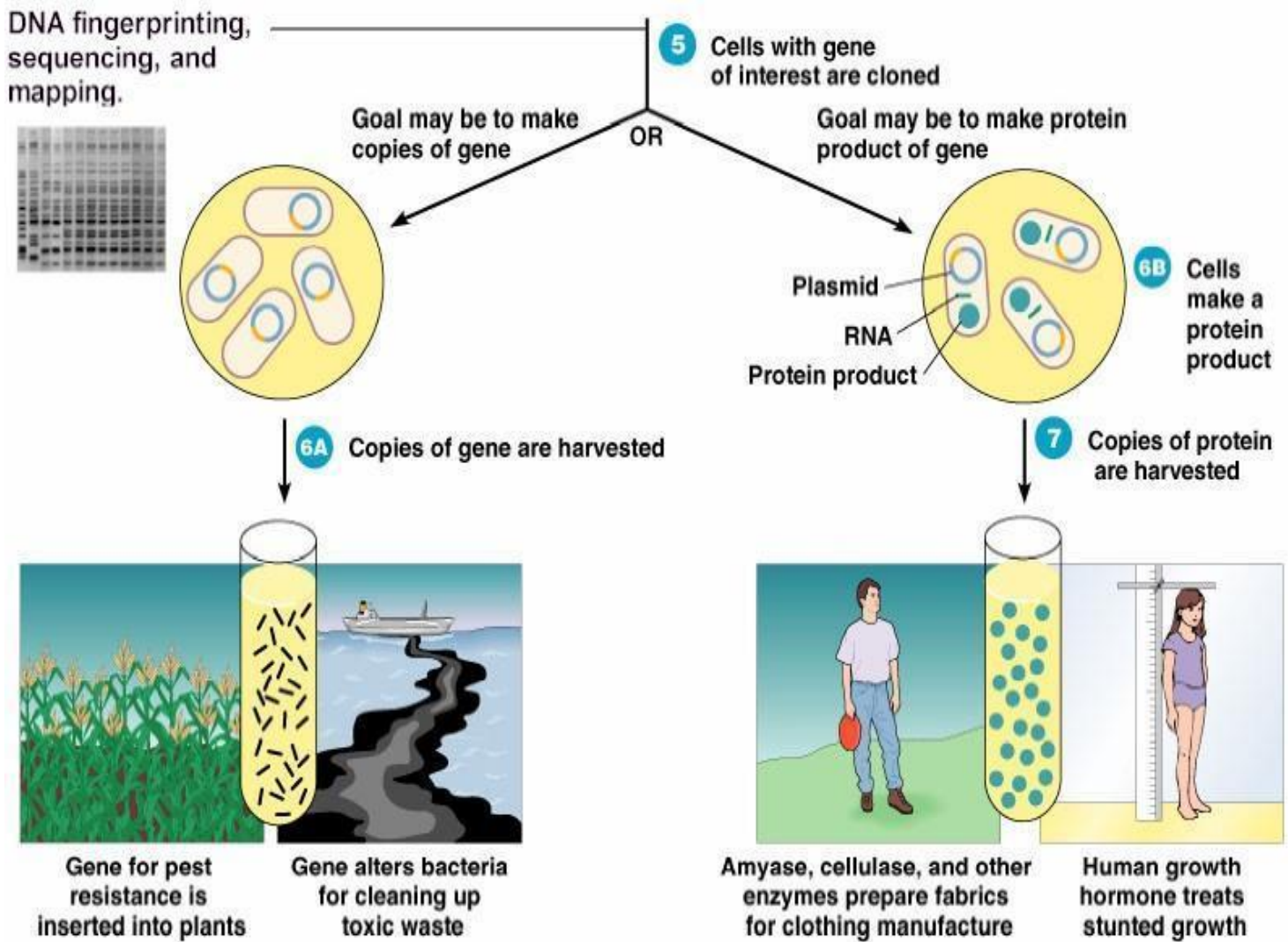


# DESIGNER GENES - BIOTECHNOLOGY

- Technology to manipulate DNA ó techniques often called **genetic engineering** or
- **Recombinant DNA Technology**-Technology used to manipulate DNA
- Procedures often called genetic engineering
- Recombinant DNA - DNA from two sources
- Transgenic individuals have DNA from another organism
- Often involve putting genes into viruses or bacteria.
- Vectors are the pieces of DNA used to transfer genes into a host cell ó often plasmids of bacteria

## Overview of Biotechnology



# Basic Tools of DNA Technology

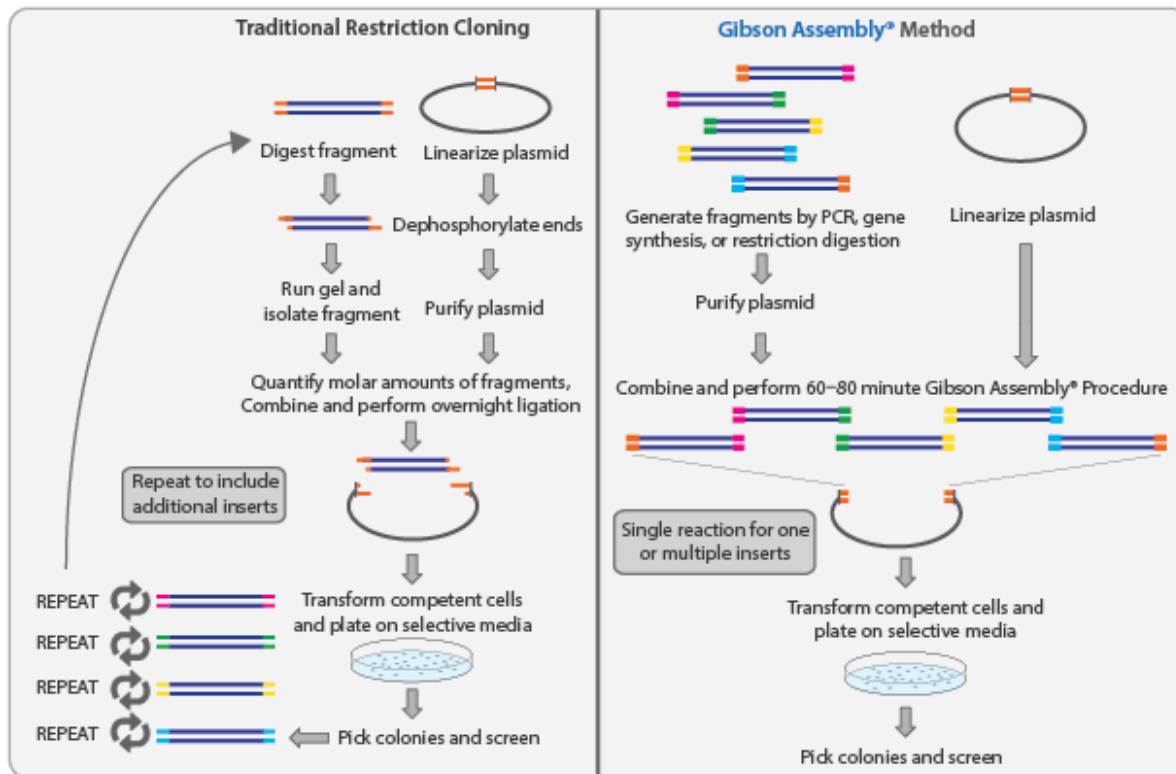
1. Identifying desired DNA
2. Cutting DNA with Restriction Enzymes
3. Inserting DNA into Vector as Plasmid
4. Connecting DNA pieces with Ligase
5. Inserting Vector into Host Cell as bacterium
6. Cloning desired DNA and Vectors
7. Storing clones in DNA Libraries
8. Identifying cloned genes with Radioactive Probes
9. Analyzing DNA by cutting fragments and separating by Electrophoresis

