Acid Detection

- Hybridization of a labeled nucleic acid to complementary sequences can identify specific nucleic acids.
- **probe –** A radioactive nucleic acid, DNA or RNA, used to identify a complementary fragment.

# Nucleic Acid Detection

- **autoradiography –** A method of capturing an image of radioactive materials on film.

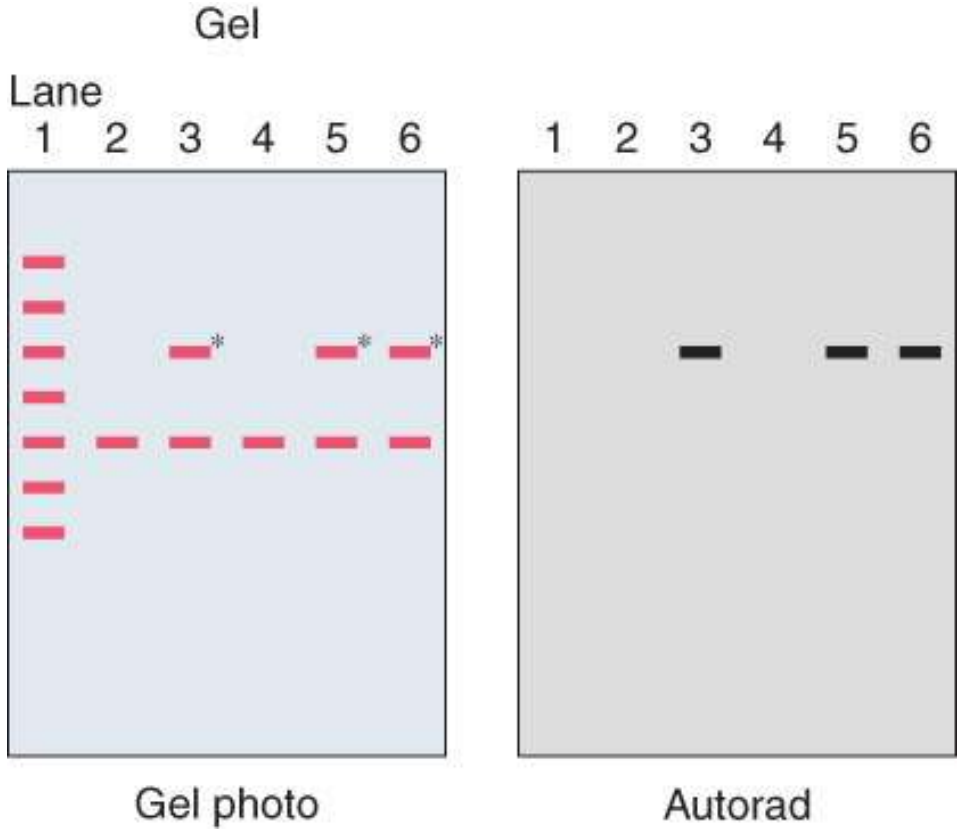



Figure : An autoradiogram of a gel prepared from the colonies described in Figure Early

# Nucleic Acid Detection

- ***in situ* hybridization** – Hybridization of a probe to intact tissue to locate its complementary strand by autoradiography.

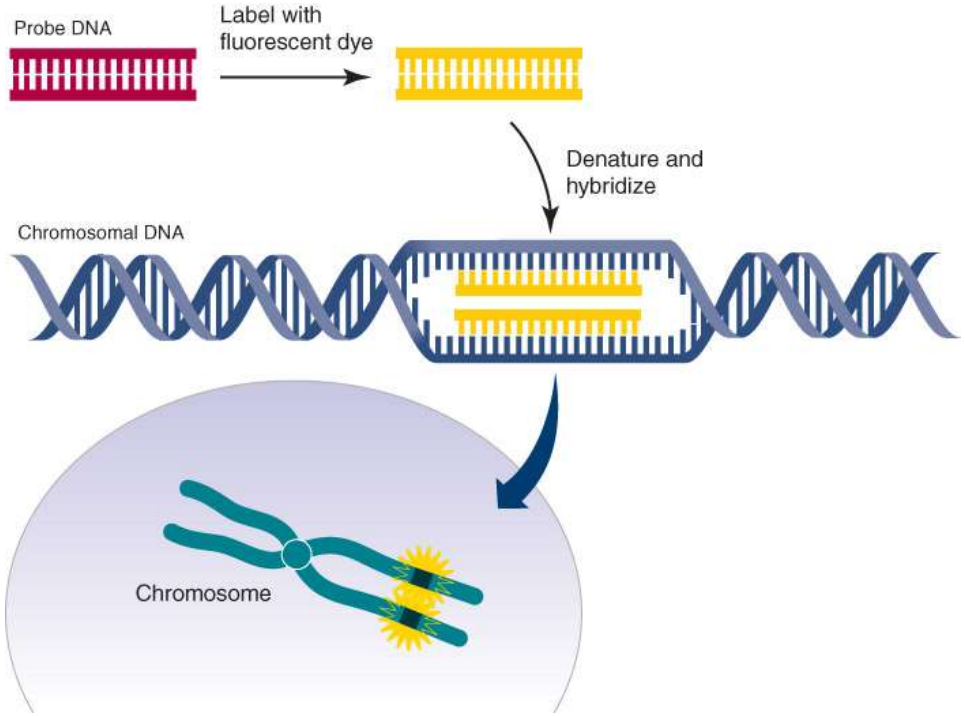



Figure : Fluorescence *in situ* hybridization (FISH).

Data from an illustration by Darryl Leja, National Human Genome Research Institute (www.genome.gov).

# DNA Separation Techniques

- Gel electrophoresis separates DNA fragments by size, using an electric current to cause the DNA to migrate toward a positive charge.

