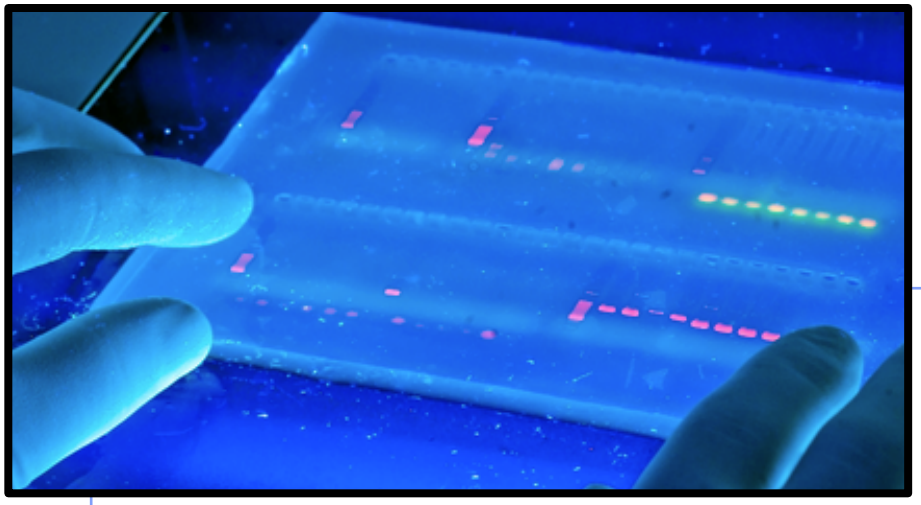
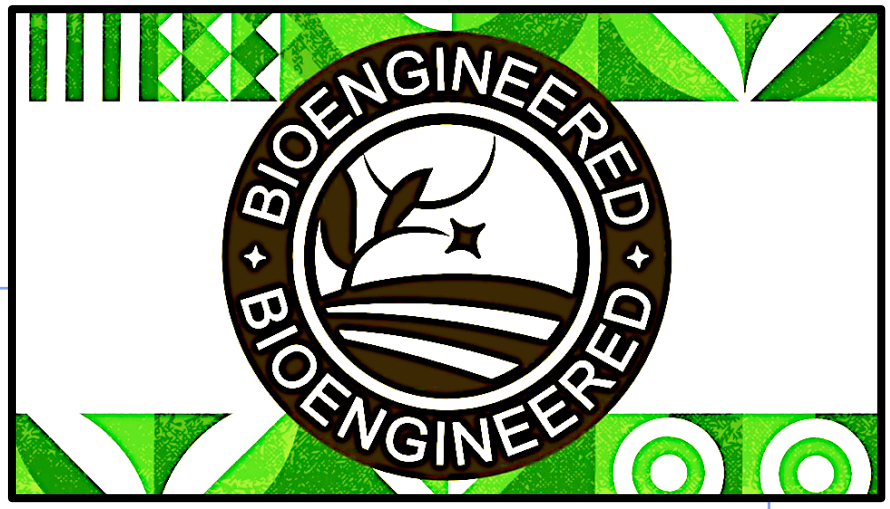
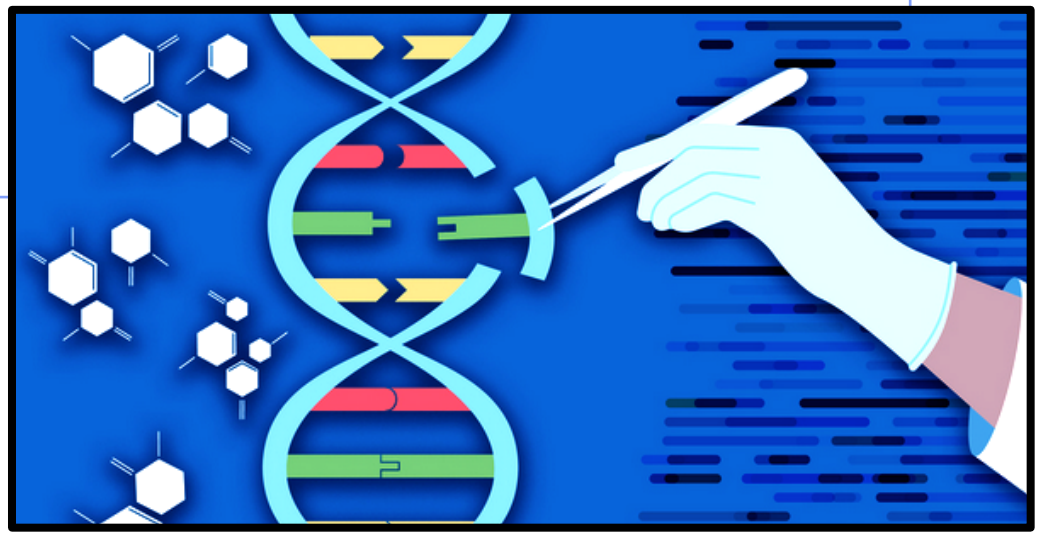
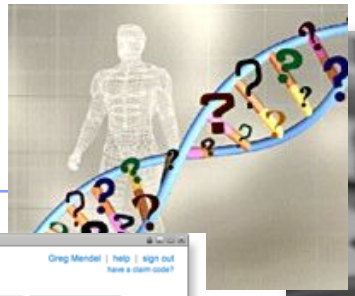


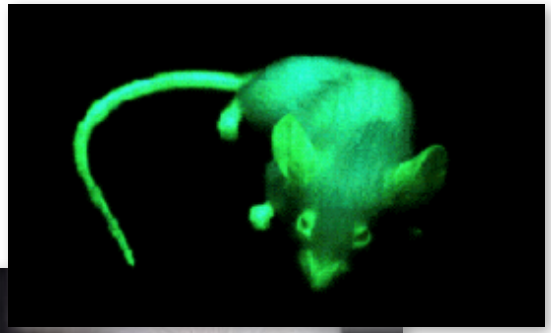


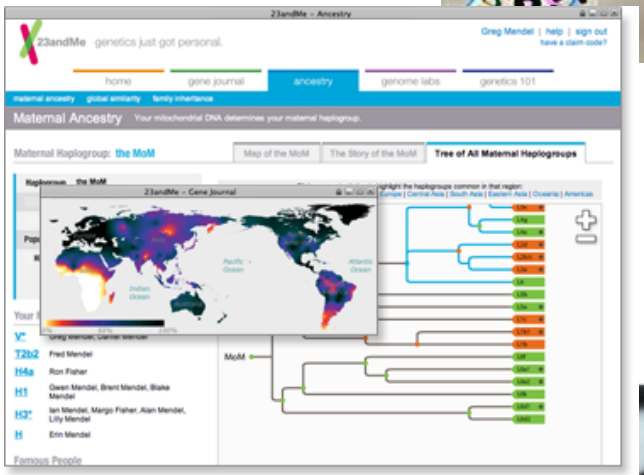
Ch.20 - Biotechnology



A Brave New World is Upon Us







23andMe

Biotechnology

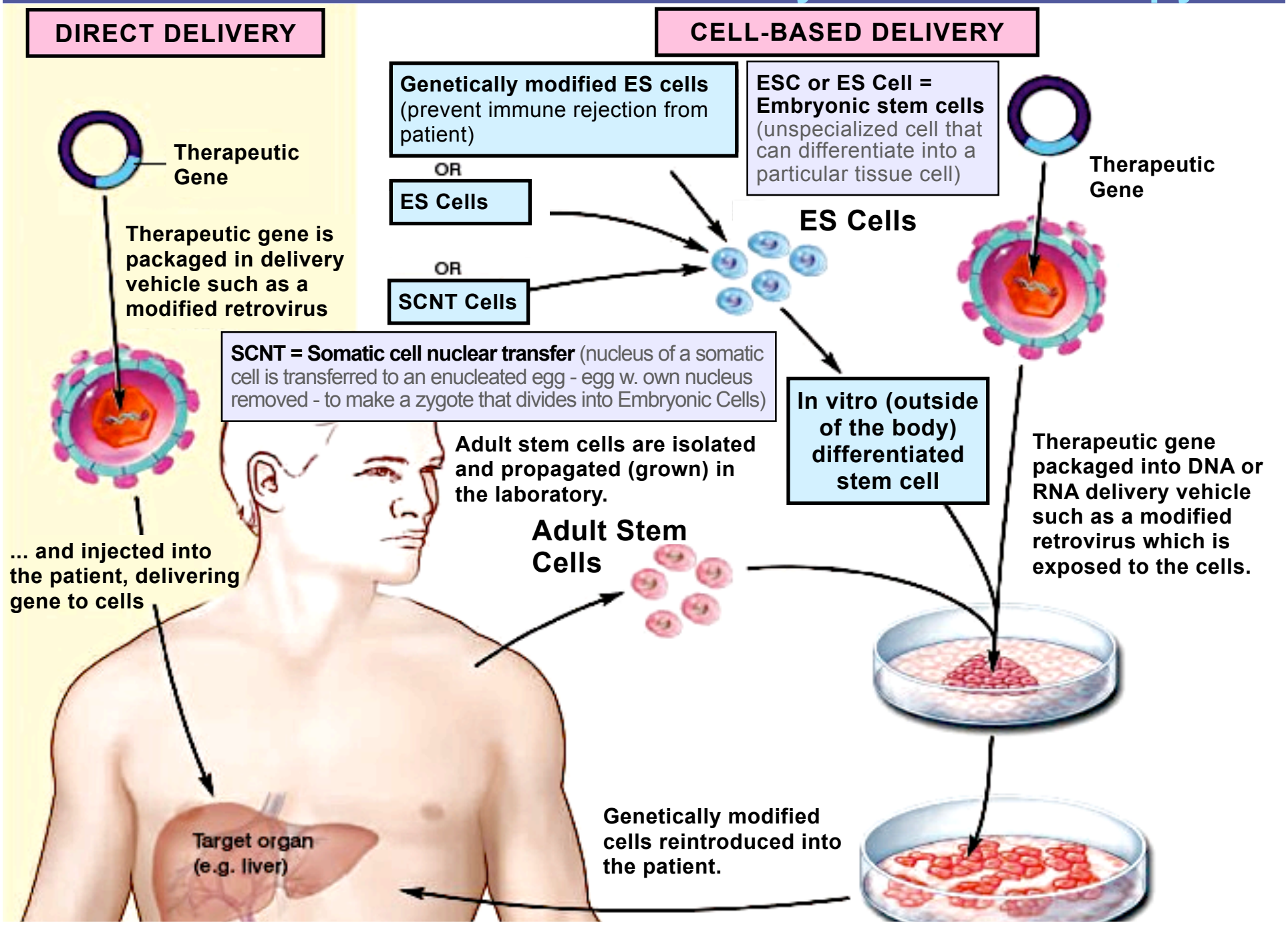
- **Biotechnology** is the use of an organism or a component of an organism or other biological system to make a product or process for a specific use.
  - Ex: In beer brewing, tiny fungi (yeasts) are added to solutions of malted barley sugar, which they metabolize through fermentation, producing alcohol for human consumption.
  - Ex: The antibiotic penicillin is generated by certain molds, the fungus being used to make a product for human use in treating bacterial infections.
  - Ex: Gene therapy is a emerging technique used to treat genetic disorders that are caused by a nonfunctional gene, which causes the person to make a nonfunctional protein. The working copy of a gene is introduced into to the cells of a person in the hopes the person will be able to make a working protein.

In cystic fibrosis, people lack functional gene for a chloride channel produced in the lungs.

In clinical trials, scientists inserted a copy of the functional gene into a circular DNA molecule called a plasmid and delivered it to patients' lung cells.

- A gene from humans and a plasmid from bacteria were combined to make a new product that helped preserve lung function in cystic fibrosis patients.

Current Avenues for Gene Delivery in Gene Therapy



Genetically Modified Organisms

- A GMO is any organism that has had its genetic material intentionally changed.
  - This could mean adding a useful gene to the organism, or removing a harmful one.
    - While the most commonly seen GMOs are vegetables and fruits, the term also refers to bacteria and viruses that have modified DNA or RNA (depending on their genetic makeup).
- Any animal whose DNA has been modified to include one or more foreign genes is a transgenic organism.
  - GMOs plants can make plants resistant to pests and environmental stresses like drought, reduce the use of pesticides, and therefore increase the yield of crops
  - GMO plants can provide added nutritional value to food to prevent disease caused by caused by its deficiency.
  - GMO bacteria can be used to manufacture a range of useful pharmaceutical products - medicines, vaccines, and enzymes.
  - GMO lab rats and mice help scientists carry out research on disease.

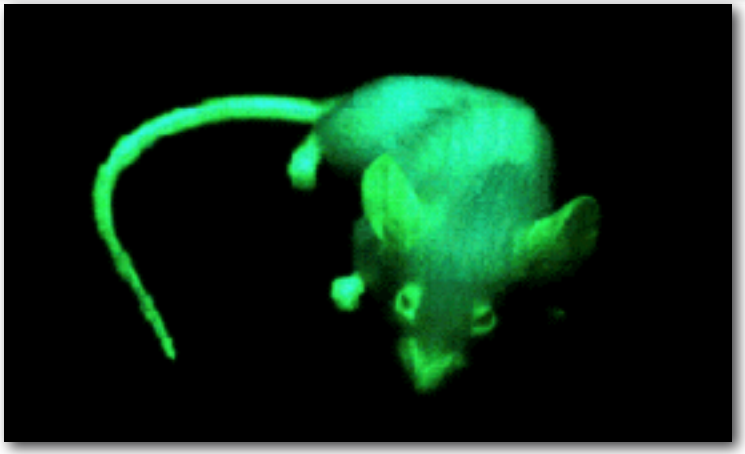
Examples of Genetic Engineering

- Making genetically modified organisms (GMO)
  - Ex: enabling plants to produce new proteins
    - Protect crops from insects: BT corn
      - corn produces a bacterial toxin that kills corn borer (caterpillar pest of corn)

    - Extend growing season: fishberries
      - strawberries with an anti-freezing gene from flounder fish

    - Improve quality of food: golden rice
      - rice producing betacarotene (that humans use to make Vitamin A) improves nutritional value
        - Currently, 70% of children under the age of 5 in Southeast Asia are deficient in vitamin A, leading to vision impairment and increased disease rates.

Green with envy??



Jelly fish “GFP”
Green Fluorescent Protein

Transformed vertebrates -

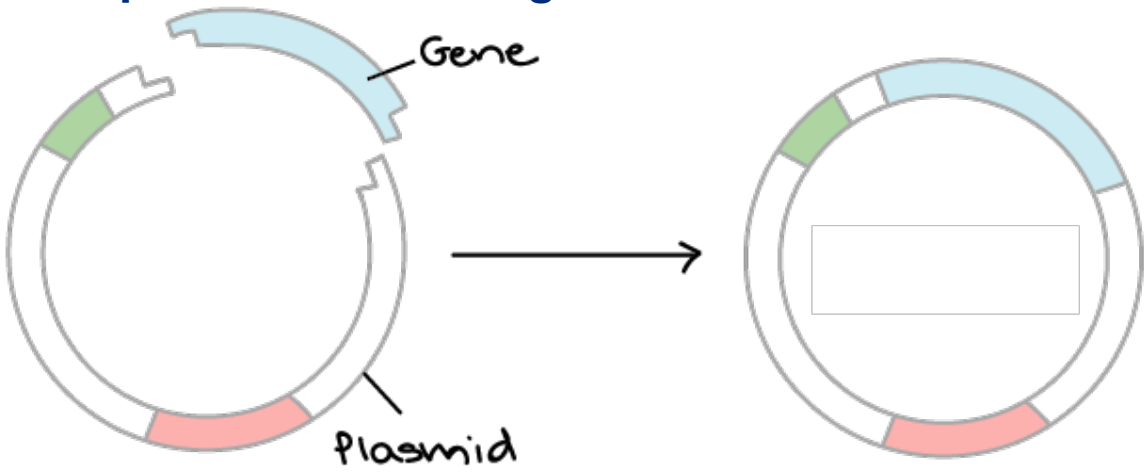
Genetically Modified Organisms

- **Why don't some trust GMOs?**
  - Some of this comes from a lack of understanding.
    - People might misunderstand the nature of GMOs and think that GM crops are harmful to health *(though many studies have shown that this not necessarily the case)*
    - There is a myth that animals or humans that ingest GM products will also become genetically modified - incorrect!

  - One concern with GMO crops is that farmers often need to buy the seeds from private companies each time, which can be expensive.
  - Another concerns includes a fear of negative consequences to still unknown targets *(Ex: GMO crops killing off useful insects instead of only pests the pests)*
  - Yet another concerns is that foreign genes could spread into the wild *(Ex: Antibiotic resistance spreading among bacteria, including harmful ones that we may want to be able to destroy).*
- **Several countries require GM products to be labeled, while some have banned their use.**
- **Research is currently ongoing to test what the effects of longterm use of GMOs are.**

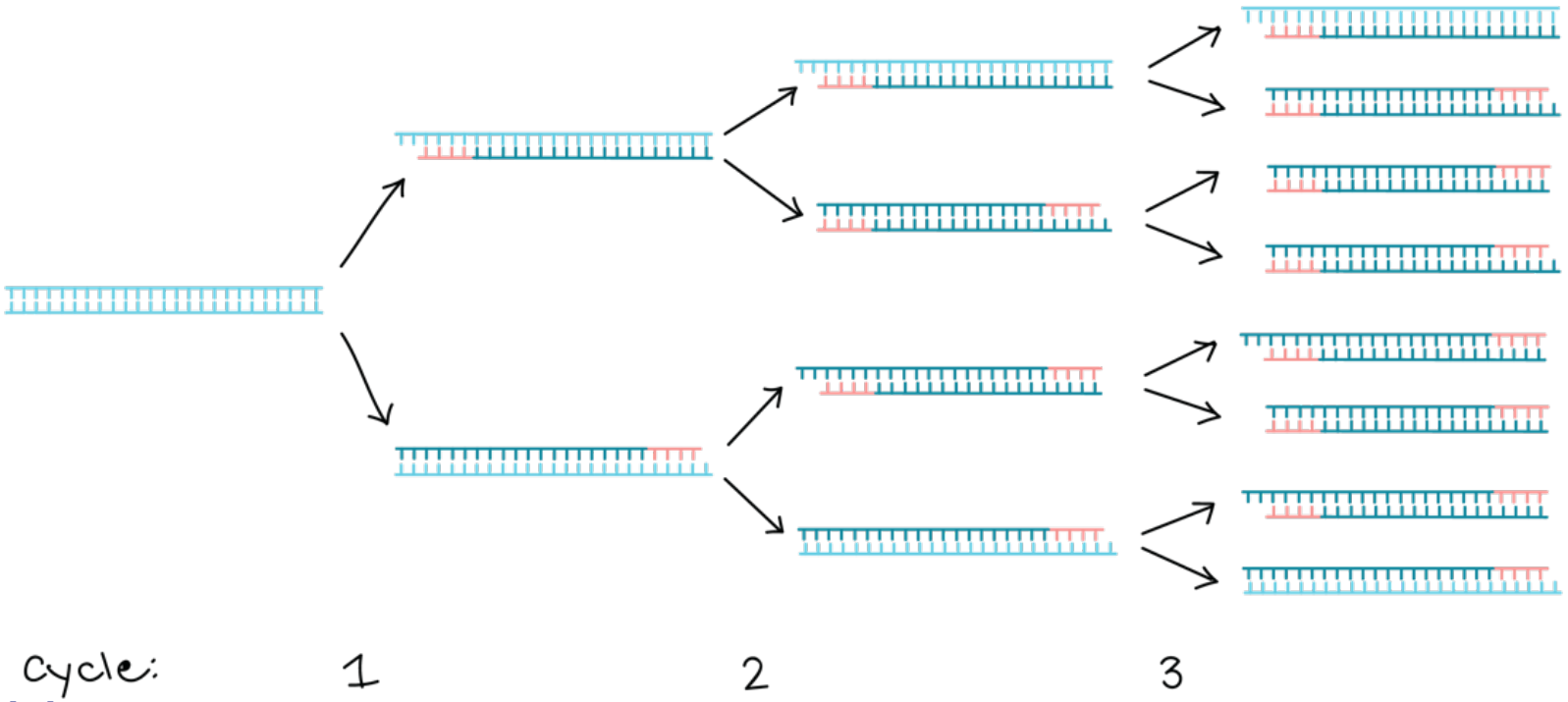
DNA Technologies

- Many examples of modern biotechnology depend on the ability to analyze, manipulate, and cut and paste pieces of DNA.
  - Approaches for the sequencing and manipulation of DNA are sometimes referred to as DNA technology
- Examples of DNA analysis and manipulation techniques:
  - In DNA cloning, researchers make many copies of a DNA fragment of interest, such as a gene, using bacteria.
    - A target gene can be inserted into a circular DNA molecule called a plasmid. The plasmid is replicated inside bacteria, making many copies of the gene of interest.
    - In some cases, the gene is also expressed in the bacteria, which make a protein out of the gene



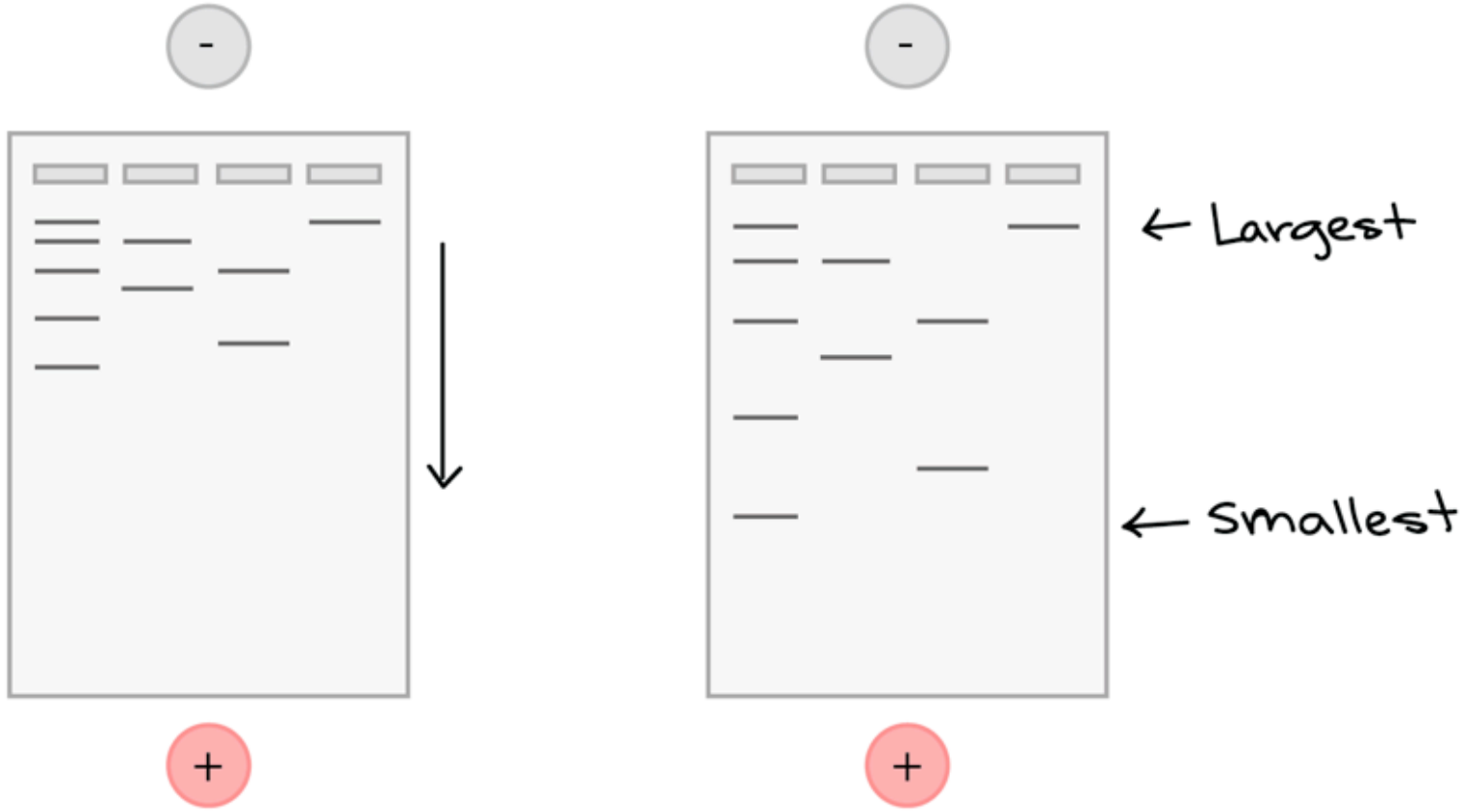
DNA Technologies

- **Researchers can use Polymerase Chain Reactions, to produce millions of copies of a target DNA sequence starting from a DNA template.**
  - **This technique can be used to make many copies of DNA that is present in trace amounts (e.g., in a droplet of blood at a crime scene).**