## DNA Analysis Technologies

- **identifying** – recognizing desired DNA fragment or plasmid using **radioactive probes**
- **cutting DNA** - using desired **restriction enzymes** or ð enzymatic scissors
- **making hybrids of DNA** using **Hybridization techniques**
- **cloning DNA** ó using other cells or in a test tube as with **PCR** – Polymerase Chain Reaction ó clones - DNA segments in a test tube quickly and inexpensively. May use very small amounts of DNA to clone
- **storing DNA** in **DNA libraries** of plasmids or bacteriophages of genome DNA or cDNA.
- **separating DNA segments** with **electrophoresis**
- **transferring DNA** using **blotting**
- **imaging DNA** with **autoradiography**
- **analyzing DNA** by **sequencing** or determining the nucleotide sequence of a gene, **microassays** analyze gene function and expression, **DNA fingerprinting techniques** such as **RFLP** or restriction fragment length polymorphism, **VNTRs** or Variable Number Tandem Repeats, **STRs** or Short Tandem Repeats, **Ribosomal DNA Analysis**, or **Y-chromosome Analysis**

## Cloning methods currently in use:

- **Traditional Restriction digestion cloning**
  - Plasmids and inserts are digested with the same restriction enzymes and then ligated together. See diagram below.
- **Gateway Recombination**
  - Regions of homology between insert DNA and plasmid are used for a recombination event that transfers the insert DNA into the plasmid.
- **Gibson assembly**
  - DNA fragments containing homologous overlapping ends are ligated together in one reaction.
- **TA cloning**
  - Linearized plasmids engineered to have single T overhangs are ligated together with a PCR product insert. Most DNA polymerases leave an A overhang on PCR products, which allows them to base pair with the T overhangs on the TA cloning plasmid.

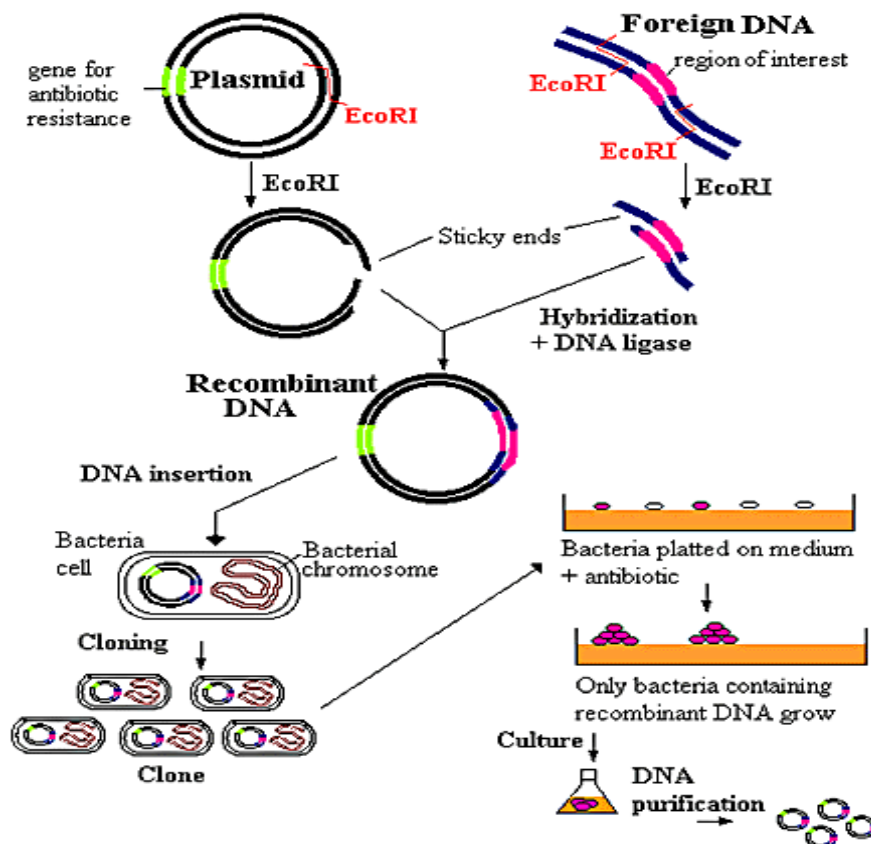
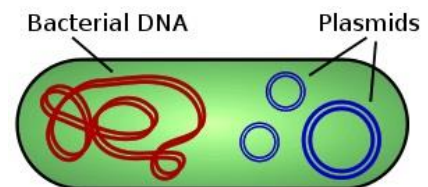


## Basic Terminology

- **Recombinant DNA** ó DNA from two different sources combined. Often involve putting genes
- into viruses or bacteria using a **vector**.
- **Inserting a gene into a bacterium** - Organism provides the desired piece of DNA which is spliced into a piece of DNA used to transfer the genes or **vector** which is inserted to a Host cell (often a bacterium)
- **Plasmids**– in bacteria, circular DNA serve as vectors. Easily taken up by bacterial cells. It is more difficult to insert vector into Eukaryotic cells.
- **Transgenic organisms** have DNA from another organism
- **Restriction enzymes** - enzymes to cut DNA at a particular spot and **DNA ligase** enzymes reattach ends.
- **Hybridization** ó process of putting pieces of DNA together.
- **Chromosome mapping** – determining the location of genes on a chromosome and making a map of restriction sites as **Restriction Maps**.

# Basic Tools of Cloning

- **Gene selection** & isolation from Donor
  - Eukaryotic genes contain introns but bacteria do not contain the necessary enzymes to remove introns
  - Eukaryotic genes that are inserted into bacteria must be inserted without introns.
  - Use *reverse transcriptase* (from retroviruses) and modified M-RNA to produce **cDNA** with introns already removed
- **Plasmid selection & isolation**
  - A small DNA molecule that is physically separate from, and can replicate independently of, chromosomal DNA within a cell such as a bacterium
  - When used in genetic engineering ó called **vectors**
  - Several methods to isolate plasmid DNA from bacteria
- **Restriction enzyme** to cut piece
- **Putting pieces together**
  - DNA hybridization
  - DNA ligase to reattach pieces
- **Insert** into Host bacteria
- **Clone** the bacteria

