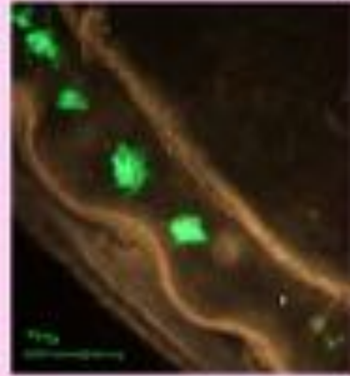
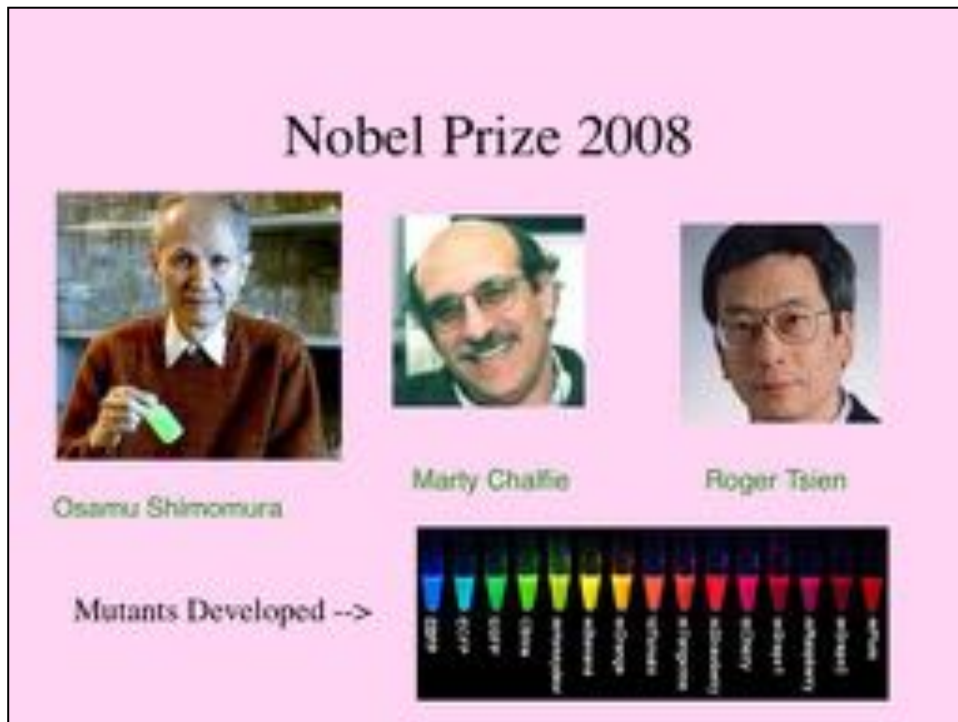
Green Fluorescent Protein

Green Fluorescent Protein (GFP) has existed for more than one hundred and sixty million years in one species of jellyfish, *Aequorea victoria*.

GFP in Photoorgans

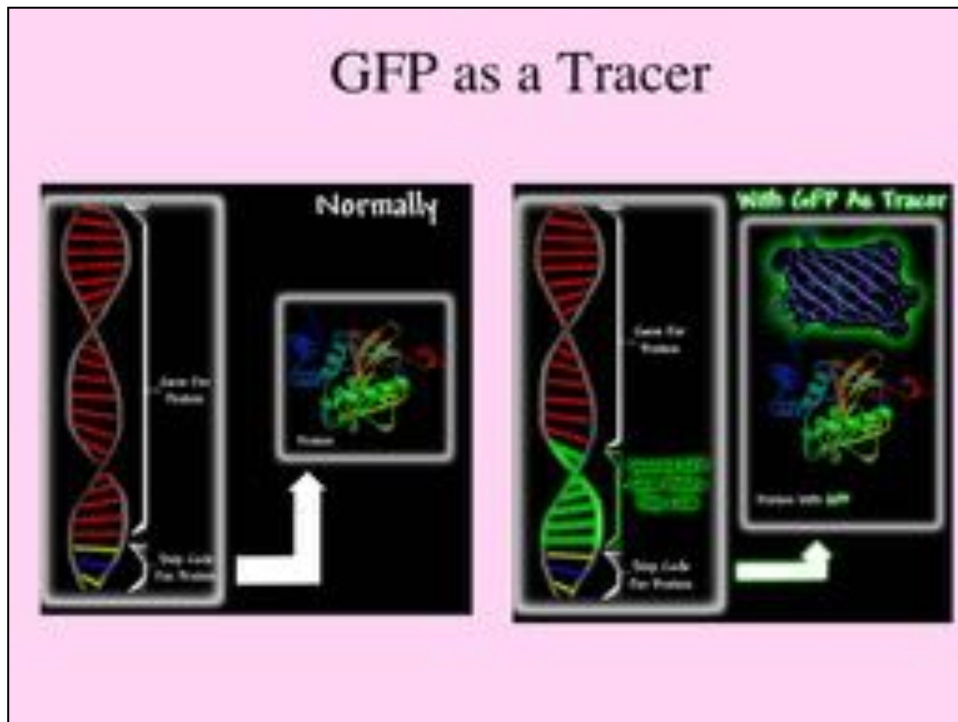


In 1994 the GFP protein was identified and cloned.

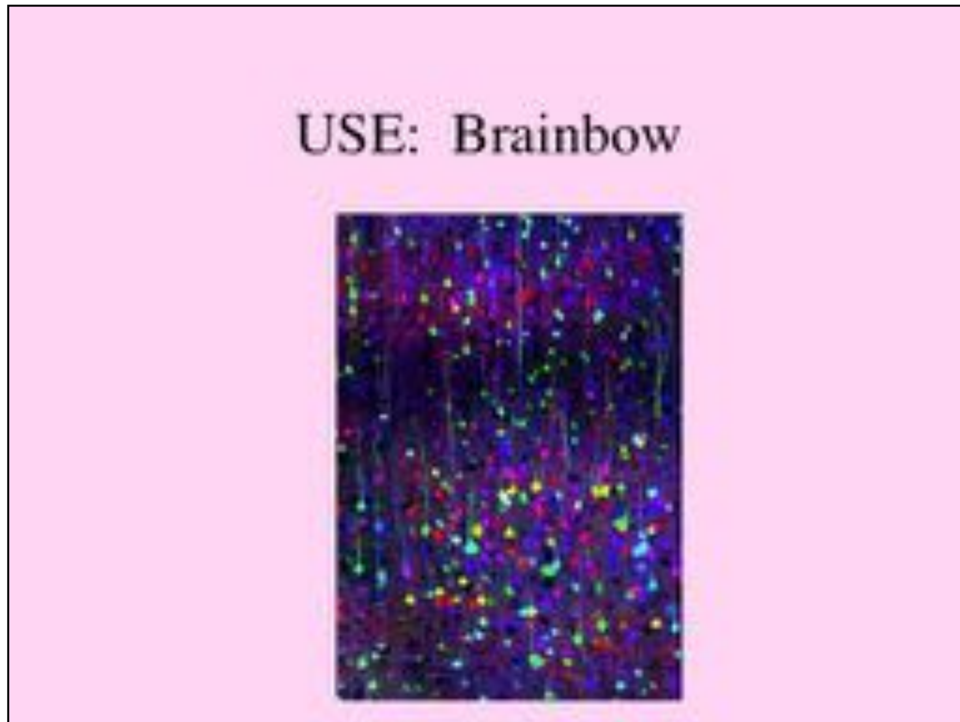


The importance of GFP was recognized in 2008 when the Nobel Committee awarded Osamu Shimomura, Marty Chalfie and Roger Tsien the Chemistry Nobel Prize "for the discovery and development of the green fluorescent protein, GFP".Why is it so popular? Well I like to think of GFP as the microscope of the twenty-first century. Using GFP we can see when proteins are made and where they can go. This is done by joining the GFP gene to the gene of the protein of interest so that when the protein is made it will have GFP hanging off it. Since GFP fluoresces one can shine light at the cell and wait for the distinctive green fluorescence associated with GFP to appear.

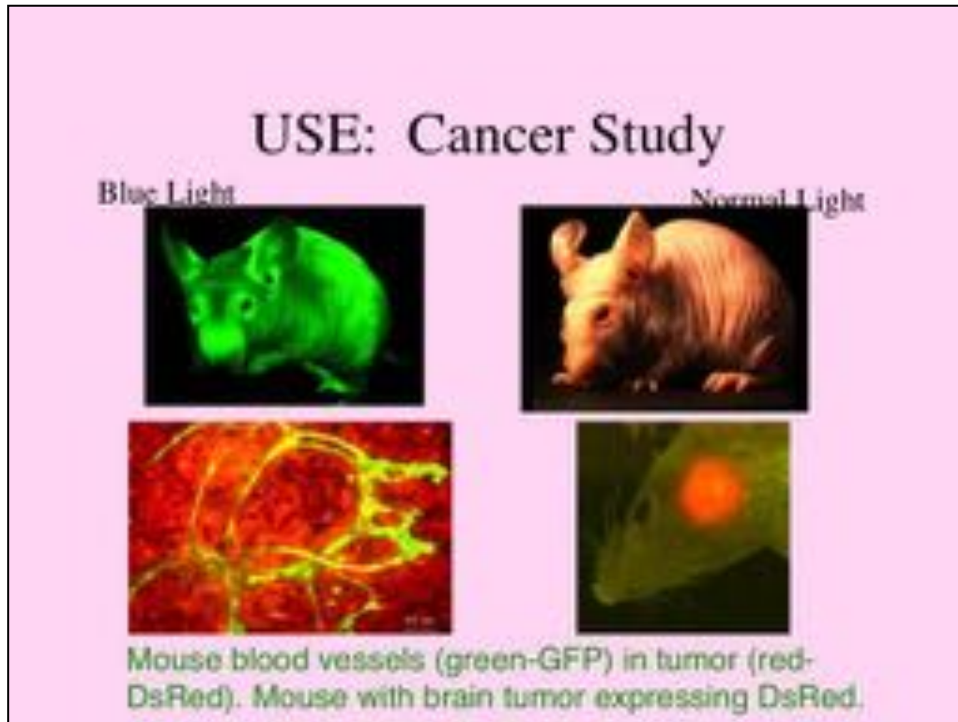
While Shimomura, Prasher and Chalfie were all instrumental in taking GFP from the jellyfish and showing that it can be used as a tracer molecule, it is Roger Tsien who is responsible for much of our understanding of how GFP works and for developing new techniques and mutants of GFP. His group has developed mutants that start fluorescing faster than wild type GFP, that are brighter and have different colors (see below, the E stands for enhanced versions of GFP, m are monomeric proteins and tdTomato is a head-to-tail dimer).