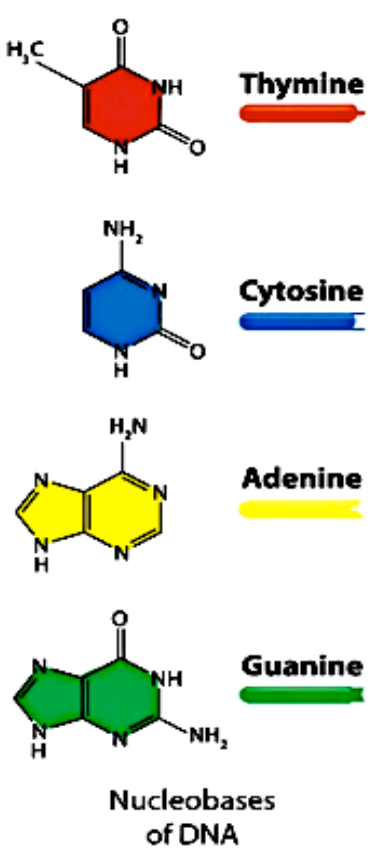
DNA Technologies

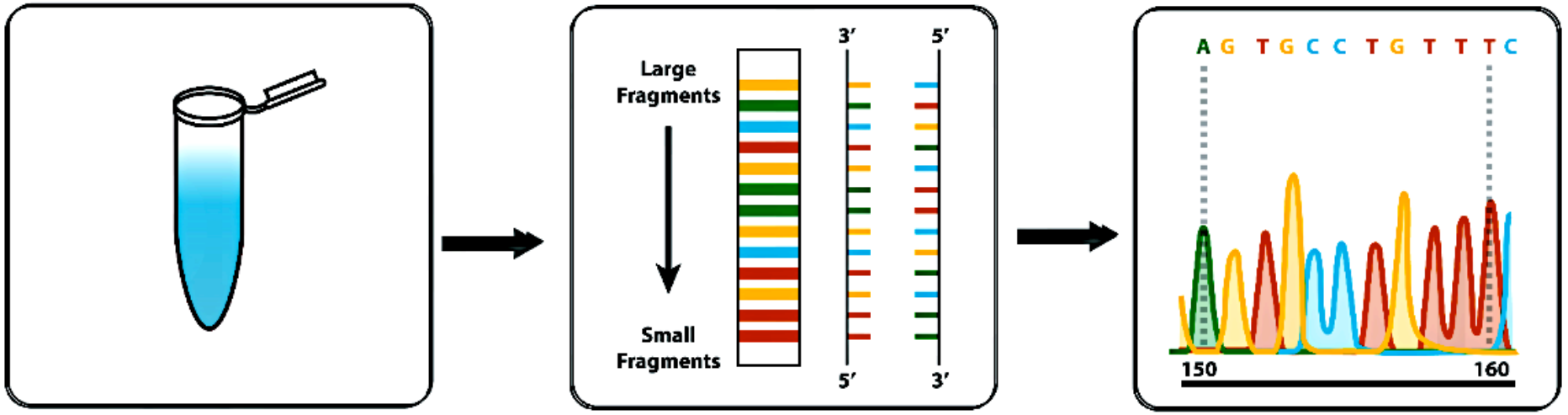
- **Gel Elctrophoresis, is used to visualize DNA fragments, which are separated by their size (lengths).**



DNA Technologies

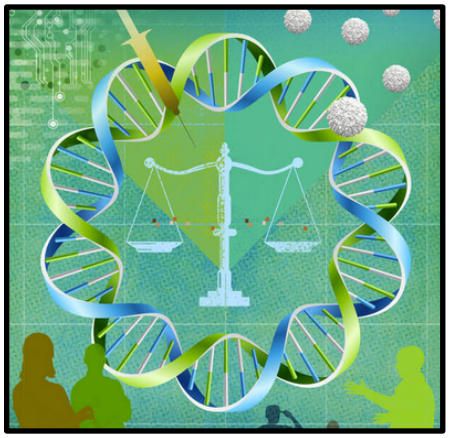
- **DNA Sequencing**, involves determining the sequence of nucleotide bases (As, Ts, Cs, and Gs) in a DNA molecule.





Biotechnology Raises Ethical Questions

- **Biotechnology can benefit society, but it can also have negative effects or unintended consequences.**
  - **Questions arise that relate to the safety, health effects, or ecological impacts of biotechnologies.**
    - **For Ex: Crops genetically engineered to make their own insecticide reduce the need for chemical spraying, but also raise concerns about plants escaping into the wild or interbreeding with local populations (potentially causing unintended ecological consequences).**



- **It is important that biotechnology innovations be carefully tested and analyzed before they are released for general use.**
- **Clinical trials and government regulation help ensure that biotechnology products placed on the market are safe and effective.**

Ethical Questions

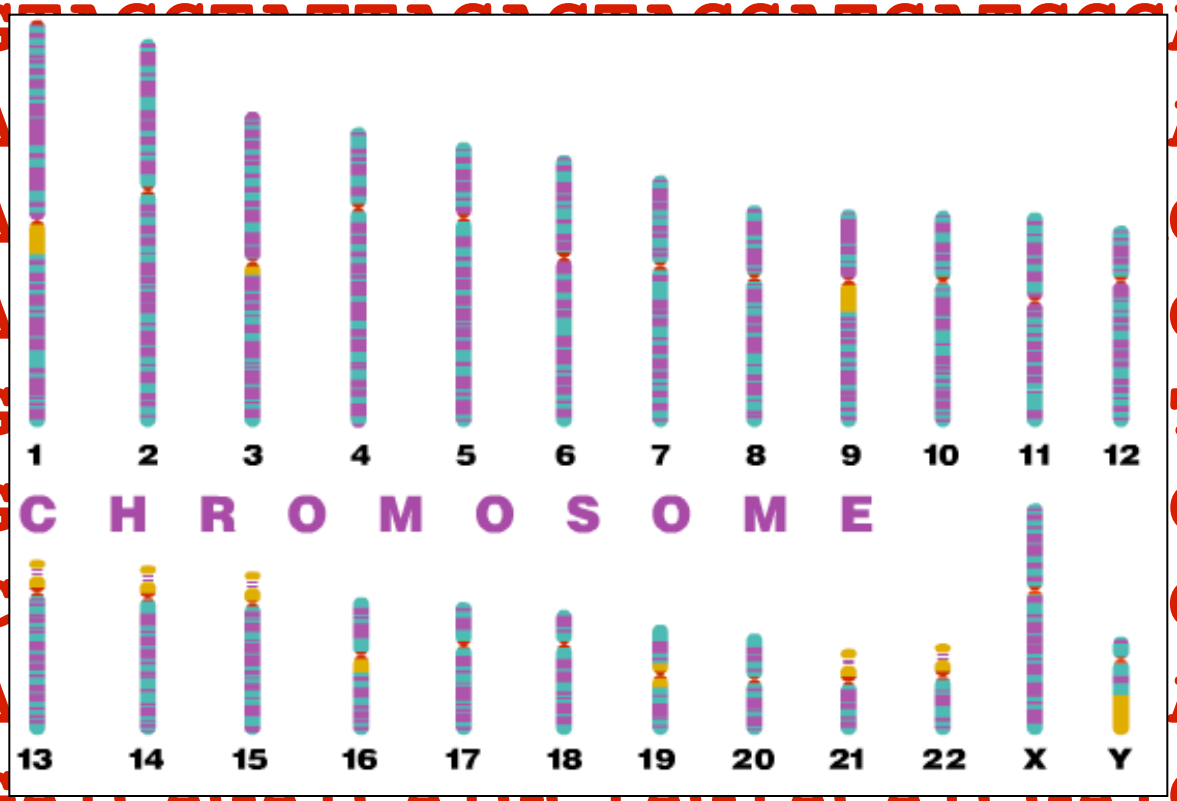
- Biotechnology innovations may raise new ethical questions about how information, techniques, and knowledge should or shouldn't be used.
  - These questions often relate to individual rights to privacy and to non-discrimination.
    - For Ex: Should your health insurance company be able to charge you more if you have a gene variant that makes you likely to develop a disease?
    - For Ex: How would you feel if your school or employer or a College Admission's office had access to your genome to see which versions of genes you carry?
  - Biotechnology may provide knowledge that creates hard dilemmas or psychological stress for individuals.

For Ex: A couple may learn via prenatal testing that their fetus has a genetic disorder.

For Ex: A person who has her genome sequenced for the sake of curiosity may learn that she is going to develop an incurable, late-onset genetic disease, such as Huntington's.

TACGCACATTTACGTACGCGGATGCCGCGACTATGATC
ACATAGACATGCTGTCAGCTCTAGTAGACTTAGCTGACT
CGACTAGCATGATGCAGCTACATGCTAGCACACYC
GTACATCGATCGTCAGATCCACCTGCTCGTACATGCTA
CTAGCTACTGACTCATGATCCAGATCACTGAAACCCTA
GATCGGGTAGCTATTAGATCATCGATGCGATCAGAT
CATGCTA ATCTAGC
TCAATCA GACTAGC
TGACTGA GGTACCT
ATTACAG TAGTACA
TCGATCG CAAACTC
TTTTTGC GATCATG
ACTCTGA AGTACGA
TCATCCGATCAGATCATGCTAGTACATCGATCGATACT

human genome
3.2 billion bases



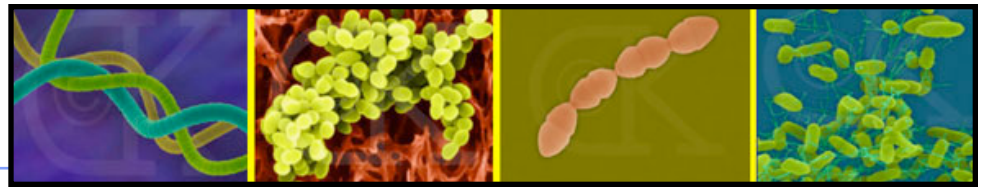
Biotechnology today

- **Genetic Engineering**
  - manipulation of DNA genes for practical purposes
- **Recombinant DNA**
  - DNA molecules formed when segments of DNA from two different sources (even two species) are combined *in vitro* (in a test tube)

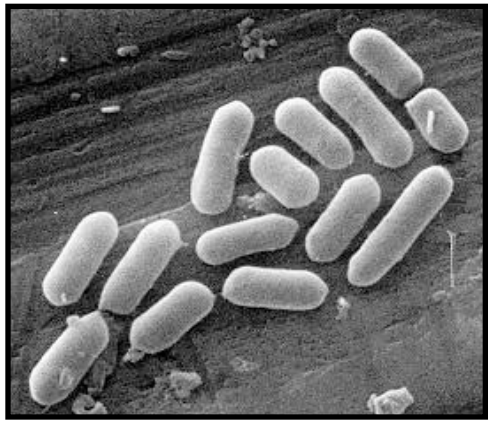
- if you are going to engineer DNA & genes & organisms, then you need a <u>set of tools</u> to work with
  - this unit is a survey of those tools…

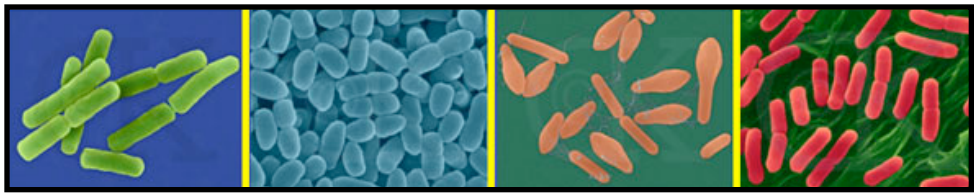
Our tool kit…

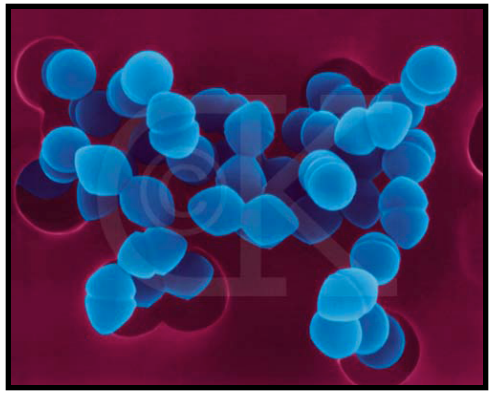
Bacteria



- Bacteria review
  - one-celled prokaryotes
  - reproduce by binary fission
  - rapid growth
    - New generation every ~20 minutes
    - 10^8 (100 million) colony overnight!
  - dominant form of life on Earth
  - incredibly diverse

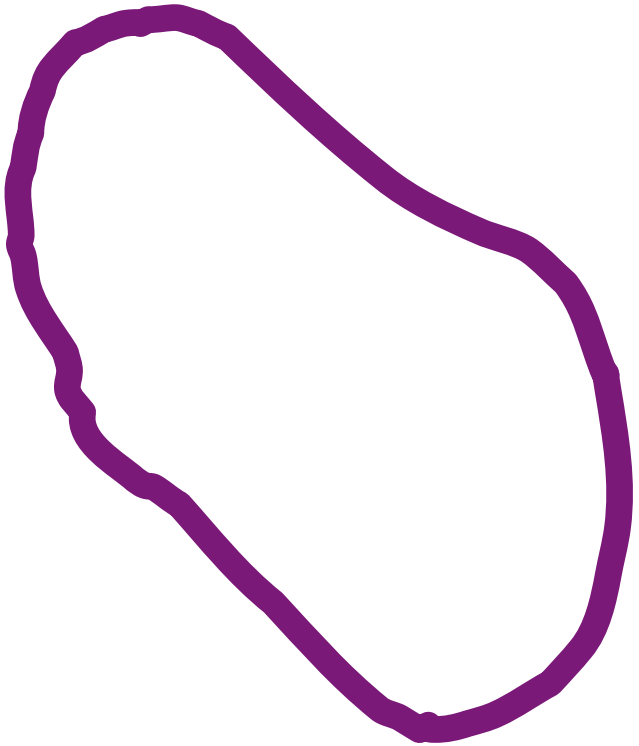


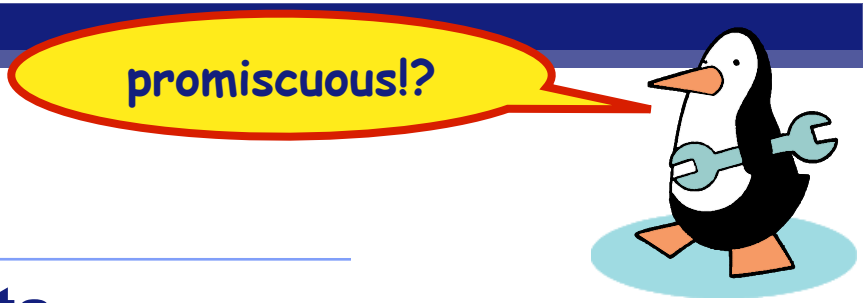




Bacterial genome

- **Single circular chromosome**
  - **Asexually reproducing**
  - **Haploid (1n)**
  - **No introns or exons** *[No mRNA Processing]*
  - **Naked DNA**
    - **no histone proteins**
  - **~4 million base pairs**
    - **~4300 genes**
    - **1/1000 DNA in eukaryote**





Transformation

- **Many bacteria are opportunists**
  - **can pick up small pieces of naked foreign DNA from their environment**
    - **have surface transport proteins that are specialized for the uptake of naked DNA**
      - **While *E. coli* lacks this specialized mechanism, it can be induced to take up small pieces of DNA if cultured in a medium with a relatively high concentration of calcium ions.**
      - **In biotechnology, this technique has been used to foreign DNA into *E. coli*.**
  - **can import bits of chromosomes from other bacteria**
  - **can incorporate the DNA bits into their own chromosome**
    - **allows them to express new genes**
      - **Process of internalizing foreign DNA= TRANSFORMATION**
        - **form of 'recombination'**

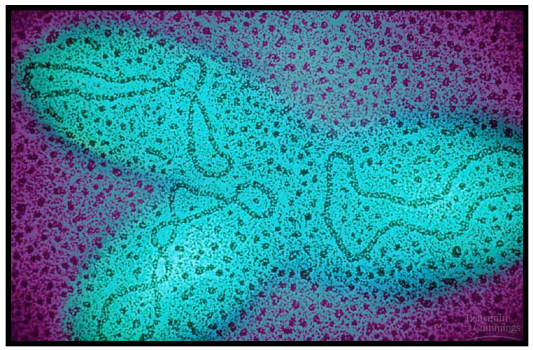
Remember Griffith?

mix heat-killed pathogenic & non-pathogenic *bacteria*

mice die

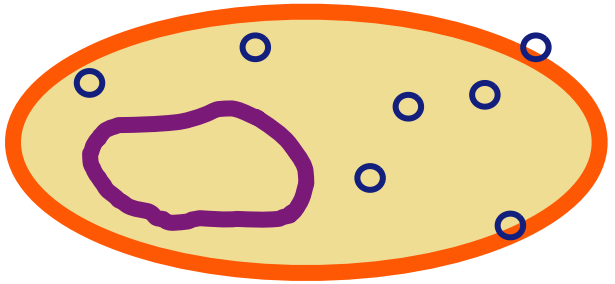
E. Coli is an organism commonly used to clone DNA

- **Bacteria contain Plasmids** *(as do few Eukaryotes- some fungi and higher plants)*
  - **Small supplemental circles of DNA**
    - 5000 - 20,000 base pairs
    - nonobligatory pieces of DNA (unlike the circular prokaryotic chromosome and linear nuclear eukaryotic chromosomes
    - self-replicating!!!
      - Since thy have an Origin of Replications replicate separately from chromosome



  - **carry extra genes**
    - 2-30 genes
      - Often also genes for antibiotic resistance

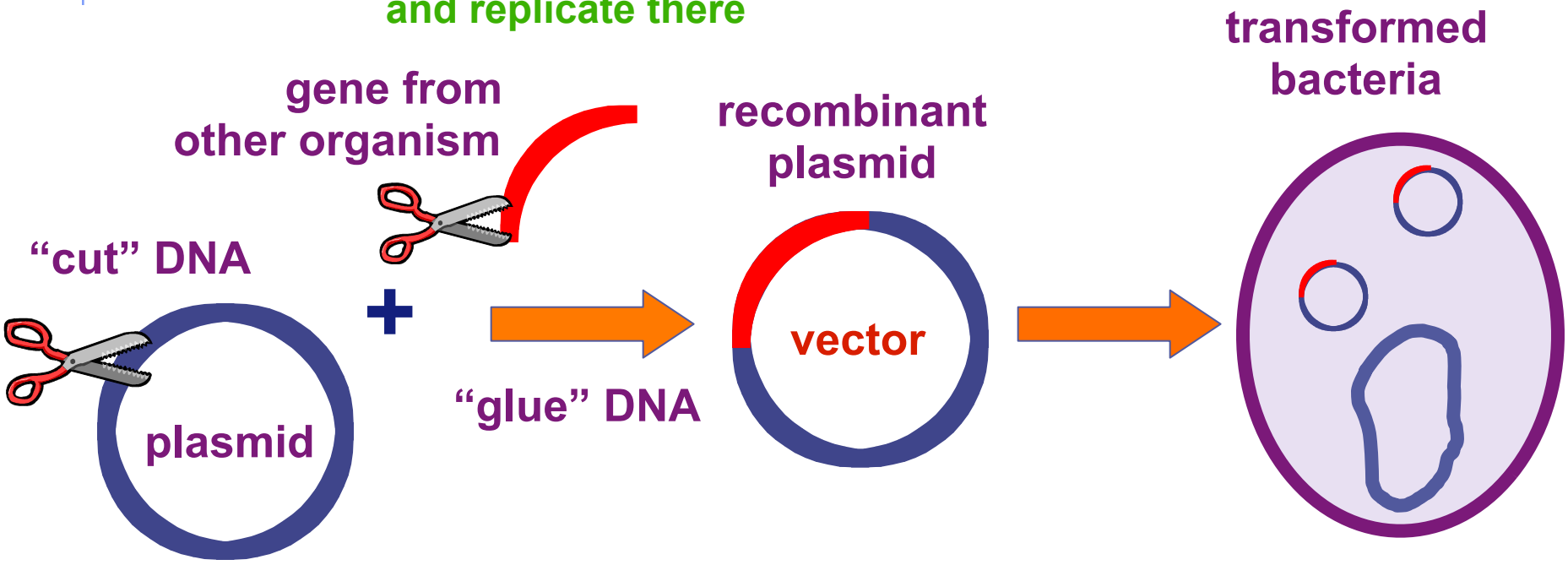
  - **can sometimes be exchanged between bacteria**
    - bacterial "sex"!! = CONJUGATION
    - can lead to rapid evolution



  - **can be imported from environment**

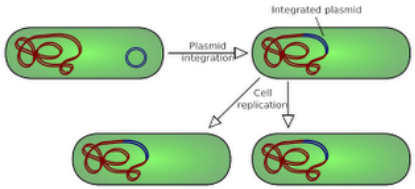
How can plasmids help us?

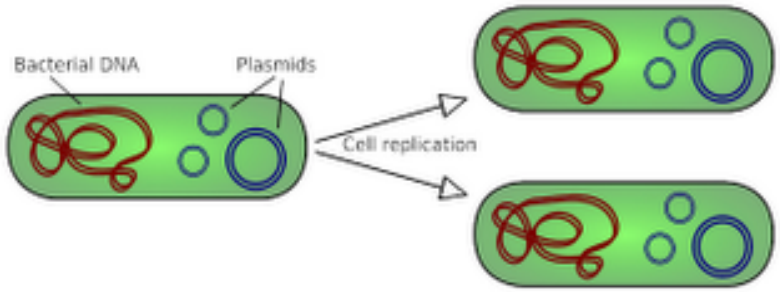
- Plasmids are a way to get genes into bacteria easily
  - Isolate a bacterial plasmid
  - insert new gene into plasmid
  - insert plasmid into bacteria (now a recombinant bacteria)
    - Such a plasmid is called a Cloning Vector
      - DNA molecule that can carry foreign DNA into a host cell and replicate there

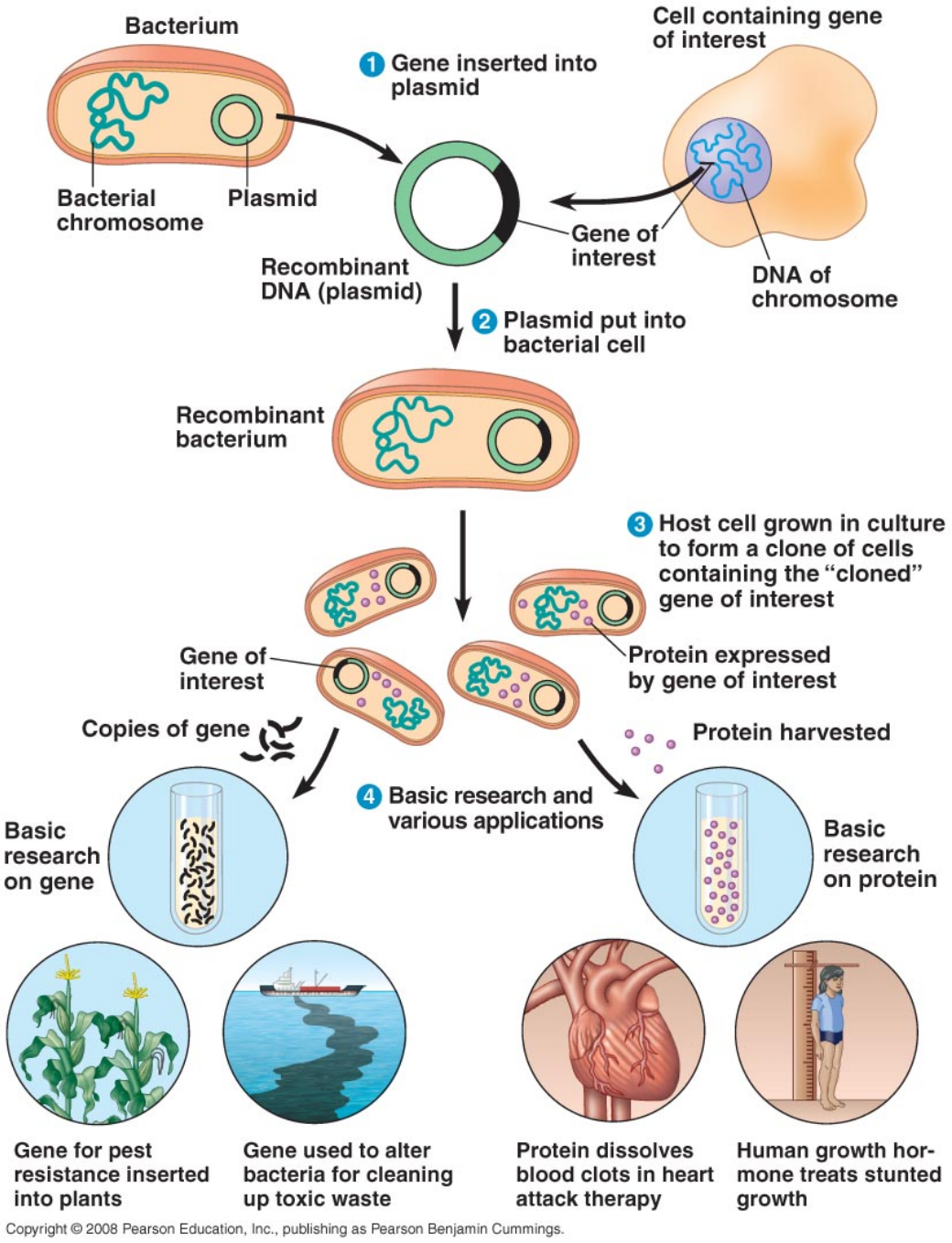


Use of plasmids

- **Two MAJOR uses:**
  1. Use this process to many many copies of a particular gene
     - Vector replicates on its own within bacteria
     - Bacteria replicate and pass copies of the plasmids on to each daughter bacterial cell