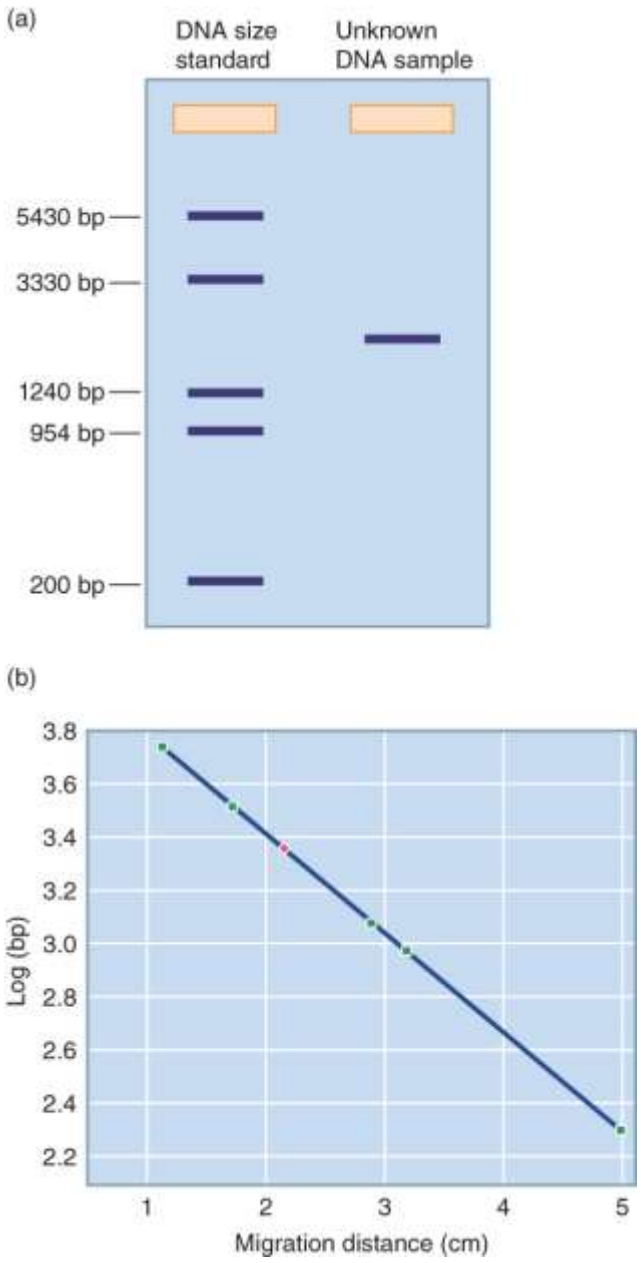
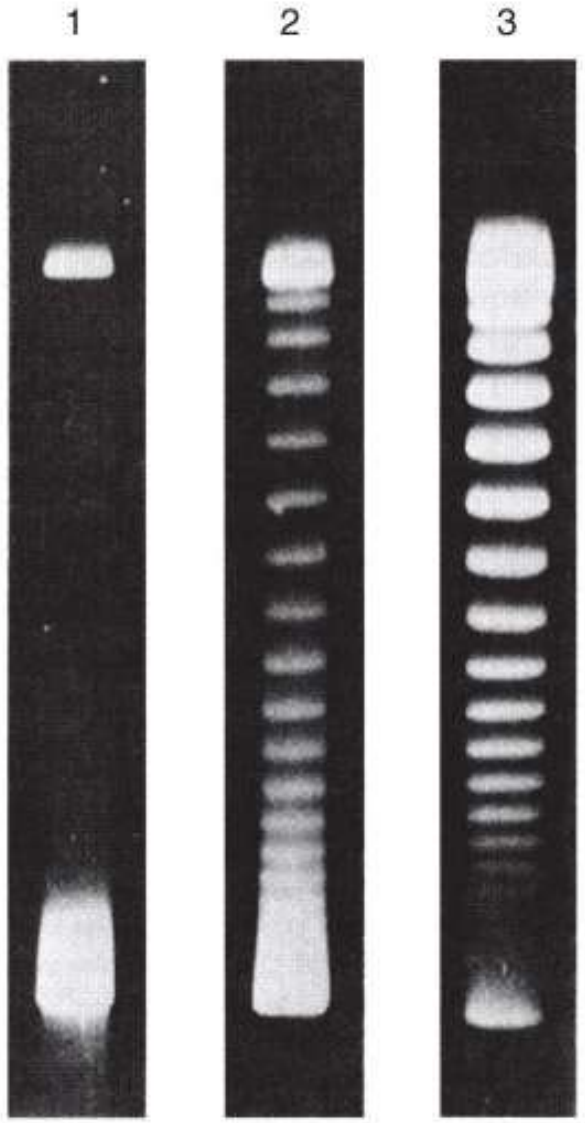



Figure : DNA sizes can be determined by gel electrophoresis.

Data from an illustration by Michael Blaber, Florida State University.




Reproduced from W. Keller, *Proc. Natl. Acad. Sci. USA* 72 (1975): 2550-2554. Photo courtesy of Walter Keller, University of Basel.

Figure : Supercoiled DNA molecules separated by agarose gel electrophoresis.

# DNA Separation Techniques

- DNA can also be isolated using density gradient centrifugation.