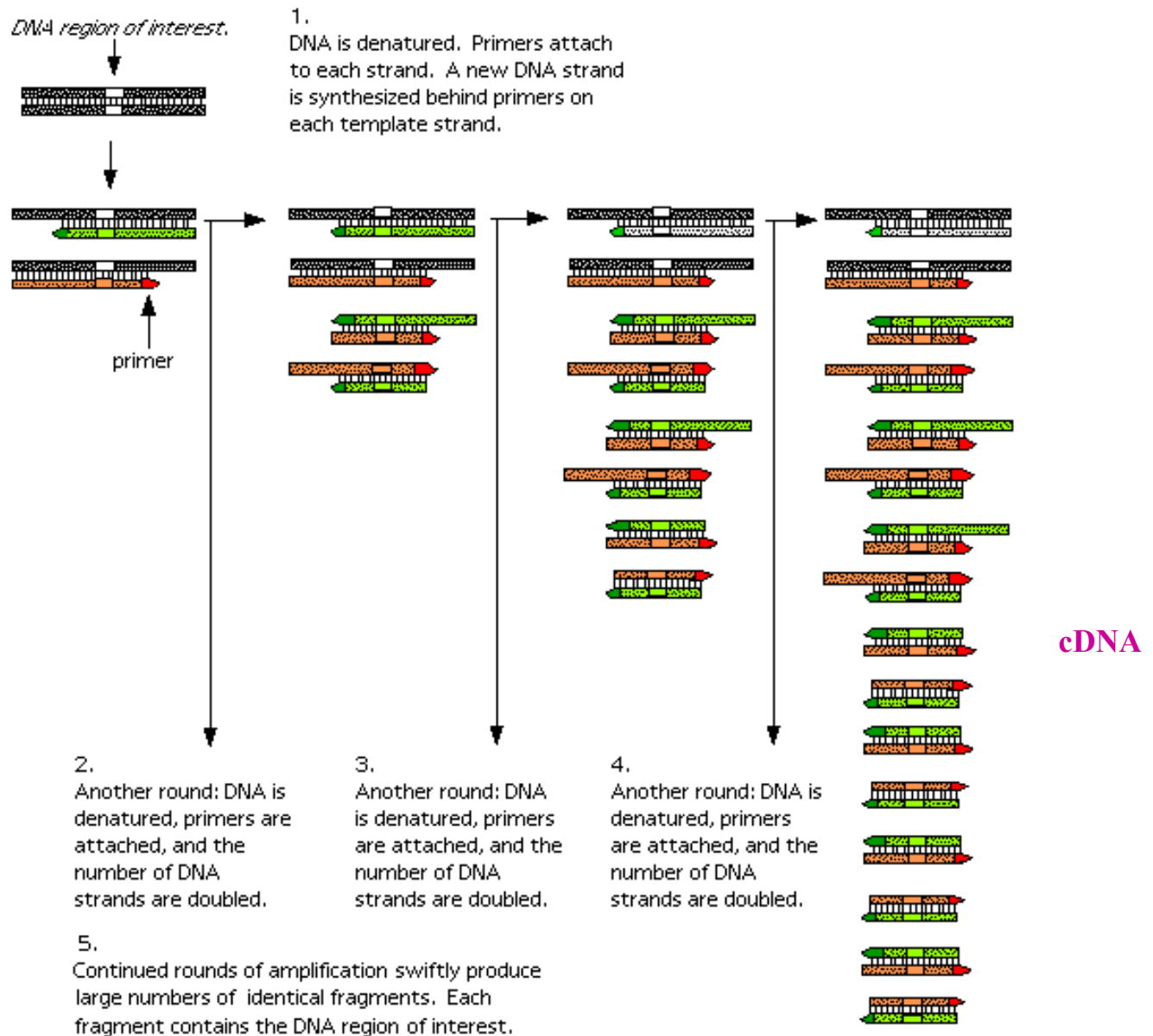


Cloning into a plasmid

## Polymerase Chain Reaction (PCR)

- Technique for quickly making an unlimited number of copies of any piece of DNA
- Sometimes called "molecular photocopying"

### POLYMERASE CHAIN REACTION



## complementary DNA

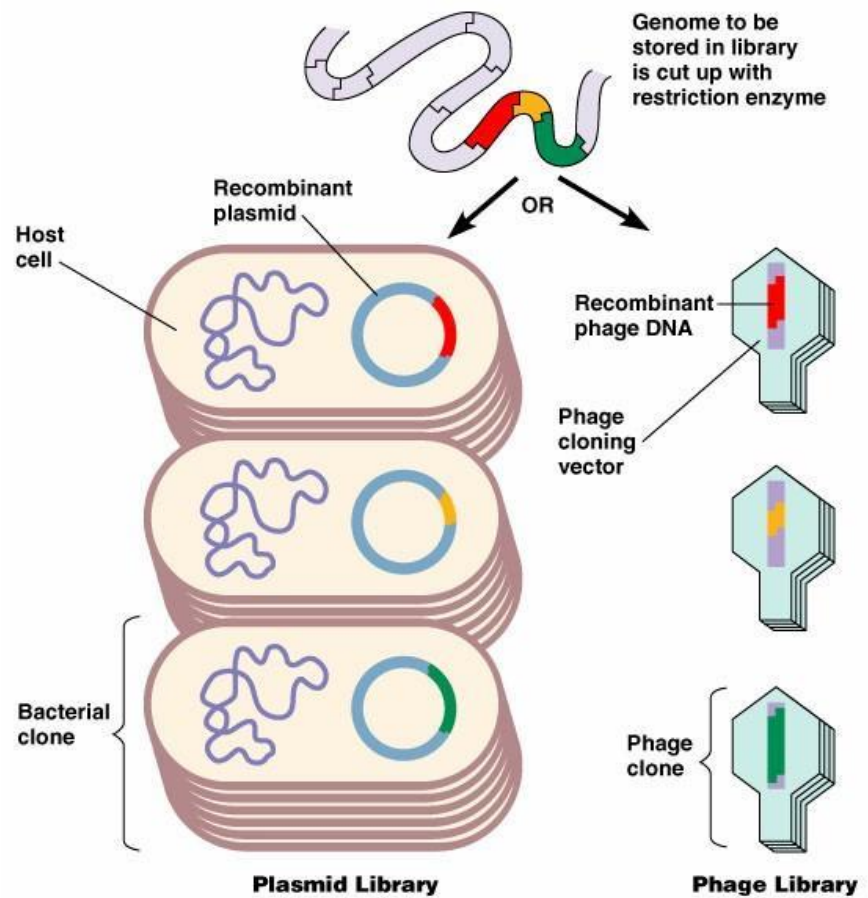
- **cDNA**- Eukaryotic genes contain introns but bacteria do not contain the necessary enzymes to remove introns
- Eukaryotic genes that are inserted into bacteria must be inserted without introns.
- Use *reverse transcriptase* (from retroviruses) and modified m-RNA to produce cDNA with introns already removed

## Techniques for Storing, Identifying, Separating Clones

- Storing clones in DNA Libraries
- Cloning within cells and with PCR
- Identifying cloned genes with Radioactive Probes
- Analyzing DNA by cutting fragments and separating by Electrophoresis/nucleic acid hybridization/DNA probes
- Transferring DNA from gel by Blotting
- Imaging with autoradiography
- DNA Sequencing to determine exact sequence
- Microassays to analyze gene function