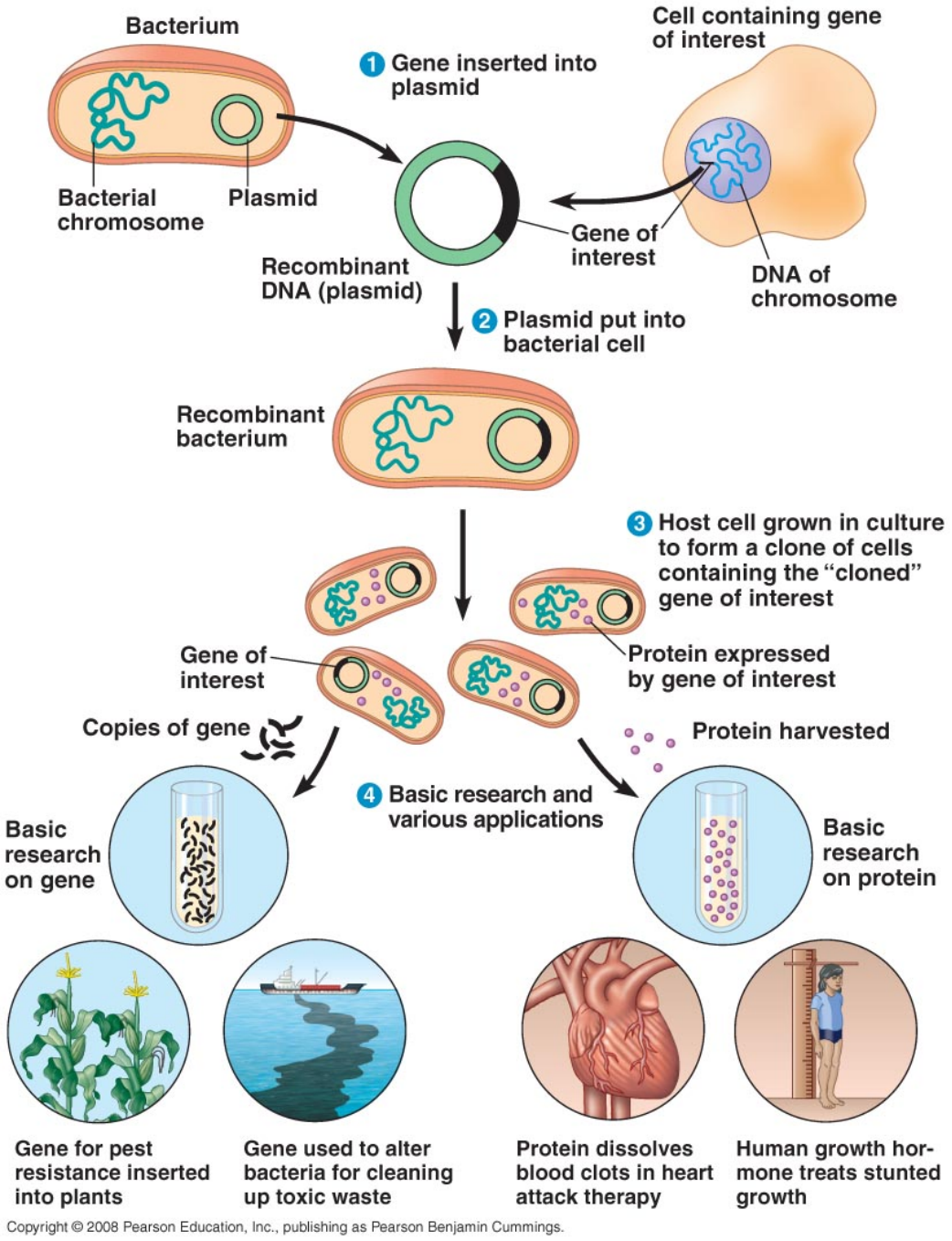




Use of plasmids

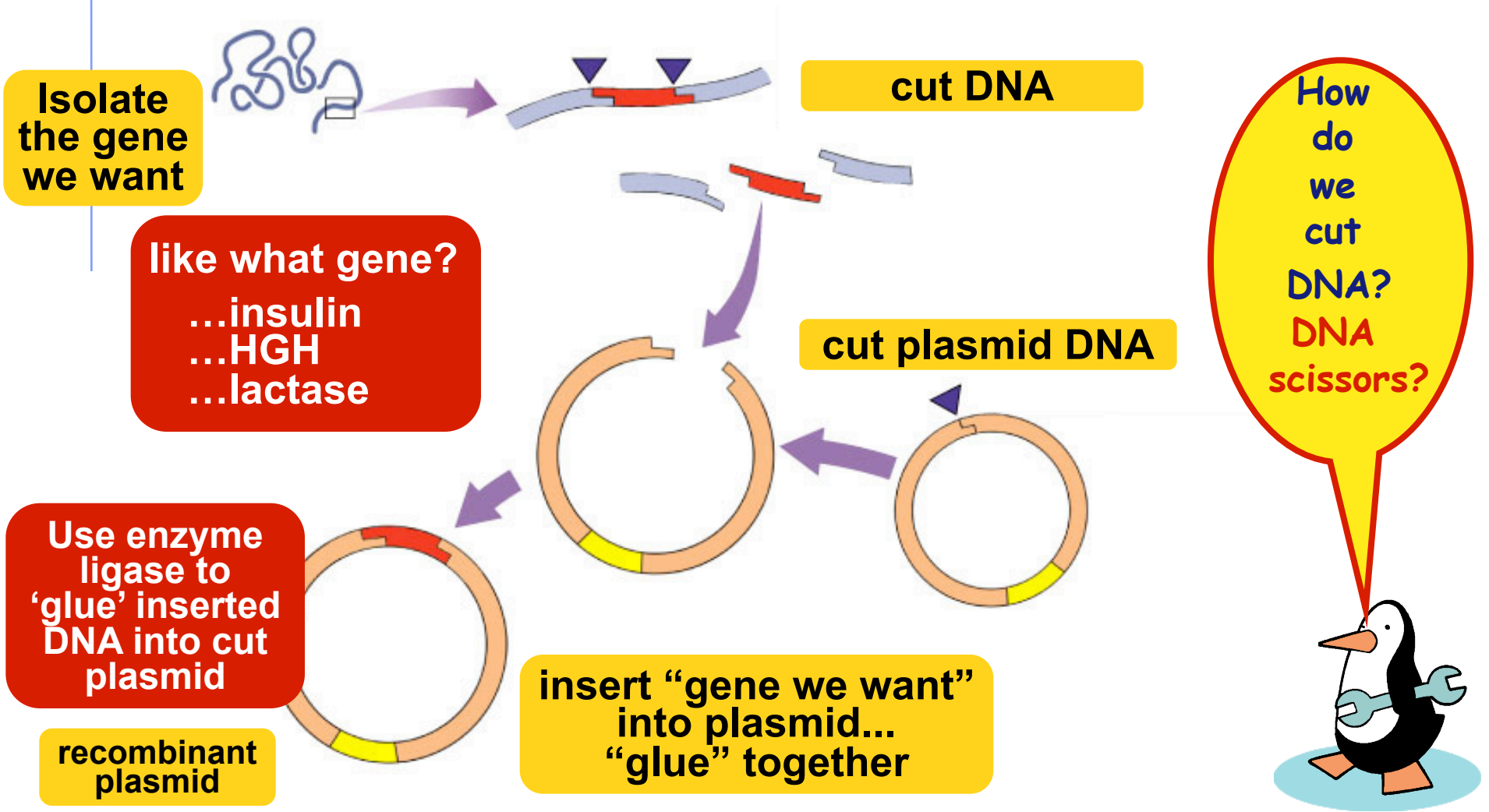
- **Two MAJOR uses:**

2. Bacteria can now express the new gene
   - Bacteria can make a new protein
   - Use process to produce large amounts of a certain protein

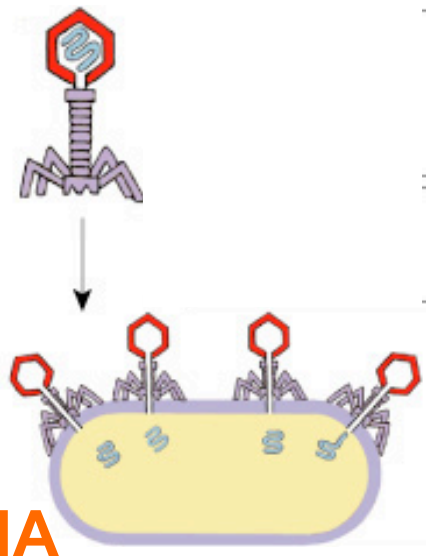


Biotechnology

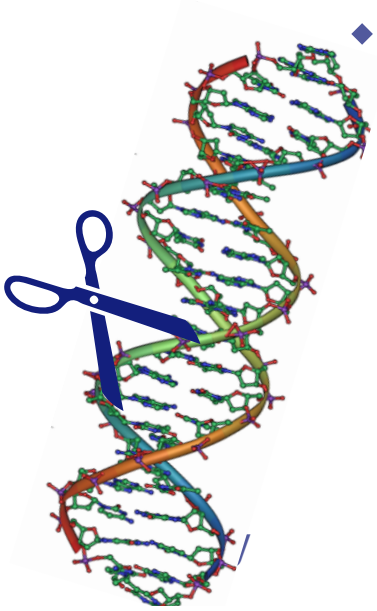
- Plasmids used to insert new genes into bacteria

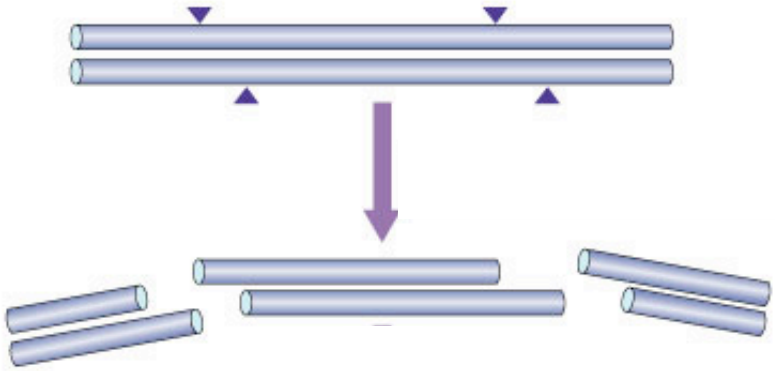


How do we cut DNA?



- **Restriction enzymes**
  - A.k.a. *restriction endonucleases*
  - discovered in 1960s
  - evolved in bacteria to cut up foreign DNA
    - "restrict" the action of the attacking organism
    - Cut foreign DNA at specifc short sequences of DNA called restriction sites
      - protection against viruses & other bacteria
        - bacteria protect their own DNA by methylation & by not using the base sequences recognized by the enzymes in their own DNA





What do you notice about these phrases?

radar

racecar

Madam I'm Adam

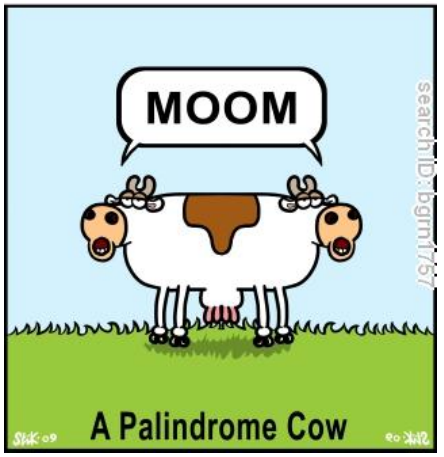
Able was I ere I saw Elba

a man, a plan, a canal, Panama

Was it a bar or a bat I saw?

go hang a salami I'm a lasagna hog

They are all:
Palindromes
(say the same thing in both directions)

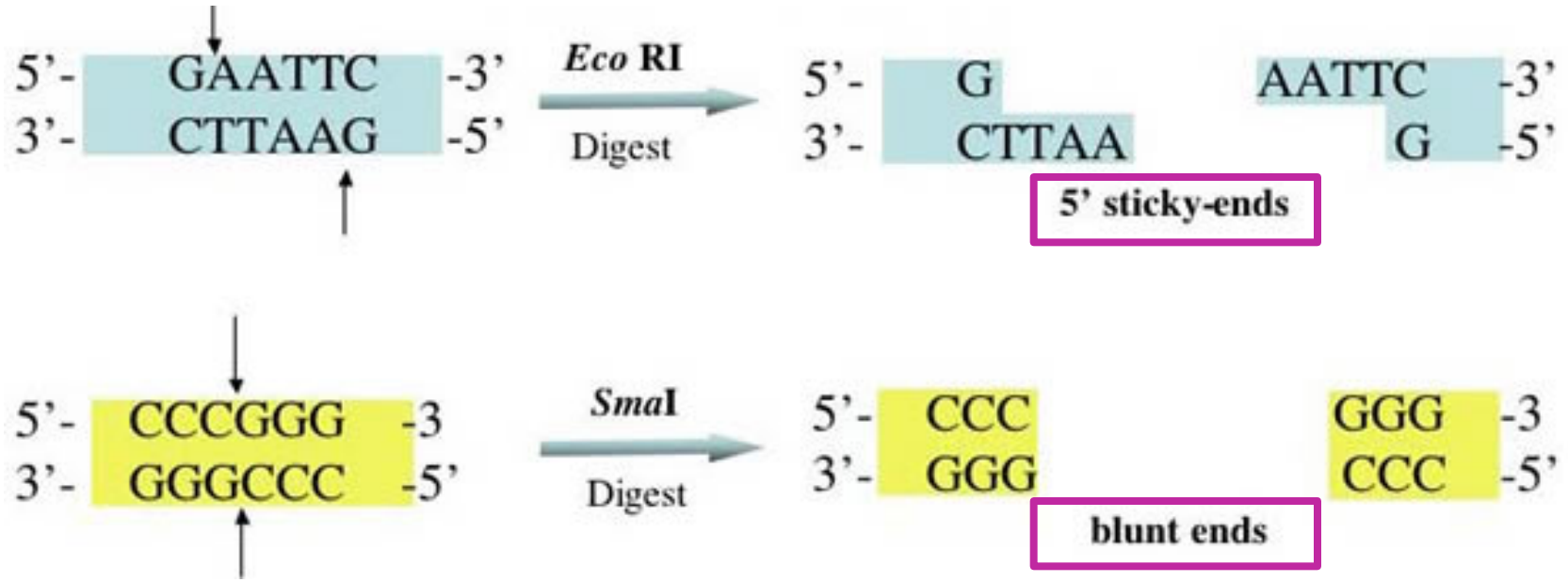


Restriction enzymes

- **Action of enzyme**
  - "cut" *[hydrolzye]* DNA at specific sequences
    - **restriction sites**
      - symmetrical "palindrome"

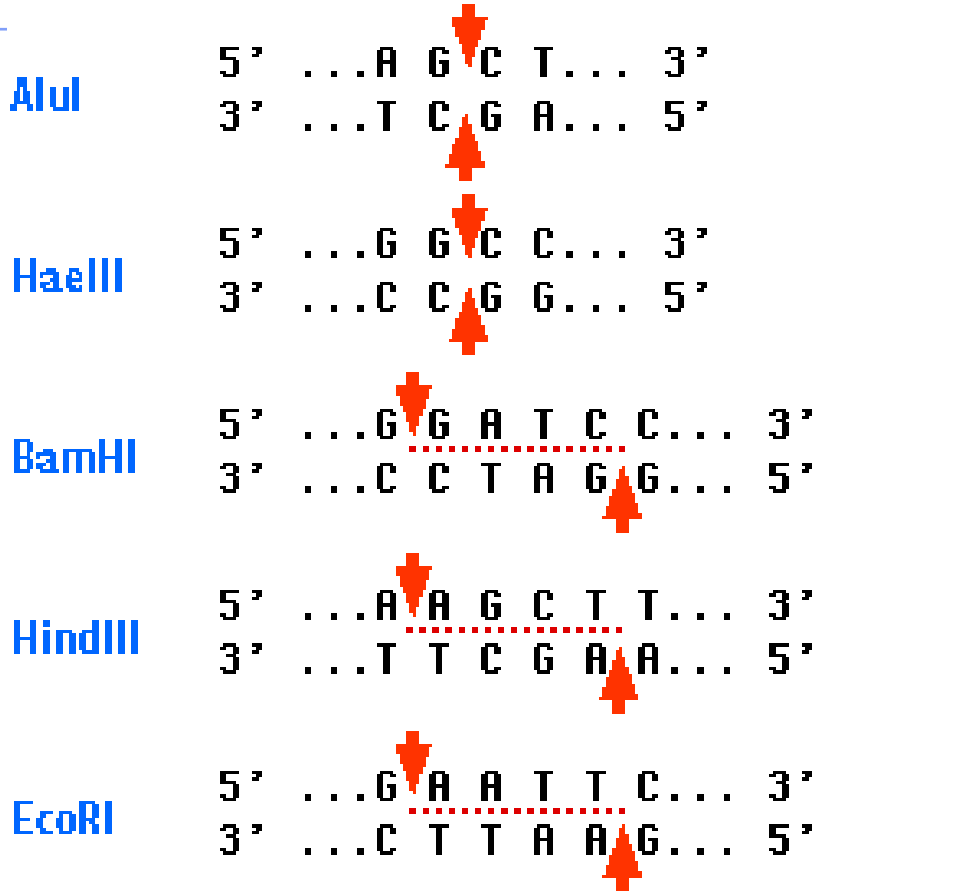
Madam I'm Adam

  - Produce two kinds of ends
    - Blunt ends or Sticky ends



Restriction Enzymes

- **Many different enzymes are used by bacteria for defense from viruses**
  - **named after organism they are found in**
    - **EcoRI, HindIII, BamHI, SmaI**
- **Each cuts DNA at a unique restriction site (sequence of DNA)**
  - **Resulting pieces of DNA after being cut are called Restriction Fragments**



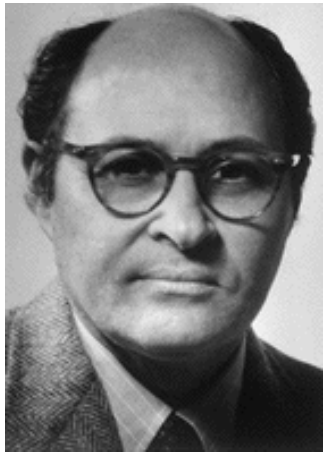
AluI and HaeIII produce blunt ends

BamHI HindIII and EcoRI produce "sticky" ends

1960s | 1978

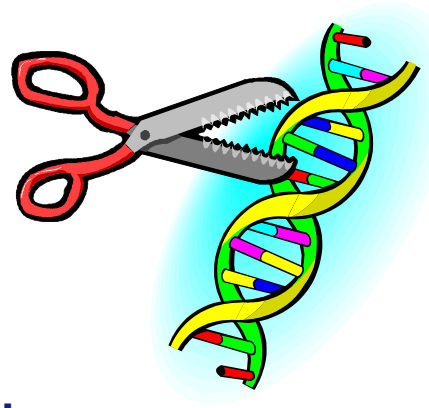
Discovery of restriction enzymes

Werner Arber



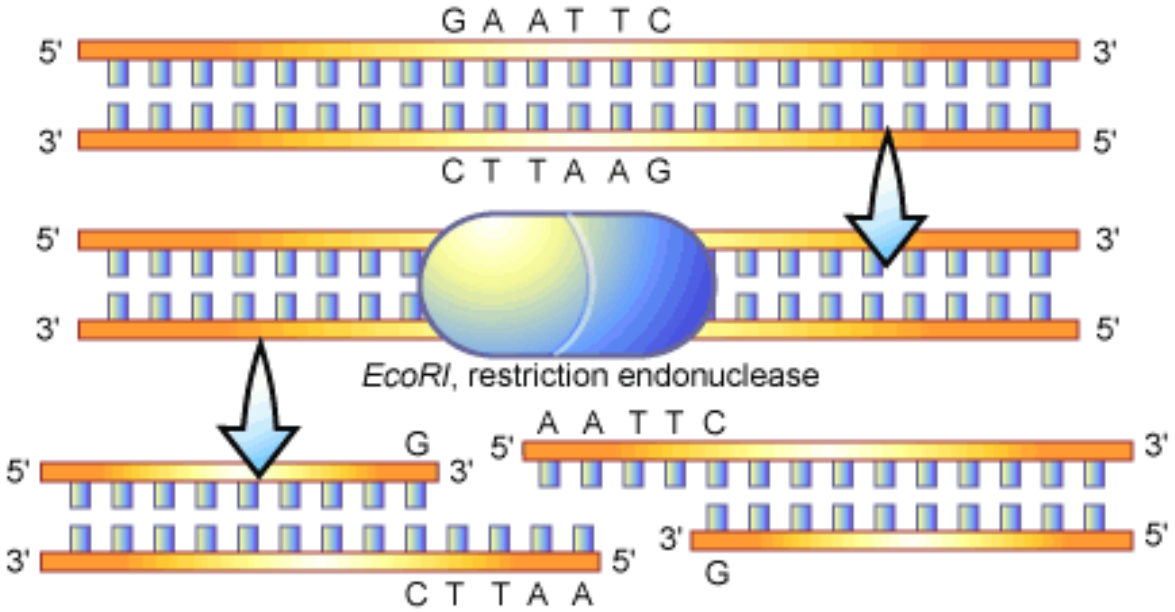
Daniel Nathans

Hamilton O. Smith



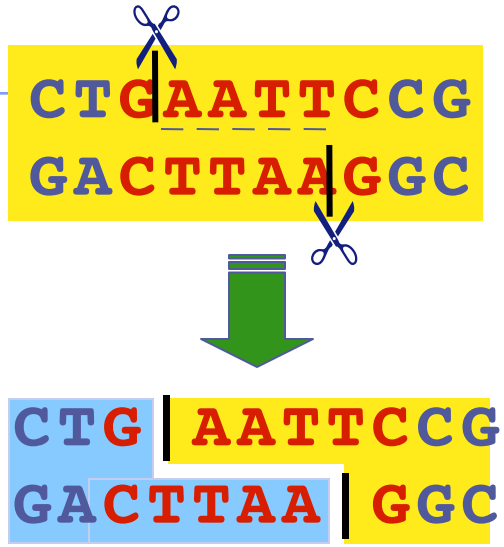
Restriction enzymes are named for the organism they come from:

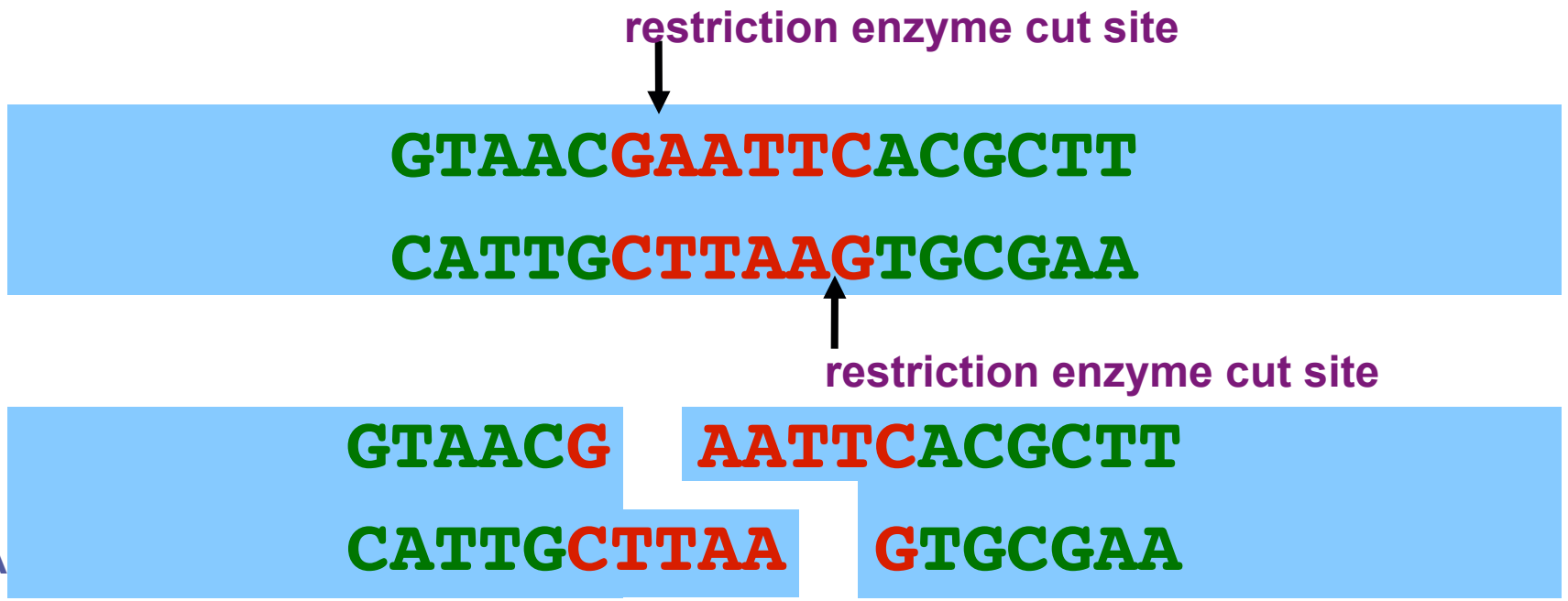
EcoRI = 1st restriction enzyme found in E. coli