Douglas Prasher was the first person to realize the potential of GFP as a tracer molecule. In 1987 he got the idea that sparked the GFP revolution. He thought that GFP from a jellyfish could be used to report when a protein was being made in a cell. Proteins are extremely small and cannot be seen, even under an electron microscope. However if one could somehow link GFP to a specific protein, for example hemoglobin, one would be able to see the green fluorescence of the GFP that is attached to the hemoglobin. It would be a bit like attaching a light bulb to the hemoglobin molecule. Hemoglobin is a vital protein in the body, since it carries the oxygen in our blood. Our bodies are continuously making new hemoglobin. Encoded in the DNA is some type of index that directs the molecular machinery to the start of the hemoglobin gene. When new hemoglobin is required protein production is activated. The gene is read and the protein is manufactured. At the end of the gene is a message called a stop codon, which ends protein production. The manufacture of proteins using the instructions from the gene is called protein expression.Doug Prasher envisioned that it would be possible to use biomolecular techniques to insert the GFP gene at the end of the hemoglobin gene, right before the stop codon. When the cell needed to make hemoglobin, it would go to the hemoglobin gene, use the information encoded in the gene to make it, but instead of



Never before has a brain been so beautiful. Jeff Lichtman and Joshua Sanes, researchers at the Harvard Brain Center, have created transgenic mice with fluorescent multicolored neurons. The photographs of the mouse brains that appear in the November 1, 2007 issue of *Nature* could be housed in the Museum of Modern Art or could be used to decorate Joseph's technicolored dream coat. But it is not their colorful splendor that makes these genetically modified mice so amazing. It is their potential to revolutionize neurobiology that excites scientists like myself and has our neurons firing away creating oodles of endorphins.The mice created by a genetic strategy termed "brainbow" will have a similar effect on neuroscience as Google Earth had on cartography. Using a brainbow of colors researchers will now be able to map the neural circuits of the brain. The individually colored neurons will help define the complex tangle of neurons that comprise the brain and nervous system. By creating a wiring diagram of the brain researchers hope to help identify the defective wiring found in neurodegenerative diseases such as Altzheimer's and Parkinson's disease.In the Brainbow mice, the Harvard researchers have introduced genetic machinery that randomly mixes green, cyan and yellow fluorescent proteins in individual neurons thereby creating a palette of ninety distinctive hues and colors. "The technique drives the cell to



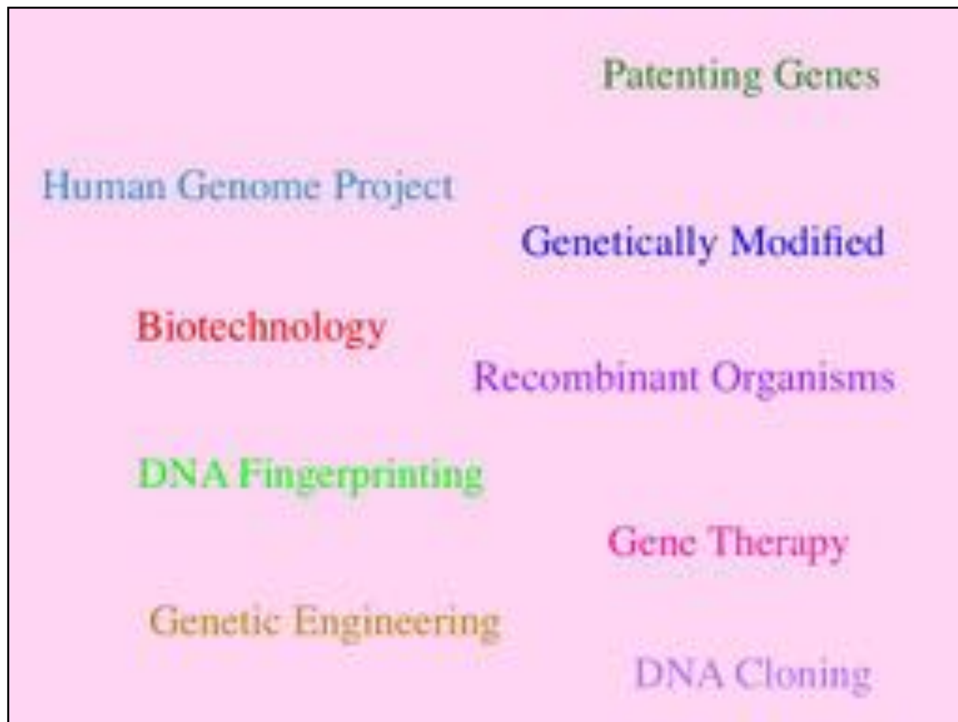
GFP has been attached ot Actin

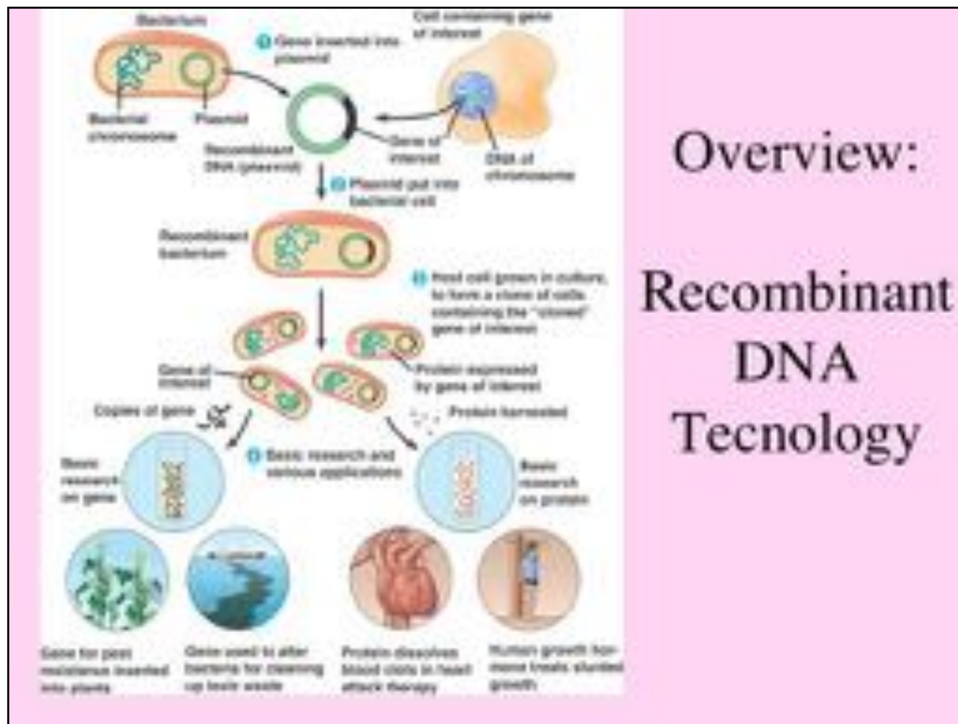
When a blue light is shone on the mouse every cell (that contains actin) in its body will fluoresce green. Human cancers that express DsRed can be implanted into these mice. The cancers will give off red fluorescence. Now the cancer cells can easily be observed and monitored in live green mice. Allowing the researchers at Anticancer Inc. to observe metastasis (cancer cells moving around the body) and angiogenesis (blood vessels growing into the cancer and supplying oxygen and food).

Randy Prather, a professor of reproductive biotechnology at the University of Missouri, Columbia, is one of the researchers in the forefront of the creation of transgenic swine for medicine. He often uses GFP and its yellow mutant YFP as a marker to show that foreign genes can be expressed in transgenic swine. The photo below shows two pigs, the one on the right is a regular piglet, a little cleaner than your typical piglet, but no different from the piglets you find on a hog farm. The one on the left is clearly very different to any pig we have ever seen before. It is a transgenic YFP cloned pig created by Professor Prather. It was formed to show that it is possible to produce a transgenic clone. In the words of Prather: "These animals prove that we can make genetic modifications to express desired traits. For xenotransplantation, this is a large step because it means it's possible to change the genetic makeup of the cells to prevent the body's rejection of transplanted organs." *Sky News* summed up the research in the following way: "Scientists have developed the first pig with a fluorescent yellow snout and trotters using jellyfish DNA. Researchers in the US say the work is a step towards growing animal organs for transplants - which could save thousands of human lives. But opponents have said the work is a freak show and a perversion of science."

Genetic Engineering and Biotechnology

What is this?