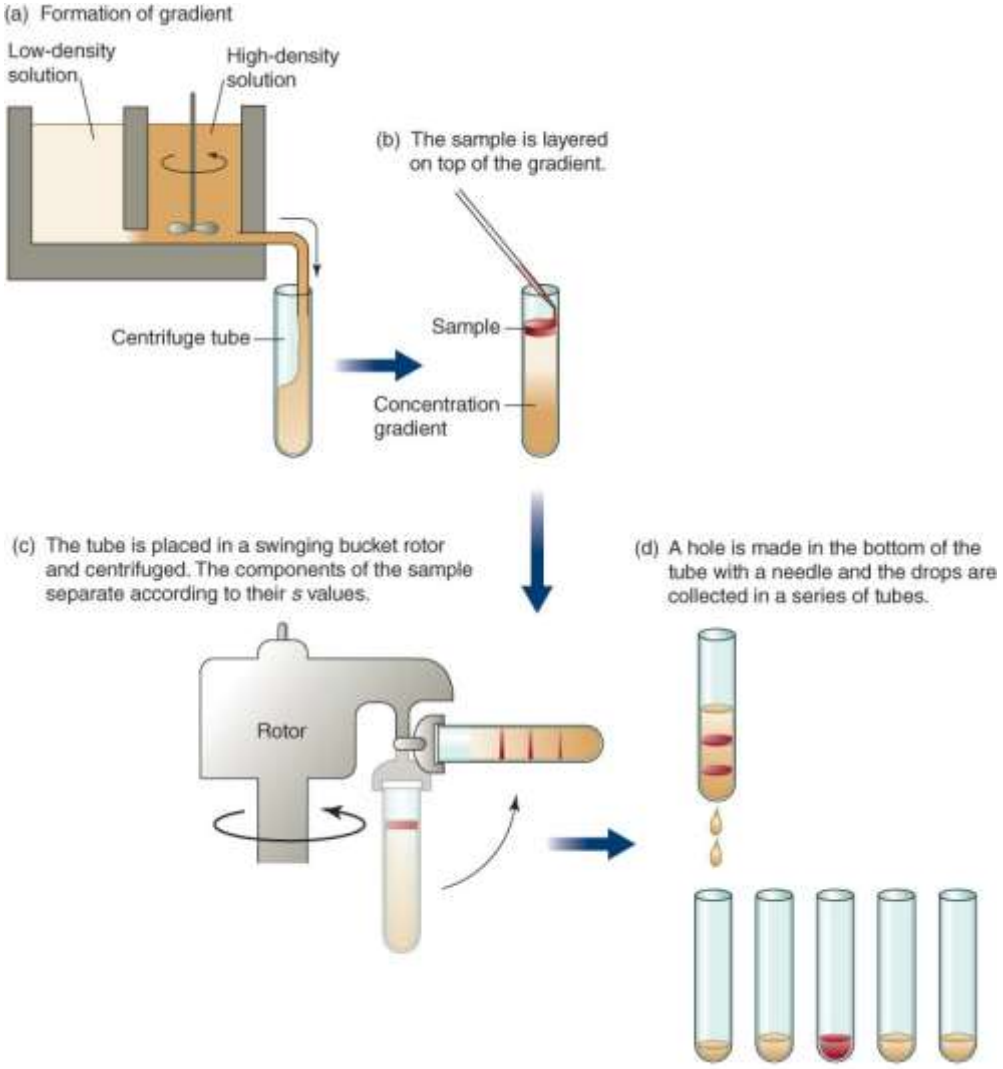



Figure : Gradient centrifugation separates samples based on their density.

# DNA Sequencing

- Classical chain termination sequencing uses **dideoxynucleotides** (ddNTPs) to terminate DNA synthesis at particular nucleotides.
- Fluorescently tagged ddNTPs and capillary gel electrophoresis allow automated, high-throughput DNA sequencing.
- The next generations of sequencing techniques aim to increase automation and decrease time and cost of sequencing.