Enzymes that cut DNA and thereby produce sticky ends are useful to us…

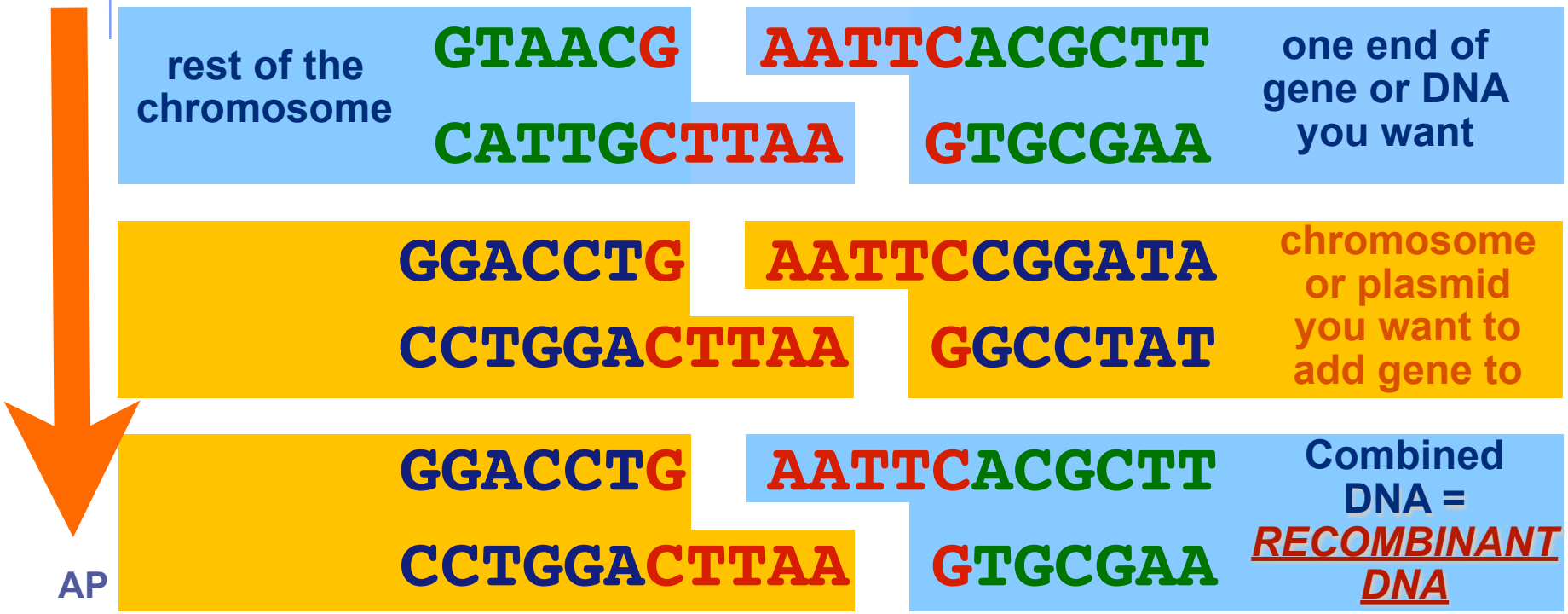
- Some enzymes cut DNA at specific sites and…
  - leave "sticky ends"
- Sticky ends are protruding s.s. ends
  - will bind to any complementary DNA



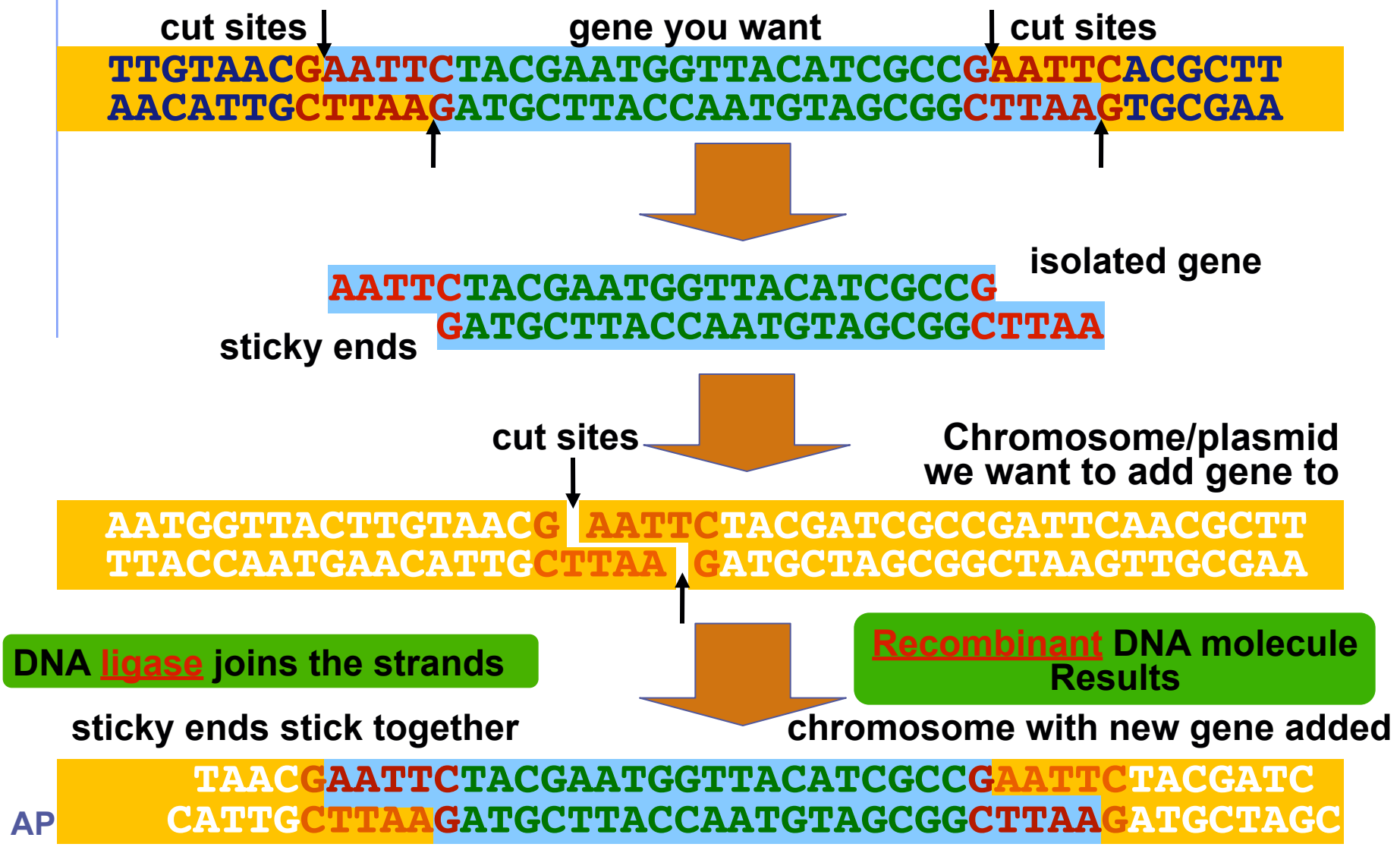


Sticky ends

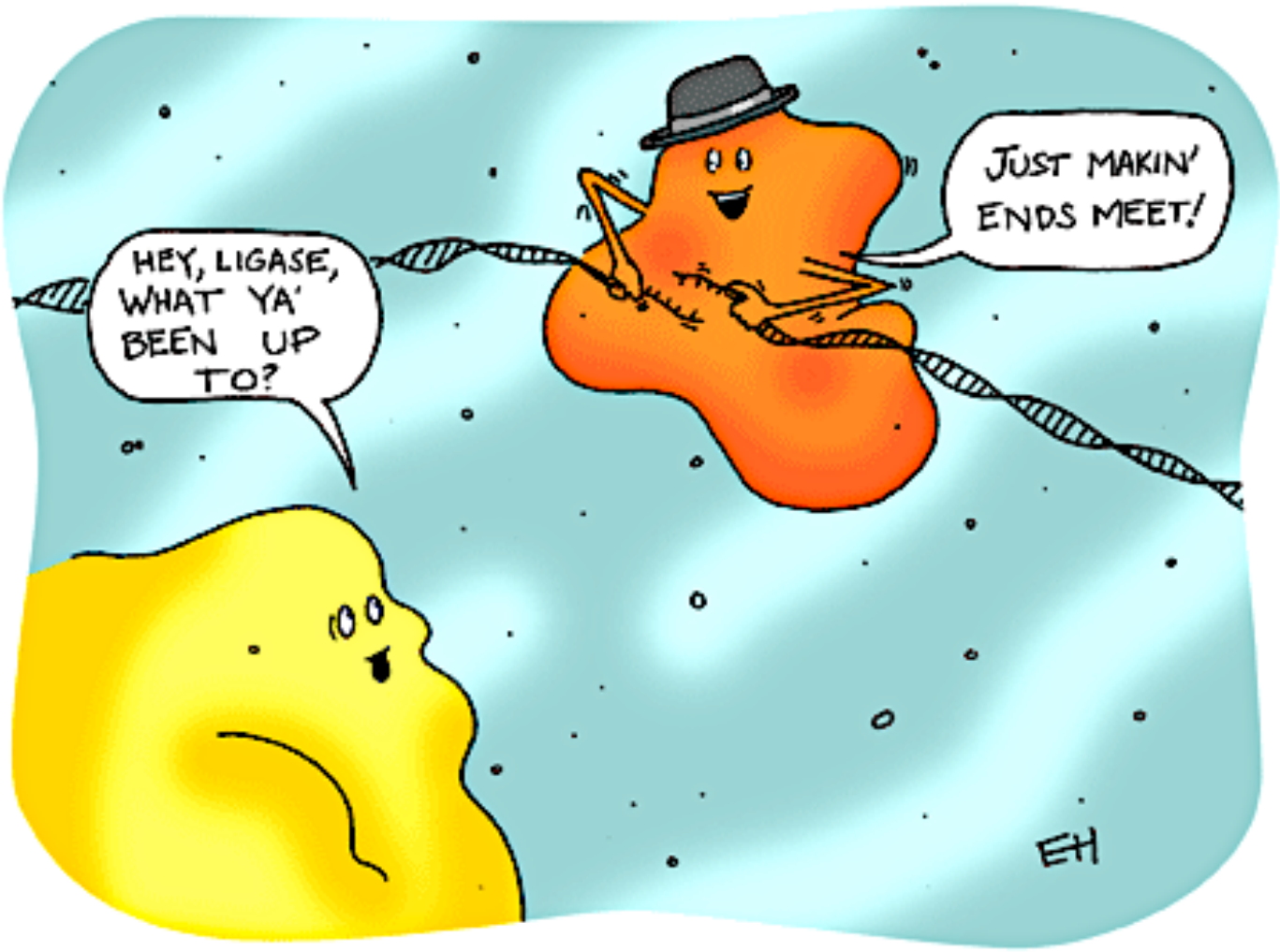
- If we cut another piece of DNA with the SAME enzymes as the first piece of DNA
  - leave complementary "sticky ends" on both DNA pieces
    - can glue DNA from different sources together at the "sticky ends" WHICH ARE COMPLIMENTARY!



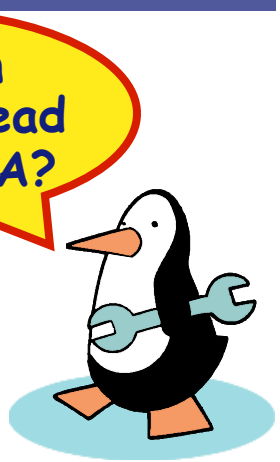
Sticky ends help glue DNA together



Ligase builds phosphodiester bonds between nucleotides of two DNA fragments



Why mix genes together?



- Gene produces protein they code for in different organism or different individual

Can put the human insulin gene in bacteria

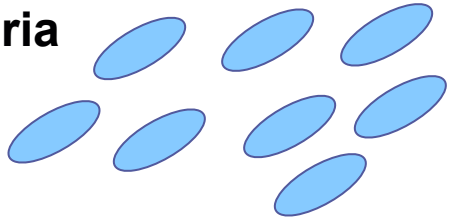
TAACGAATTCTACGAATGGTTACATCGCCGAATTCTACGATC
CATTGCTTAAGATGCTTACCAATGTAGCGGCTTAAGATGCTAGC

Bacteria can then express this human gene!

"new" protein from organism

ex: human insulin from bacteria

bacteria



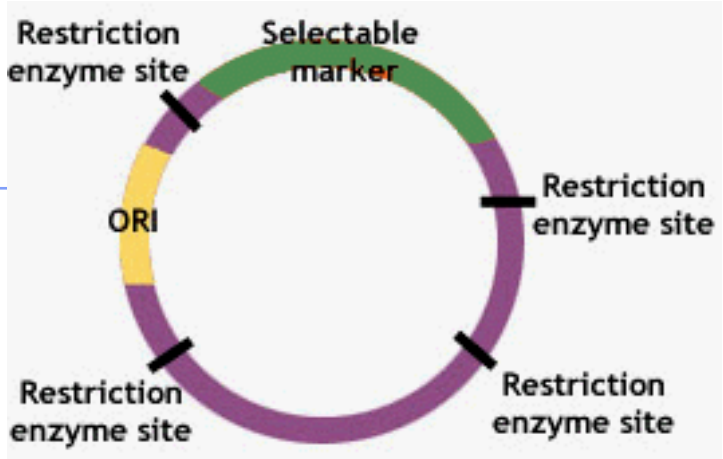
human insulin (protein) produced

The code is universal

- Since all living organisms…
  - use the same DNA
    - *use the same "code book"*
  - read their genes the same way and translate nucleotide sequences into amino acids in the same order

First base (5′ end)	Second base: U	Second base: C	Second base: A	Second base: G	Third base (3′ end)
U	UUU Phe	UCU Ser	UAU Tyr	UGU Cys	U
	UUC Phe	UCC Ser	UAC Tyr	UGC Cys	C
	UUA Leu	UCA Ser	UAA Stop	UGA Stop	A
	UUG Leu	UCG Ser	UAG Stop	UGG Trp	G
C	CUU Leu	CCU Pro	CAU His	CGU Arg	U
	CUC Leu	CCC Pro	CAC His	CGC Arg	C
	CUA Leu	CCA Pro	CAA Gln	CGA Arg	A
	CUG Leu	CCG Pro	CAG Gln	CGG Arg	G
A	AUU Ile	ACU Thr	AAU Asn	AGU Ser	U
	AUC Ile	ACC Thr	AAC Asn	AGC Ser	C
	AUA Ile	ACA Thr	AAA Lys	AGA Arg	A
	AUG Met or start	ACG Thr	AAG Lys	AGG Arg	G
G	GUU Val	GCU Ala	GAU Asp	GGU Gly	U
	GUC Val	GCC Ala	GAC Asp	GGC Gly	C
	GUA Val	GCA Ala	GAA Glu	GGA Gly	A
	GUG Val	GCG Ala	GAG Glu	GGG Gly	G

Copy (& Read) DNA



- **Transformation**
  - insert recombinant plasmid into bacteria
  - grow recombinant bacteria in agar cultures
    - bacteria make lots of copies of plasmid & pass plasmid to daughter cells
    - bacteria "clone" the plasmid
      - Used for the production of many copies of an inserted gene
  - If the plasmid has a high active bacterial promoter then the plasmid can be induced to express the gene it carries and is called and EXPRESSION VECTOR!
    - Used for the production of "new" protein
      - transformed phenotype

DNA → RNA → protein → trait

Grow bacteria…make more

gene from other organism

plasmid

recombinant plasmid

vector

By placing bacteria in concentrated calcium solution or Electroporation = using electricity to make plasma membrane of bacteria porous

transformed bacteria

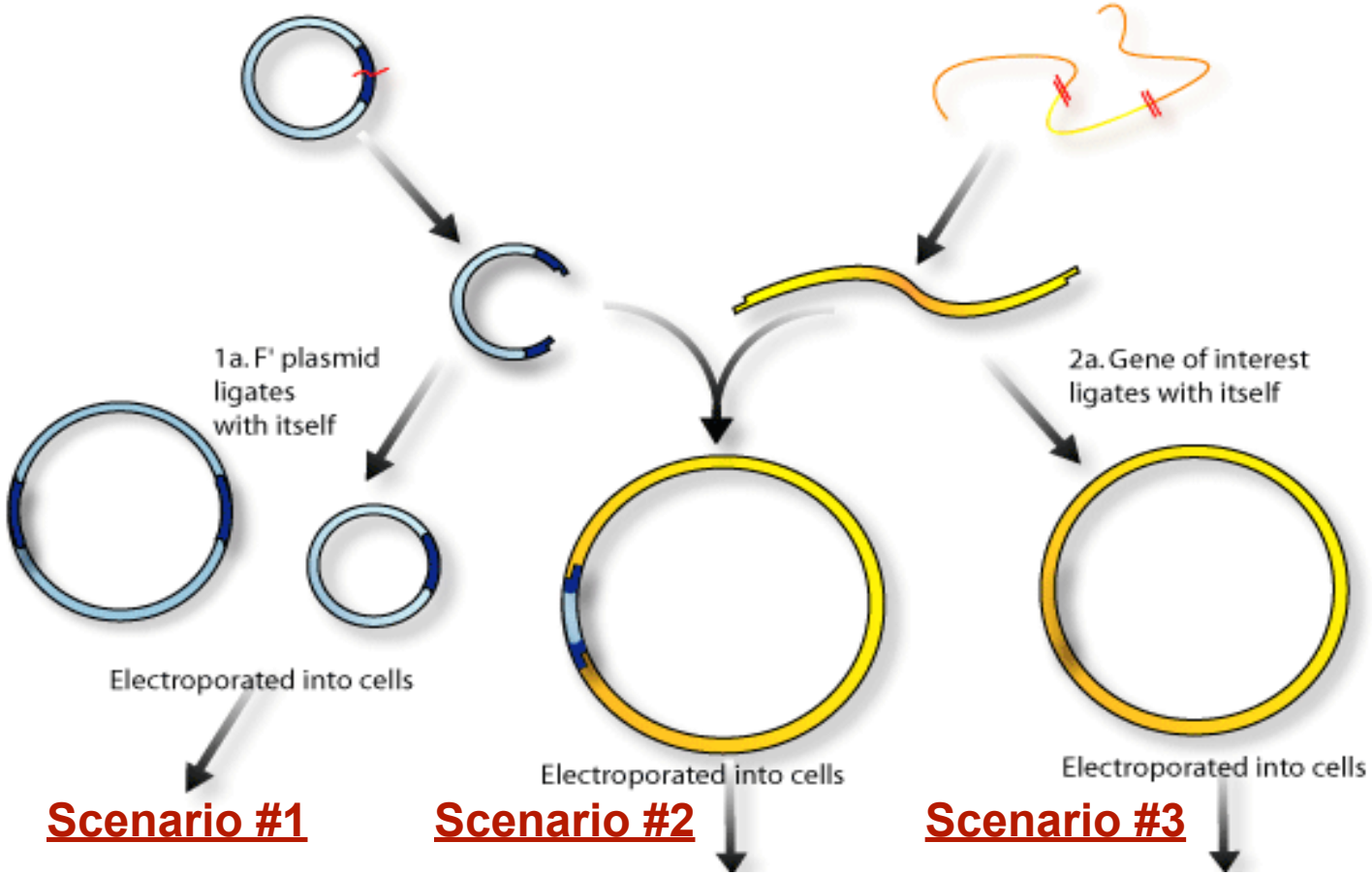
grow bacteria

harvest (purify) protein

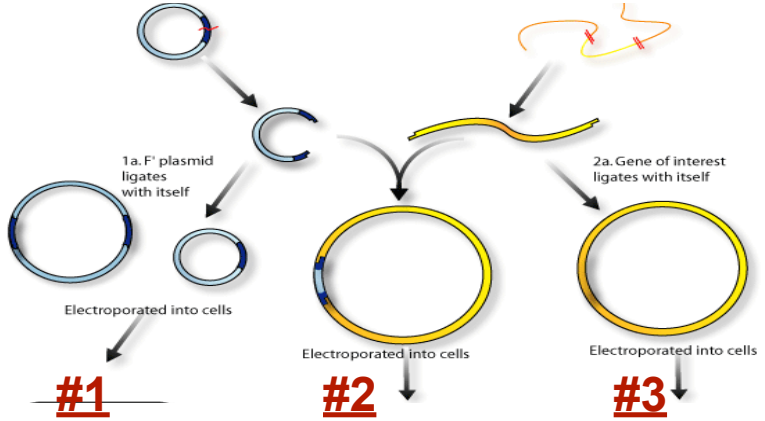
Humulin®

Did the cloning procedure work?

- **When DNA is cut with a restriction enzyme, restriction fragments are produced.**
  - **These fragments are mixed with cut plasmids and ligase which completes the phosphodiester bond between nucleotides, *ANY nucleotides!***
  -



Did the cloning procedure work?

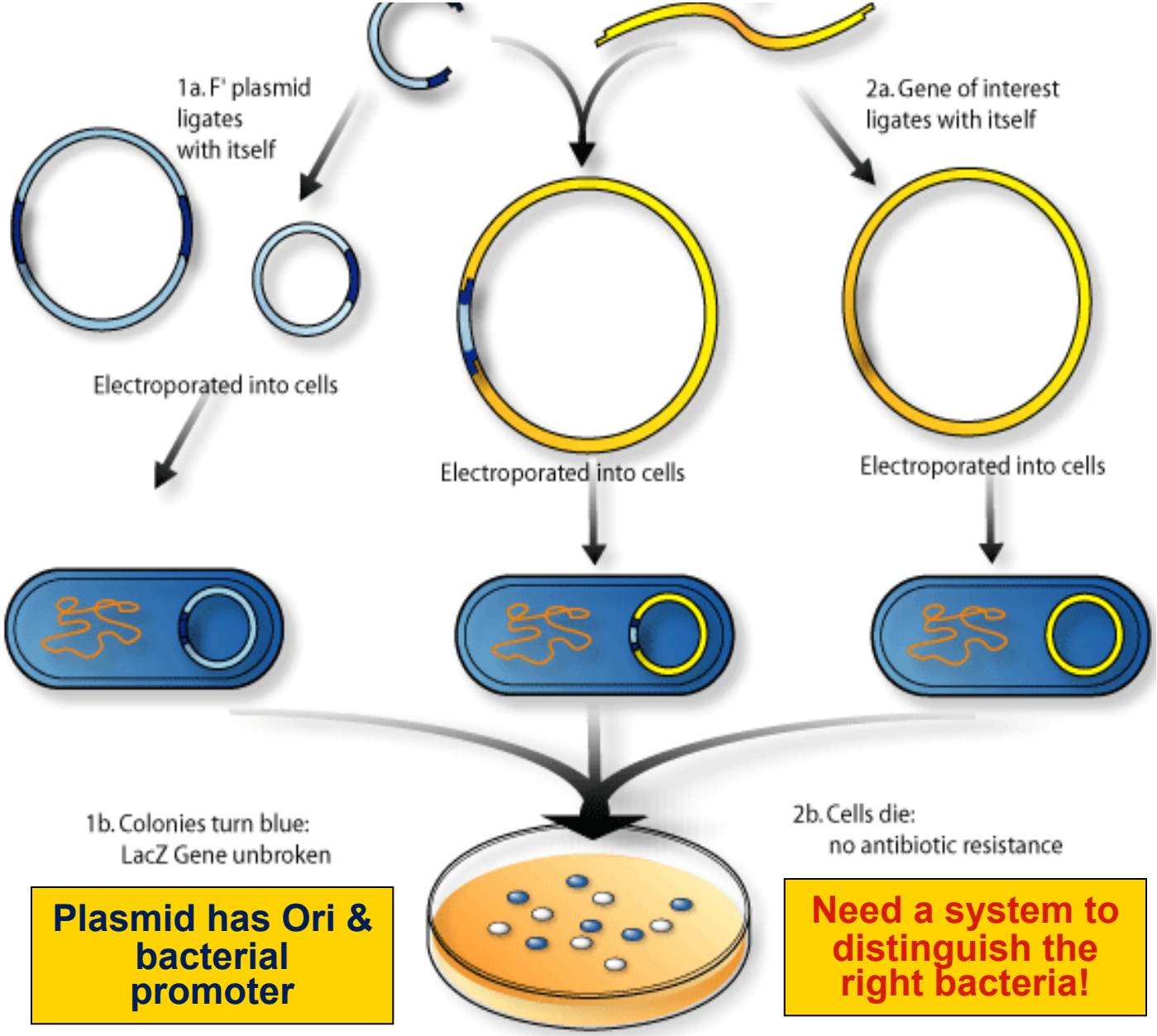


- **A researcher would want to make sure the plasmid was taken up by the bacteria after the solution of plasmids and foreign DNA were mixed with ligase and then exposed to the bacteria. In the solution, three events took place but only one event produces the desirable plasmid.**
  - **Scenario #1: If the plasmid closes up *without* the foreign DNA inserted, the plasmid can be copied once inside the bacteria, but you don't have the foreign DNA in the bacteria so you *won't* be cloning that foreign DNA and *won't* be able to express that foreign gene**
  - **Scenario #3: If the foreign DNA closes up without getting attached to the plasmid, then, if a bacteria takes up this foreign DNA, the bacteria will *not* be able to copy *nor* express this foreign DNA as the bacterial origin of replication and promotor have not been added to the foreign DNA.**
  - **Scenario #2: This is the desired scenario. The foreign DNA has been inserted successfully into a plasmid. The plasmid has the bacterial origin of replication which allows for the recombinant plasmid to be replicated inside the bacteria and contains the foreign DNA we wanted to clone *(have the bacteria make many copies of)* and possibly express *(have the bacteria build proteins from the encoded information).***

Did the cloning procedure work?