Patenting Genes
Human Genome Project
Genetically Modified
Biotechnology
Recombinant Organisms
DNA Fingerprinting
Gene Therapy
Genetic Engineering
DNA Cloning

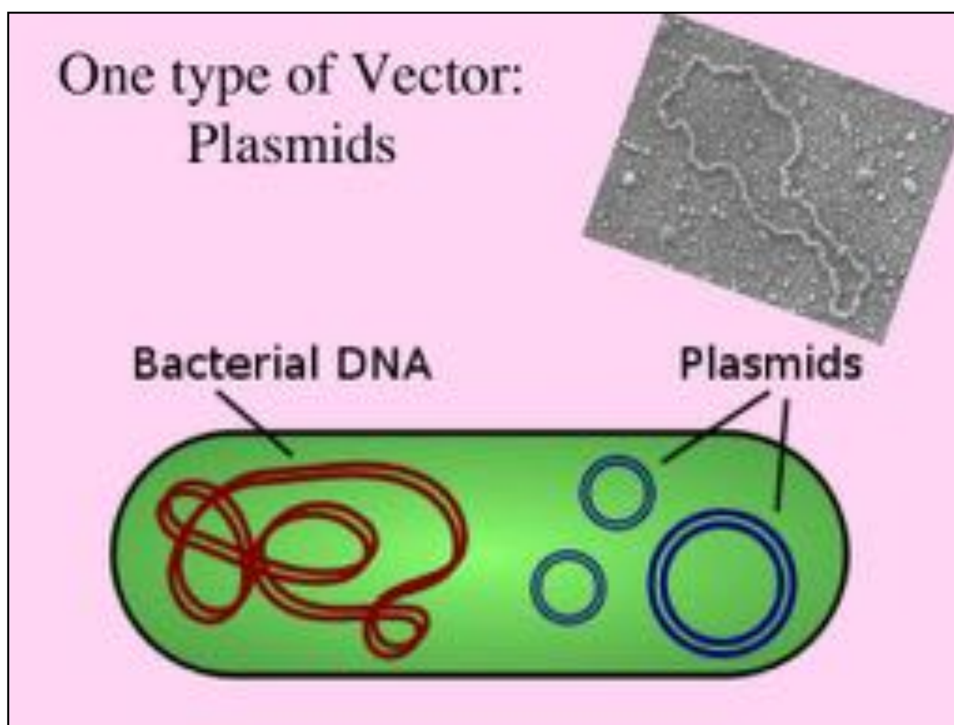


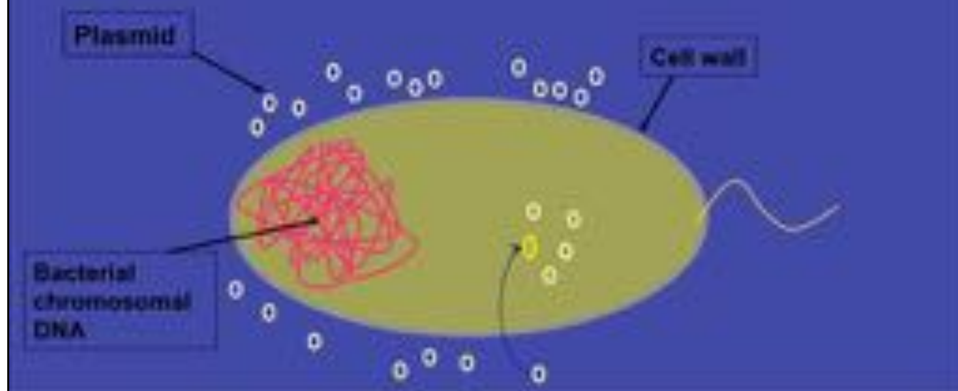
Plasmids

Recombinant DNA

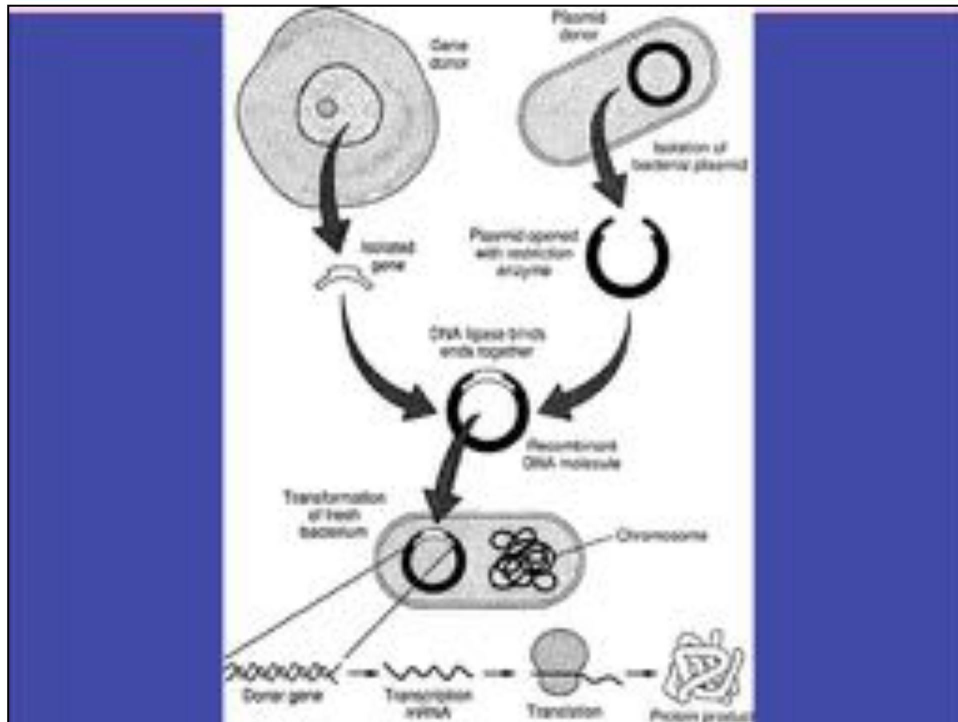
Transformation

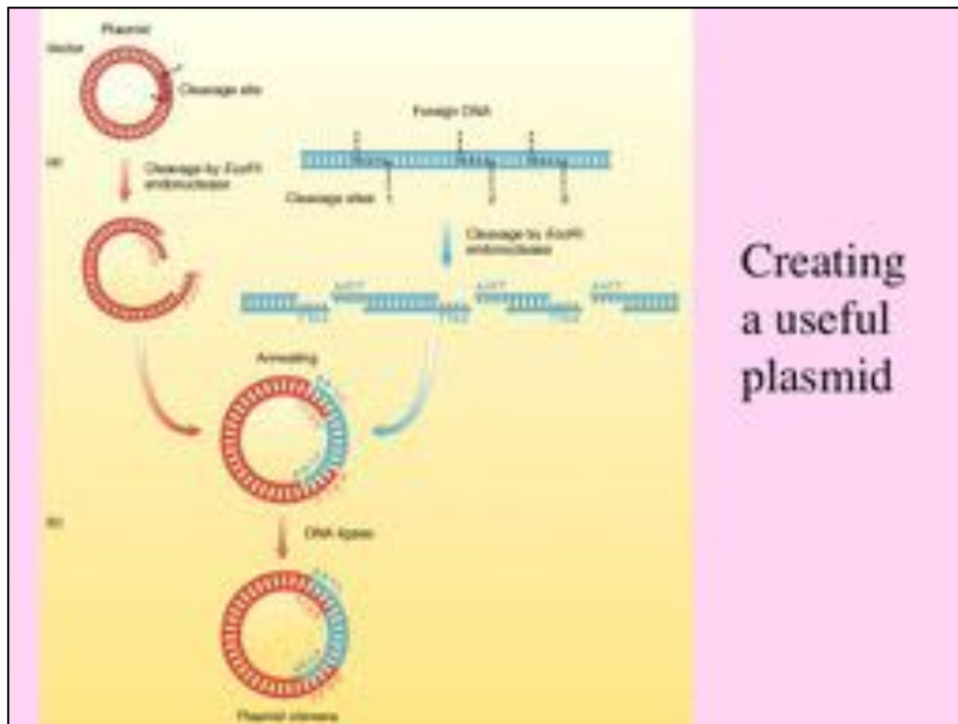
Gene Expression

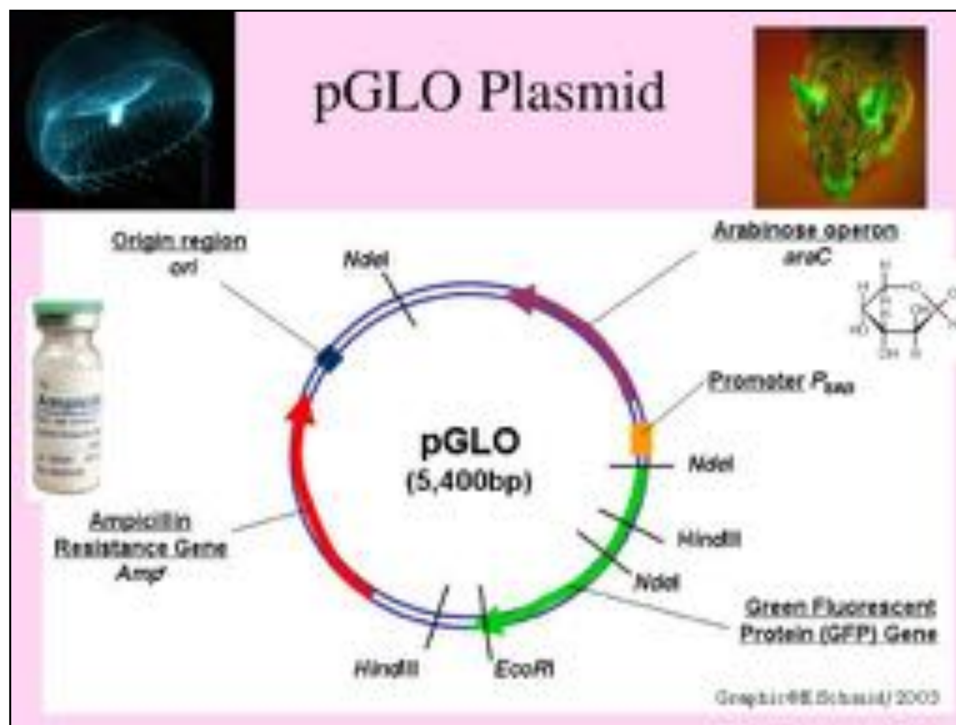

One type of Vector:
Plasmids
Bacterial DNA
Plasmids



Genetic **transformation** is taking genes from one organism and putting them in another. A **gene** is a piece of DNA that gives the instructions for making a protein. This **protein** gives an organism a certain trait. A gene is inserted into an organism in order to change the organism's trait. Genetic transformation is used in many areas of biotechnology. In **agriculture**, genes coding for traits such as frost, pest, or spoilage resistance can be inserted into plants. In **medicine**, gene therapy treats diseases caused by defective genes by inserting healthy copies of the defective gene in a sick person's cells.