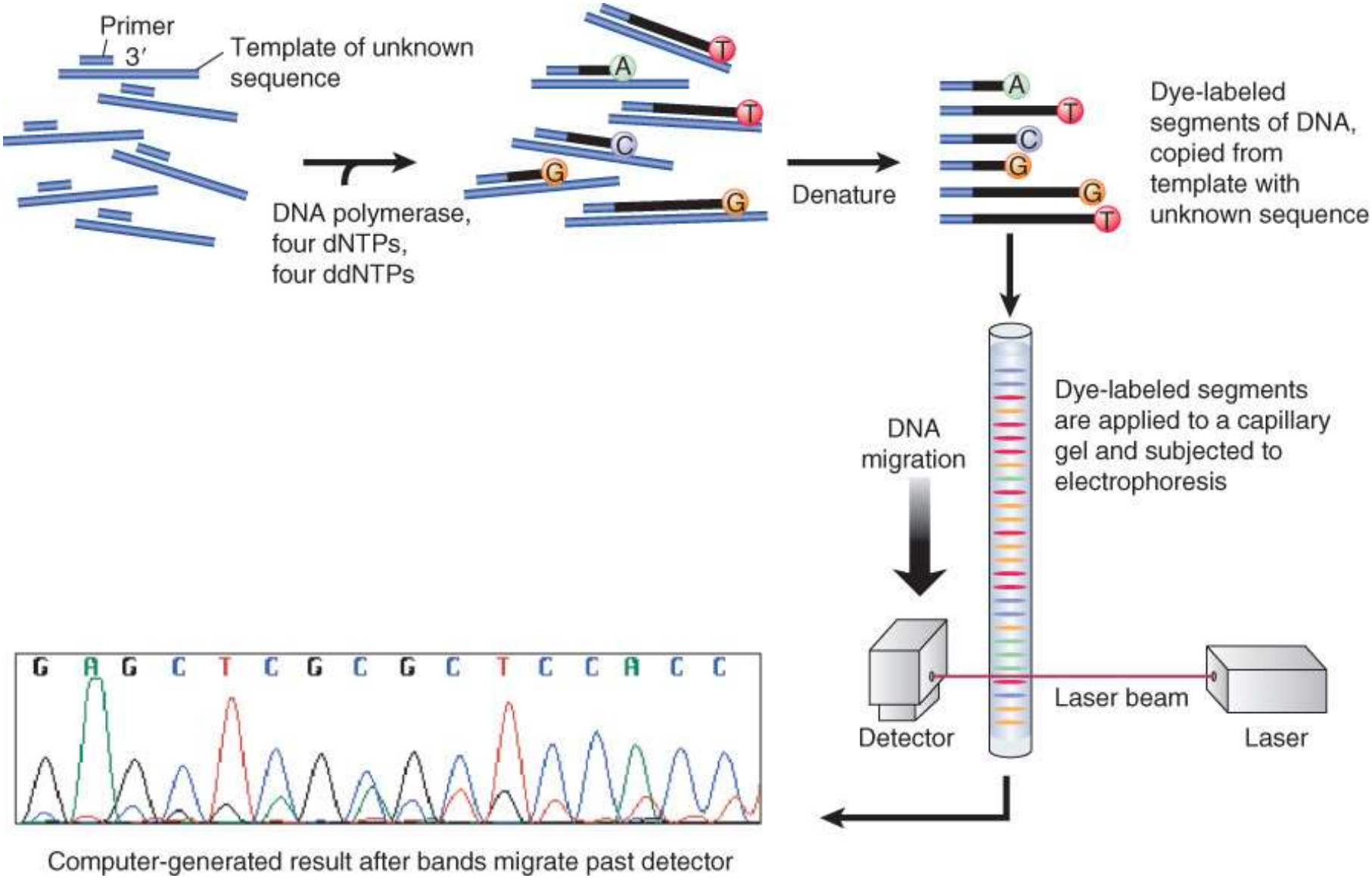



Figure : DideoxyNTP sequencing using fluorescent tags.

# CC9TH 4TH SEMESTER METHODOLOGY

- Polymerase Chain Reaction Basic Concepts
- Principles of Western Southern and Northern Blotting Techniques

