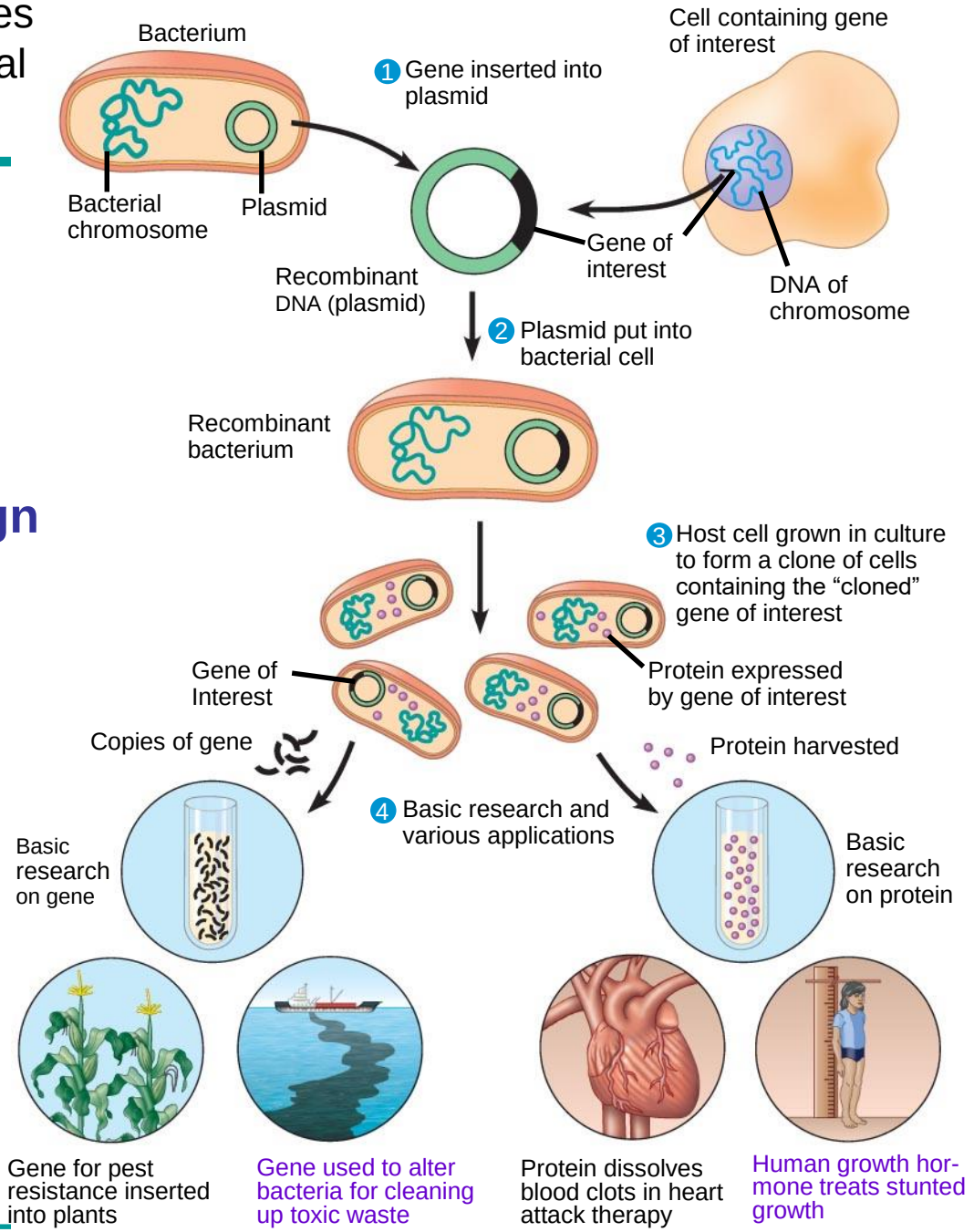
Plasmids are small circular DNA molecules that replicate separately from the bacterial chromosome

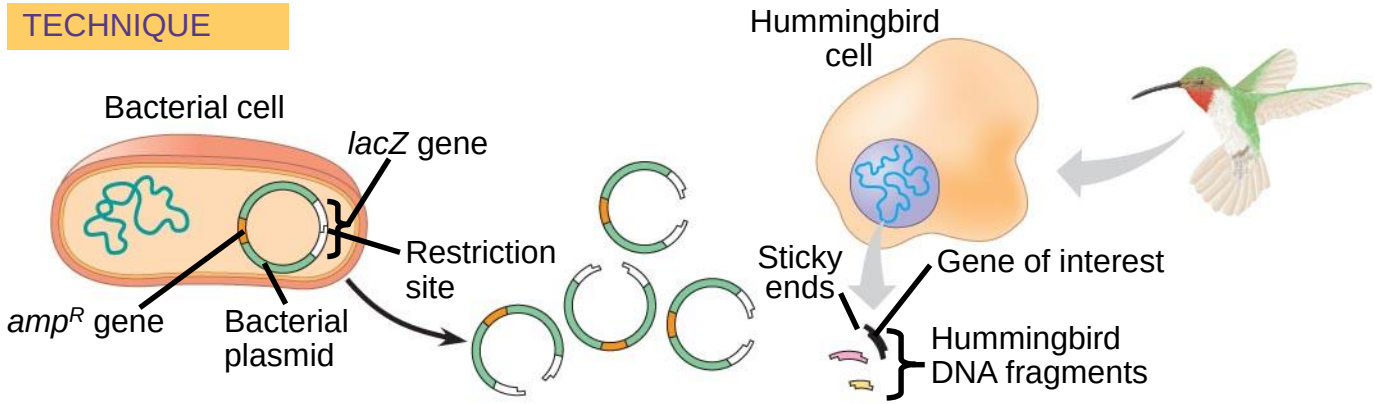
**Replication, transcription and translation machinery in bacterial cell responsible for production of protein of foreign gene**