Gene donor
Plasmid donor
Isolation of bacterial plasmid
Isolated gene
Plasmid opened with restriction enzyme
DNA ligase binds ends together
Recombinant DNA molecule
Transformation of fresh bacterium
Chromosome
Donor gene
Transcription mRNA
Translation


Creating
a useful
plasmid


pGLO Plasmid
Origin region
ori
NdeI
Arabinose operon
araC
Promoter P_{BAD}
pGLO
(5,400bp)
NdeI
HindIII
NdeI
Ampicillin
Resistance Gene
Amp^r
Green Fluorescent
Protein (GFP) Gene
HindIII
EcoRI

Transformation Procedure: Overview

- Suspend bacterial colonies in **Transformation Solution**
- Add **pGLO plasmid DNA**
- Place tubes on **ice**
- **Heat shock** at 42°C and place on **ice**
- Incubate with **LB nutrient broth**
- **Streak** plates

Transformation Solution: Used because it is a sterile solutions and it contains calcium chloride which renders bacteria competent and able to uptake the plasmid

Addition of pGlo: students must ensure film of pGlo is on the loop (like blowing bubbles) or not enough plasmid will be used.

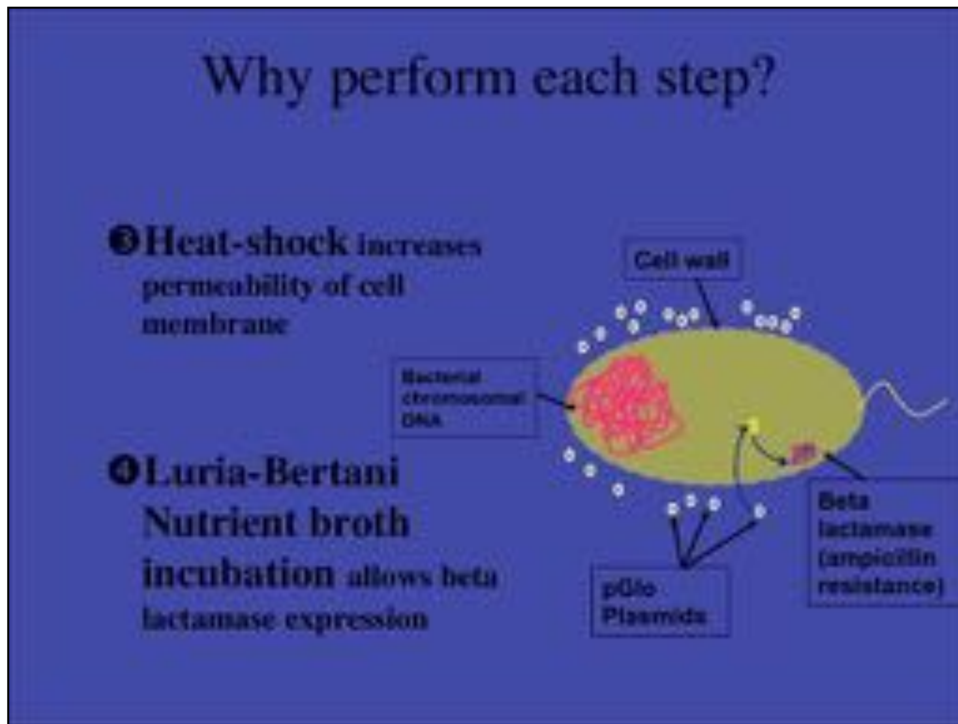
Ice→Heat→Incubate: Important to follow timing guidelines to give best results

Streak plates: Follow directions; too much bacteria or insufficient spreading could lead to poor results.

Why perform each step?

1. **$CaCl_2$ treatment on ice** crytallizes fluid membranes and stabilizes distribution of charged molecules
2. **$CaCl_2$ Transformation solution provides Ca^{++} cations** that neutralize the repulsive negative charges of the phosphate backbone of the DNA and the phospholipids of the cell membrane, allowing the DNA to enter the cells

The bacterial cell membrane is permeable to chloride ions, but is non-permeable to calcium ions. As the chloride ions enter the cell, water molecules accompany the charged particle. This influx of water causes the cells to swell and is necessary for the uptake of DNA. The exact mechanism of this uptake is unknown. It is known, however, that the calcium chloride treatment be followed by heat.



Heat Shock:

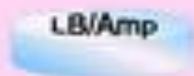
When E. coli are subjected to 42degC heat, a set of genes are expressed which aid the bacteria in surviving at such temperatures. This set of genes are called the heat shock genes. The heat shock step is necessary for the uptake of DNA. At temperatures above 42degC, the bacteria's ability to uptake DNA becomes reduced, and at extreme temperatures the bacteria will die.

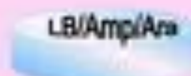
Incubation:

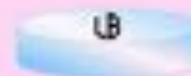
After the heat shock step, intact plasmid DNA molecules replicate in bacterial host cells. To help the bacterial cells recover from the heat shock, the cells are briefly incubated in LB Nutrient Broth, a solution that provides nutrients for the bacteria. As the cells recover, plasmid genes are expressed, including those that allow the replication of plasmids which will end up in new, dividing bacterial cells.

Gene selection

- Grow transformed bacteria and control bacteria under various conditions.







- On which plates will colonies **grow?**
- Which colonies will glow?

Not all cells will have the plasmid and not all newly produced plasmids will end up in new bacteria cells. So, it is necessary to select for bacterial cells which contain the plasmid. This is commonly performed with antibiotic selection. Some E.coli strains cannot grow in the presence of common antibiotics like ampicillin. Plasmids used for the cloning and manipulation of DNA have been engineered to contain the genes for antibiotic resistance. Thus, if the bacterial transformation is plated onto media containing ampicillin, only bacteria which have the plasmid DNA will have the ability to metabolize ampicillin and form colonies. In this way, bacterial cells containing plasmid DNA are selected.

The pGlo System

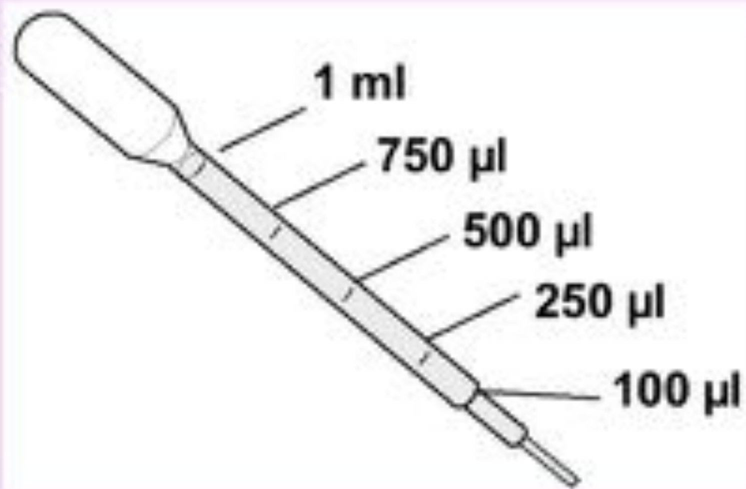
A film of plasmid must be on the loop!

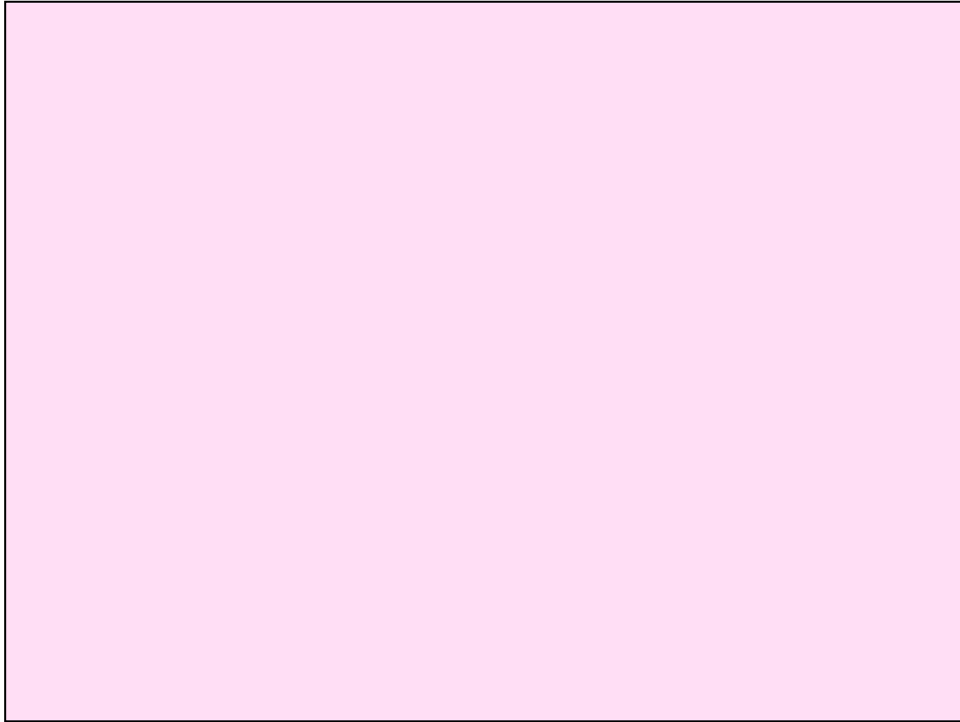
Timing is important…be efficient!!