# PCR and RT-PCR

- **Polymerase chain reaction (PCR)** permits the exponential amplification of a desired sequence, using primers that anneal to the sequence of interest.

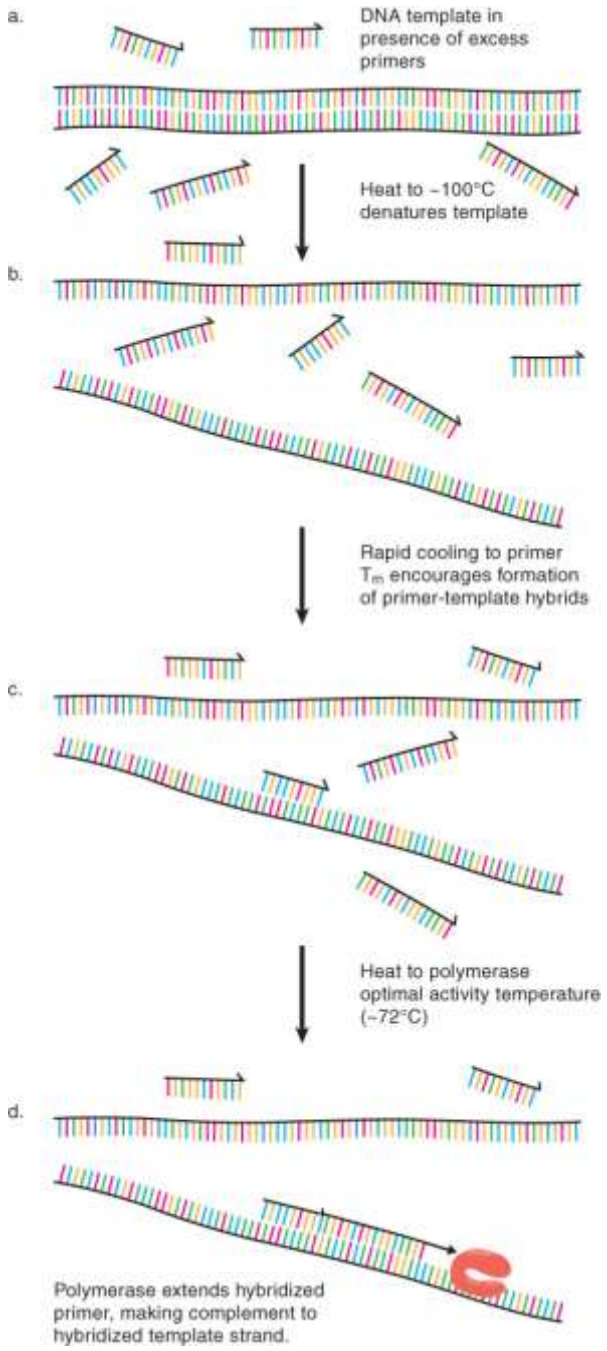
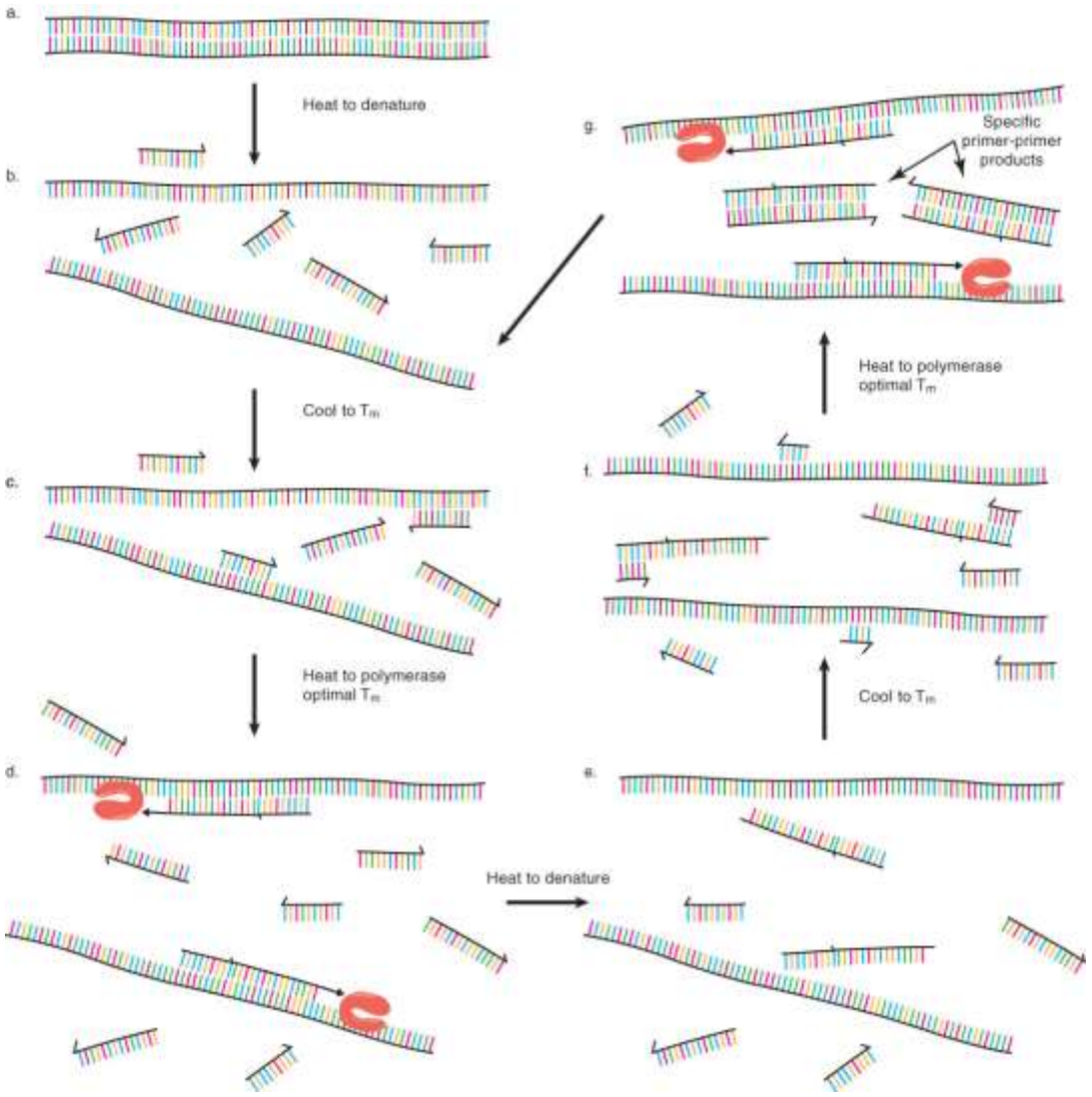