## DNA Libraries

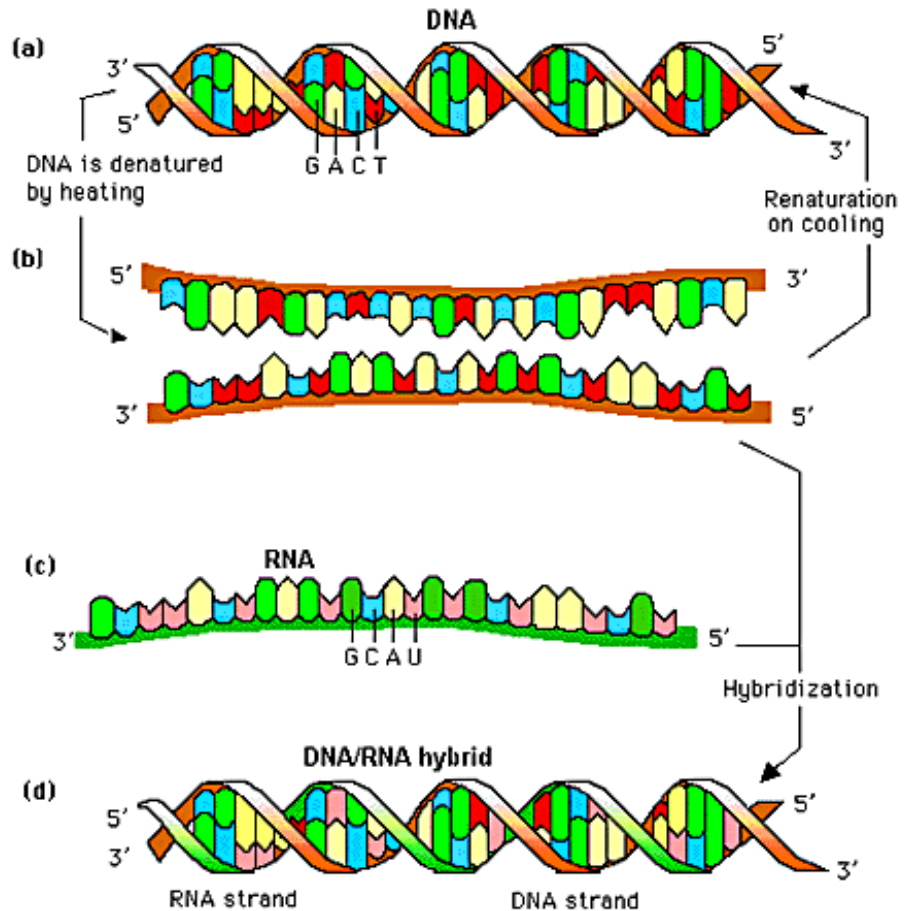
- **Genomic** ó normal DNA
- **cDNA** ó modified to remove introns
- Fragments are stored in plasmids or bacteriophages





## DNA Hybridization

- Base pairing of two single strands of DNA or RNA.
- Can be DNA-DNA, DNA-RNA
- Can be a radioactive probe



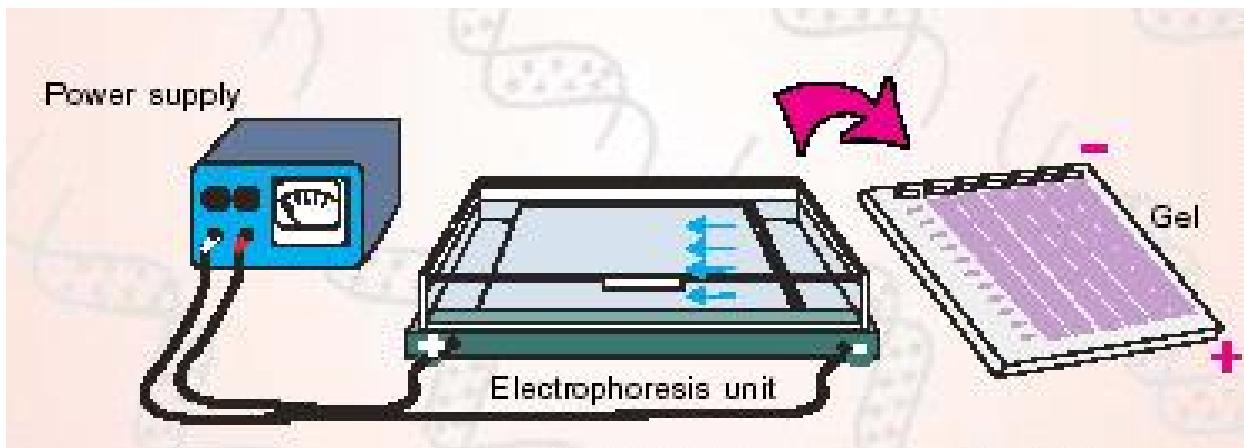
## Radioactive Probes

- short, radioactive strands of DNA
- will pair up with complementing strands of DNA
- fragments that contain the labeled pieces will show up on an x-ray film

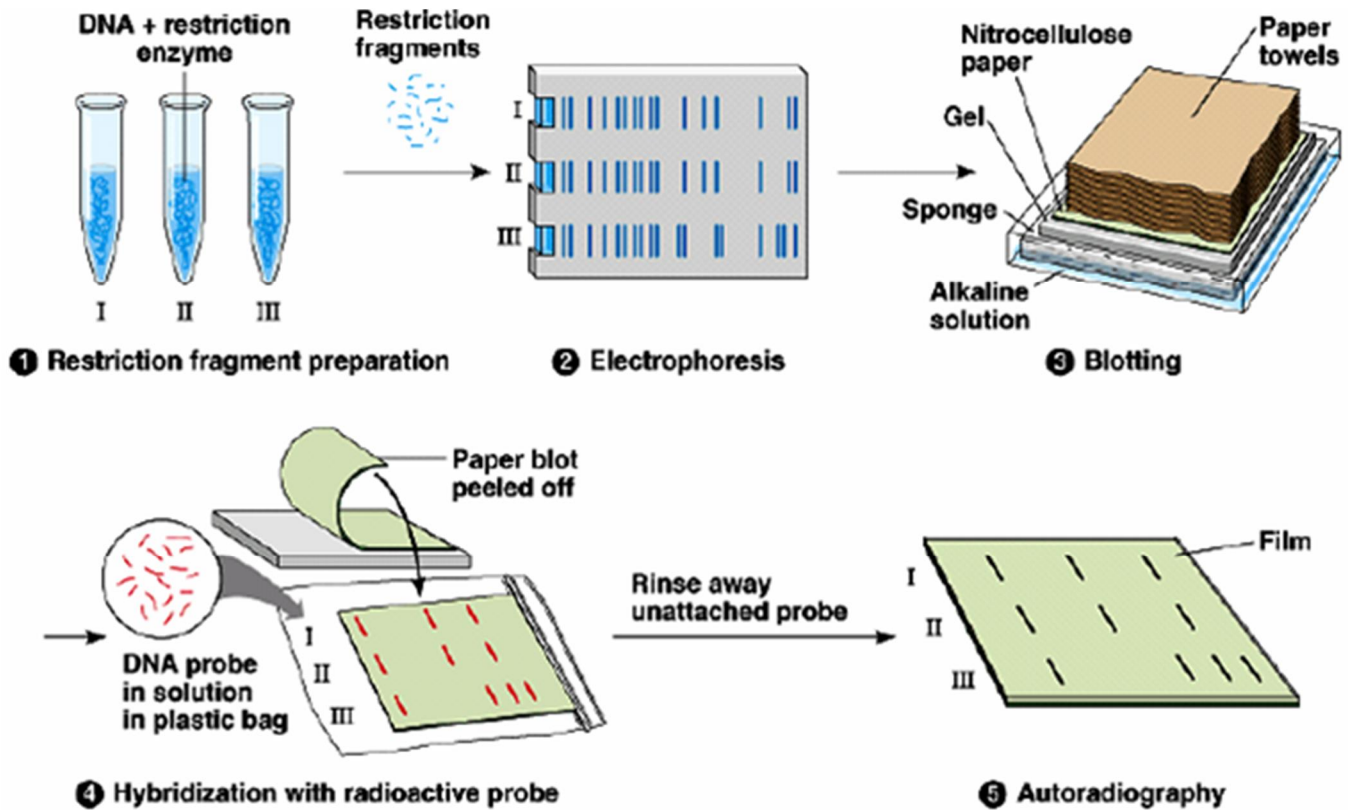
## Nucleic Acid Hybridization

## Electrophoresis

A process in which molecules (such as proteins, DNA, or RNA fragments) can be separated according to size and electrical charge by applying an electric current to them.