The results of ligase treatment, including the plasmid *(now cloning vector),* are introduced into a bacteria via electroporation or treatment with a concentrated calcium solution, the bacteria to be grown on a medium

But how do we know if the fragment we wanted to clone attached itself to a plasmid or if a plasmid entered a bacteria successfully?

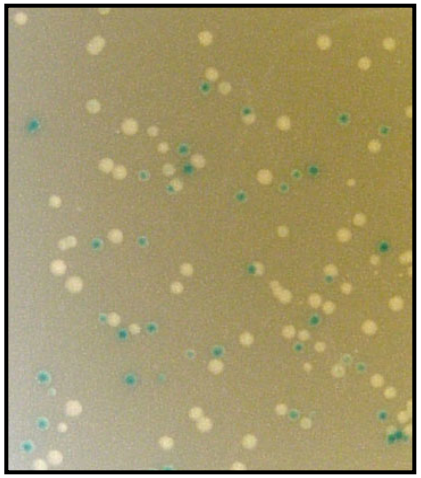


Plasmid has Ori & bacterial promoter

Need a system to distinguish the right bacteria!

Use Bacterial Plasmids with lacZ gene

- **To identify the bacteria that took up the plasmid *(with the origin of replication and possibly a bacterial promoter)* and the plasmid with the desired foreign DNA inserted, we use plasmids that have been engineered to carry two useful genes *(two "selectable markers")*:**



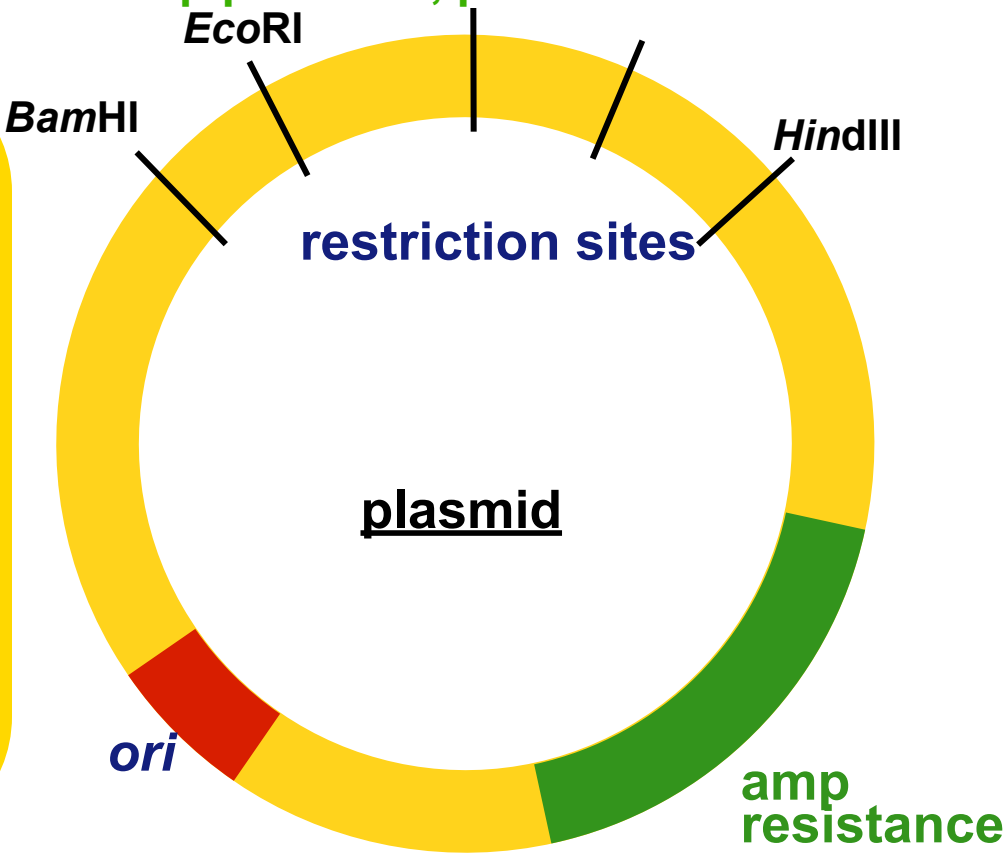
1. **LacZ gene codes for an enzyme that breaks down lactose and X-gal.**
   - **Medium containing X-gal turns blue when bacteria produces the enzyme to hydrolyze it**
   - **Medium containing X-gal stays white if bacteria lacks this enzyme**
2. **amp^R gene allows a bacteria to break down the antibiotic ampicillin, making bacteria resistant and able to grow in a medium containing this antibiotic.**

Engineering plasmids

- **Building custom plasmids that have features we want like:**
  - selectable markers
  - high transcription rates *(bacterial promoters with high affinities for bacterial RNA polymerase)*
  - high copy rates inside bacteria *(very effective origins of replication)*
  - convenient restriction sites
    - Biotech companies exist that develop plasmids, patent them & sell them.

Selectable marker

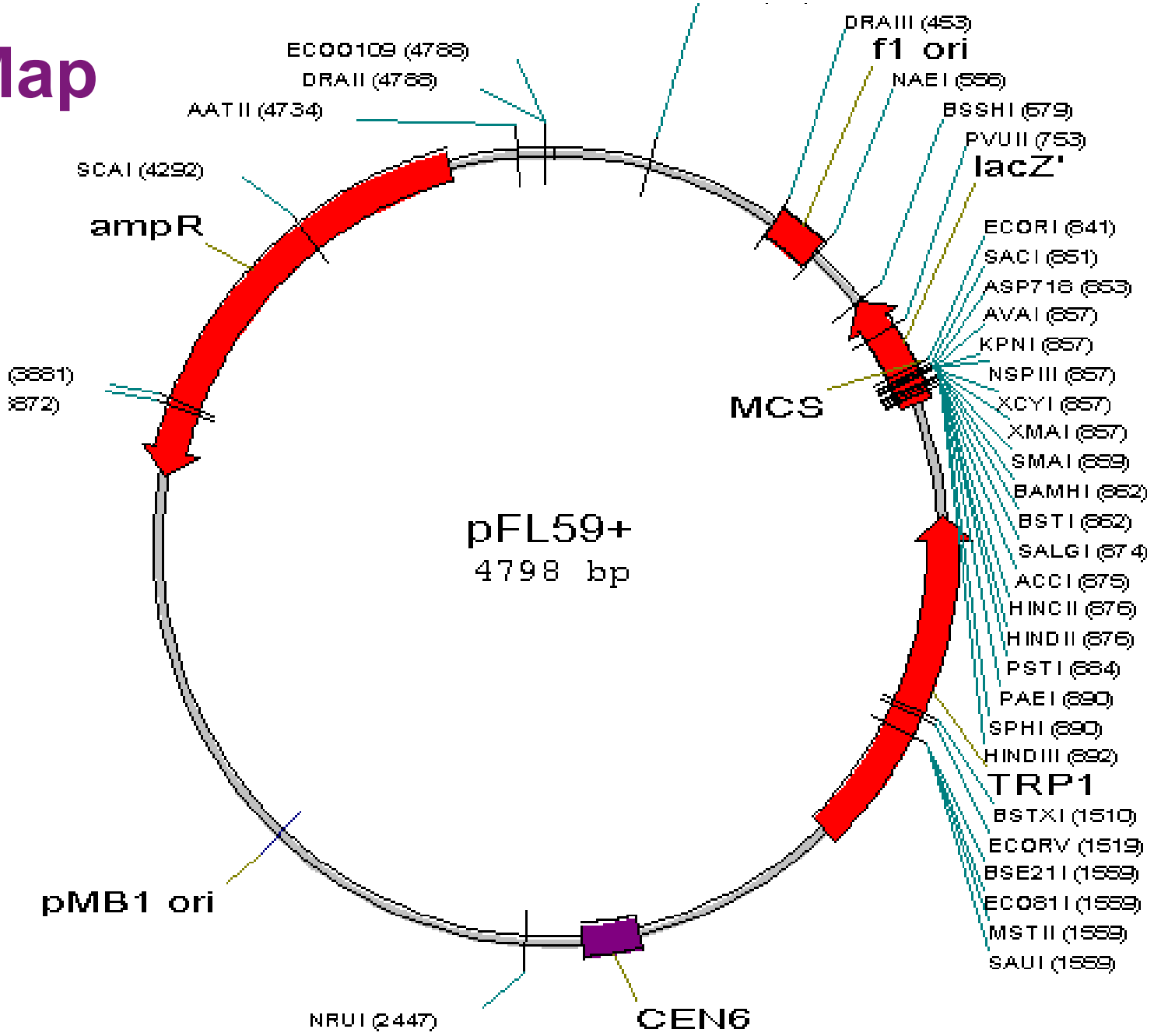
- Used to I.D. bacteria which were successfully transformed
  - Check for the successful uptake of recombinant plasmid
    - antibiotic resistance gene on plasmid is often used as a selectable marker *(a marker we can select for)*
      - Ex: ampicillin resistance



Vector Map

- Map shows restriction sites for different restriction enzymes

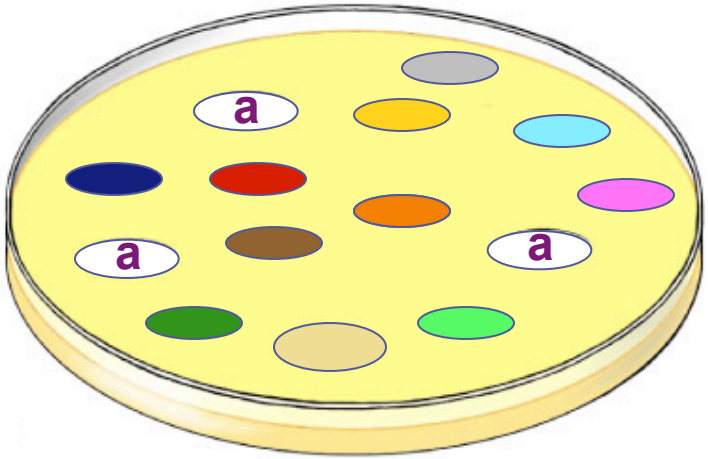
- If fragments inserts itself in a gene, gene becomes nonfunctional
- If fragment does not insert itself in a gene, gene is functional



Selection for plasmid uptake

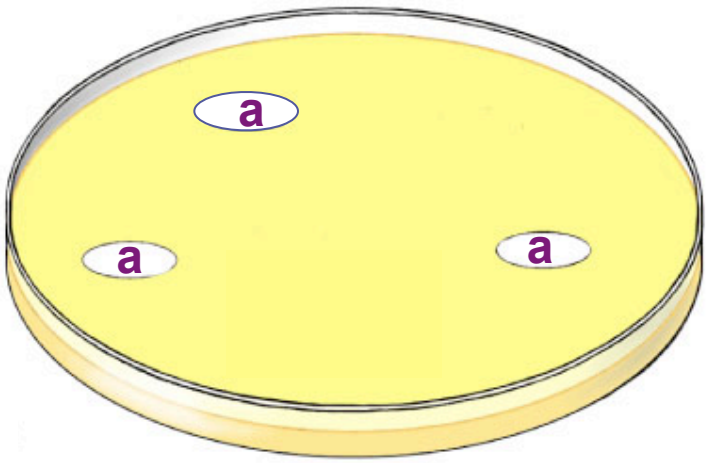
- Antibiotic becomes a selecting agent added to the bacterial food source (the medium or "agar")
  - ONLY bacteria with the plasmid will grow on antibiotic-containing (ampicillin or "amp") plate or petri dish medium/LB agar.

all bacteria grow



LB agar medium plate

only transformed bacteria grow
(bacteria that gained the ampicillin resistance gene)

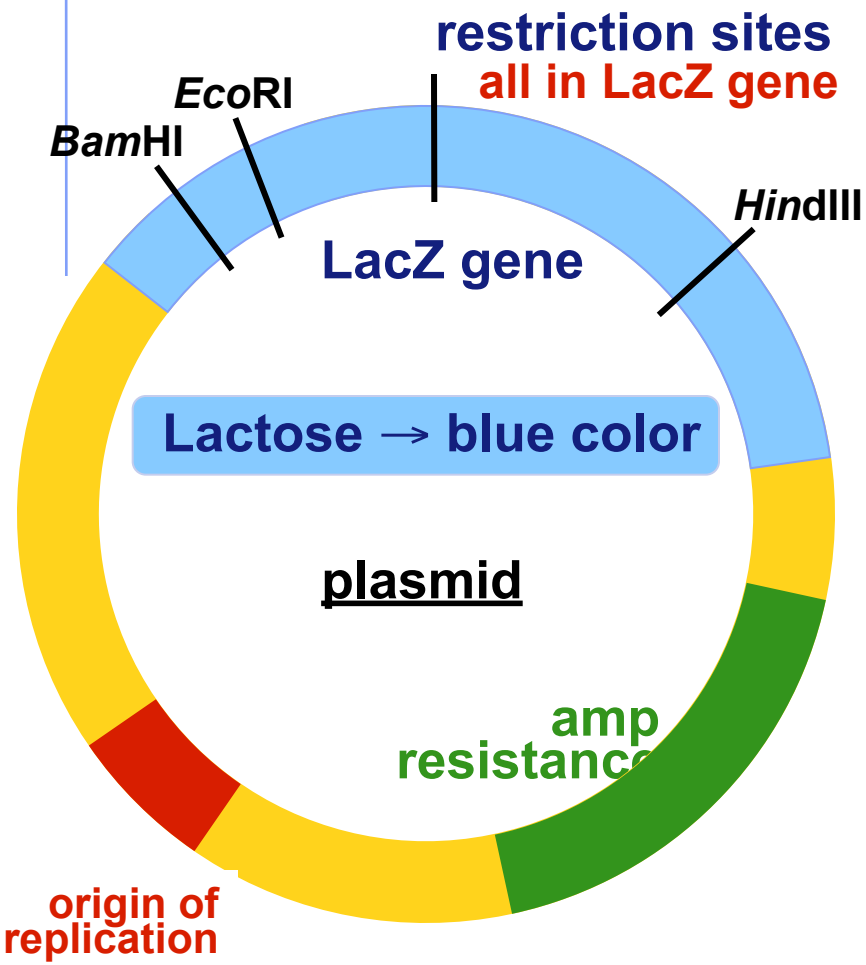


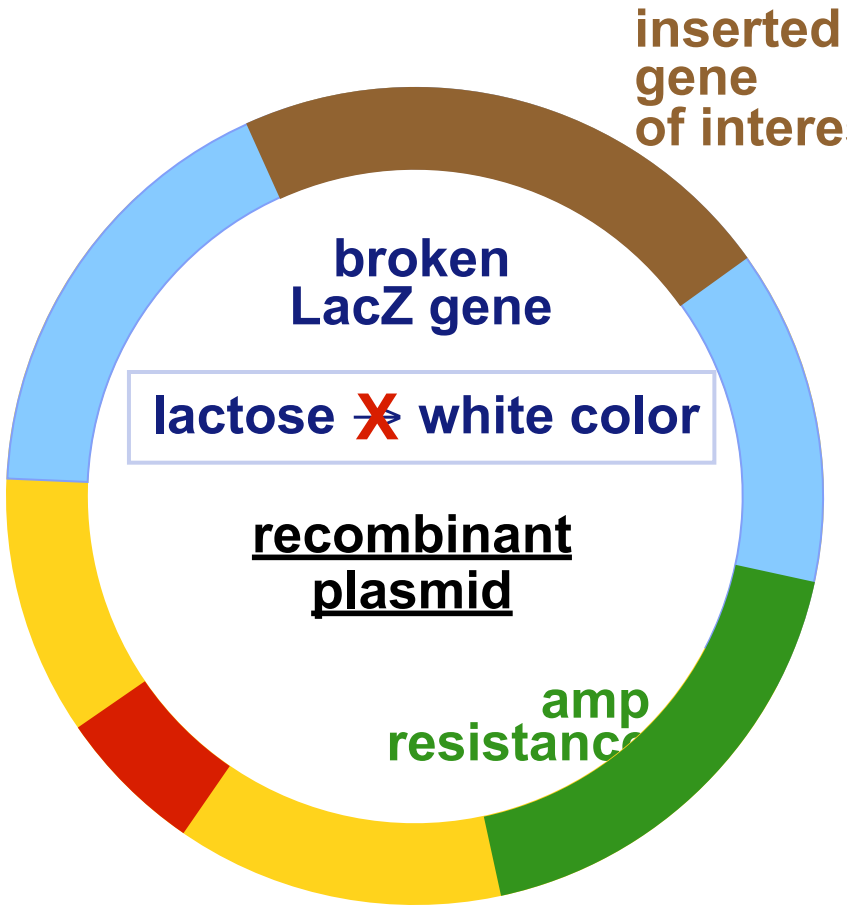
LB + *amp* plate

Used in cloning

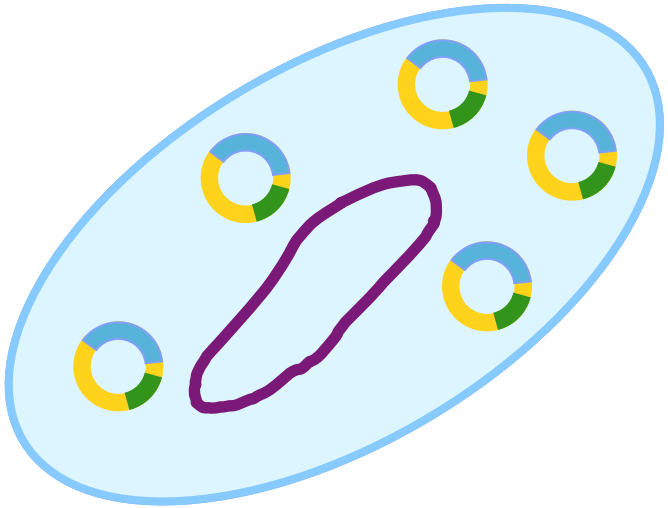
Once you determined which bacteria took up a plasmid, you now need to screen plasmids

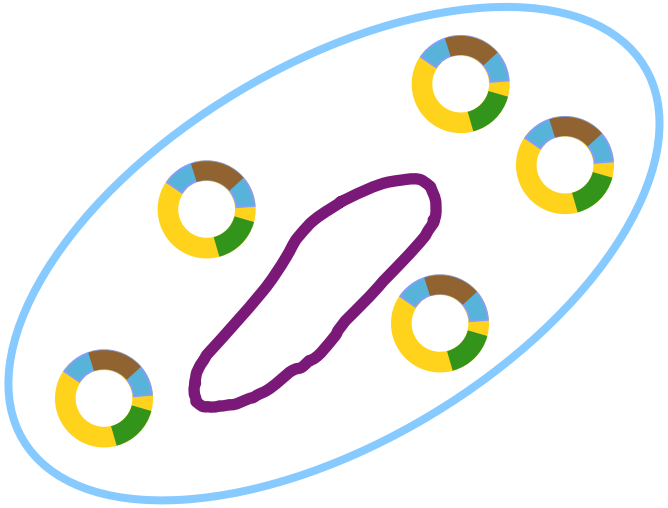
- Need to make sure bacteria have a recombinant plasmid





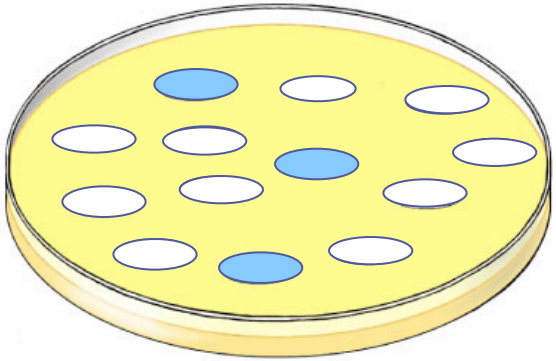
Screening for recombinant plasmid





- Bacteria take up plasmid (no inserted DNA)
- Functional LacZ gene
- Bacteria make blue color

- Bacteria take up recombinant plasmid
- Non-functional LacZ gene
- Bacteria stay white color



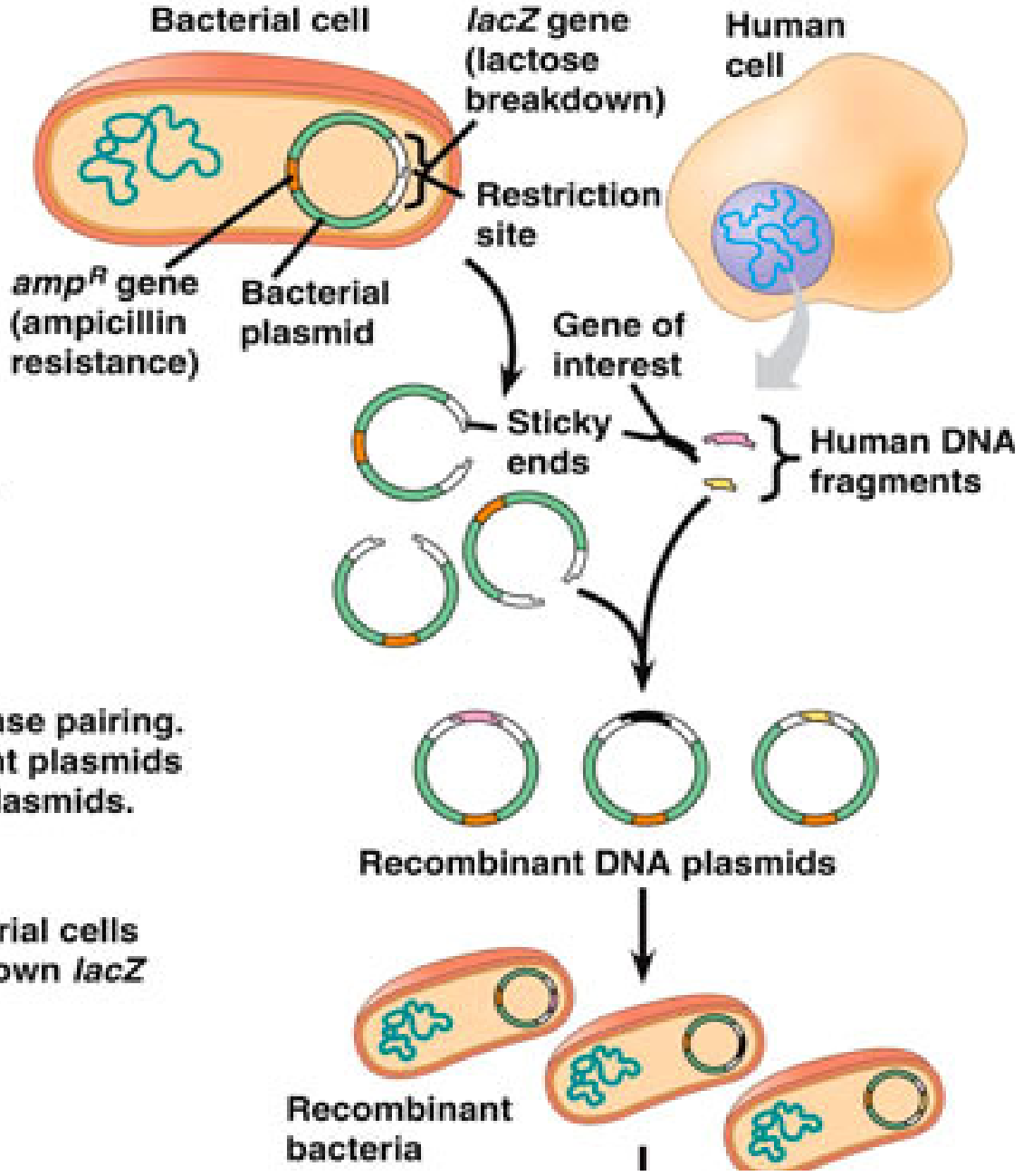
Cloning Procedure (Steps 1 - 5)