Figure : Denaturation (a) and rapid cooling (b) of a DNA template molecule in the presence of excess primer.

# PCR and RT-PCR




- **RT-PCR** uses reverse transcriptase to convert RNA to DNA for use in a PCR reaction.

Figure : Exponential production of the short, primer-to-primer–defined sequence (the “amplicon”).

# PCR and RT-PCR

- **Real-time**, or **quantitative**, **PCR** detects the products of PCR amplification during their synthesis, and is more sensitive and quantitative than conventional PCR.
- PCR depends on the use of thermostable DNA polymerases that can withstand multiple cycles of template denaturation.

# PCR and RT-PCR

- **fluorescence resonant energy transfer** (**FRET**) **–** A process whereby the emission from an excited fluorophore is captured and reemitted at a longer wavelength by a nearby second fluorophore whose excitation spectrum matches the emission frequency of the first fluorophore.

# Blotting Methods

- **Southern blotting** involves the transfer of DNA from a gel to a membrane, followed by detection of specific sequences by hybridization with a labeled probe.

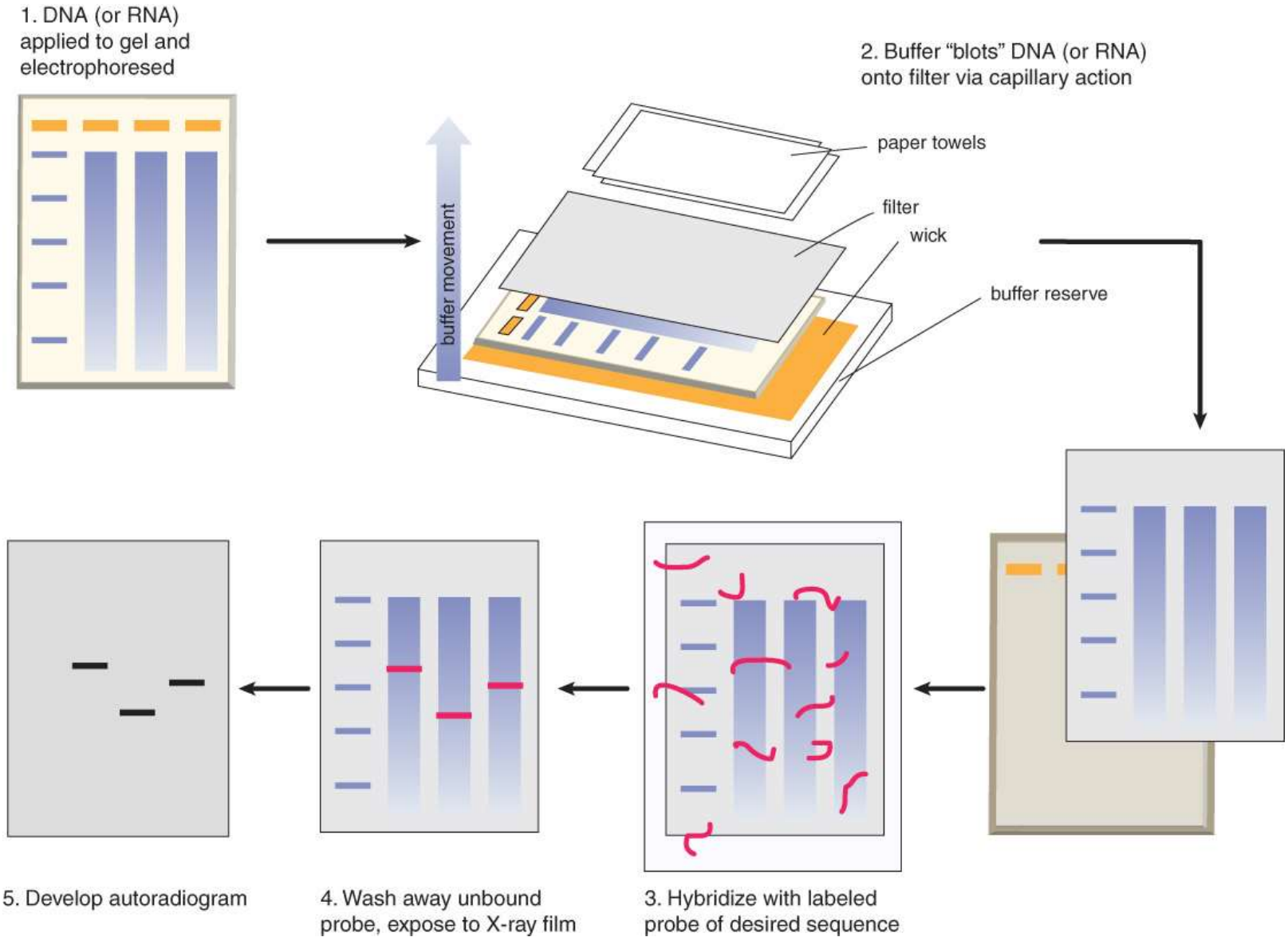



Figure : Southern blot.

# Blotting Methods

- **Northern blotting** is similar to Southern blotting, but involves the transfer of RNA from a gel to a membrane.
- **Western blotting** entails separation of proteins on a sodium dodecyl sulfate (SDS) gel, transfer to a nitrocellulose membrane, and detection of proteins of interest using antibodies.

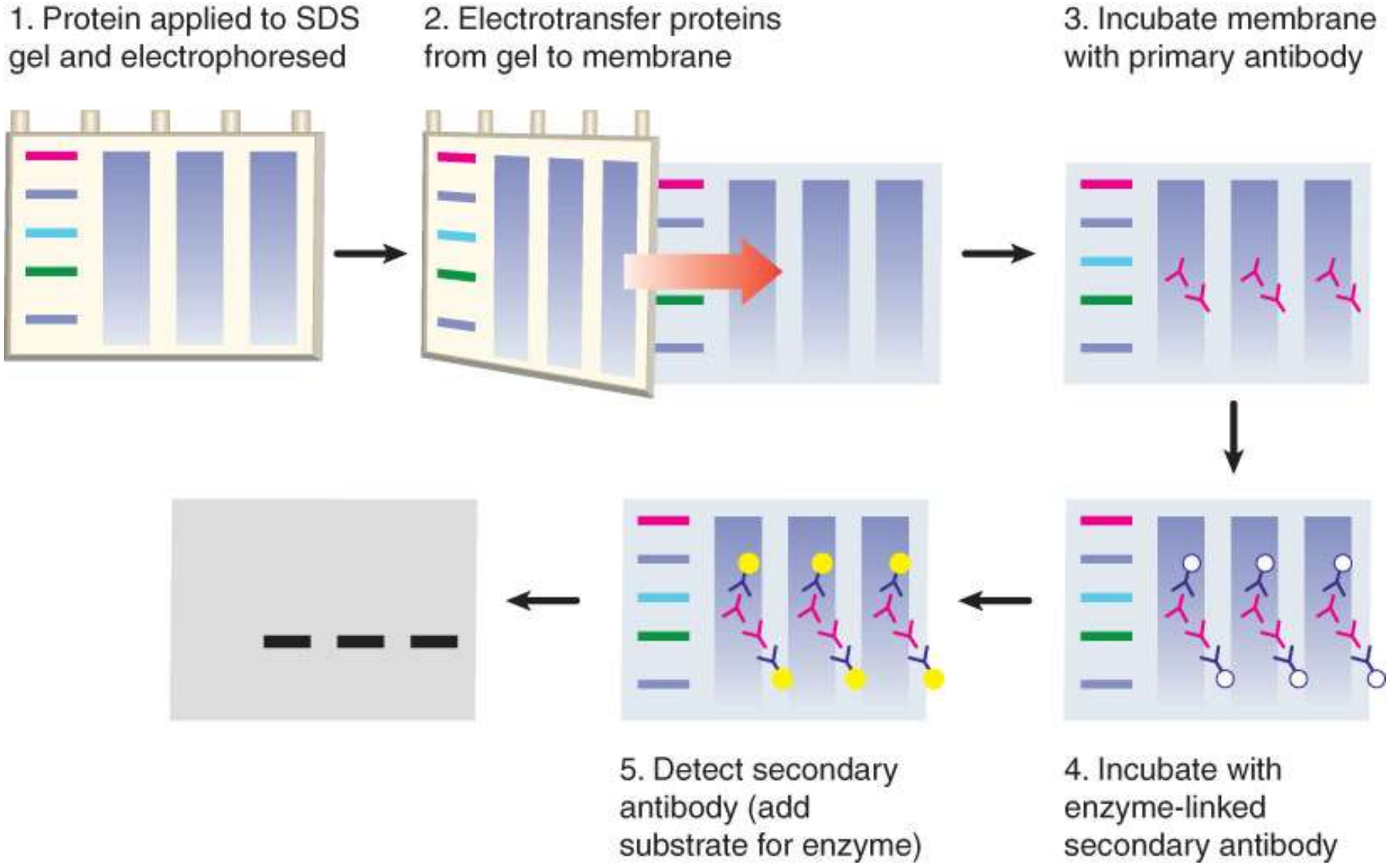



Figure : In a Western blot, proteins are separated by size on an SDS gel, transferred to a nitrocellulose membrane, and detected by using an antibody.