# DNA Analysis



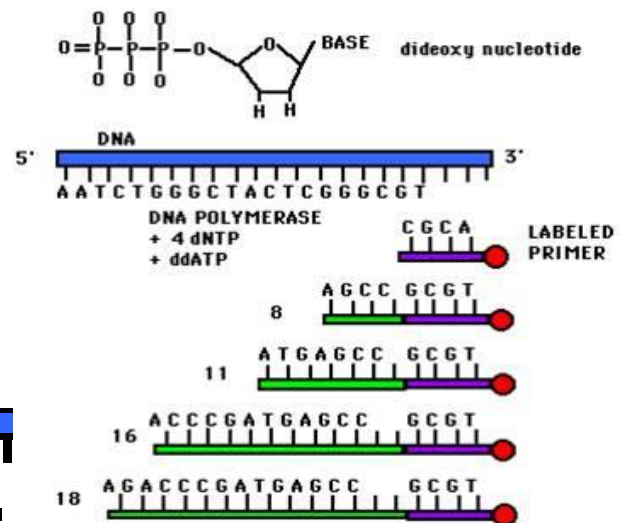
## Analysis of DNA Fragments

- When a plasmid is digested by restriction enzymes, the length of each fragment can be analyzed on a gel
- Then the physical map of the plasmid can be constructed.
- The DNA on a gel can be analyzed by hybridization after transfer onto a membrane, this is called a **Southern blot**.
- A similar procedure called a **Northern blot** is used to detect mRNA on a membrane.
- **Reverse transcription mediated PCR** can also be used to analyze mRNA from cells.



## Sanger Method of DNA Sequencing

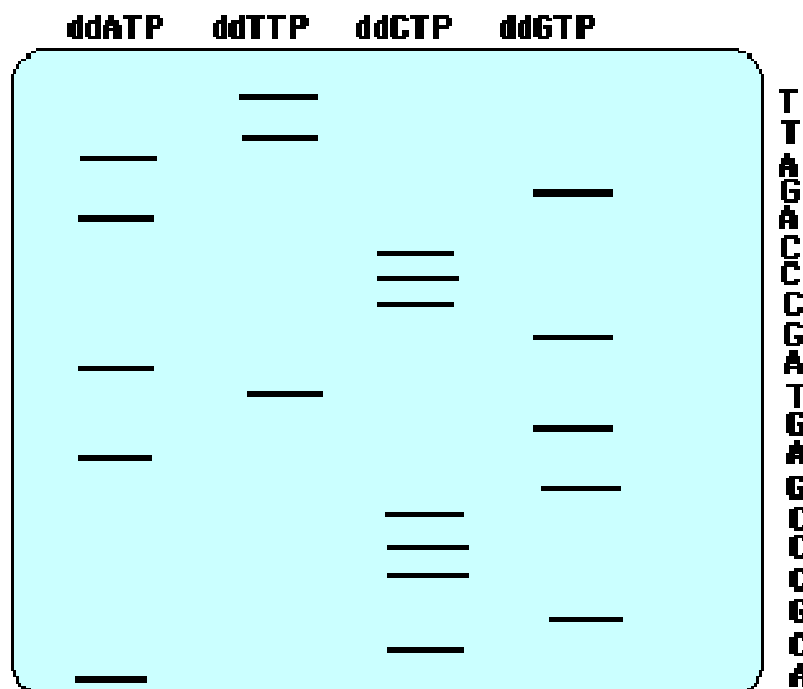
- Analysis of genes at the nucleotide level
- Tool has been applied to many areas of research
- Polymerase chain reaction (PCR) - quickly making an unlimited number of copies of any piece of DNA requires knowing the sequence of the piece to be copied
- Amino acid sequences can be determined more easily by sequencing a piece of cDNA
- Can utilize sequencing to identify the site of a point mutation
- Utilizes 2',3'-dideoxynucleotide triphosphates (ddNTPs)



- First convert double stranded DNA into single stranded DNA
- Determine the **exact nucleotide sequence**
- Columns for A,T,C, and G
- The **sequence of the sequenced strand, 5' to 3'**, is  
**AATCTGGGCTACTCGGGCGT**
- The sequence of the **strand of DNA complementary to**



the sequenced strand is 5' to 3'  
**ACGCCCCGAGTAGCCCCAGATT**

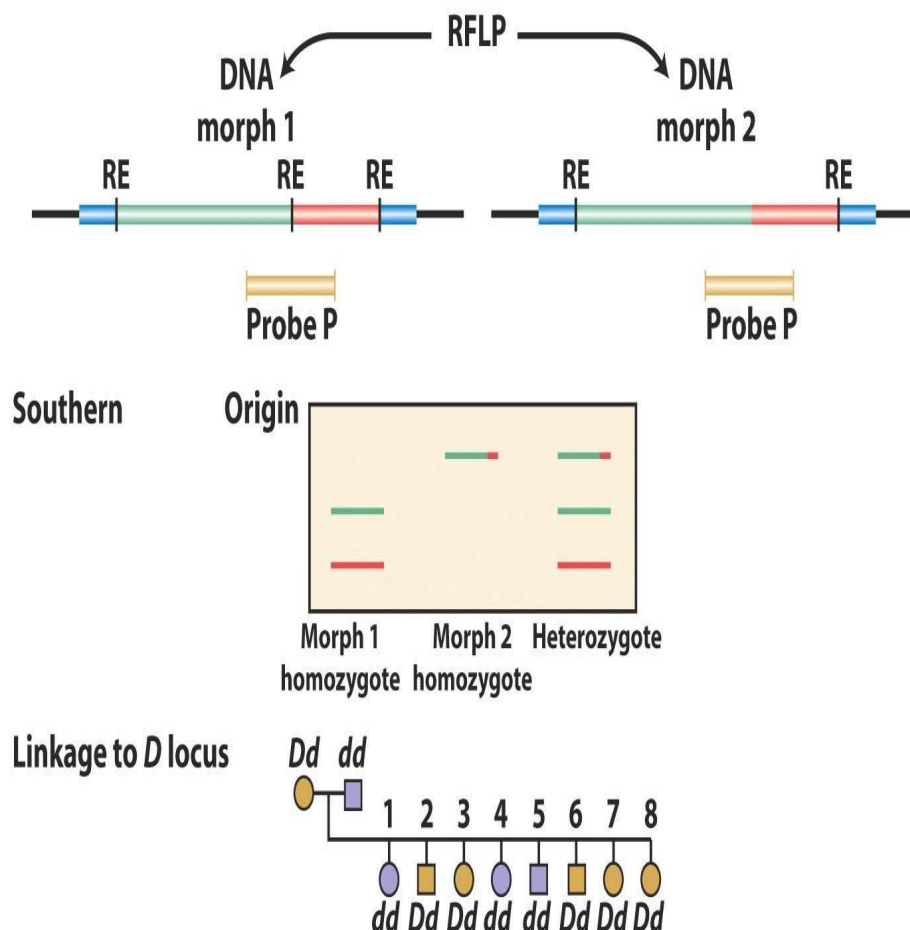


## DNA Fingerprinting Techniques

- RFLP ó Restriction Fragment Length Polymorphism (original)
- PCR ó Polymerase Chain Reaction
- VNTRs ó Variable Number Tandem Repeats
- STRs - Short Tandem Repeats
- Ribosomal DNA analysis
- Y-chromosome analysis