**Almost everyday we do gene cloning**

**How to cut the plasmid DNA at precise position to insert the foreign gene?**



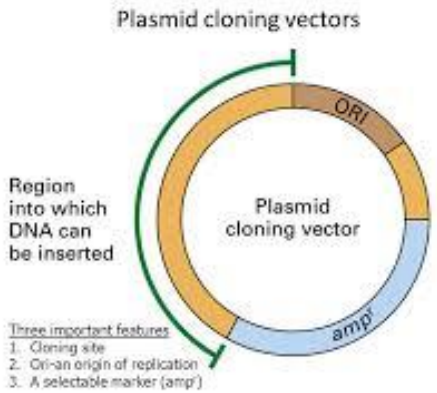
# Cloning a Eukaryotic Gene in a Bacterial Plasmid

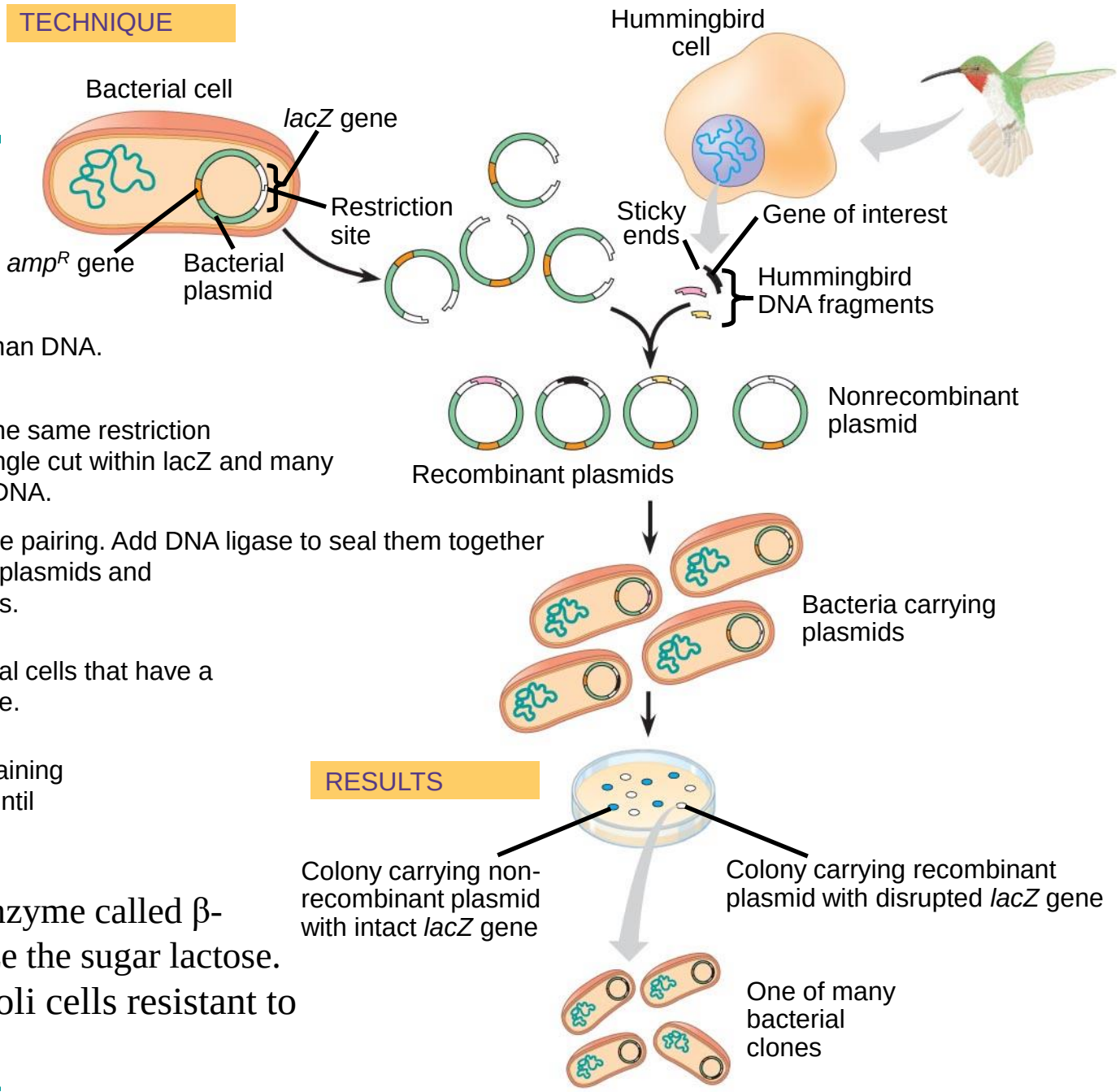



Cloning is used to prepare many copies of a gene of interest for use in sequencing the gene, in producing its encoded protein, in gene therapy, or in basic research.

Plasmids are small circular DNA molecules that replicate separately from the bacterial chromosome

A cloning vector is a DNA molecule that can carry foreign DNA into a host cell and replicate there.