# Blotting Methods

- **epitope tag –** A short peptide sequence that encodes a recognition site (“epitope”) for an antibody, typically fused to a protein of interest for detection or purification by the antibody.

| Vector | Features | Isolation of DNA | DNA limit |
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# Gene Knockouts, Transgenics, and Genome Editing

- **transgenics –** Organisms created by introducing DNA prepared in test tubes into the germline.
  - The DNA may be inserted into the genome or exist in an extrachromosomal structure.

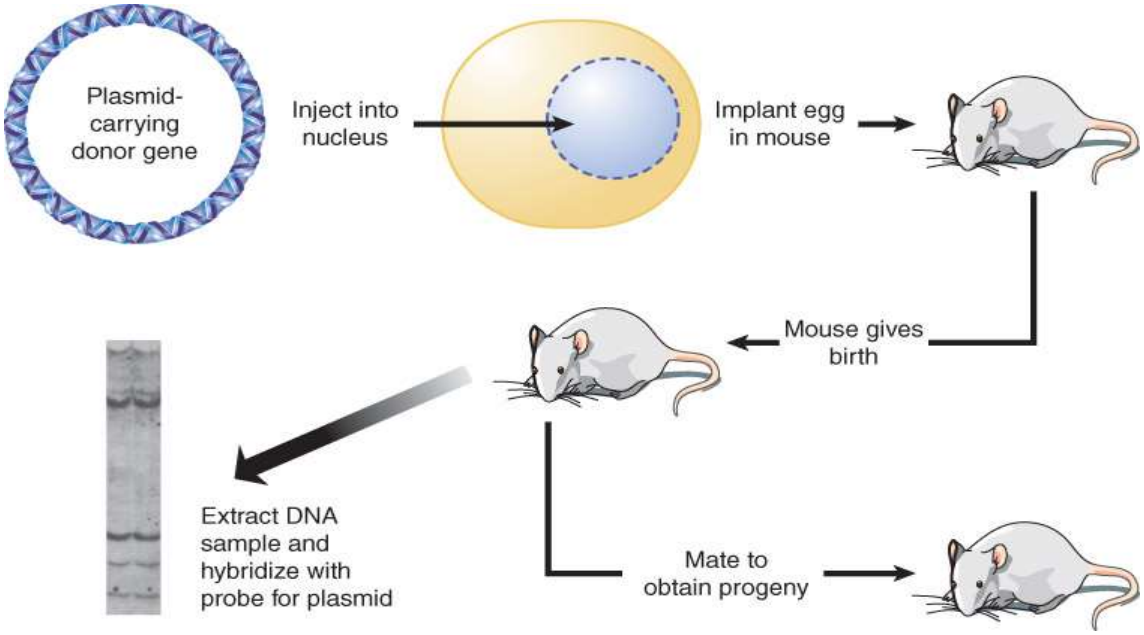


Figure : Transfection can introduce DNA directly into the germline of animals.

# Gene Knockouts, Transgenics, and Genome Editing

- Embryonic stem (ES) cells that are injected into a mouse blastocyst generate descendant cells that become part of a chimeric adult mouse.
  - When the ES cells contribute to the germline, the next generation of mice may be derived from the ES cell.
  - Genes can be added to the mouse germline by transfecting them into ES cells before the cells are added to the blastocyst.