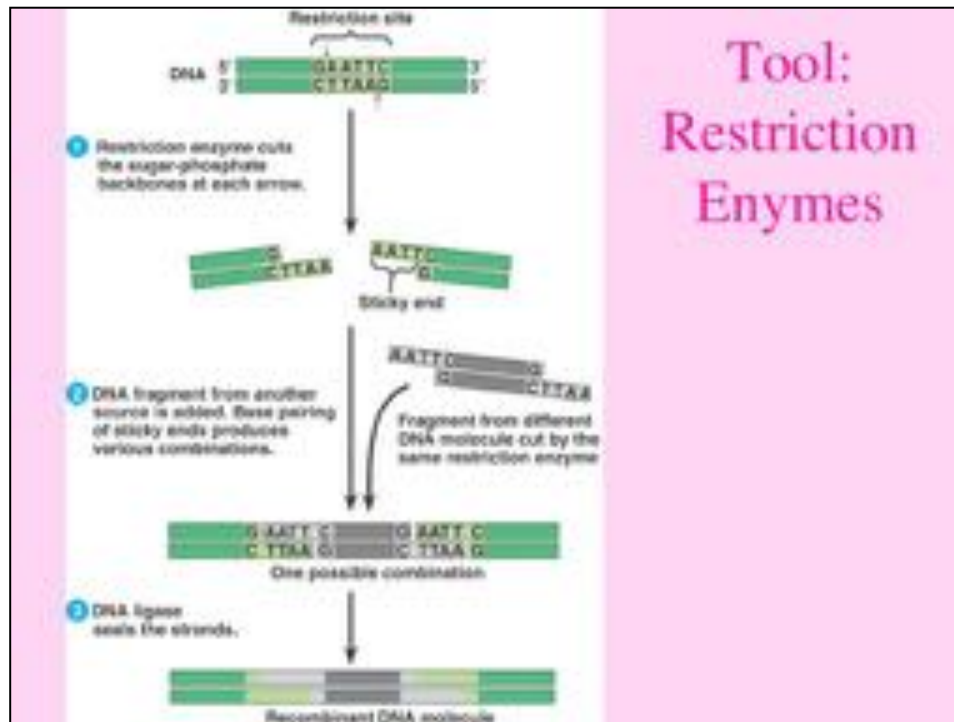
Mix contents before pipetting!!!

Sterile Technique

- Bacteria are UBIQUITOUS…they are found EVERYWHERE!
- Sterile technique refers to procedures that reduce the possibility of contamination…these techniques protect YOU, your CULTURES and REAGENTS, and LAB EQUIPMENT


Volume Measurement
1 ml
750 µl
500 µl
250 µl
100 µl





3 Tools: Restriction Enzymes, Vectors, Host Organism

Cut DNA

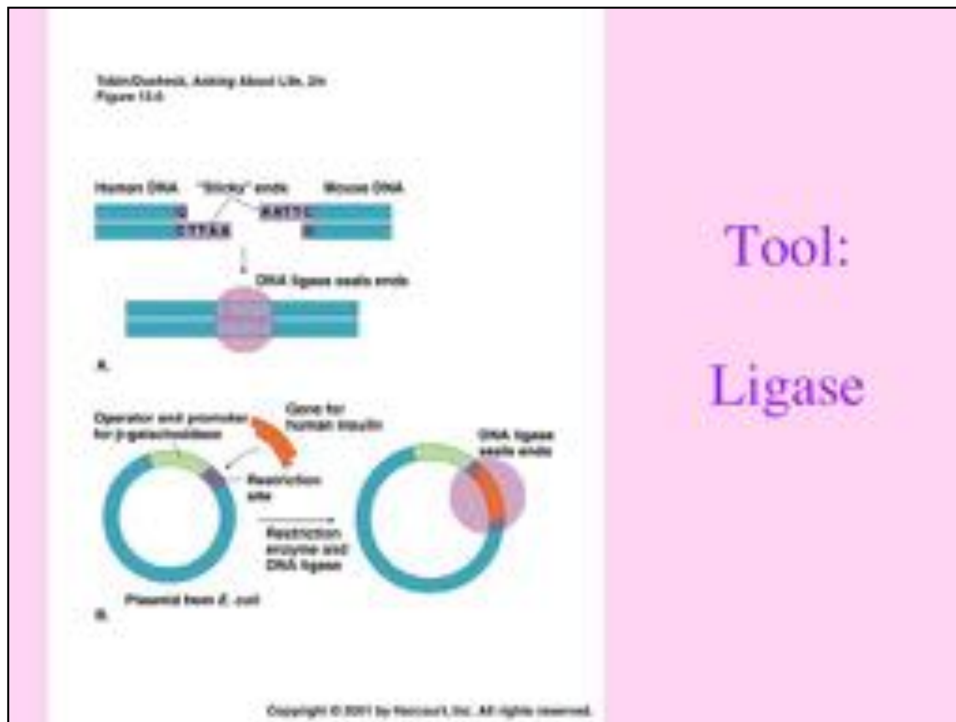
Found in Bacteria to get rid of foreign DNA

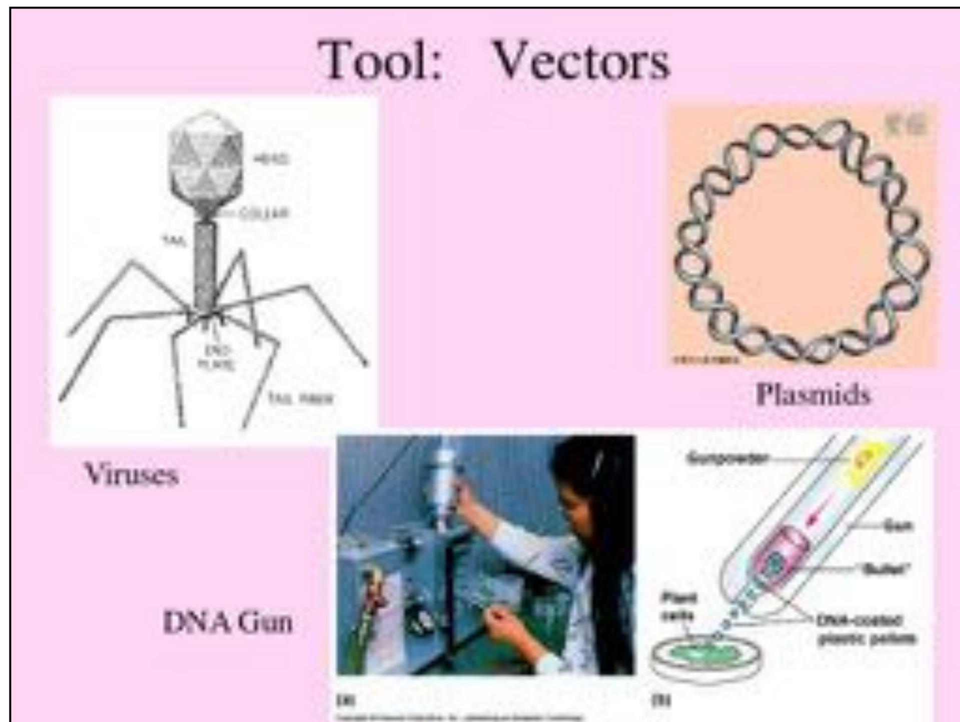
Restriction Sites

Available commercially

Symmetrical - produce restriction fragments with sticky ends

Add ligase to seal these unions


Tool:
Ligase

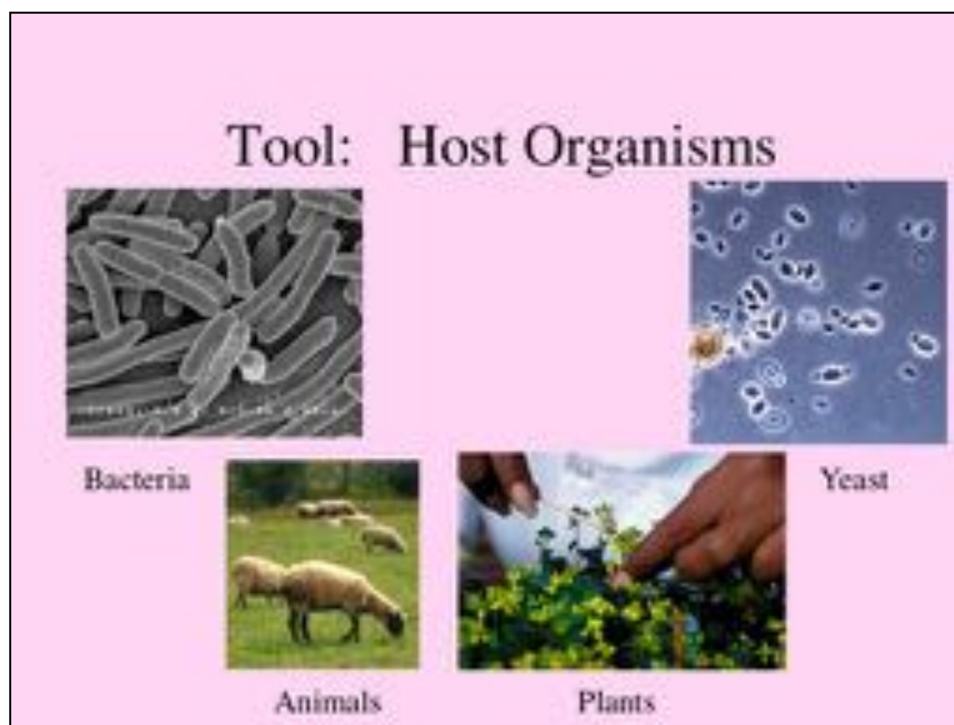


Plasmids

Some Viruses - Lysogenic, bacteriophages, retrovirses (animal cells)