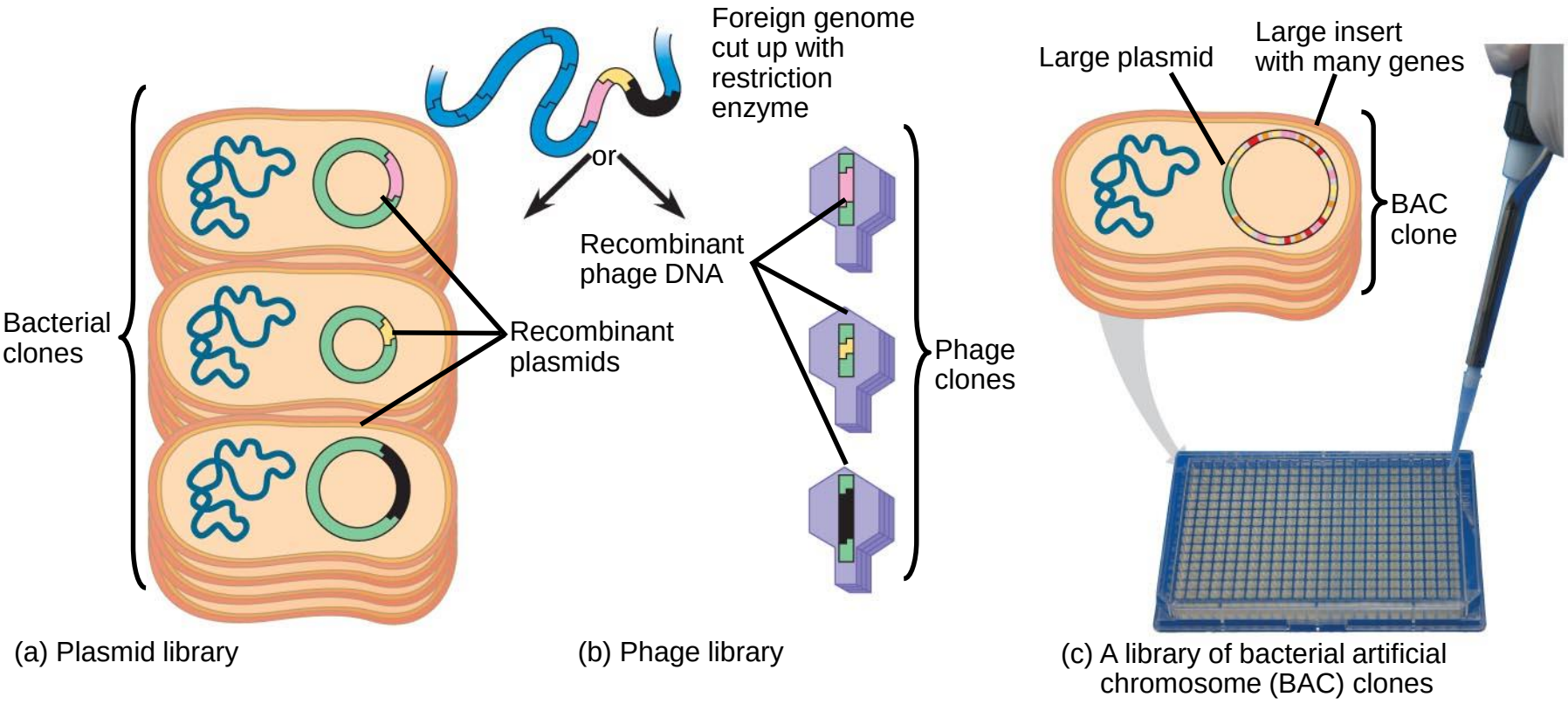

- lacZ, which encodes an enzyme called β-galctosidase that hydrolyze the sugar lactose.
- ampR, which makes E coli cells resistant to antibiotic Amp

# Storing Cloned Genes in DNA Libraries

- A **genomic library** that is made using bacteria is the collection of recombinant vector clones produced by cloning **DNA fragments from an entire genome**
- A genomic library that is made using bacteriophages is stored as a collection of phage clones
- Each plasmid clone in a library is **like a book containing specific information**. Today scientists often obtain **such libraries (even particular cloned gene) from another researcher, a commercial source, or a sequencing center.**

**Approximate number of plasmid vectors for cloning entire human genome**

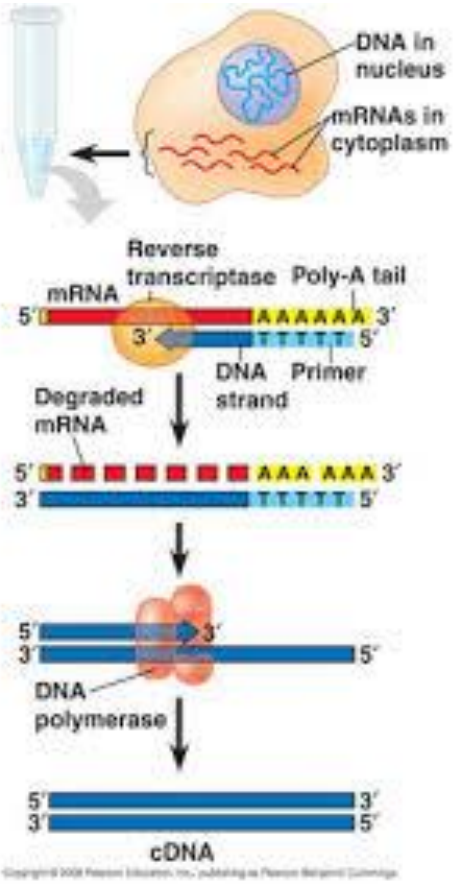
$$= 3 * 10^9/10^4$$



# Storing Eukaryotic DNA code for the mRNA

- A **complementary DNA (cDNA)** library is made by cloning DNA made *in vitro* (test tube) by reverse transcription (using reverse transcriptase) of all the mRNA produced by a particular cell
- A **cDNA library** represents only part of the genome—only the subset of genes transcribed into mRNA in the original cells

Ex: cDNA library of liver, pancreases, lungs etc




Only about 1 **percent** of DNA is made up of **protein-coding** genes !!!!!!

# Identifying Clones Carrying a Gene of Interest

We can detect the gene's DNA by its ability to base-pair with a complementary sequence of another nucleic acid molecule, **using nucleic acid hybridization**