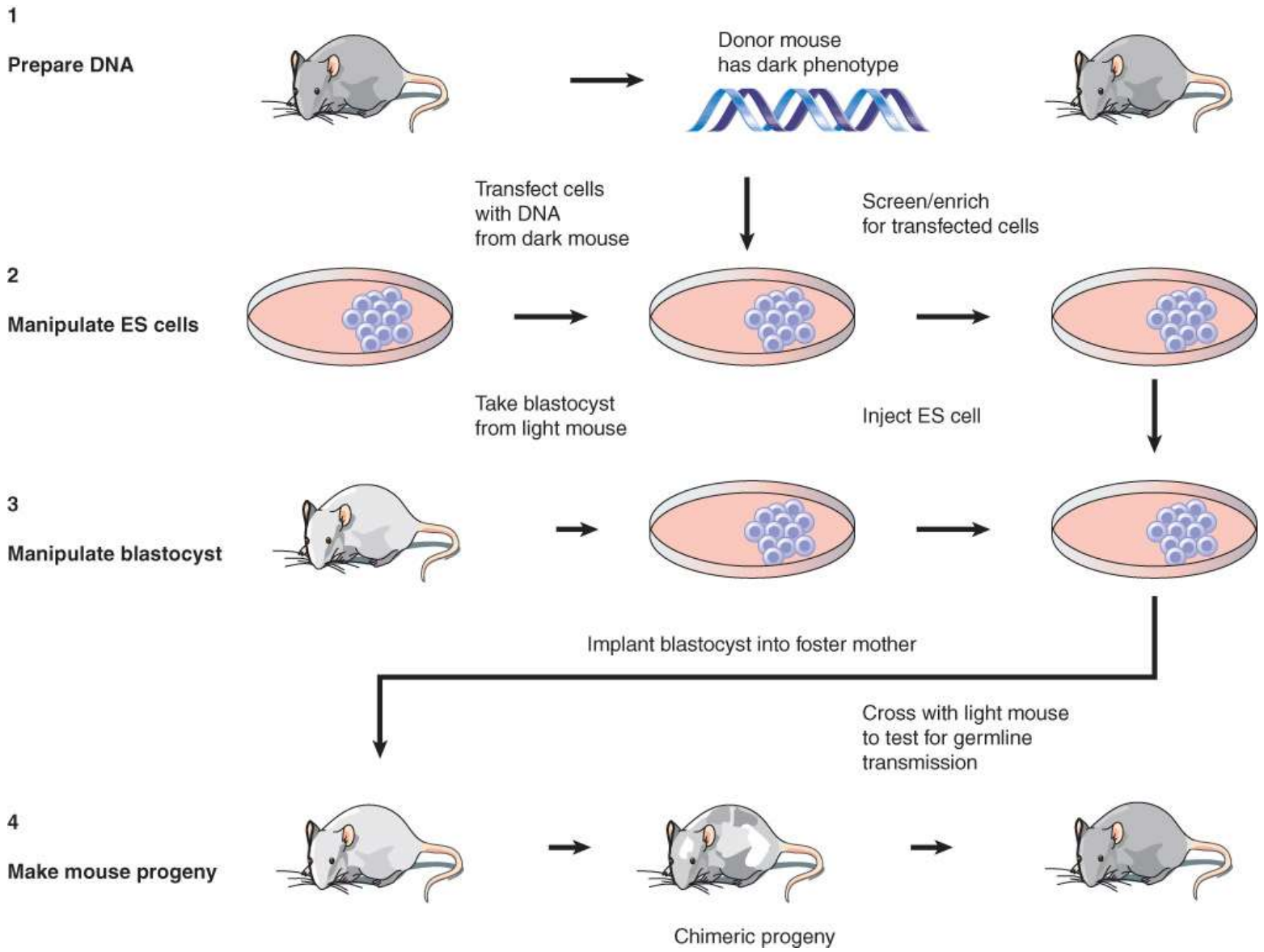


Figure : ES cells can be used to generate mouse chimeras.

# Gene Knockouts, Transgenics, and Genome Editing

- An endogenous gene can be replaced by a transfected gene using homologous recombination.
- The occurrence of successful homologous recombination can be detected by using two selectable markers, one of which is incorporated with the integrated gene, the other of which is lost when recombination occurs.

# Gene Knockouts, Transgenics, and Genome Editing

- The Cre/*lox* system is widely used to make inducible **knockouts** and **knock-ins**.
  - **knockout –** A process in which a gene function is eliminated, usually by replacing most of the coding sequence with a selectable marker *in vitro* and transferring the altered gene to the genome by homologous recombination.
  - **knock-in –** A process similar to a knockout, but more subtle mutations are made.

# Gene Knockouts, Transgenics, and Genome Editing

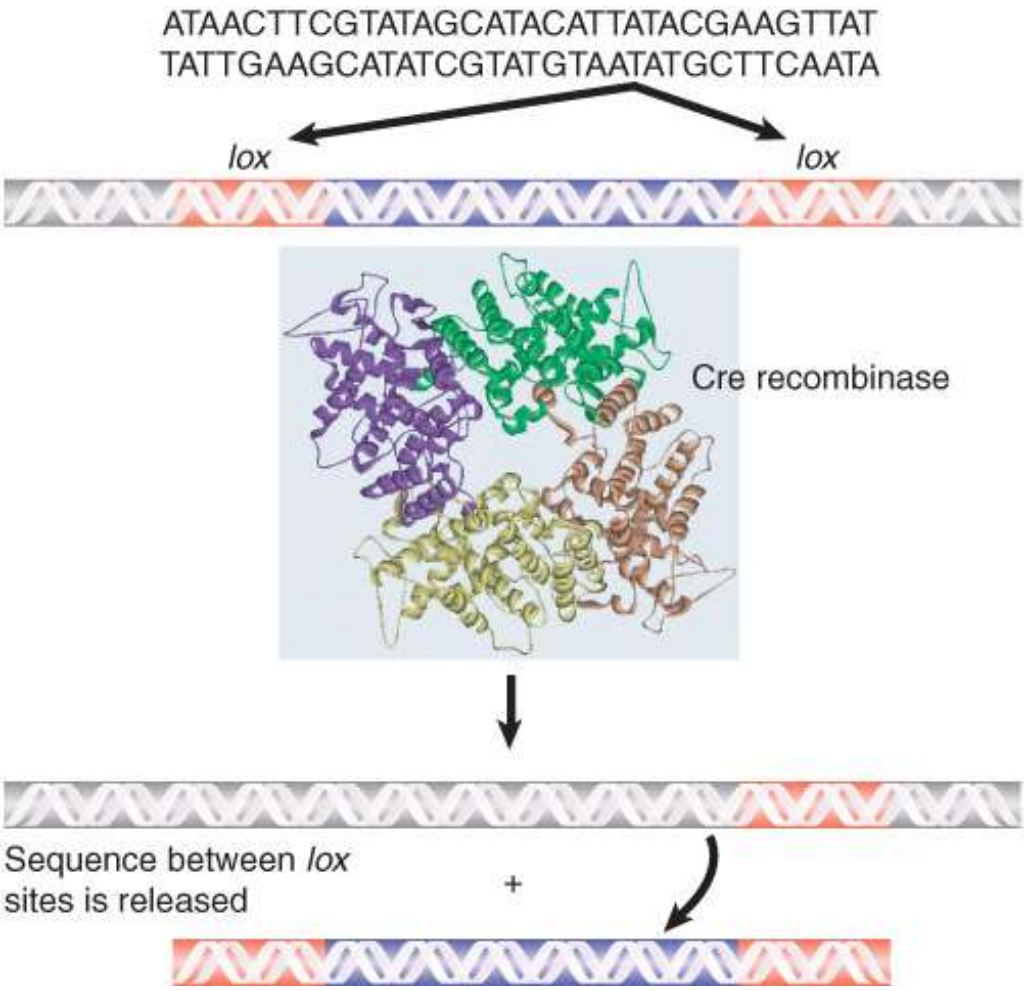


Figure: The Cre recombinase catalyzes a site-specific recombination between two identical *lox* sites, releasing the DNA between them.