1. Isolate plasmid DNA and human DNA.

2. Cut both DNA samples with the same restriction enzyme.

3. Mix the DNAs; they join by base pairing. The products are recombinant plasmids and many nonrecombinant plasmids.

4. Introduce the DNA into bacterial cells that have a mutation in their own *lacZ* gene.

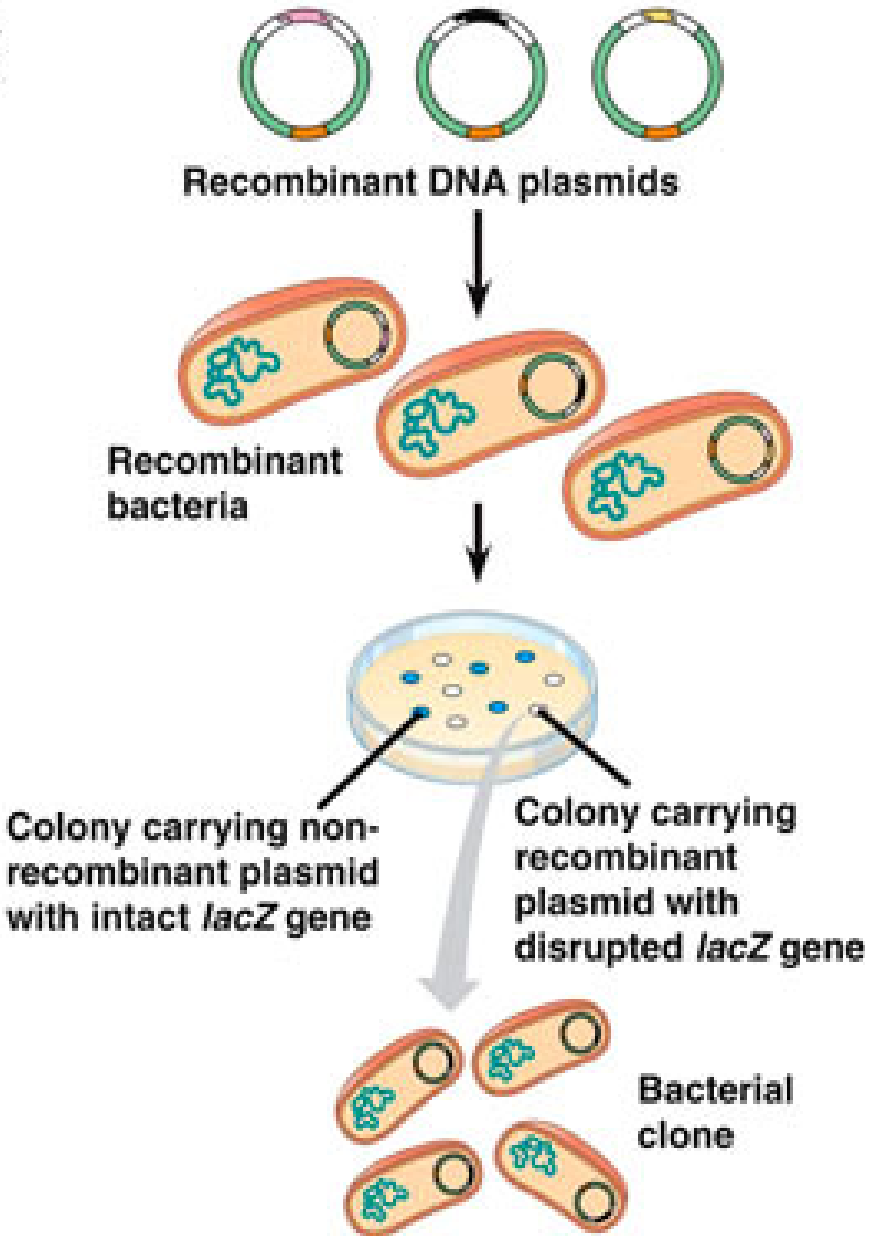


Identifying recombinant bacteria

3. Mix the DNAs; they join by base pairing. The products are recombinant plasmids and many nonrecombinant plasmids.

4. Introduce the DNA into bacterial cells that have a mutation in their own *lacZ* gene.

5. Plate the bacteria on agar containing ampicillin and X-gal. Incubate until colonies grow.



★Only bacteria with plasmid *(that contains amp^R gene)* can survive on ampicillin-containing medium: Nontransformed bacteria DIE!

★Plasmids with a restriction fragment inserted into their lacZ gene can no longer hydrolyze X-gal, and so form white colonies.

Problems…

What problem may arise when letting a bacteria make a fully functional human **(eukaryotic)** protein directly from <u>eukaryotic DNA</u>?

- **When making a Human Genome Library**
  - **A lot of DNA that does not get expressed!**
  - **Human genomic library has more noncoding DNA than coding DNA**
- **To get bacteria to build correct amino acid sequences in eukaryotic polypeptides, one must clean up the "junk" within genes*!***
  - **if you want to clone a human gene in bacteria and have the bacteria produce the correct 3-D protein, you can't have:**

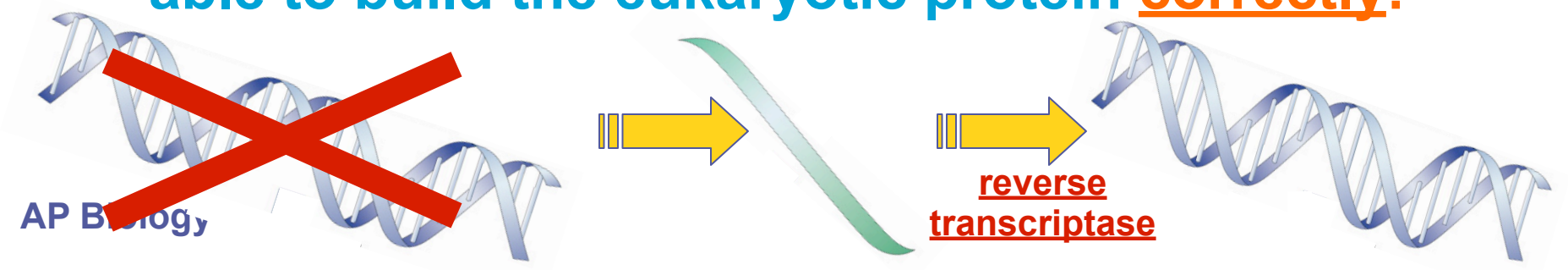
INTRONS!!! **(remember, bacteria <u>CANNOT</u> do RNA Splicing to remove eukaryotic intronic sequences from mRNA prior to translation)**

How do you clean up the gene so the bacteria only transcribes what must be translated by its ribosomes?

- Don't start with DNA…
- Use eukaryotic mRNA
  - Represents a copy of the gene without the introns since the eukaryotic cell removes introns and splices together the wanted exons in pre-mRNA!
- But in the end, you need double-stranded DNA to clone into a plasmid…
- How do you go from ssRNA → dsDNA?

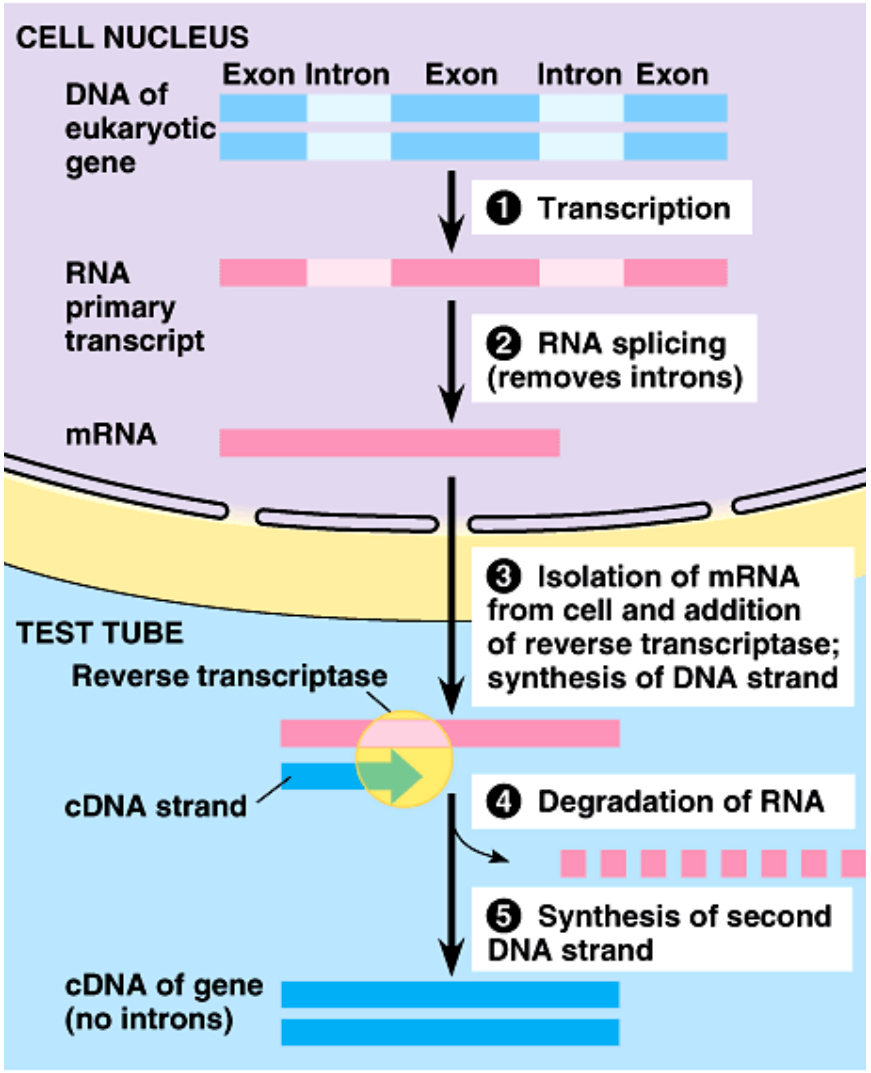
Use reverse transcriptase from RNA retroviruses

- Now insert cDNA into plasmids and bacteria will be able to build the eukaryotic protein correctly!



Now we can make cDNA (copy DNA) libraries

- Collection of only the coding sequences of expressed eukaryotic genes
  1. extract mRNA from cells
  2. use reverse transcriptase to make dsDNA from mRNA
     - RNA → dsDNA (cDNA)
  3. splice into cDNA plasmids
  4. clone in bacteria
- Applications
  - If you need edited DNA for correct expression of a gene in bacteria
    - Ex: human insulin
  - To study all the genes that are expressed in a particular cell or time in an organisms
    - Ex: Which genes are expressed only in the brain?
    - Ex: Which genes are turned on and off throughout different periods of gestation?



We can clone genes and DNA in eukaryotic cells which can do RNA processing!

- A yeast artificial chromosome (YAC) is a vector used to clone large DNA fragments
  - 100 kb - 3000 kb
  - It is an artificially constructed LINEAR chromosome and contains the telomeric, centromeric, and replication origin sequences needed for replication and preservation in yeast cells.
- Extremely useful as one can get eukaryotic protein products with post-translational modifications as yeasts are themselves eukaryotic cells which can conduct RNA Splicing
  - YACs have been found to be more unstable than BACs, producing chimeric effects (rearrangements of yeast DNA with YAC or one piece of YAC with another)

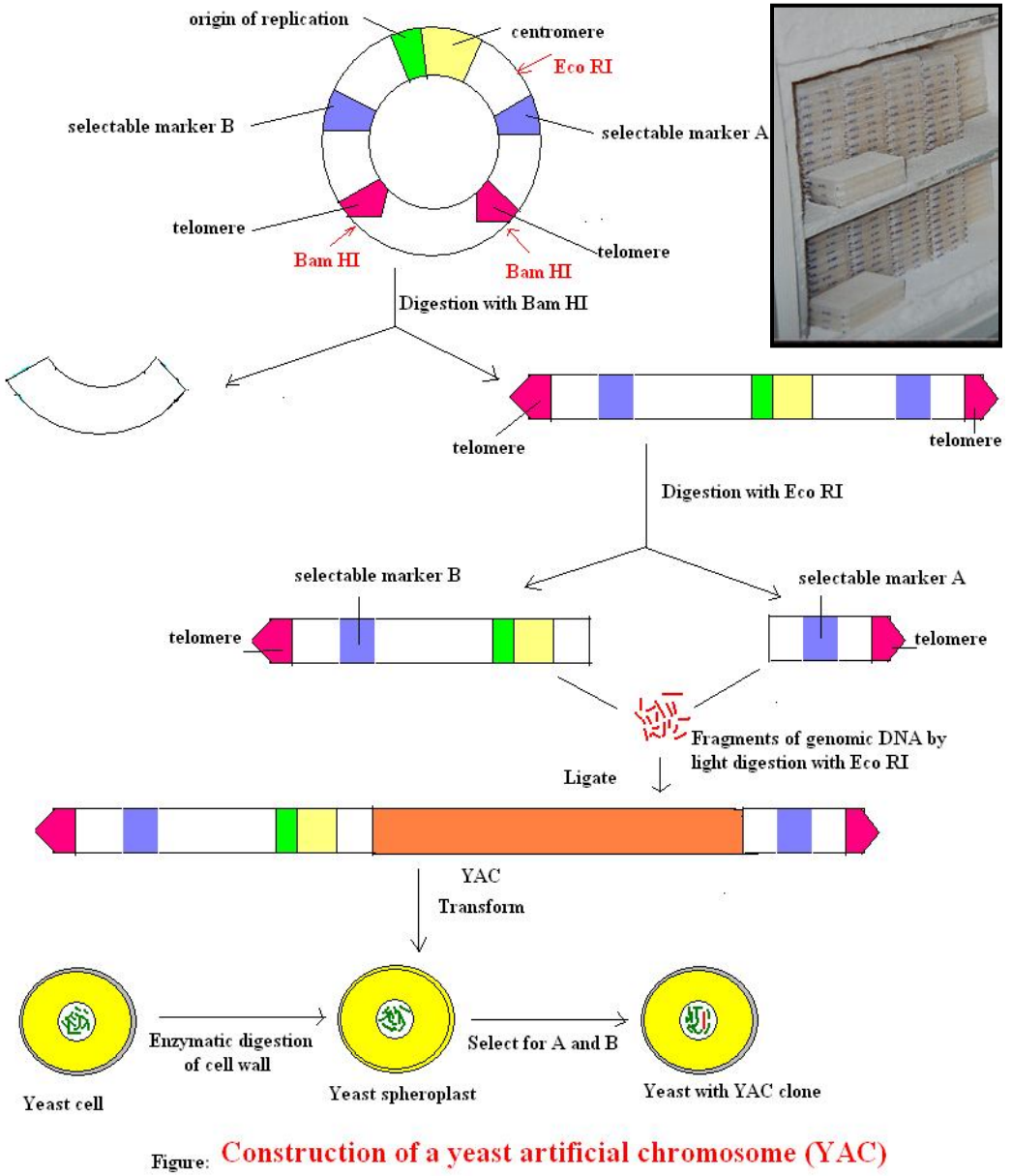
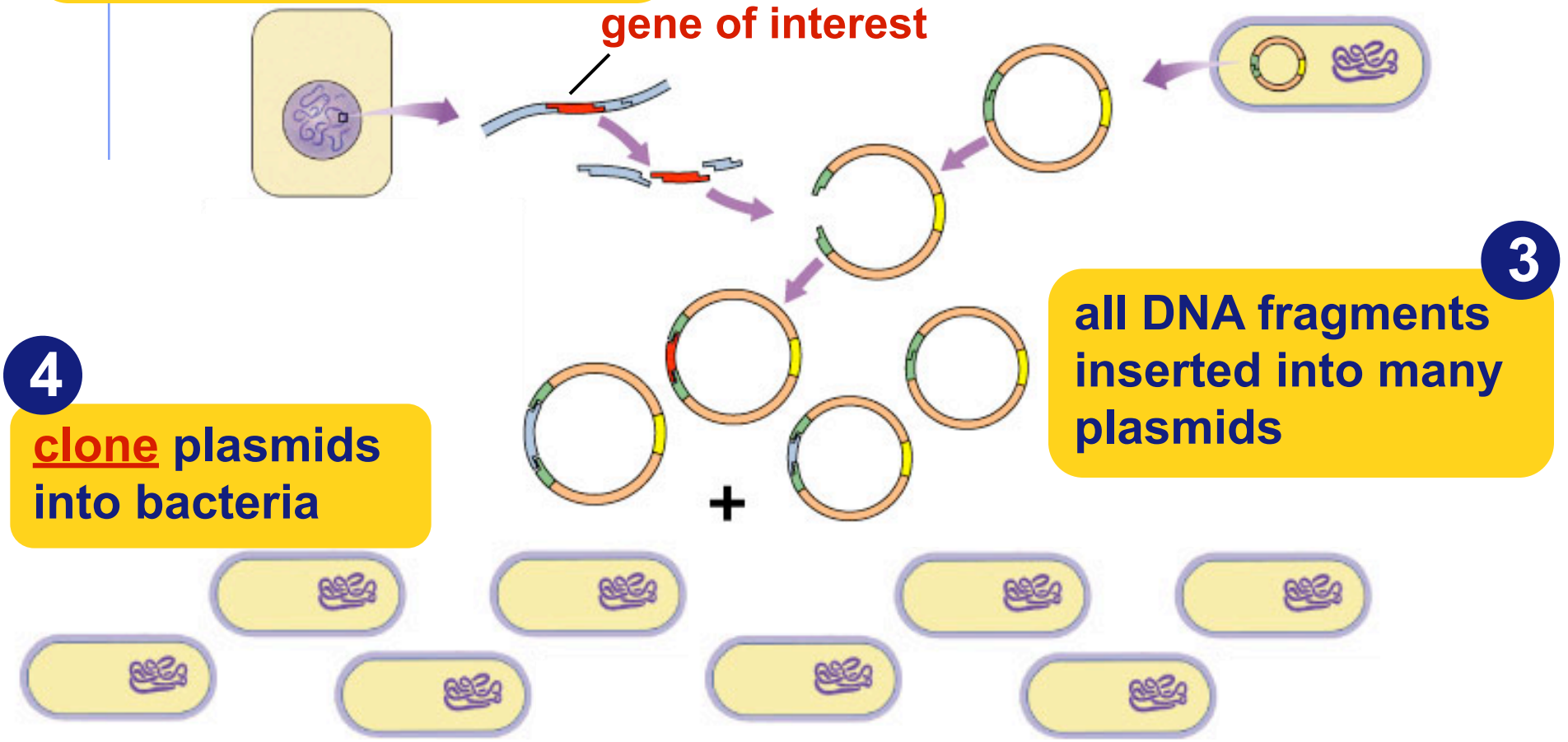


Figure: Construction of a yeast artificial chromosome (YAC)

Overview: Making a DNA library

1. all DNA from many cells of an organism is cut with restriction enzymes

2. engineered plasmid with selectable marker & screening system

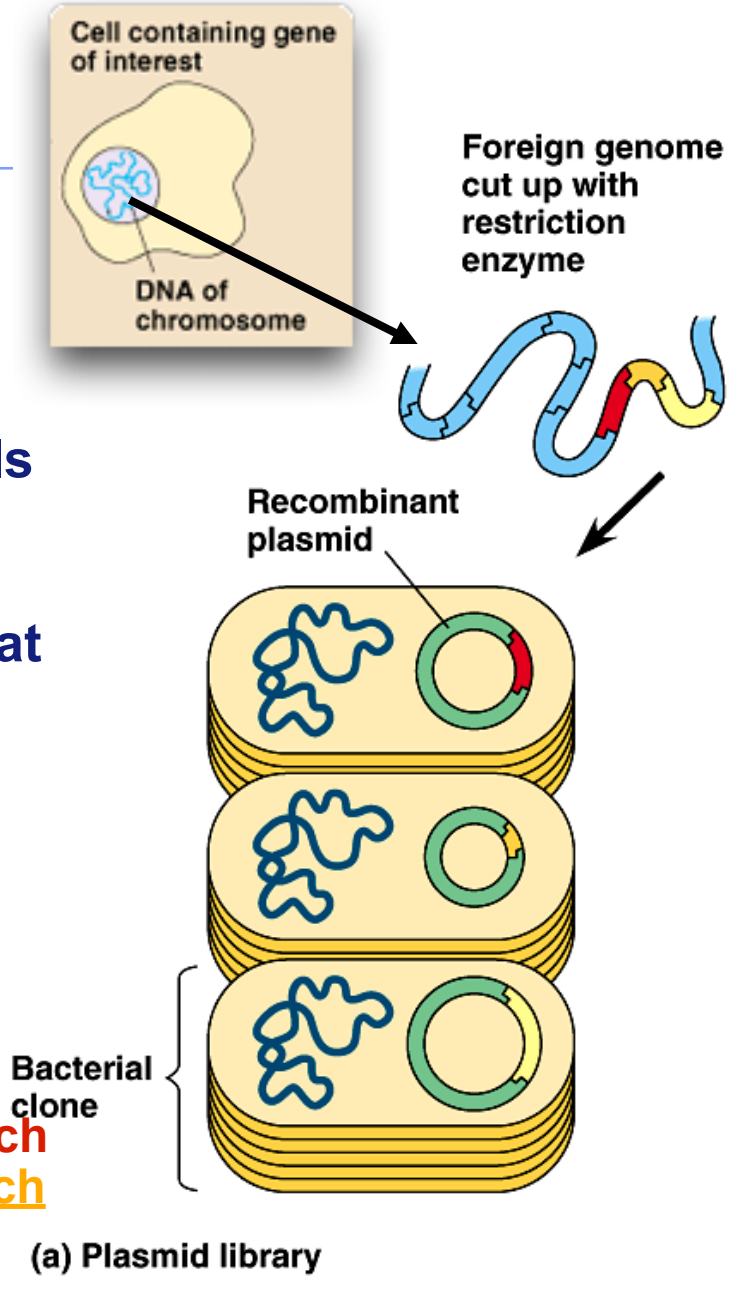


3. all DNA fragments inserted into many plasmids

4. clone plasmids into bacteria

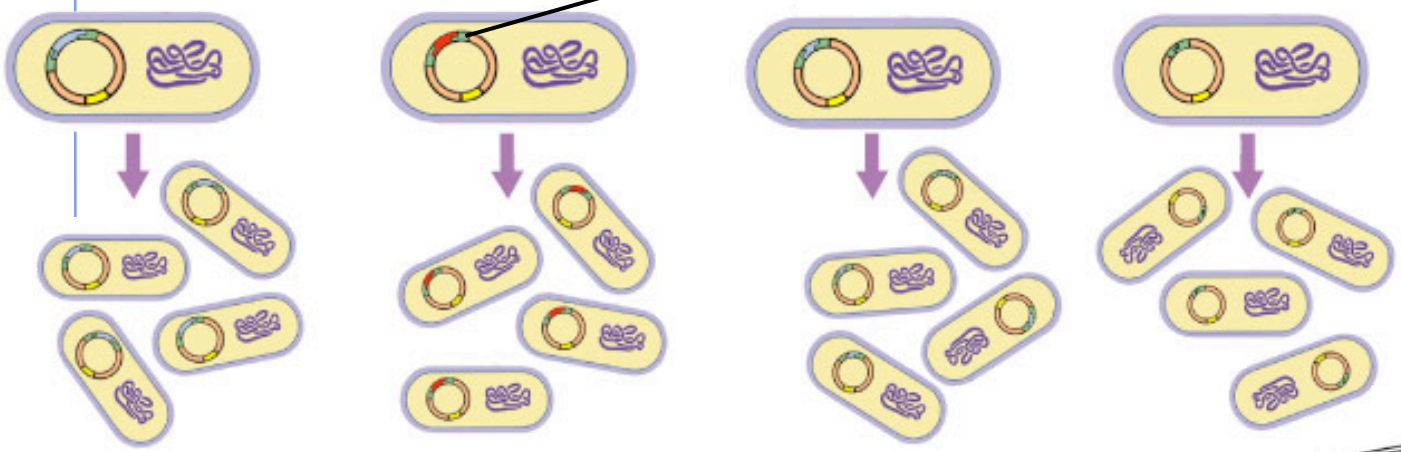
DNA libraries
(plasmid & phage libraries)