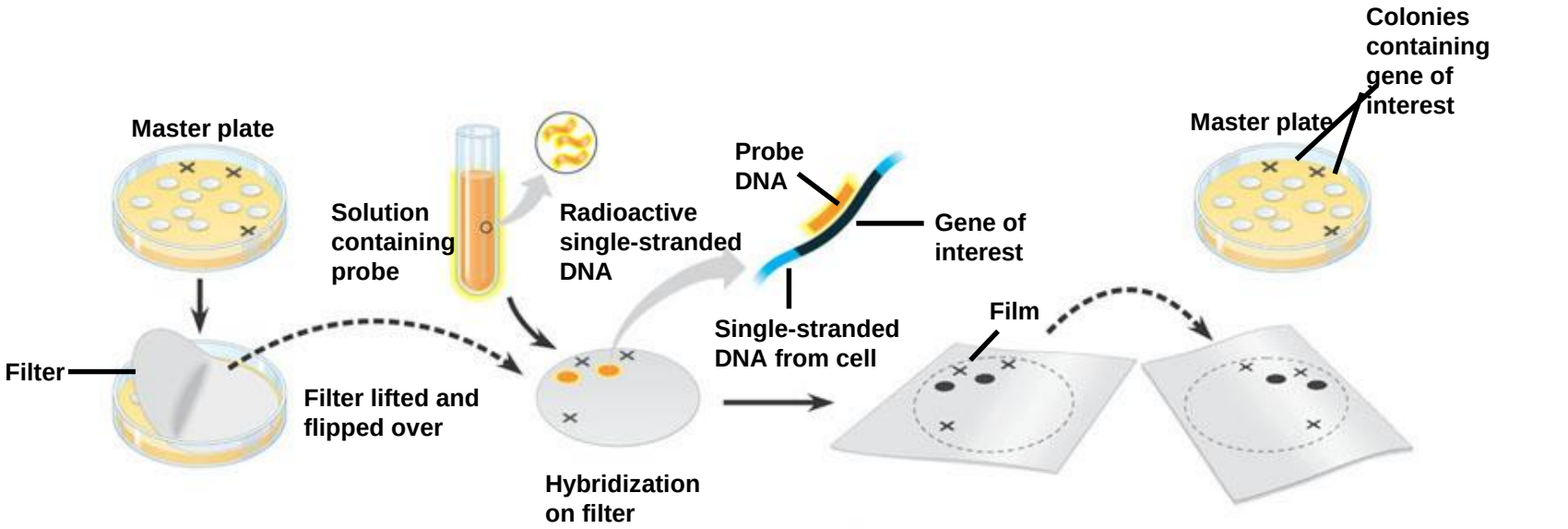
- A clone carrying the gene of interest
  - Can be identified (detected) with a radioactively labeled nucleic acid probe that has a sequence complementary to the gene, a process called nucleic acid hybridization

For example, if the desired gene (ex: hummingbird β-globin gene) has following sequence



Then we would synthesize this probe

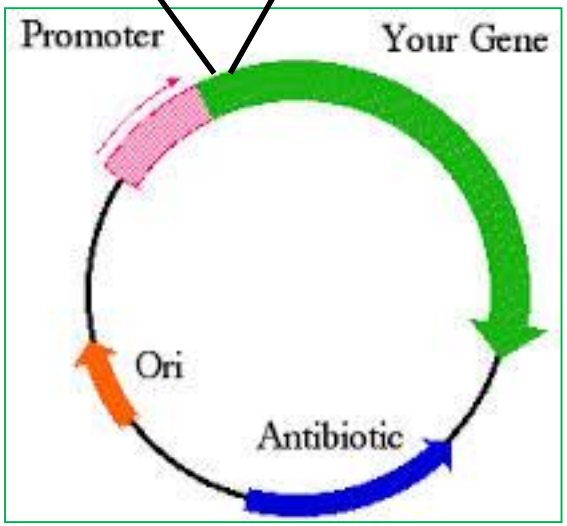
# Nucleic acid probe hybridization




1. A special filter paper is pressed against the master plate, transferring cells to the bottom side of the filter.
2. The filter is treated to break open the cells and denature their DNA; the resulting single-stranded DNA molecules are treated so that they stick to the filter.
3. The filter is laid under photographic film, allowing any radioactive areas to expose the film (autoradiography).
4. After the developed film is flipped over, the reference marks on the film and master plate are aligned to locate colonies carrying the gene of interest.

# Expression of Cloned Eukaryotic (Human) gene in Bacterial Expression Systems

Multiple restriction site
(Multiple cloning site (MCS)




## Expression vector

-After a gene has been cloned, its protein product can be produced in larger amounts for research
-Cloned genes can be expressed as protein in either bacterial or eukaryotic cells

A expression vector is a DNA molecule that can carry foreign DNA into a host cell and replicate then transcribe there.

- Cloning vector that contains highly **active bacterial** promoter just upstream of the restriction site where the eukaryotic gene is inserted **in the correct reading frame.**
- The bacterial host cell will recognize the promoter and proceeds to express **the foreign gene now linked to that promoter**. Such expression vector allows the synthesis of many eukaryotic proteins in bacterial cells.

**Genetic code is universal**

**Cloning vector = Expression vector - promoter**

# Amplifying DNA in Vitro: The Polymerase Chain Reaction (PCR)

When the sources of DNA is scanty or impure the polymerase chain reaction or PCR is quicker and more selective to clone the gene.

- The **polymerase chain reaction, PCR**, can produce many copies of a specific target segment of DNA
- A three-step cycle—heating (Denaturation), cooling (Annealing), and replication (Extension)—brings about a chain reaction that produces an exponentially growing population of identical DNA molecules

PCR is being used increasingly to make enough of a specific DNA fragment to insert it directly into a vector, entirely skipping the steps of making and screening a library.