Some yeast have plasmids

LAST TOOL: Host Organisms: Bacteria, Yeast, plants, animals, humans



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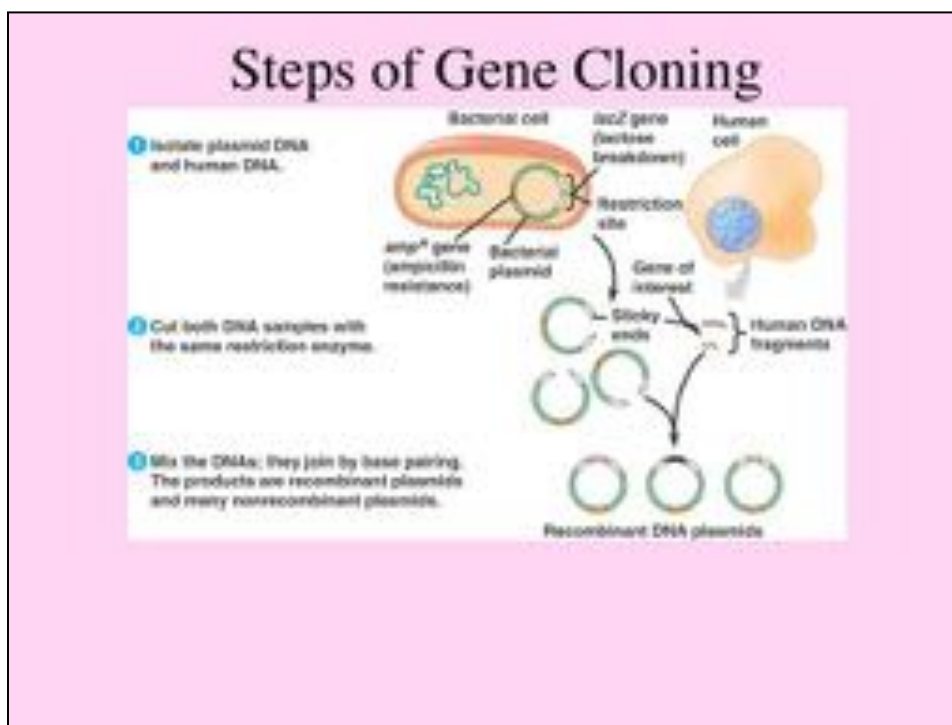
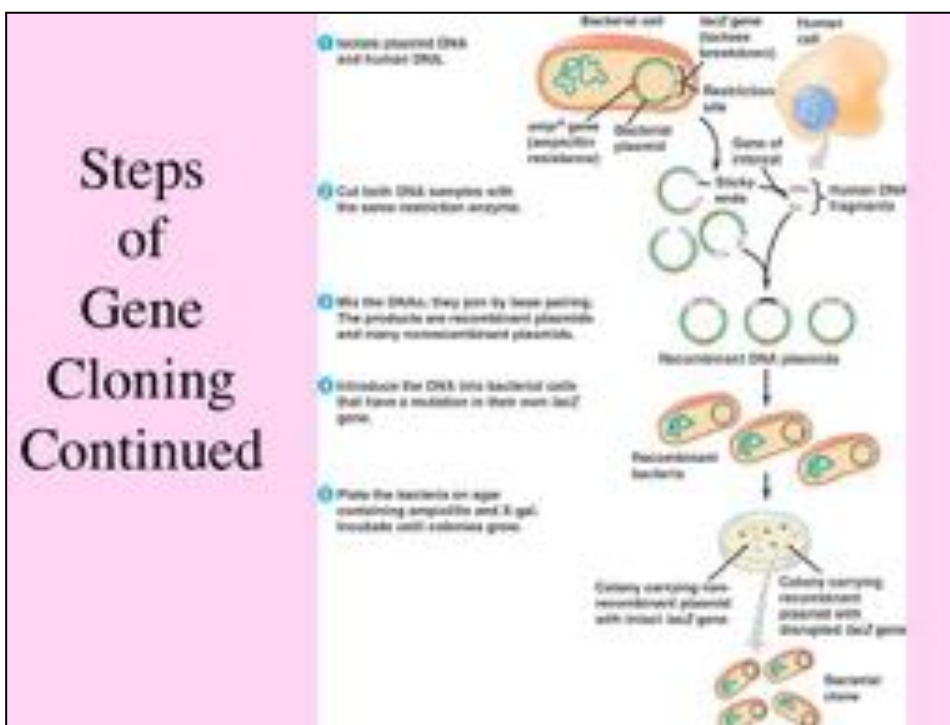


1. Isolate from human cells grown in culture; isolate the bac. Plasmid; engineer plasmid to have (a) amp resistance and the lacZ gene that make enzyme to digest lactose AND synthetic sugar called X-gal (X-gal turns blue when metabolized); lac z gene has restriction site
2. Digest plasmid and human gene with SAME restriction enzyme; one of the human fragments carries the gene of interest; the plasmid has only the ONE restriction site
3. Mix the plasmid and the human DNA; they will join at their sticky ends; add ligase; lots of plasmids are formed: some have the gene we're interested in; some just recl-ose; some take up other fragments of the human DNA we originally cut up
4. Mix the plasmids with the host bacteria; the host has a mutant lacZ gene and thus can't met. Lactose or X-gal; heat sock so to increase the transformation frequency
5. Plate the bacteria on medium with amp and X-gal:
6. Only cells with the plasmid will reproduce (amp kills all others); each bacterium forms a clone of 10^5 cells which is seen as a colony; the plasmids are copied when each bacterium reproduces. Colonies without the human gene will be blue because they can metabolize the X-gal; colonies with the human gene will be white because they CAN'T use X-



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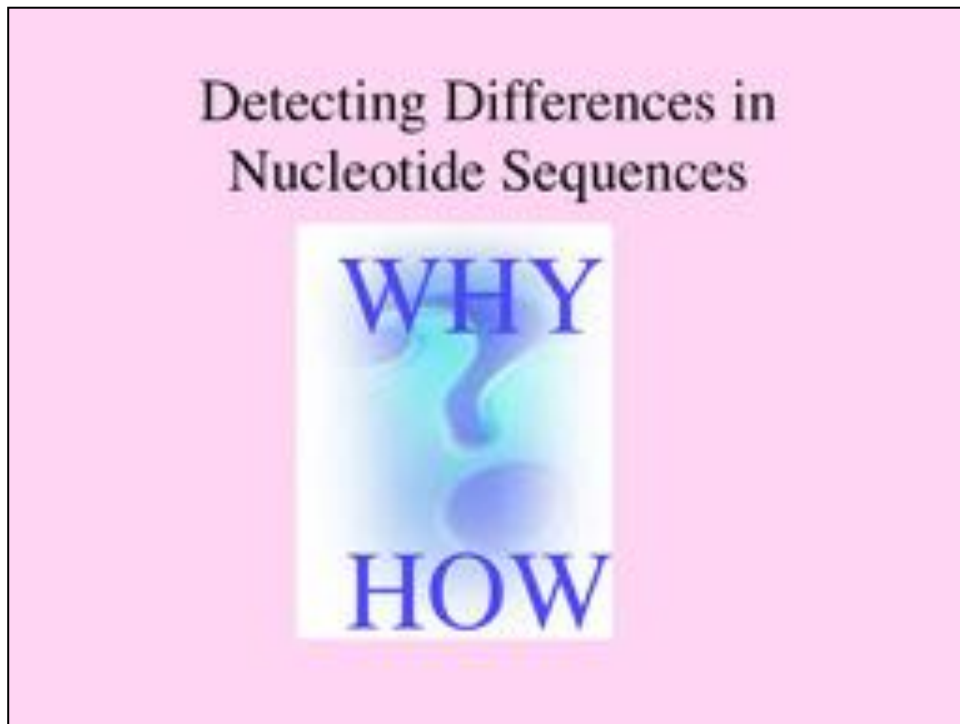


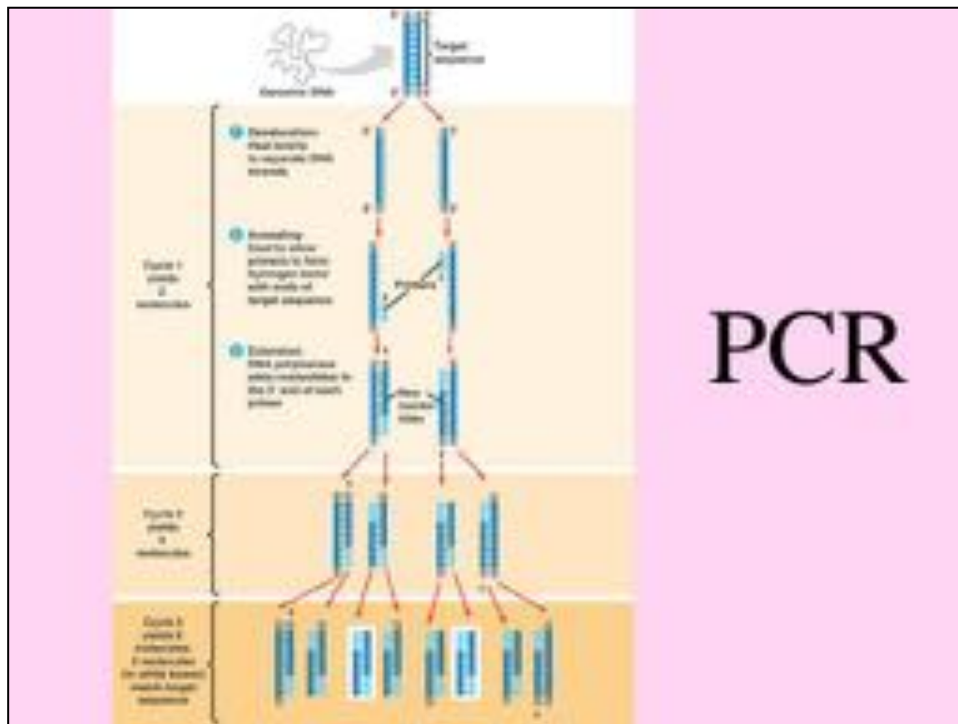
Does a gene differ from person to person?