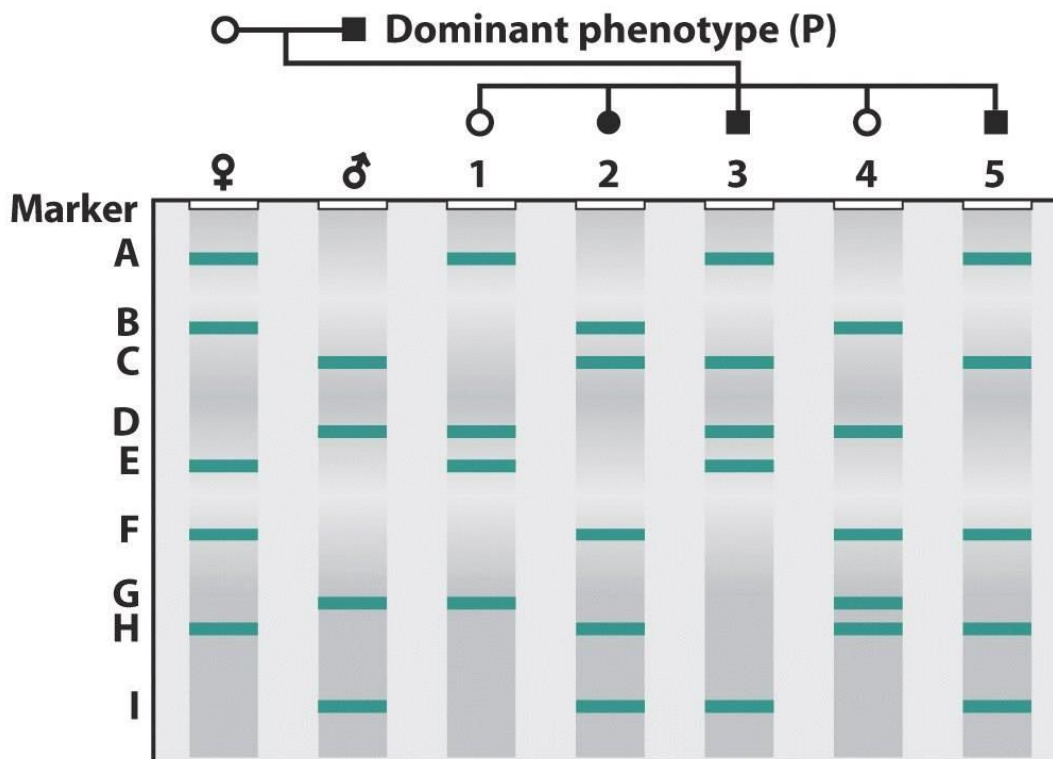
## Restriction Fragment Length Polymorphism – RFLP

- The DNA of an organism is cut up into fragments using restriction enzymes.
- A large number of short fragments of DNA will be produced. (RFLPø)
- Electrophoresis is a technique used to separate the DNA fragments according to their size.
- Uses- identification of diseased genes including oncogenes, identification of viral infections, determining family relationships among individuals, and identifying tissue found at a crime scene.
- Genetic variations at the site where a restriction enzyme cuts a piece of DNA.
- Such variations affect the size of the resulting fragments.
- These sequences can be used as markers on physical maps and linkage maps.



## VARIABLE NUMBER TANDAM REPEATS (VNTR's)

- Short nucleotide sequences
- Organized in clusters of tandem repeats
- VNTR = 14-100 base pairs
- SNR = 2-10 base pairs



### ANALYSIS EXAMPLES

**F and H** Always inherited together — linked?

**A and B** In progeny, always either A or B — “allelic”?

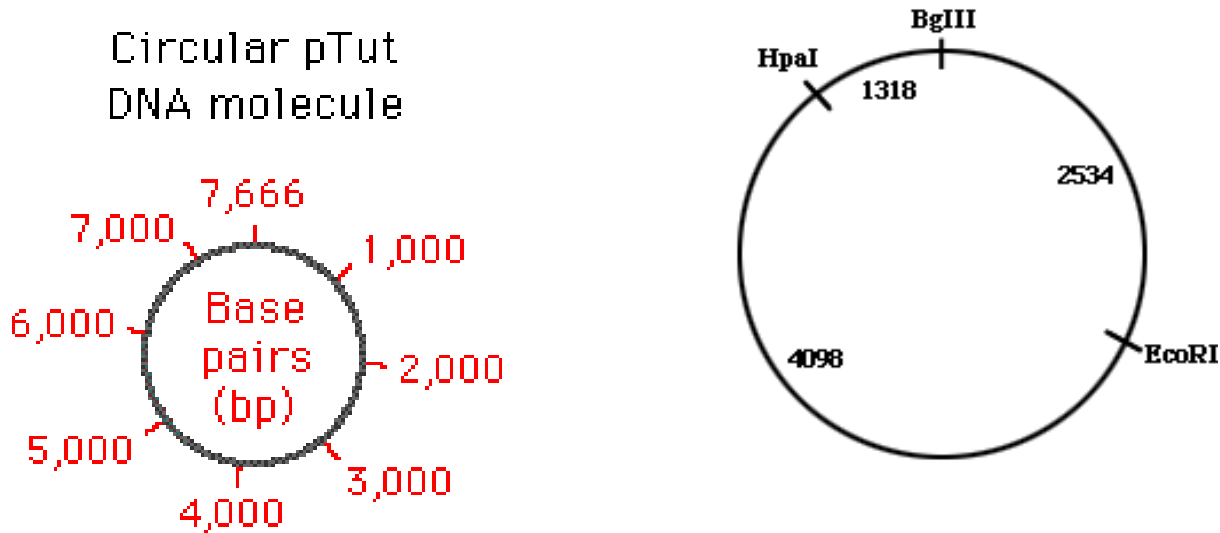
**A and D** Four combinations; A and D, A, D, or neither — unlinked?

**F, H, and E** Always *either* F and H *or* E — closely linked in trans?

**Allele P** Possibly linked to I and C.

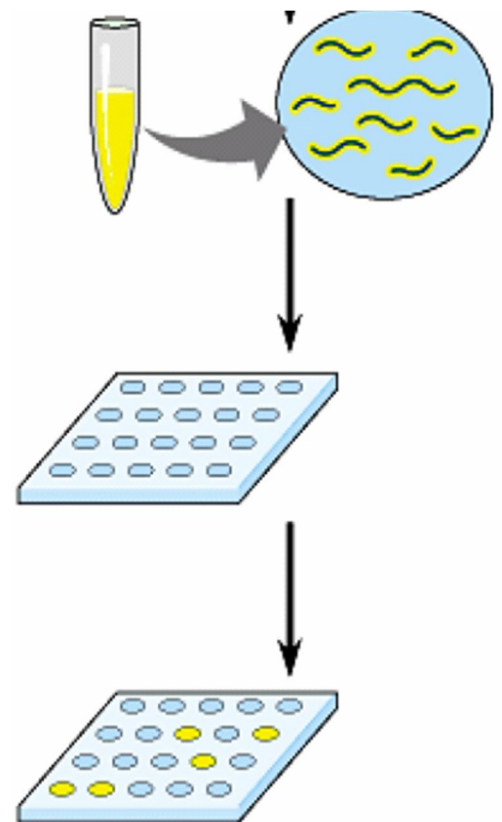
## Restriction Mapping

- Description of **restriction enzyme cleavage sites** within a piece of DNA
- Use of **different restriction enzymes** to analyze and generate a physical map of genomes, genes, or other segments of DNA



## DNA Microassay

- studying **how large numbers of genes interact with each other**
- precisely apply tiny droplets containing functional DNA to glass slides
- attach fluorescent labels to DNA from the cell they are studying.
- labeled probes are allowed to bind to complementary DNA strands on the slides
- slides are put into a scanning microscope that can measure the brightness of each fluorescent dot
- brightness reveals how much of a specific DNA fragment is present, an indicator of how active it is.
- **Advantages of using microarray technology:**
  - Readily available mature technology
  - Standard methods available
  - Relatively inexpensive
- **Limitations of microarray technology:**
  - Dynamic range of measurement is limited:
    - Intensity of fluorescent dyes
    - Sensitivity of scanning instruments
    - Non-specific hybridization
  - Known genome.