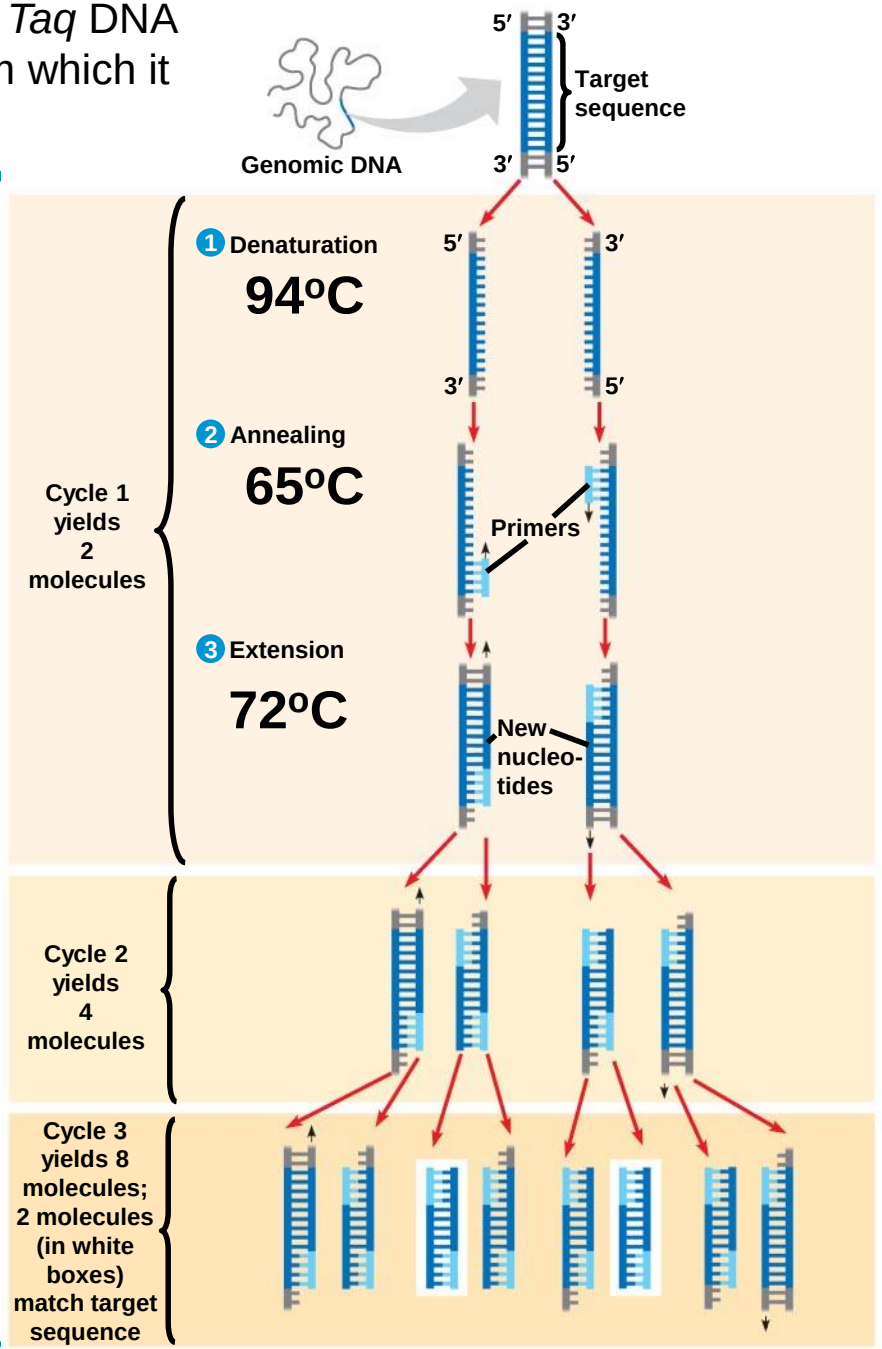
PCR is like a photocopying just one page rather than checking out all the books in a library.

Hoffmann-La Roche eventually bought the PCR and *Taq* DNA polymerase patents from Cetus for $330 million, from which it may have received up to $2 billion in royalties.

- That spring, according to Mullis, he was driving his vehicle late one night with his girlfriend, when he had the idea to use a pair of primers to bracket the desired DNA sequence and to copy it using DNA polymerase, a technique which would allow a small strand of DNA to be copied almost an infinite number of times.
- PCR is now a common and often indispensable technique used in medical and biological research labs for a variety of applications. DNA cloning for sequencing, functional analysis of genes; the diagnosis of hereditary diseases (breast cancer, sickle cell anemia, hemophilia etc); DNA fingerprinting (used in forensic sciences and paternity testing); and the detection and diagnosis of infectious diseases.

The 1993 Nobel Prize in Chemistry was awarded to Kary Mullis for the invention of PCR, a method that made it possible to copy a large numbers of DNA fragments in only a few hours.

**Michael Smith**
Canada, for his fundamental contributions to the establishment of oligonucleotide-based, site-directed mutagenesis and its development for protein studies



# DNA Technology: DNA Sequencing

**Relatively short DNA fragments can be sequenced by the *dideoxy chain termination method***

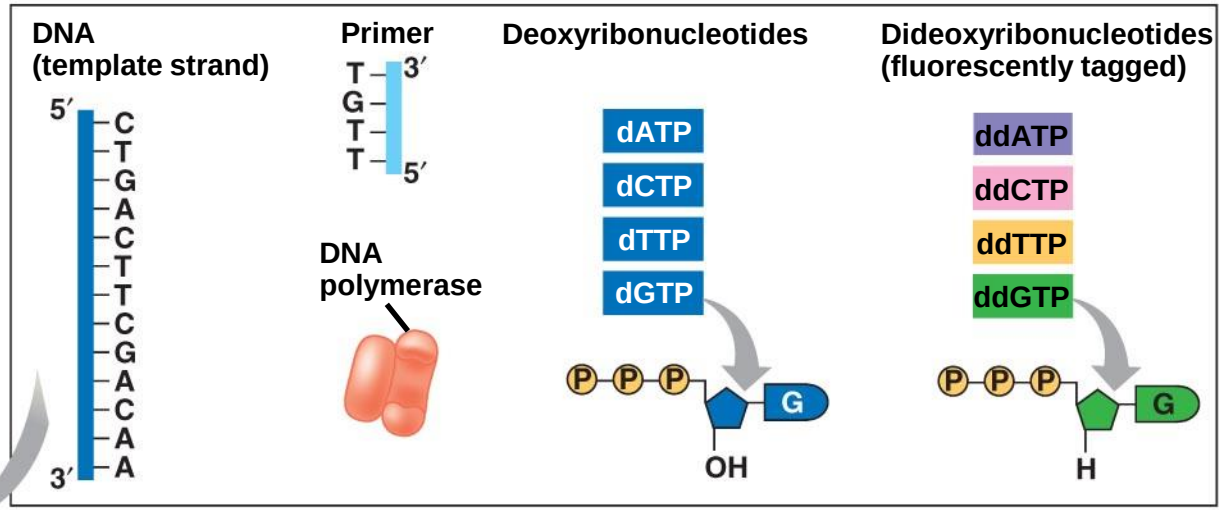
**Modified nucleotides called dideoxyribonucleotides (ddNTP) attach to synthesized DNA strands of different lengths**

**Each type of ddNTP is tagged with a distinct fluorescent label that identifies the nucleotide at the end of each DNA fragment**

**The DNA sequence can be read from the resulting spectrogram**

If no 3' OH group on the sugar does DNA polymerization happens?