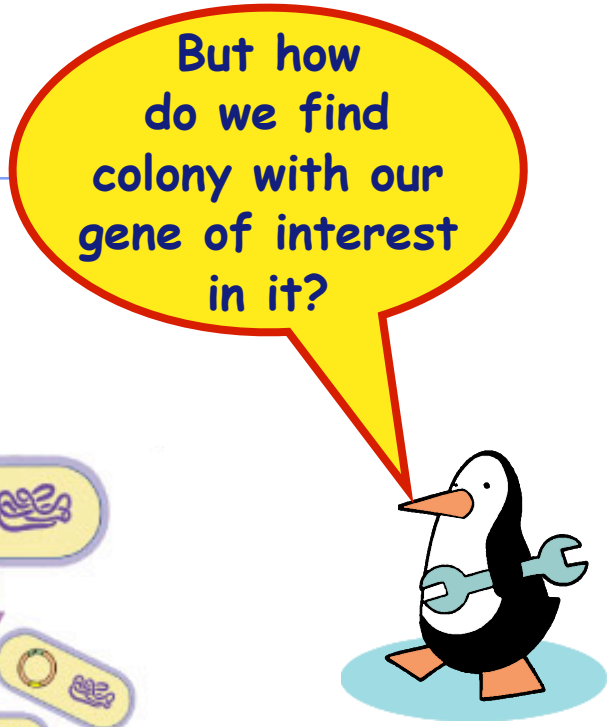
- **Genomic library** = the complete set of plasmid-containing cell clones, each carrying copies of a particular segment from the initial genome of an organism
- Cut up all of nuclear DNA from many cells of an organism
  - Using restriction enzyme
- Clone all fragments into many plasmids at same time
  - "shotgun" cloning = no single gene is targeted for cloning
- Create a stored collection of DNA fragments from the organisms
  - Each petri dish has many bacterial colonies
    - Each colony holds consists of a bunch of clones of one type of bacteria which has a specific plasmid with specific DNA fragment of our organisms in it



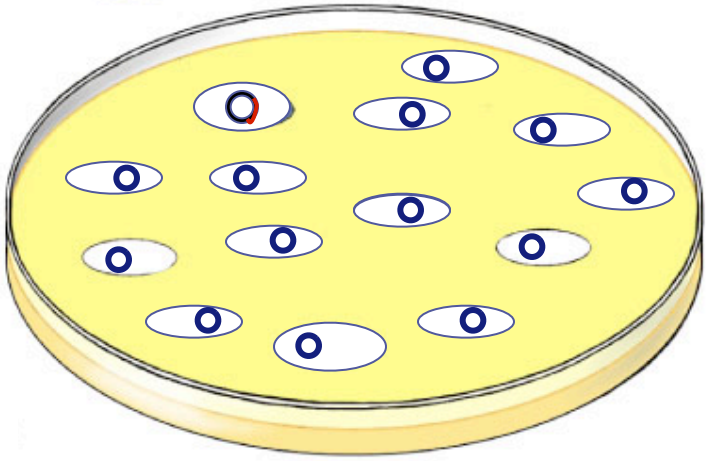
DNA library

recombinant plasmids
inserted into bacteria

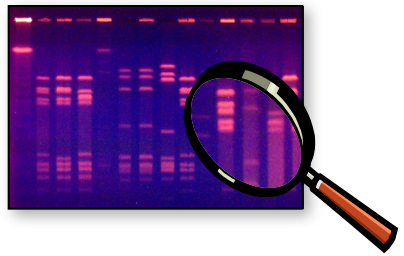




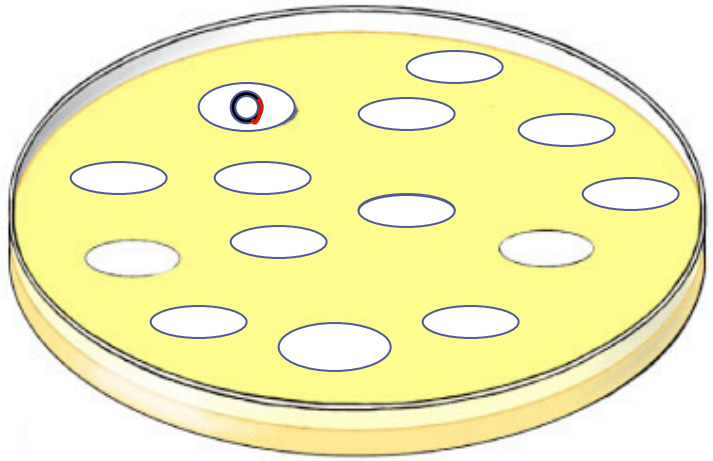
DNA Library
plate of bacterial colonies storing & copying all genes from an organism (ex. human)



Find your gene in DNA library

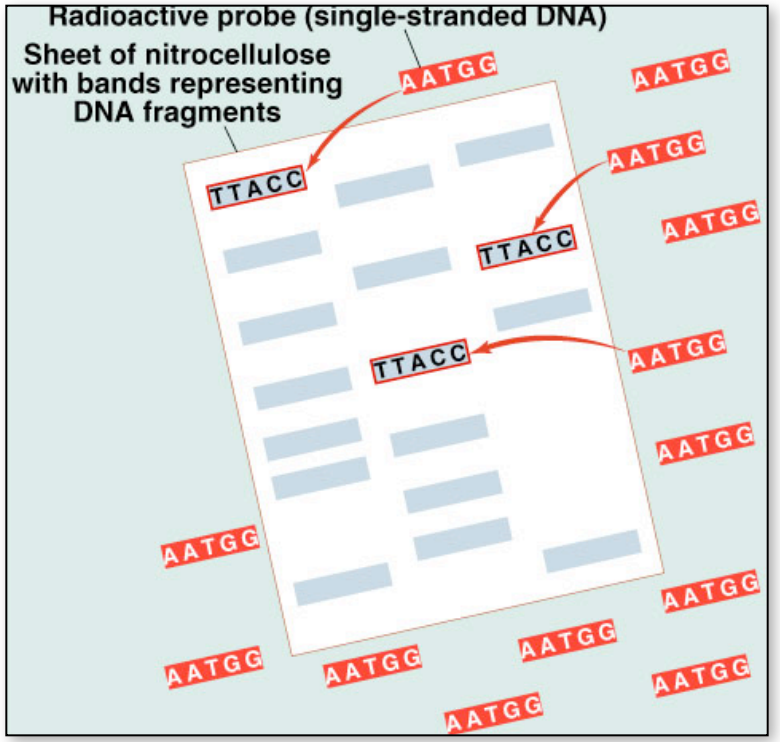


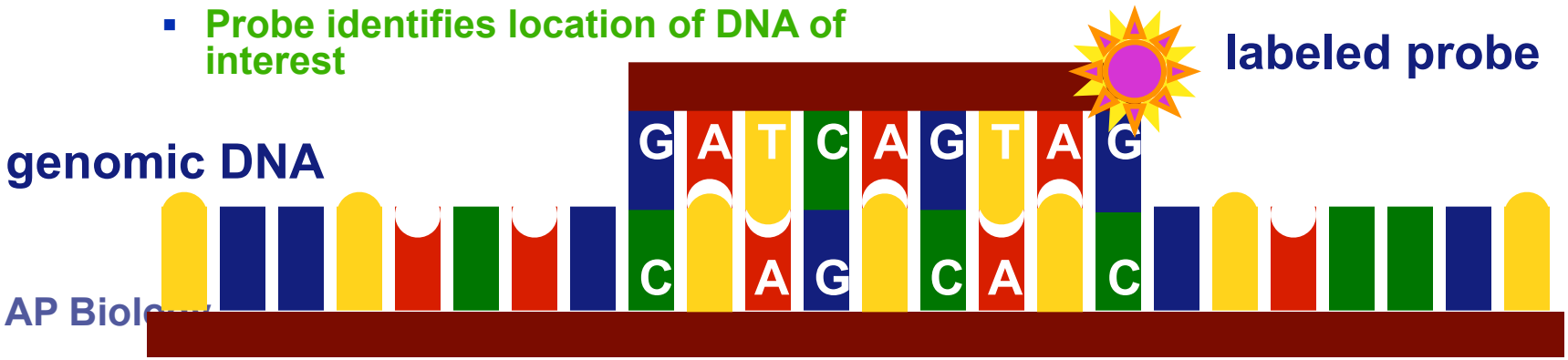
- **Locate Gene of Interest**
  - **to find your gene you need some of gene's sequence**
    - **if you know sequence of protein…**
      - **can "guess" part of DNA sequence that coded for the protein**
      - **"back translate" protein to DNA**
    - **if you have sequence of similar gene from another organism…**
      - **use part of this sequence**



Finding your DNA of interest

- **Nucleic Acid hybridization:**
  - find sequence of DNA using a **labeled probe**
    - short, single stranded DNA molecule
    - complementary to part of gene of interest
    - labeled with radioactive P^{32} or fluorescent dye
  - heat treat DNA in a gel or on nitrocellulose paper
    - Denatures (unwinds and makes single-stranded) strands
  - wash gel or paper with solution containing the labeled probe
    - probe hybridizes with denatured DNA
    - Probe identifies location of DNA of interest

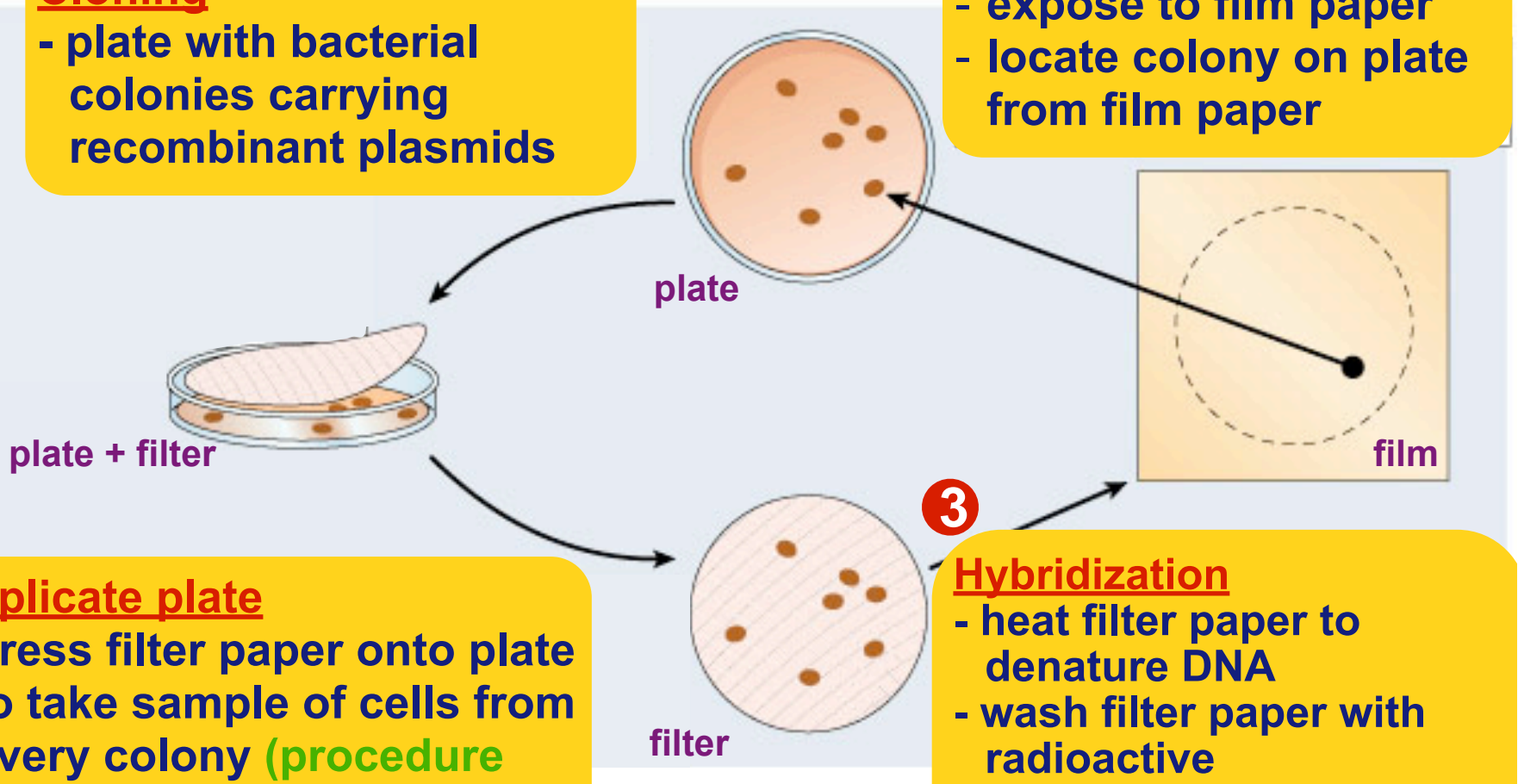




Colony Blots, followed by DNA Hybridization

1 **Cloning**
- plate with bacterial colonies carrying recombinant plasmids

4 **Locate hybridized Probe**
- expose to film paper
- locate colony on plate from film paper



2 **Replicate plate**
- press filter paper onto plate to take sample of cells from every colony (procedure called "blotting")

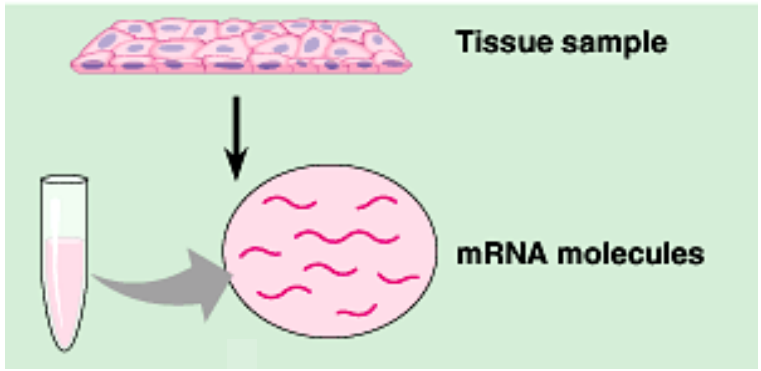
3 **Hybridization**
- heat filter paper to denature DNA
- wash filter paper with radioactive complimentary ssDNA probe which will only attach to gene of interest

How else can a colony blot and DNA hybridization be used?

DNA → RNA → protein → trait

- **When a gene is turned on, it creates a trait**
  - **If you want to know what gene is being expressed in a particular cell or at a particular time…**

extract mRNA from cells
mRNA = active genes



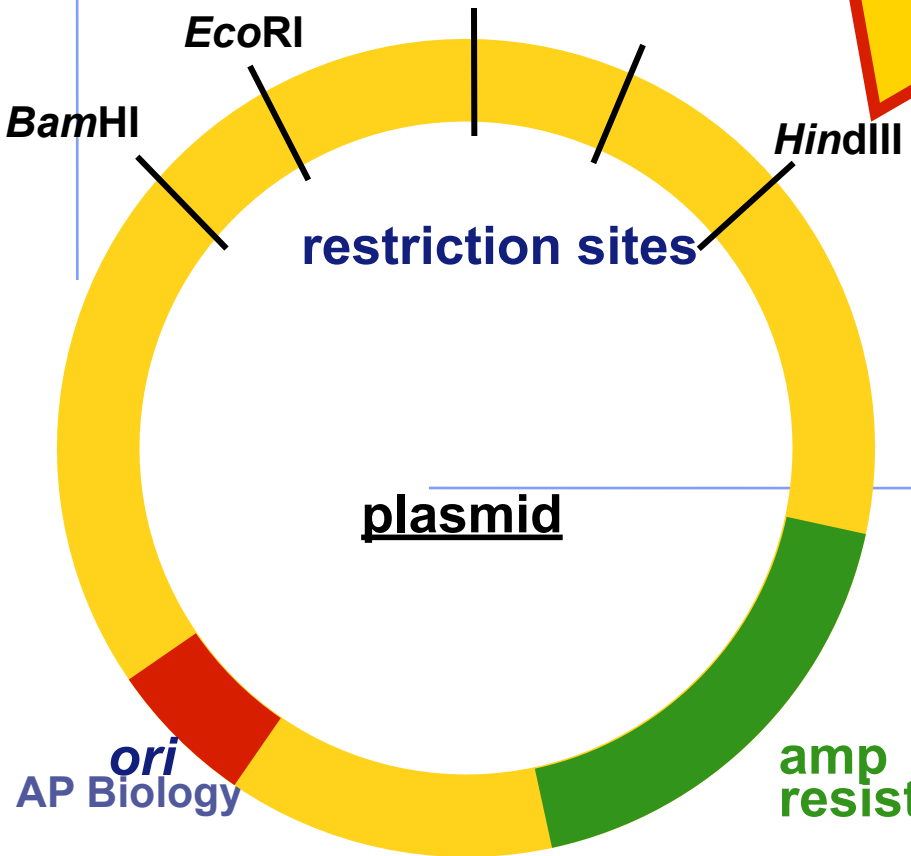
How do you match mRNA back to DNA in cells???

Use *reverse transcriptase* to make a DNA probe and you can hybridize this probe to you're the DNA in your library to identify which DNA is being expressed

AP

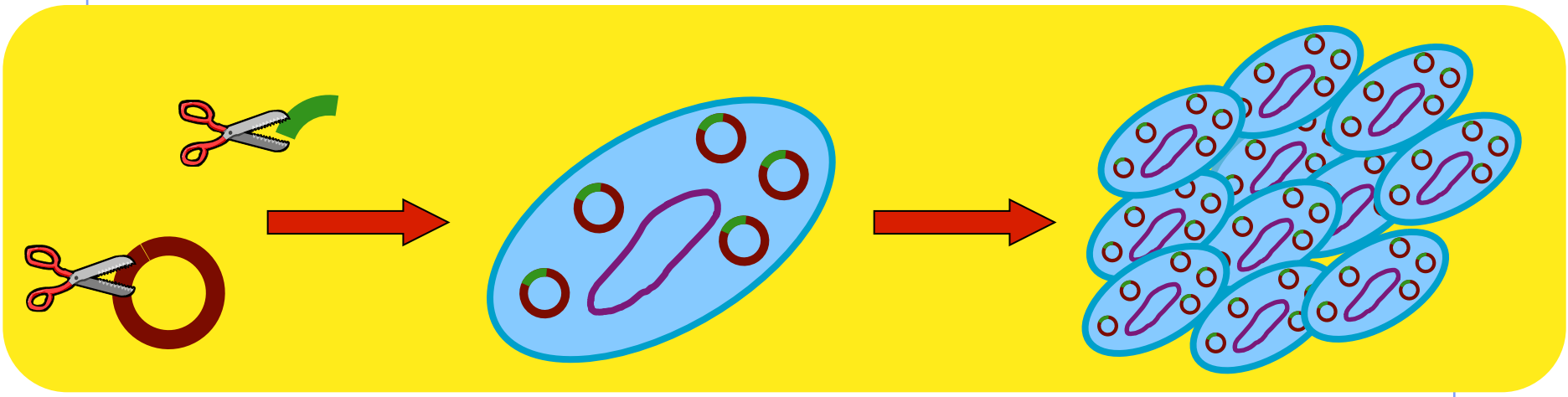
I may be very selective…

But still Ask Questions!



Let's return to the idea of making lots of copies of DNA…

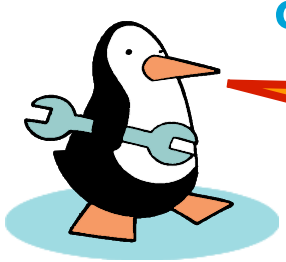
Yes, we can use bacteria…



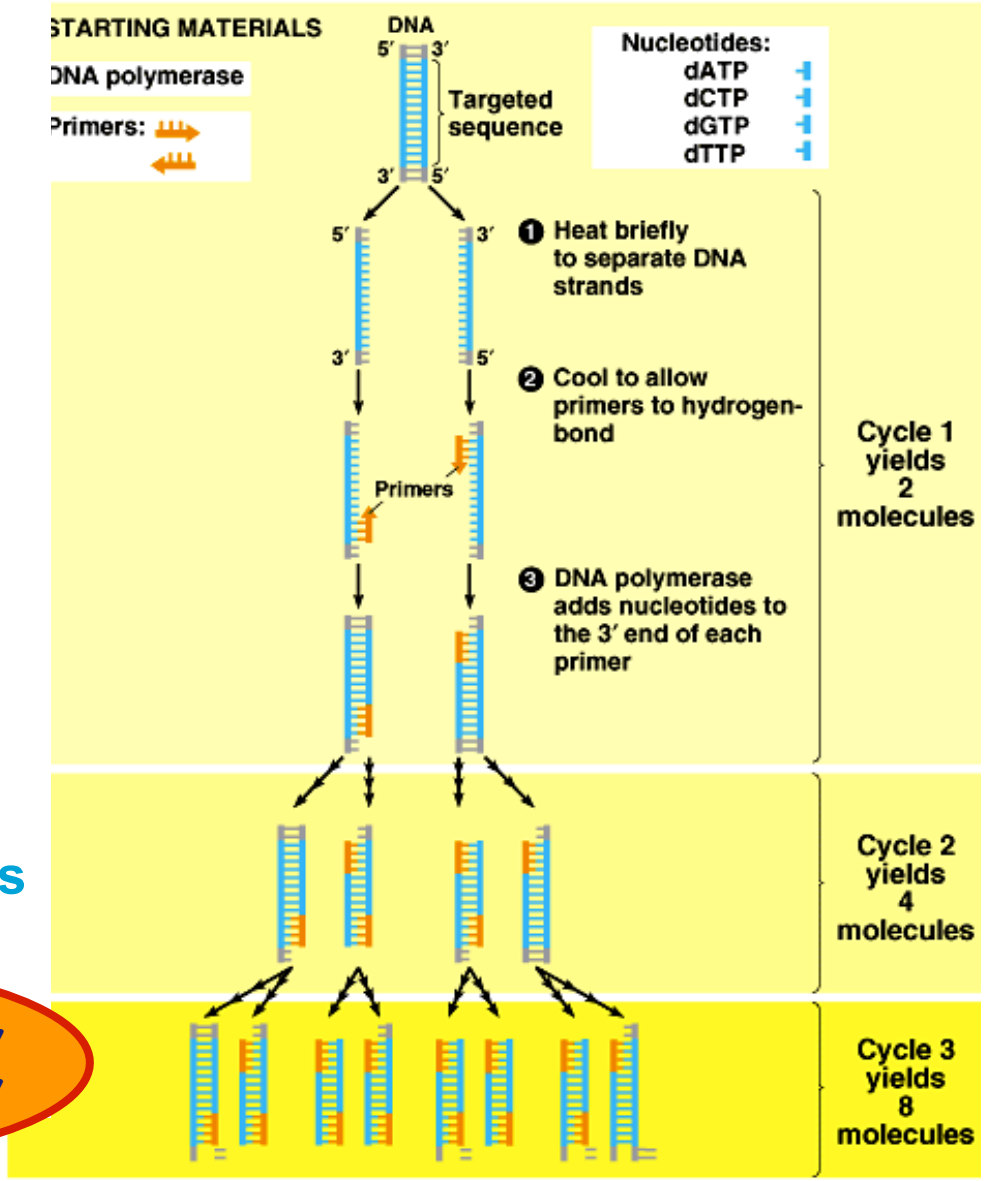
But it would be so much easier & faster if we didn't have to use bacteria every time…

Copy DNA without plasmids? PCR!

- **Polymerase Chain Reaction**
  - method for making many, many copies of a specific segment of DNA
  - ~only need 1 cell of DNA to start
  - Faster (< 1 hr) & more selective than cloning in bacteria using restriction enzymes!
    - The scientist can decide the exact section of chromosomal DNA to "amplify" or copy millions of times over!!!!!!



No more bacteria,
No more plasmids,
No more E. coli!



PCR process

- It's copying DNA in a test tube!
- What do you need?