## RNA-Seq

- RNA-seq refers to the method of using Next Generation Sequencing (NGS) technology to sequence RNA which can then be used to quantify relative levels of transcription for every gene in an organism's genome across different conditions
- NGS technology is an ultra-high-throughput technology to measure DNA sequences.
- **Advantages of RNA-seq over microarray** include:
  - Wider measurable range of expression levels
  - Not dependent on known genome
  - Free of hybridization artifacts
  - Can be used to identify and quantify small regulatory RNAs
  - Possibility of one platform for all applications

## Next Generation Sequencing Platforms

- Roche 454 sequencer
- Illumina Genome Analyzer (Solexa sequencing)
- Applied Biosystems SOLiD sequencer
- Ion Proton sequencing
- PacBio real-time sequencing
- Comparison of the Second-generation DNA sequencing technologies

Platform	Chemistry	Read Length	Run Time	Gb/Run	Advantage	Disadvantage
454 GS Junior (Roche)	Pyro-sequencing	500	8 hrs.	0.04	Long Read Length	High error rate in homopolymer
454 GS FLX+ (Roche)	Pyro-sequencing	700	23 hrs.	0.7	Long Read Length	High error rate in homopolymer
HiSeq (Illumina)	Reversible Terminator	2*100	2 days (rapid mode)	120 (rapid mode)	High-throughput / cost	Short reads Long run time (normal mode)
SOLiD (Life)	Ligation	85	8 days	150	Low Error Rate	Short reads Long run time
Ion Proton (Life)	Proton Detection	200	2 hrs.	100	Short Run times	New*
PacBio RS	Real-time Sequencing	3000 (up to 15,000)	20 min	3	No PCR Longest Read Length	High Error Rate

# Applications of Biotechnology Techniques

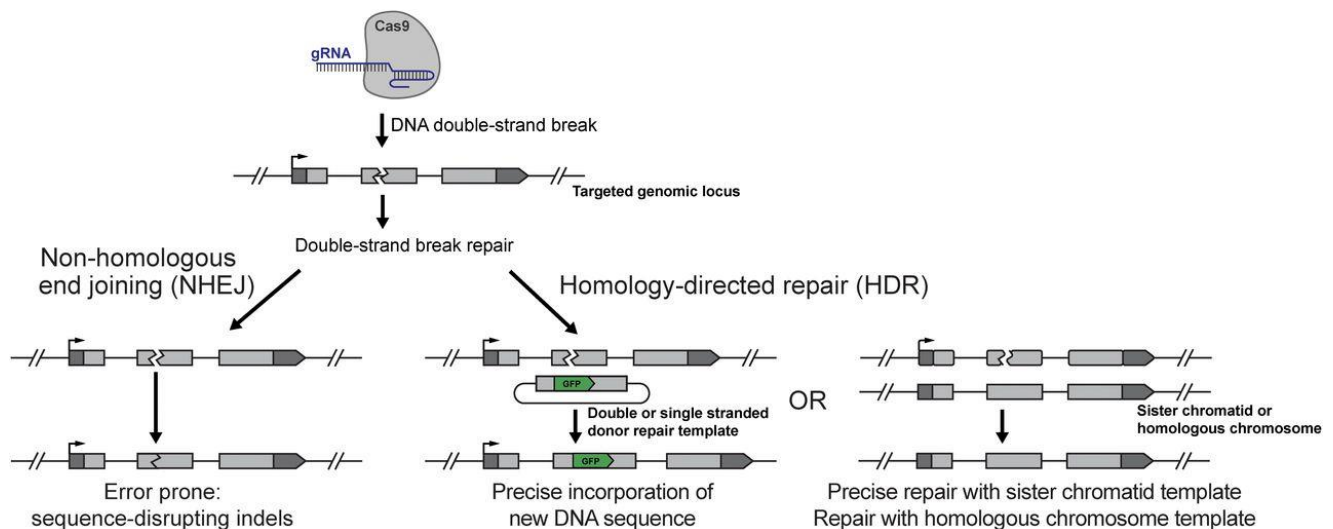
- Human Genome Project - entire genome made up of humans
- Diagnosis of Disease ó PCR & DNA probes
- Human Gene Therapy
- Vaccines & Pharmaceutical Products
- Forensics ó DNA Fingerprints (RFLP & VNTR)
- Environmental ó Recycling & detoxification
- Agricultural ó transgenic organisms

**Gene Therapy** - changing the expression of a person's genes - body (somatic) or germ cells done *in vitro* or *ex vitro*.

## CRISPR Cas9 gene editing

CRISPR are Clustered Regularly-Interspaced Palindromic Repeats.

- They are comprised of repeated DNA sequences separated by spacers.
- Originally discovered in bacteria, they became more interesting to microbiologists when it was discovered that the spacer DNA between the repeats are derived from foreign DNA (like that of a bacteriophage) that has previously infected the bacterium or one of its ancestors.
- Having the foreign DNA inserted into the bacterial genome allows for the production of anti-sense RNAs that can be used to guide attacks on future infections with the same phage DNA.
- This has been compared to the function antibodies play in the adaptive immune system of higher organisms.



- Integrating these foreign DNAs into the bacterial genome to generate CRISPR spacers requires specialized enzymes that are coded in genes near the CRISPR called CRISPR associated (Cas) genes.
- Cas9 is an example of one of these genes that is being extensively used to edit DNA in the lab.
- Cas9 is an endonuclease that cuts DNA at a very specific site near a 20bp sequence that is complementary to a guide RNA present within the Cas9 protein.
- The guide RNA can be altered by an experimenter to very specifically target a cut site in any DNA sequence.