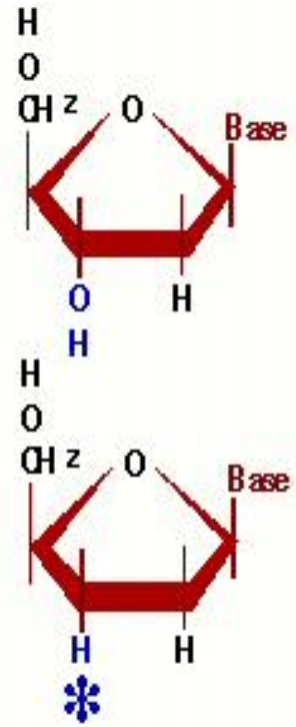
The **Sanger method**, in mass production form, is the technology which produced the **first human genome in 2001**, ushering in the age of genomics. However, later in the decade, radically different approaches reached the market, bringing the **cost per genome down from $100 million (~Rs 600 crore) in 2001** to $10,000 (Rs. 7 lakh) in 2011.

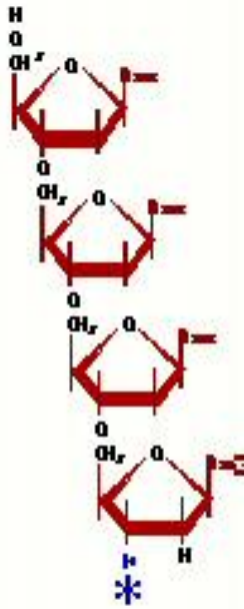





Normal nucleotides:

Dideoxy Chain Terminators:



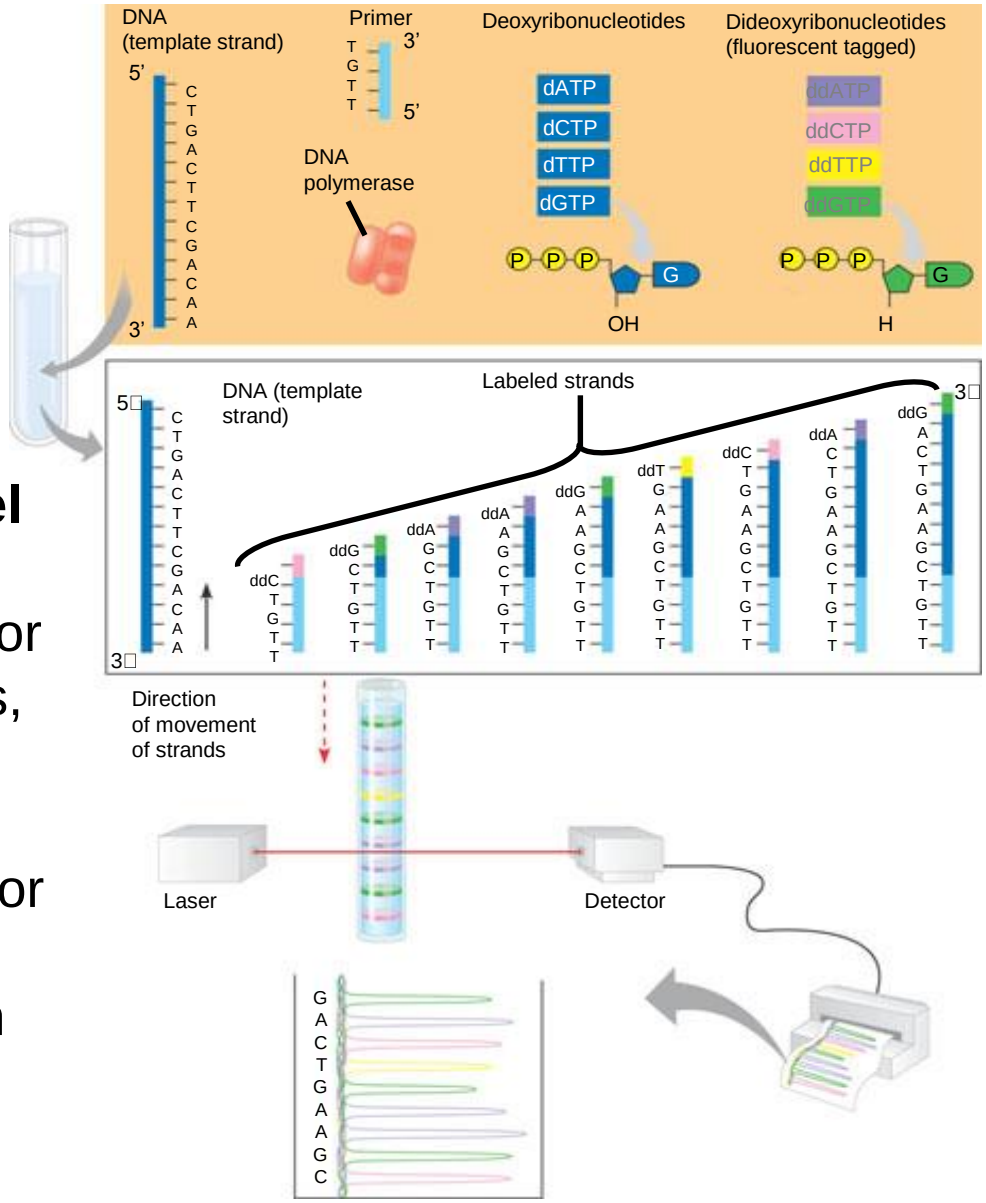



# Dideoxy chain-termination method for sequencing DNA

**Frederick Sanger: Two time Nobel prize winner:**

In 1958, Nobel Prize in chemistry "for his work on the structure of proteins, especially that of insulin".

In 1980, Walter Gilbert and Sanger shared half of the chemistry prize "for their contributions concerning the determination of base sequences in nucleic acids".