Are certain alleles associated with genetic disorders?

Where is the gene located in the genome?

How does a gene differ from one species to another? -- unravel evolutionary history

HOW: Restriction Fragment Analysis



DON' T DO TECHNIQUE

Polymerase Chain Reaction

1985 - devised technique

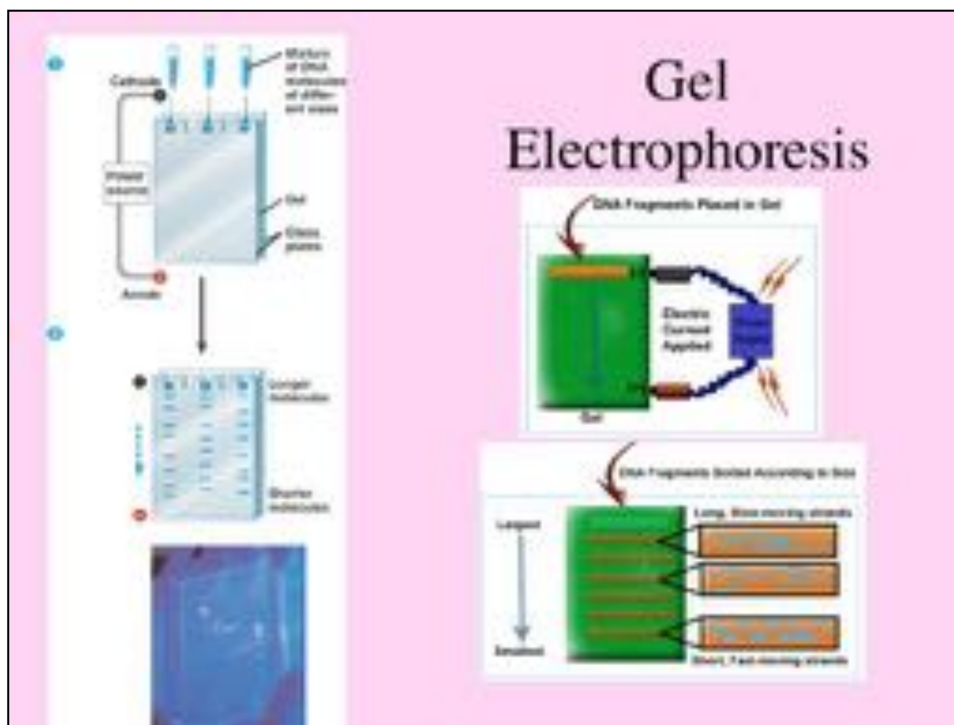
Used to amply scanty amounts of DNA

e.g. Fragments of DNA from a wooly mammoth

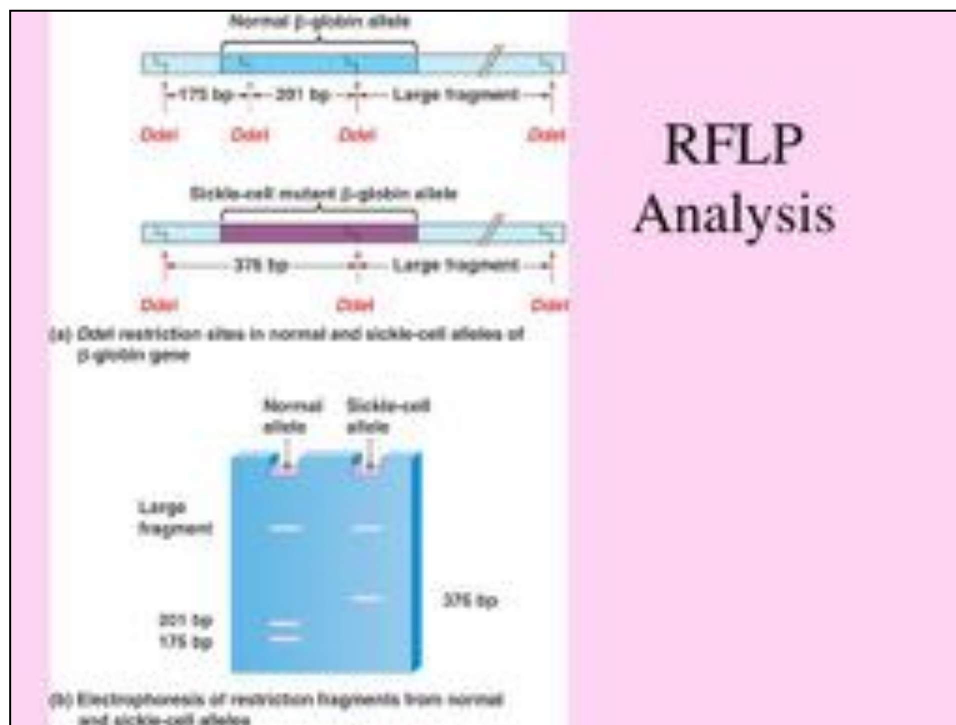
e.g. DNA from blood, semen or tissue at a crime scene

e.g. DNA from a single embryonic cell for prenatal diagnosis of genetic disorders

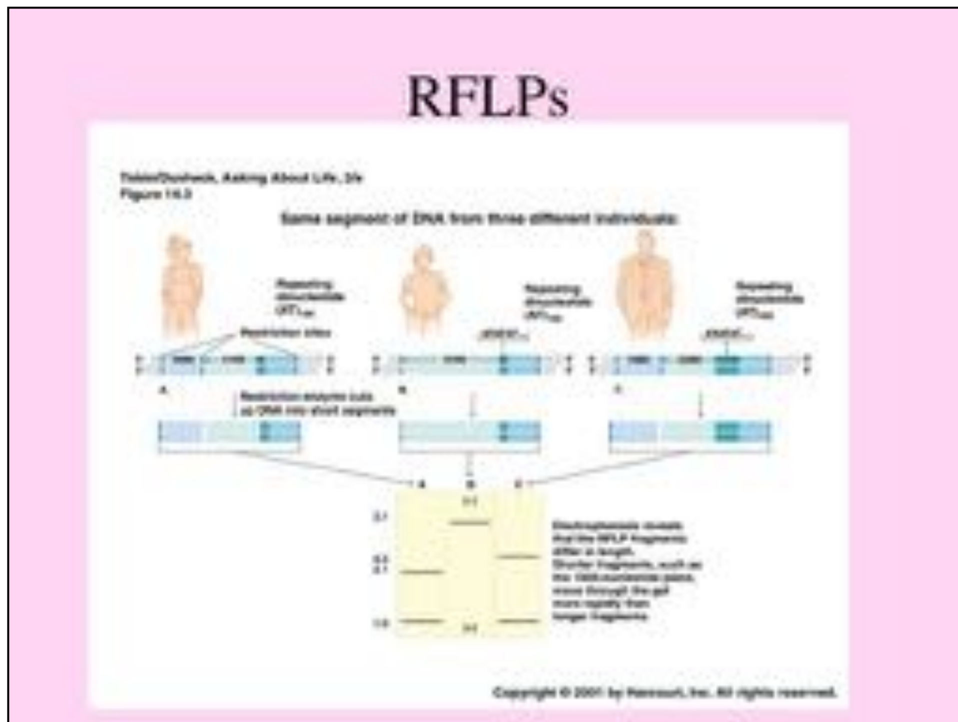
e.g. DNA from cells infected with a virus like HIV