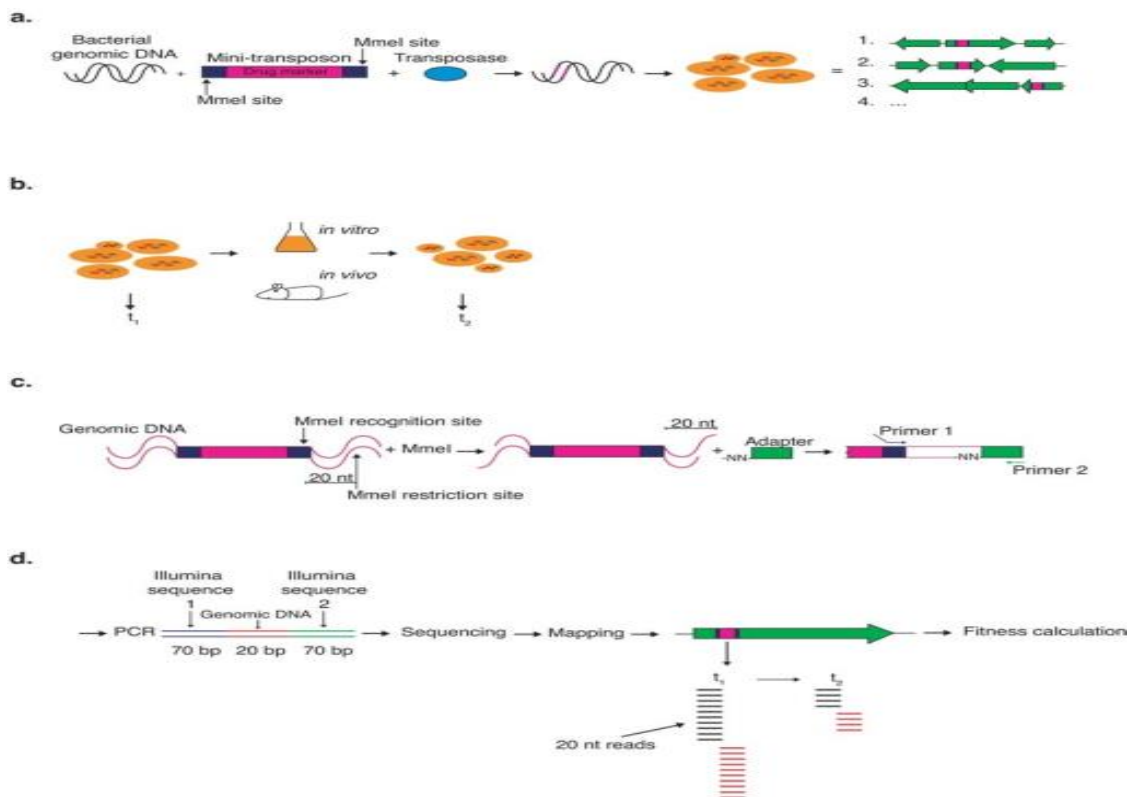


- When Cas9 cuts, it forms a double strand break that can be repaired by non-homologous end joining which results in a small deletion of DNA near the cut site.
- Alternatively when homologous DNA is present, homology directed repair occurs.
- This homologous DNA could be provided by a sister chromosome; however, an experimenter could also provide the DNA that will be used to repair the break.
- If this DNA contains small changes, then the changes will be included in the repair, which is why this technology holds much promise for genetic engineering.

## TN-Seq

TN-Seq is a popular modern technology for determining genes that contribute to an organism's fitness in any particular environment. The following letters refer to the figure below.

- A very large pool of mutants is made, each with a single transposon inserted randomly into its genome. The transposon disrupts the gene into which it inserts, generally rendering it non-functional.
- The pool of mutants is exposed to some sort of selective conditions like growth in a particular medium or animal host. Samples of the mutant pool are collected before exposure to the condition ( $t_1$ ) and after exposure ( $t_2$ ).
- Chromosomal DNA is purified from the samples of mutants and prepared for rapid sequencing using one of the available next generation sequencing technologies such that the DNA adjacent to the transposon is sequenced.
- Sequencing reads are mapped to the genome of the organism that was mutated. Comparing the maps of transposon mutants before and after exposure to the condition can yield information about which genes contribute to the organism's ability to survive the condition. These genes are called fitness determinants.



The scheme summarizes what TN-Seq results can mean:

- Decreased numbers of transposon mutants in the output ( $t_2$ ) sample means that the genes that were disrupted are important for surviving the condition.
- Having the transposon disrupt those genes made the organism less fit, so they are underrepresented in the pool of mutants after exposure to the condition.
- Increased numbers of transposon mutants in the output ( $t_2$ ) sample means that the genes that were disrupted are not important for surviving the condition.
- Having the transposon disrupt those genes made the organism more fit so they are overrepresented in the pool of mutants after exposure to the condition.
- No changes in numbers of transposon mutants in the output ( $t_2$ ) sample means that the genes that were disrupted are not involved in surviving the condition.
- Having the transposon disrupt those genes didn't make the organism any more or less fit so they are unchanged in their representation in the pool of mutants after exposure to the condition.

## Bioethics

Major concerns concerning safety and ethics of recombinant DNA technology.

- Potential Hazards vs. Potential Gains
- Concerns:
  - genetically modified foods
  - genetically engineering microbes
  - cloning whole organisms
  - embryonic stem cell research
  - gene therapy
  - genetic testing
  - bioterrorism

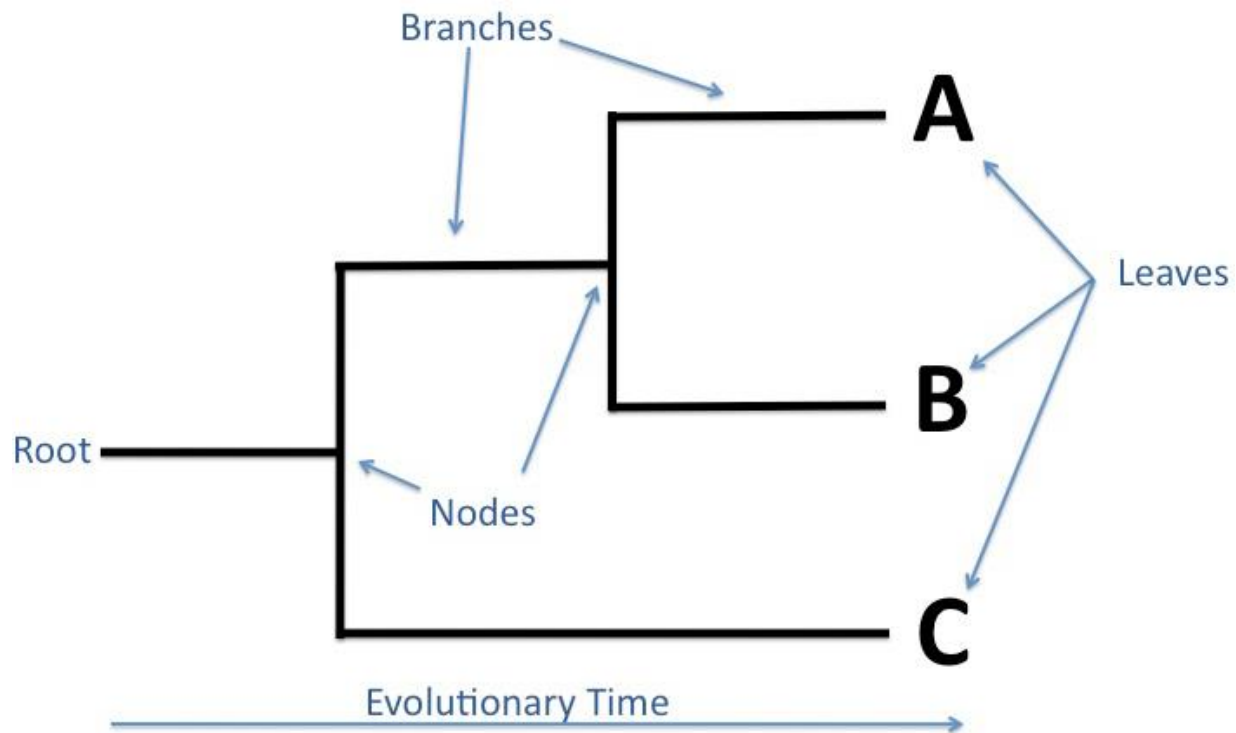
## Epigenetics

The study of heritable changes in gene activity that occur without a change in the sequence of the genetic material. Epigenetics literally means -in addition to genetics-

- Epigenetic factors can regulate the amount of gene activity, influencing the growth and appearance of an organism
- There are several epigenetic ways in which gene activity can be prevented or controlled, including
  - modification of histone proteins
  - DNA methylation
  - RNA interference
- For any of these methods of gene regulation, the absence of the protein product of the gene causes a change in the function or development of the cell
- malfunctions in epigenetic control of gene activity have been implicated in cancer, cardiovascular disease and several inherited genetic conditions

# Phylogenetics

- Study of evolutionary relationships among groups of organisms based upon their genetics
- Has taxonomy folks in a turmoil ó they can't agree so we have national lists for our taxonomy events



- Relationships between organisms are often represented using phylogenetic trees.
- Organisms more closely related to each other are physically closer on the tree.
- Common ancestors are represented by nodes that connect branches together.