# Determining Gene Function

One way to determine function is to disable the gene and observe the consequences

Using ***in vitro* mutagenesis**, mutations are introduced into a cloned gene, altering or destroying its function

**Gene "Knocks Out":** When the mutated gene is returned to the mice (cell), the normal gene's function might be determined by examining the mutant's phenotype

Mutant cell might help to reveals the function of the missing normal protein

Researchers can even generate mice with any given gene disabled, in order to study the role of that gene in development and in adult

**The Nobel Prize in Physiology or** Medicine **2007 was awarded jointly to Mario R. Capecchi, Sir Martin J. Evans and Oliver Smithies "for their discoveries of principles for introducing specific gene modifications in mice by the use of embryonic stem cells".**

# RNA interference

- Gene expression can also be silenced using **RNA interference (RNAi)**
- Synthetic double-stranded RNA molecules matching the sequence of a particular gene are used to break down or block the gene's mRNA

RNAi technique has been used successfully to reduce (**Knock down**) the expression of specific genes (reduction in mRNA production) in mammalian cells, including human cells in culture

-Analyzing functions of gene in large scale

-**System Biology:** Interaction between genes in the system as a whole- the basis for system biology

**The Nobel Prize in Physiology or Medicine 2006 was awarded jointly to Andrew Z. Fire and Craig C. Mello *"for their discovery of RNA interference - gene silencing by double-stranded RNA".***

# Cloning organisms

A **stem cell** is a unspecialized cell that can reproduce itself indefinitely and differentiate into specialized cells (liver, root etc) of one or more types : Embryonic and adult stem cells in mammals; callus and meristem in plants

**Differentiation** is the process by which stem cell becomes a specialized cell type (liver, root etc).

**Dedifferentiation** is a cellular process in which a specialized cells (differentiated cell) reverts to an earlier developmental stage (Stem cell or callus)

**Redifferentiation** is Transformation of dedifferentiated cells into differentiated cells

A cell that can differentiate into all cell types is known as **totipotent**.
Ex: Embroyonic stem cells in mammals

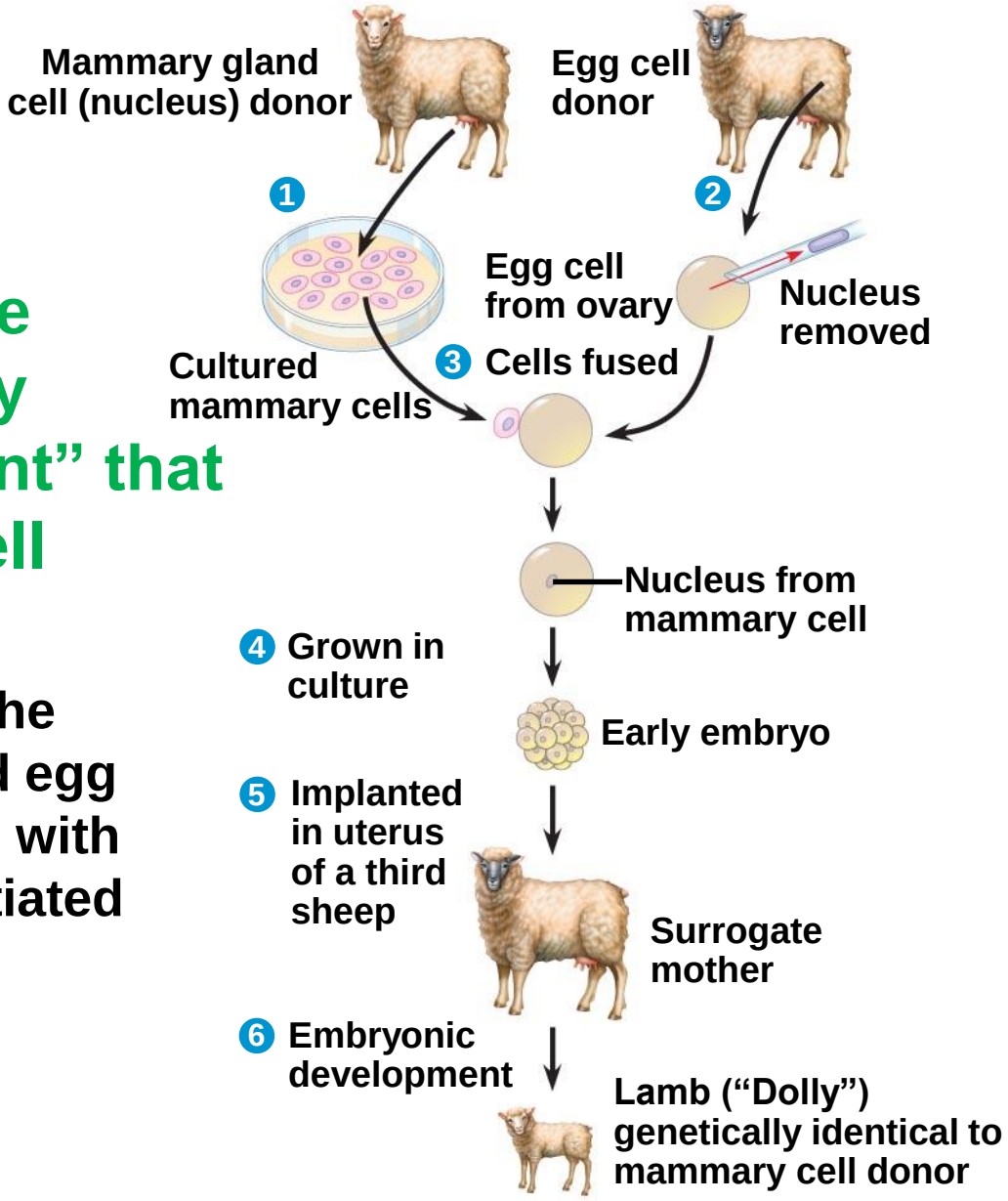
A cell that can differentiate into many cell types of the adult organism is known as **pluripotent.**

**Organismal cloning produces one or more organisms genetically identical to the "parent" that donated the single cell**

# Reproductive Cloning of Mammals by Nuclear Transplantation

**Organismal cloning produces one or more organisms genetically identical to the "parent" that donated the single cell**

**In nuclear transplantation, the nucleus of an unfertilized egg cell or zygote is replaced with the nucleus of a differentiated cell**