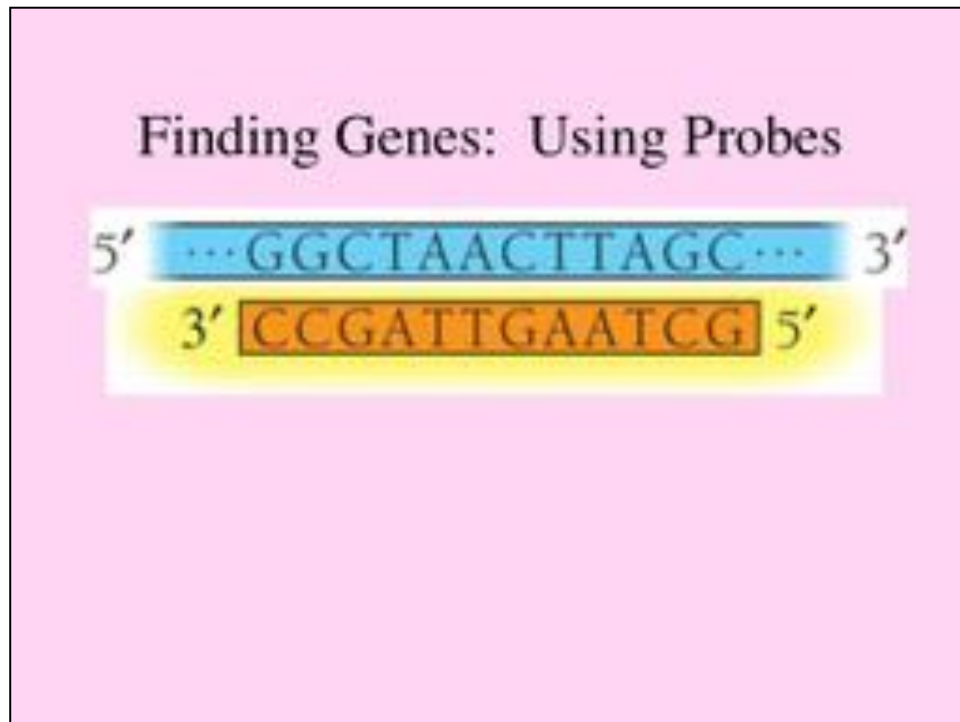


Explain how you would use this to identify a person who is a carrier for sickle cell anemia --See next slide



Restriction Frgment Length Polymorphisms


RFLPs

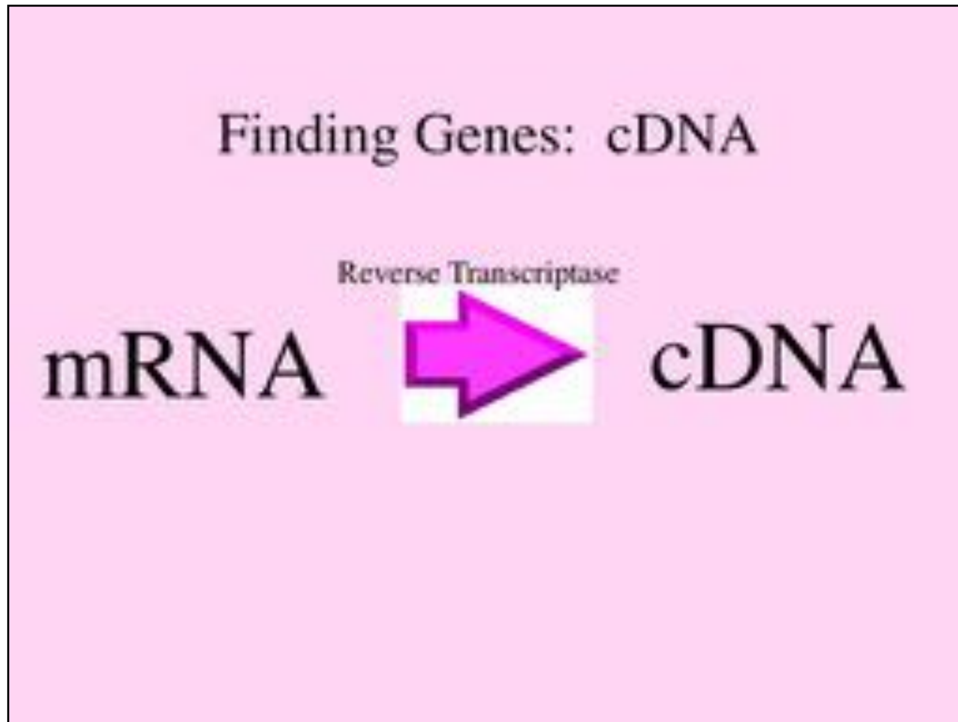


Nucleic acid hybridizaton

Use a probe for a known segment of the gene (maybe from knowing the protein product)

Label the probe with either radio activity or fluorescent tag to track it

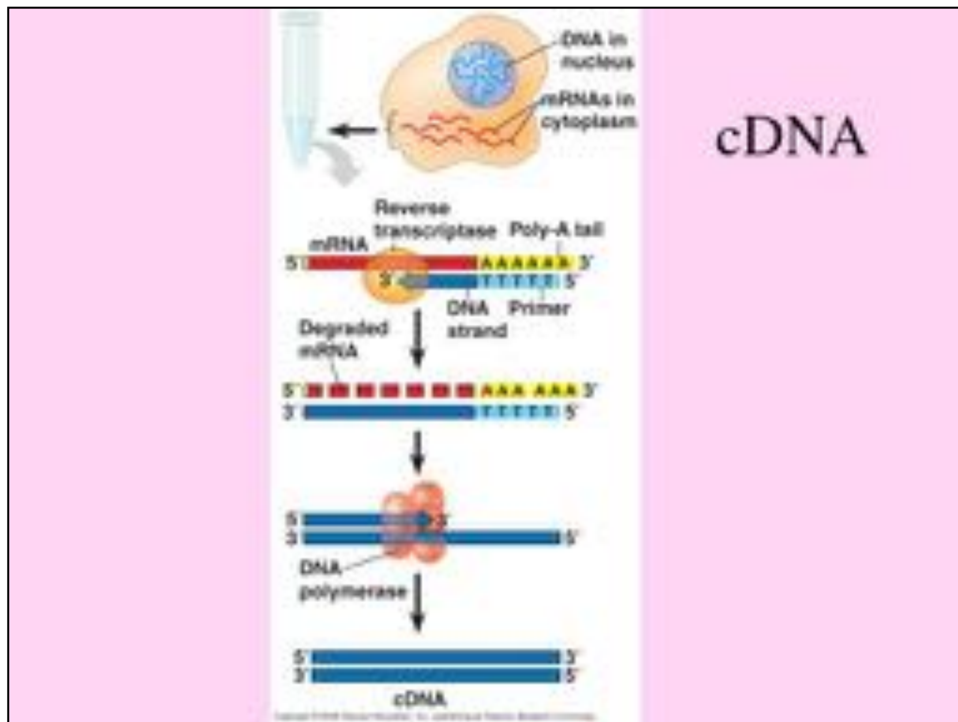
Skip the procedural details of this process

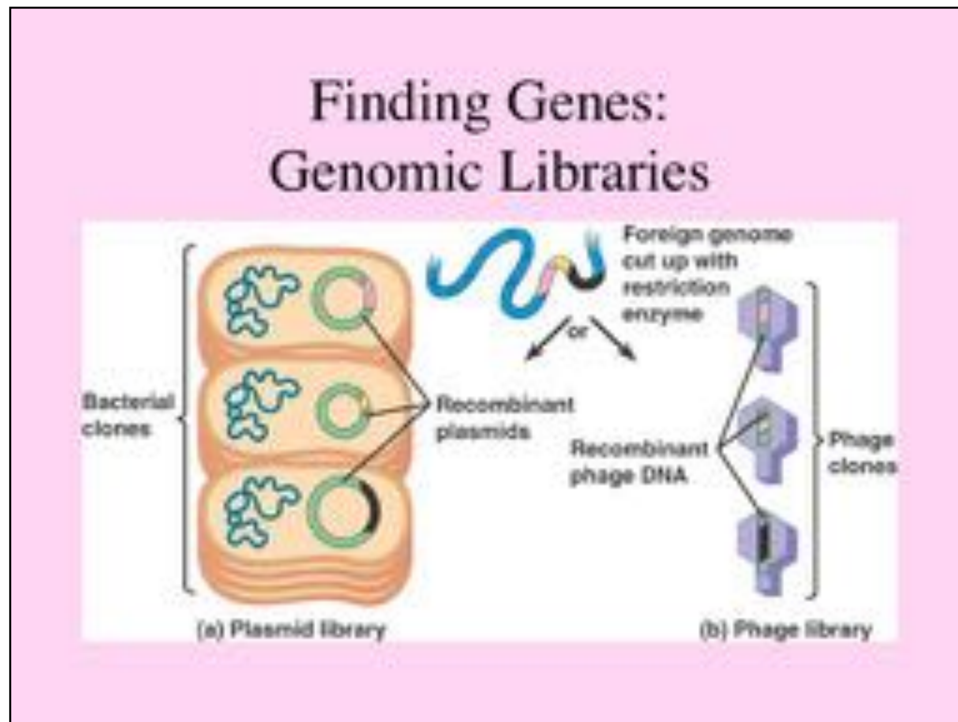


mRNA from a cell of a certain type, say pancreas cell, which would likely have mRNA for insulin.

Extract the mRNA, use reverse transcriptase to make single stranded DNA, digest away the mRNA, use DNA polymerase to make opposite side of DNA, now you have cDNA

These cDNA are many kinds but much more limited than from all the genome


cDNA
DNA in nucleus
mRNAs in cytoplasm
Reverse transcriptase
Poly-A tail
mRNA
5'
AAAAAA
3'
TTTTT
DNA strand
Primer
Degraded mRNA
DNA polymerase
cDNA



Shot gun approach - just take a source of DNA (human) cut into pieces and insert into plasmids or viruses -- don't really know what you've got

Uses of Cloned Genes & Techniques of Biotechnology

- Produce a Protein Product
  - Human Growth Hormone; Blood Clotting Factors
  - Insulin; Vaccines
- Give an Organism a New or Improved Capability
  - Pest & Herbicide resistance in crops
  - Environmental Clean-up - digest oil, extract heavy metals
  - Increased Nutritional value - Golden Rice
- Make Copies of Gene for Research
- DNA Fingerprinting
  - Forensics/ Crime scene investigation
  - Paternity Identification
  - Disease diagnosis
  - Determine gene carrier status - self, spouse, child, embryo
- Gene Sequencing
  - Evolutionary relationships
  - Finding genes
- Replace Defective Genes -- Human Gene Therapy