# Stem Cells of Animals

***Embryonic stem cells (ES)***; these are able to differentiate into all cell types

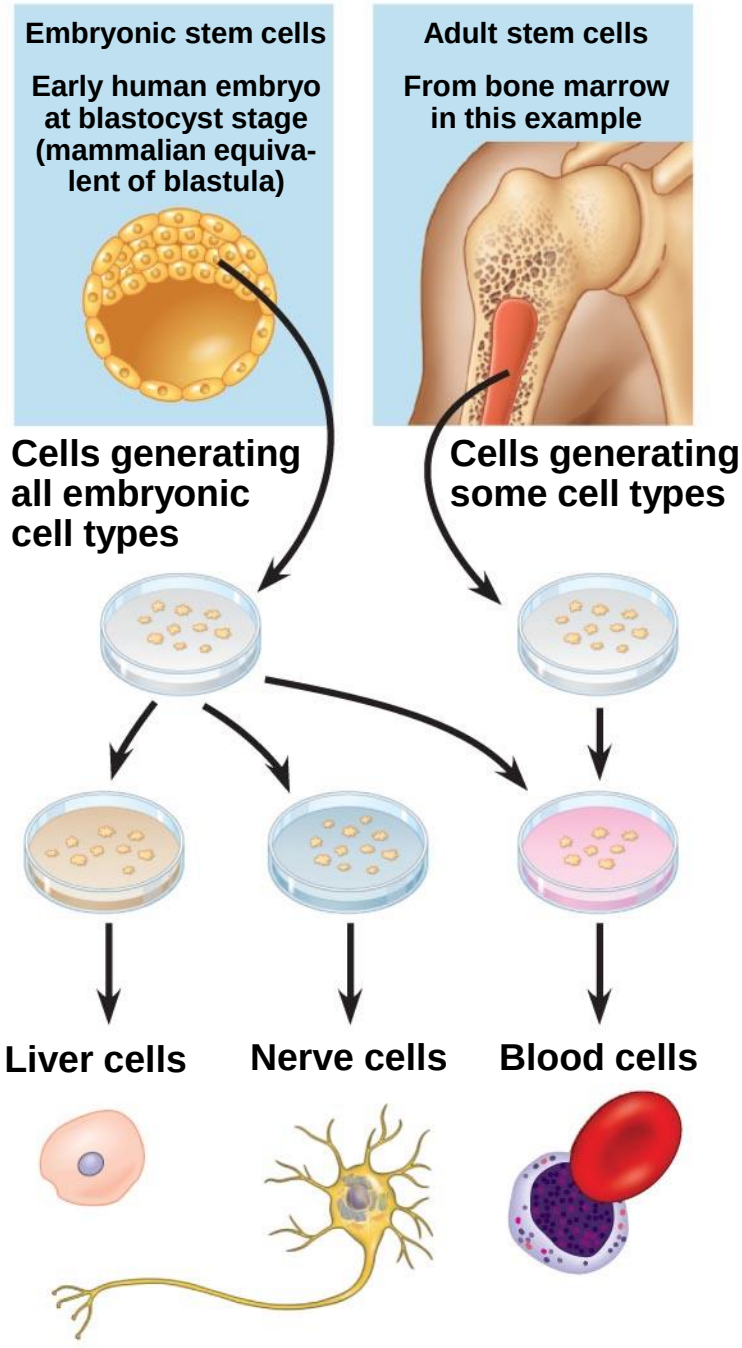
The adult body also has stem cells, which replace nonreproducing specialized cells

Stem cell bank: Umbilical cord blood cells (rich source of stem cells like bone marrow) are extracted immediately after birth

**Approved Treatments Using Cord Blood Stem Cells**

1. Leukemia – A type of cancer affecting the leukocytes of the blood immune system. To treat acute, chronic and juvenile forms of leukemias.
2. Lymphoma – A type of blood cancer affecting the leukocytes that circulate in the blood and lymph nodes.
3. Other blood proliferated disorders – This include anaemia, sickle cell anaemia, Beta Thalassemia major, severe combined immunodeficiency (SCID) condition, red cell aplasia, multiple myeloma, plasma cell leukemia etc.
4. Inherited disorders of immune and metabolic disorders – Some of the disorders include hurler syndrome, hunter syndrome, ALD, Lesch Nyhan syndrome, Osteopetrosis etc.
5. Tumors (cancers) including neuroblastoma, retinoblastoma and medulloblastoma.

**Umbilical cord blood stem cells have been used in over 30,000 stem cell transplants to treat around 80 medical conditions.**



# Applications of Stem cell Research

The aim of stem cell research is to supply cells for the repair of damaged or diseased organs

- The main of cloning is to produce ES cells to treat disease (ex: insulin-producing pancreatic cells for people suffering fro diabetes, creating kind of brain cells for the people suffering from Parkinson disease) , the process is called as therapeutic cloning

**Induced pluripotent stem cells** (iPS) are a type of pluripotent stem cell that can be generated directly from adult cells.

The iPSC technology was pioneered by Shinya Yamanaka's lab in Kyoto, Japan, who showed in 2006 that the introduction of **four specific genes** could convert adult cells to pluripotent stem cells. He was awarded the 2012 Nobel Prize along with Sir John Gurdon "for the discovery that mature cells can be reprogrammed to become pluripotent."

# Human Gene Therapy

- **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 specific types of cells, for example bone marrow
- Gene therapy raises ethical questions, such as whether human germ-line cells should be treated to correct the defect in future generations

**In one study, all five adults and 19 of 22 children with acute lymphocytic leukemia (ALL) were cleared of the cancer. A few have relapsed since the study was done.**

The gene therapy must be made individually for each patient, and lab costs now are about $25,000, without a profit margin,

# Applications of DNA Technology: Pharmaceutical Products

## Synthesis of Small Molecules for Use as Drugs

- The drug imatinib is a small molecule that inhibits overexpression of a specific leukemia-causing receptor

## Protein Production in Cell culture

•Pharmaceutical products that are proteins can be synthesized on a large scale

Ex: Insulin, Human Growth Hormone, Tissue Plasminogen Activator, vaccines

# DNA Technology : Other Applications

**Forensic Evidence and Genetic Profiles:**

Center for DNA Finger Printing Diagnostics (CDFD)

Ex: Paternity dispute, criminal cases, Victims of mass causalities

**Environmental cleanup:**

Genetically engineered microbes: Mining minerals and cleaning of highly toxic mineral wastes

Chlorinated Hydrocarbon

Biodiesel

# DNA Technology : Agriculture Applications

Genetic engineering in plants has been used to transfer many useful genes; Ex: Herbicide resistance, increased resistance to pests, increased resistance to salinity, and improved nutritional value of crops (Golden rice), C4 Rice project, Rice harboring genes for milk proteins (Dehydration formula)

# Pomato (TomTato)

**'Fruit salad'** trees: Citrus fruits