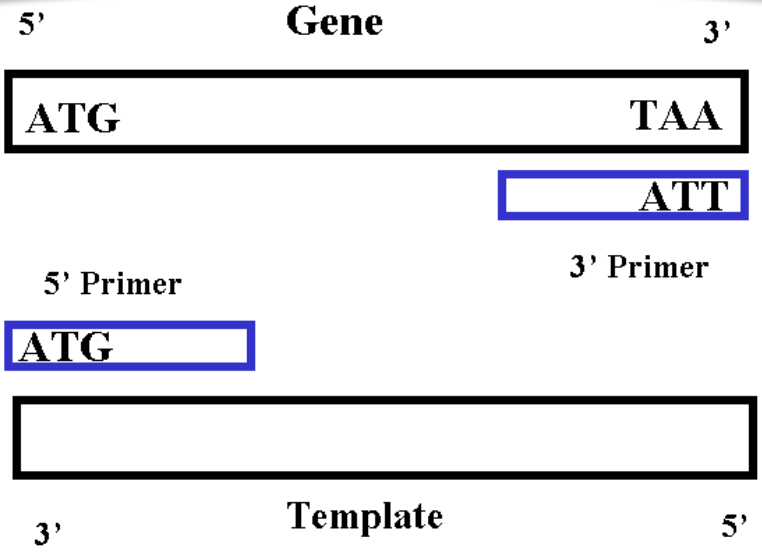
1. Double stranded DNA with the target sequence *(which will contain the template strands)*
2. Heat-resistant DNA polymerase enzyme
3. All four nucleotides
   - dATP, dGTP, dCTP, dTTP (deoxynucleotides)
4. Two single-stranded 15-20 base pair length DNA strands that serve as primers
   - One primer is complementary to one end of the target sequence
   - The second primer is complimentary to the other end of the sequence on the opposite target DNA strand

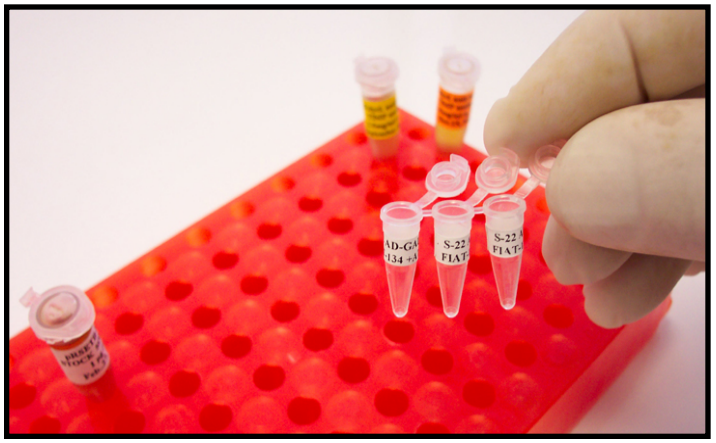
Thermocycler

PCR primers

Two different primers are needed to bind to the two strands of dsDNA. DNA polymerase will elongate the primers in the 5' to 3' direction.

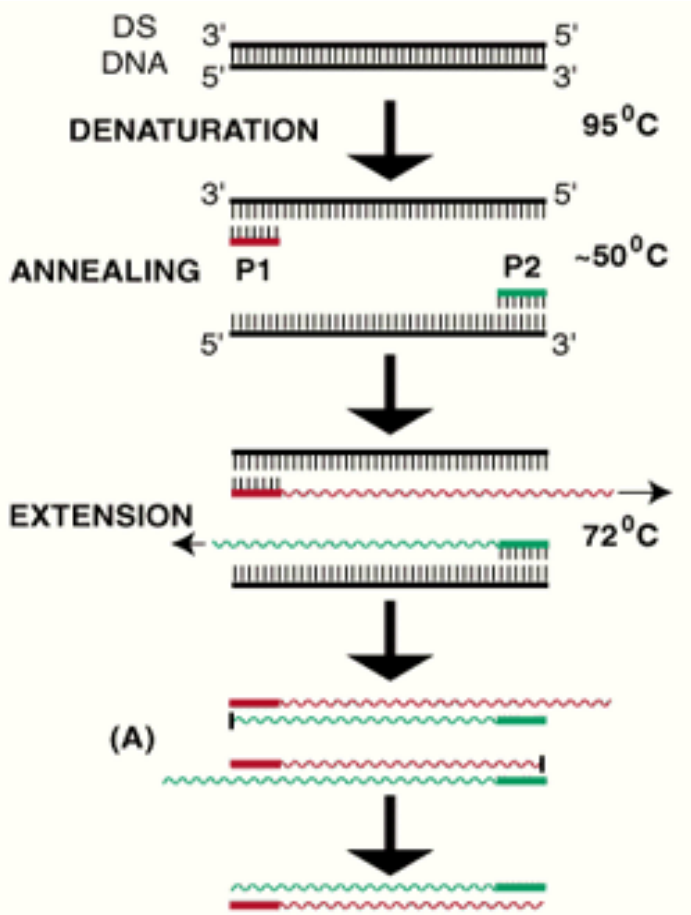
- The primers are critical!
  - need to know a bit of sequence to make proper single-stranded DNA primers
    - Must design primers complimentary to the ends of the sequence you wish to 'clone'
    - Primers will be extended by DNA Polymerase
  - primers can bracket target sequence
    - In PCR you start with long piece of DNA (like all the DNA from a cell) & make multiple copies of a specified shorter segment
      - primers define section of DNA to be cloned





The three automated steps of PCR

1. **Denaturation**
   - **Heat reaction mixture (95°)**
     - **Double-stranded DNA denatures (separates)**
2. **Annealing**
   - **Reaction mixture is cooled (50°)**
     - **Primers anneal (stick to) to complimentary template strands through hydrogen bonding**
3. **Extension**
   - **Reaction mixture warmed (72°)**
     - **Heat stable DNA polymerase extends the primers in 5' to 3' direction**

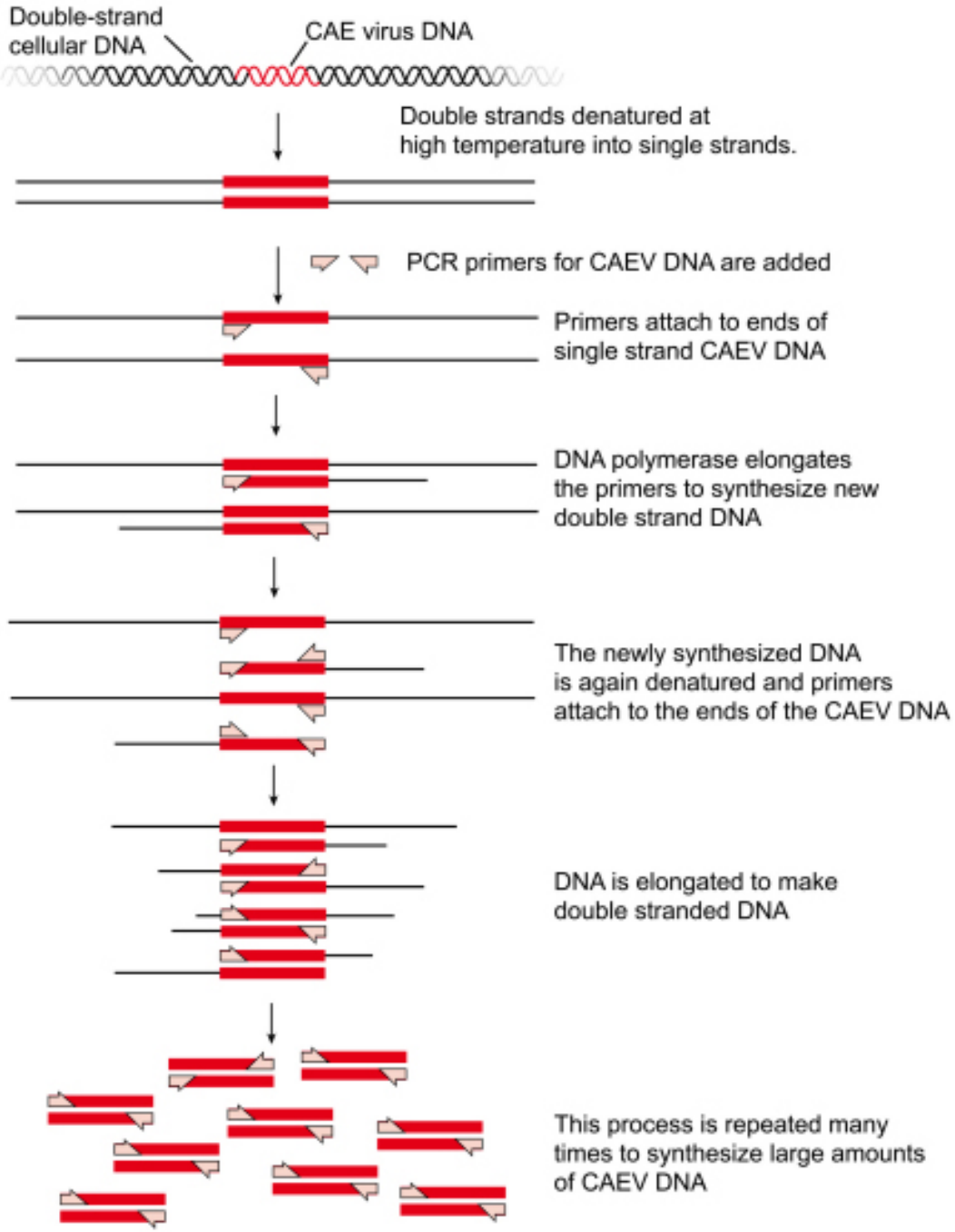


The whole process involves:
Repeating 20-30 cycles of 3 steps each cycle
(30 seconds per step)

PCR's diversity

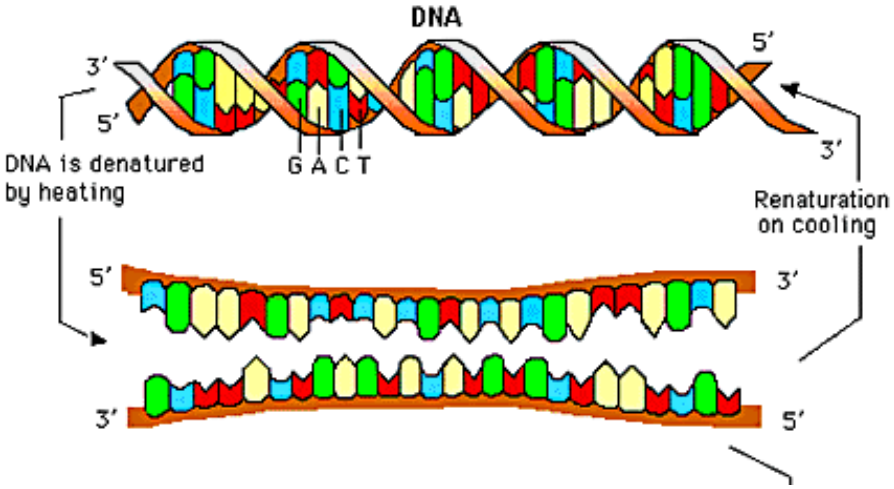
An important use of PCR now is to "pull out" a piece of DNA sequence, like a gene, from a larger collection of DNA, like the whole cellular genome.

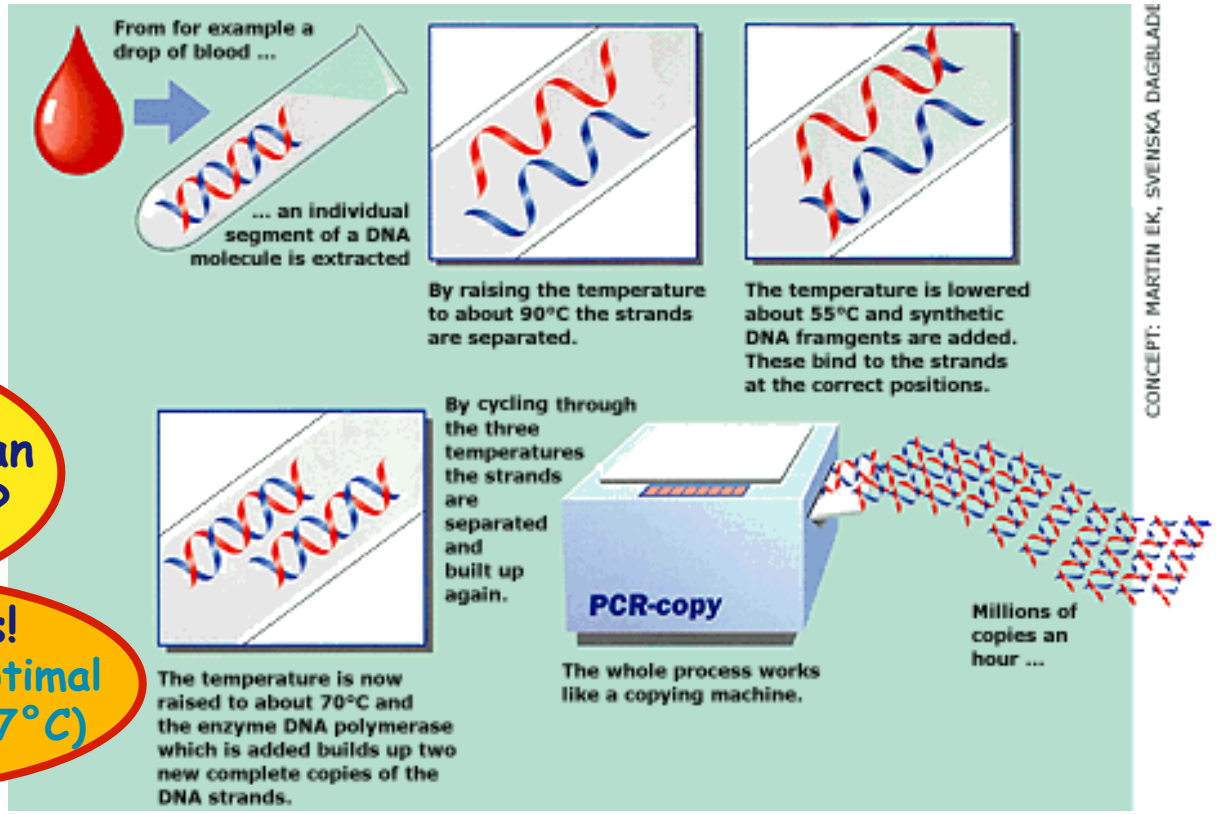
- **You don't have to go through the process of restriction digest anymore to cut the gene out of the cellular DNA.**
- **You can just define the gene with "flanking" primers and get a lot of copies in 40 minutes through PCR.**
  - **Note: You can also add in a restriction site into to the copies of the gene made by PCR (if these sites don't naturally exist) by adding a restriction site sequence as part of the primer which will become part of the new DNA fragments being copies. Now, each fragment has also the restriction site so you can insert that fragment into a plasmid as desired.**

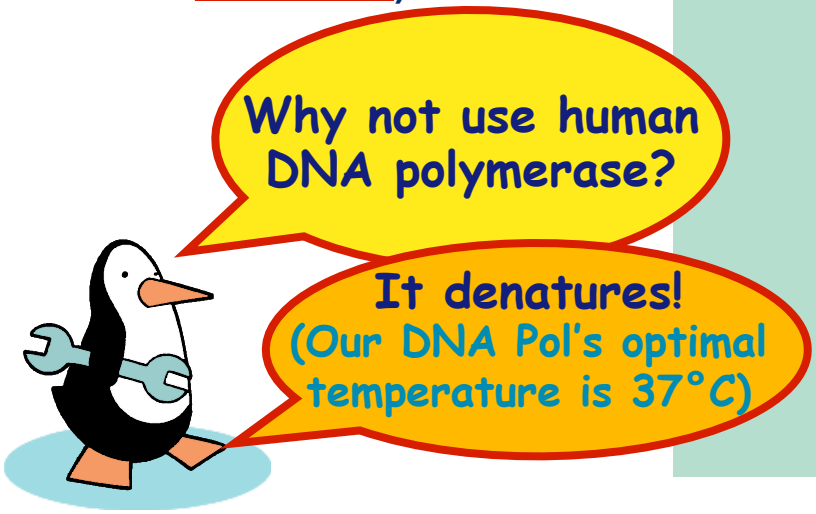


PCR process

- What do you need to do?
  - In tube: Mix DNA, DNA polymerase, primer, DNA nucleotides
  - denature DNA: heat (95°C) DNA to separate strands
  - anneal DNA: cool to hybridize with primers &
  - Build DNA (DNA extension)



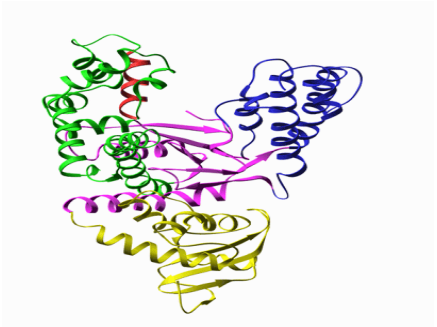




The polymerase problem

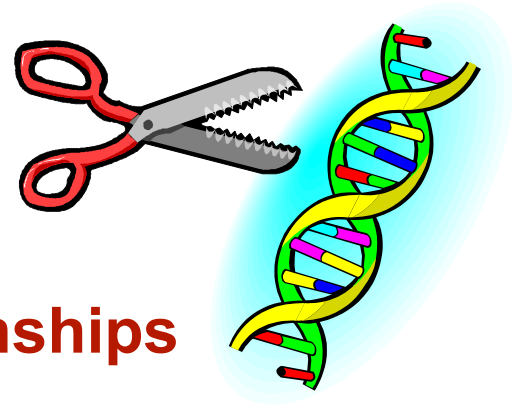
PCR
20-30 cycles
3 steps/cycle
30 sec/step

- **Heat DNA to denature (unwind) it**
  - **95°C destroys DNA polymerase**
  - **You would have to add new enzyme every cycle**
    - **almost impractical!**
- **Need enzyme that can withstand 95°C…**
  - **Use special DNA polymerase called Taq polymerase**
    - **from hot springs bacteria**
      - ***Thermus aquaticus***
        - **Highly thermostable**



Many uses of restriction enzymes & PCR…

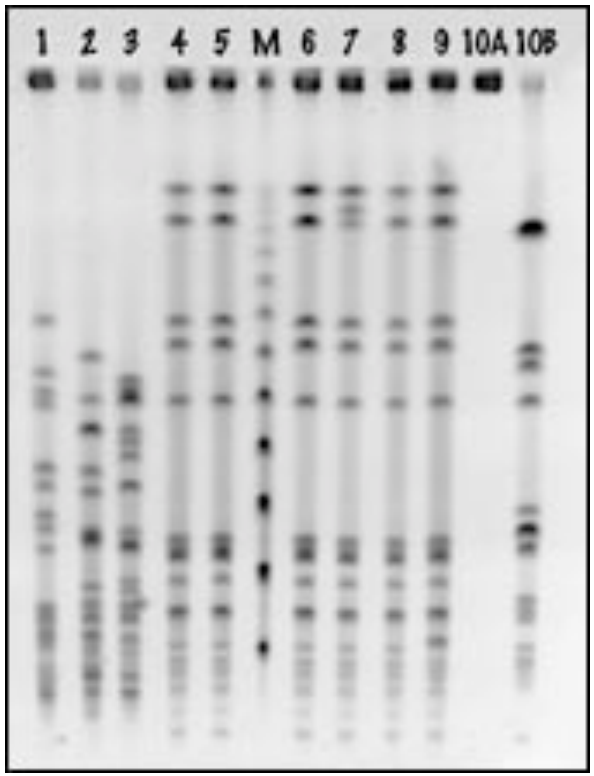
- Now that we can cut DNA with restriction enzymes…
  - we can cut DNA with restriction enzymes at restriction sites or amplify sections of DNA with PCR from different people… or different organisms, and compare the fragments obtained
  - why?
    - forensics
    - medical diagnostics
    - paternity testing
    - Determining evolutionary relationships
    - and more…



Comparing DNA fragments

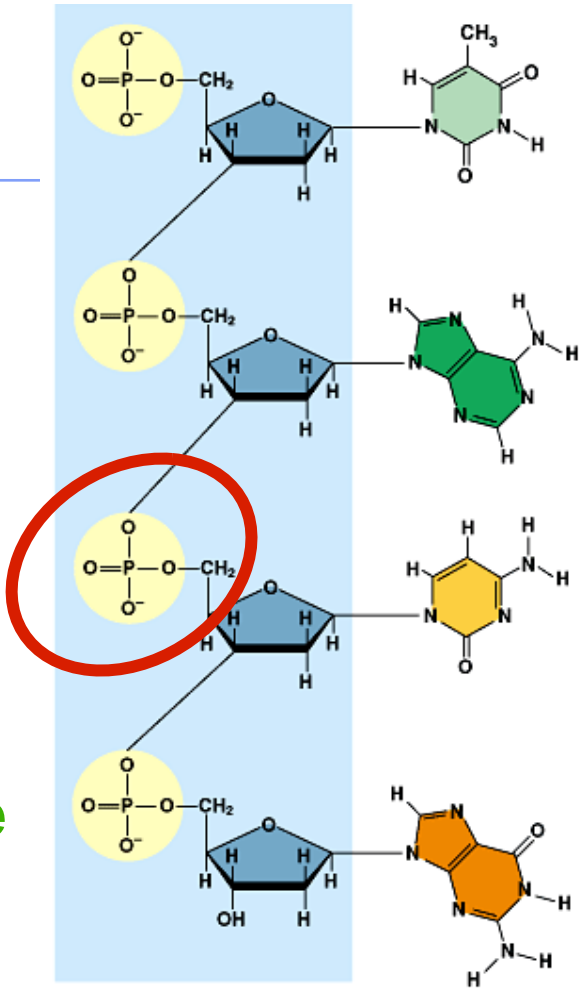
- How do we compare DNA fragments?
  - Can separate fragments based on size!!!
- How do we separate DNA fragments?
  - run it through a porous gelatin
    - Made of polymer agarose
      - Made from algae
  - Process called

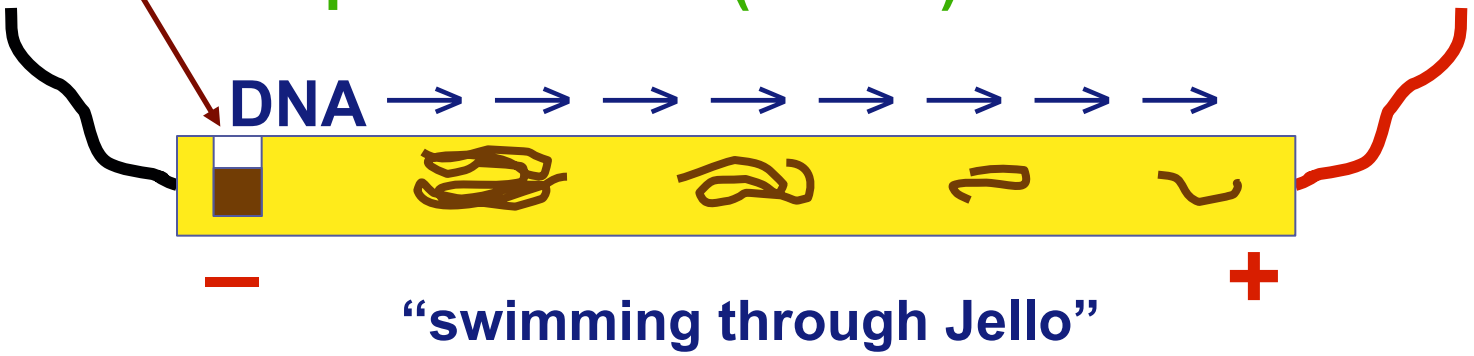
    gel electrophoresis



Gel electrophoresis

- A method of separating DNA in a gelatin-like material using an electrical field
  - DNA is negatively charged
    - when it's in an electrical field DNA moves from the negative end (cathode) toward the positive side (anode)

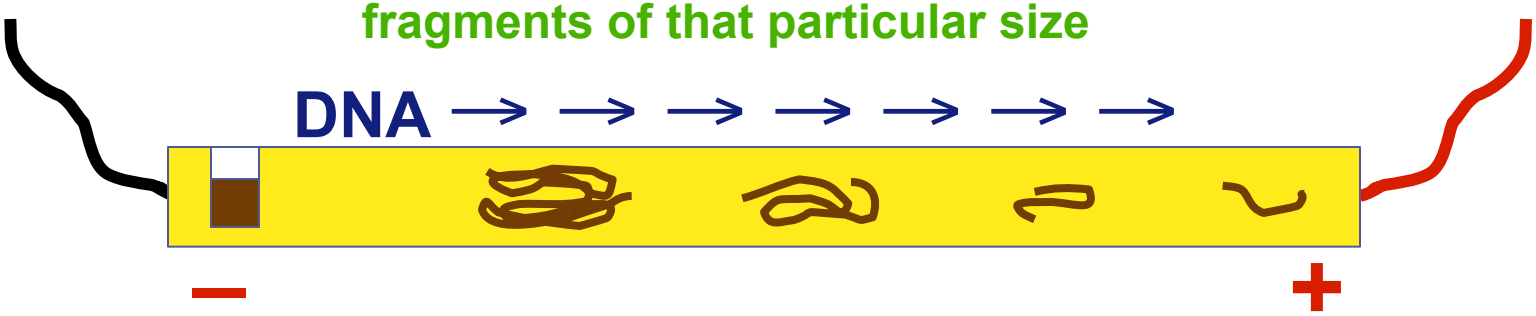




"swimming through Jello"

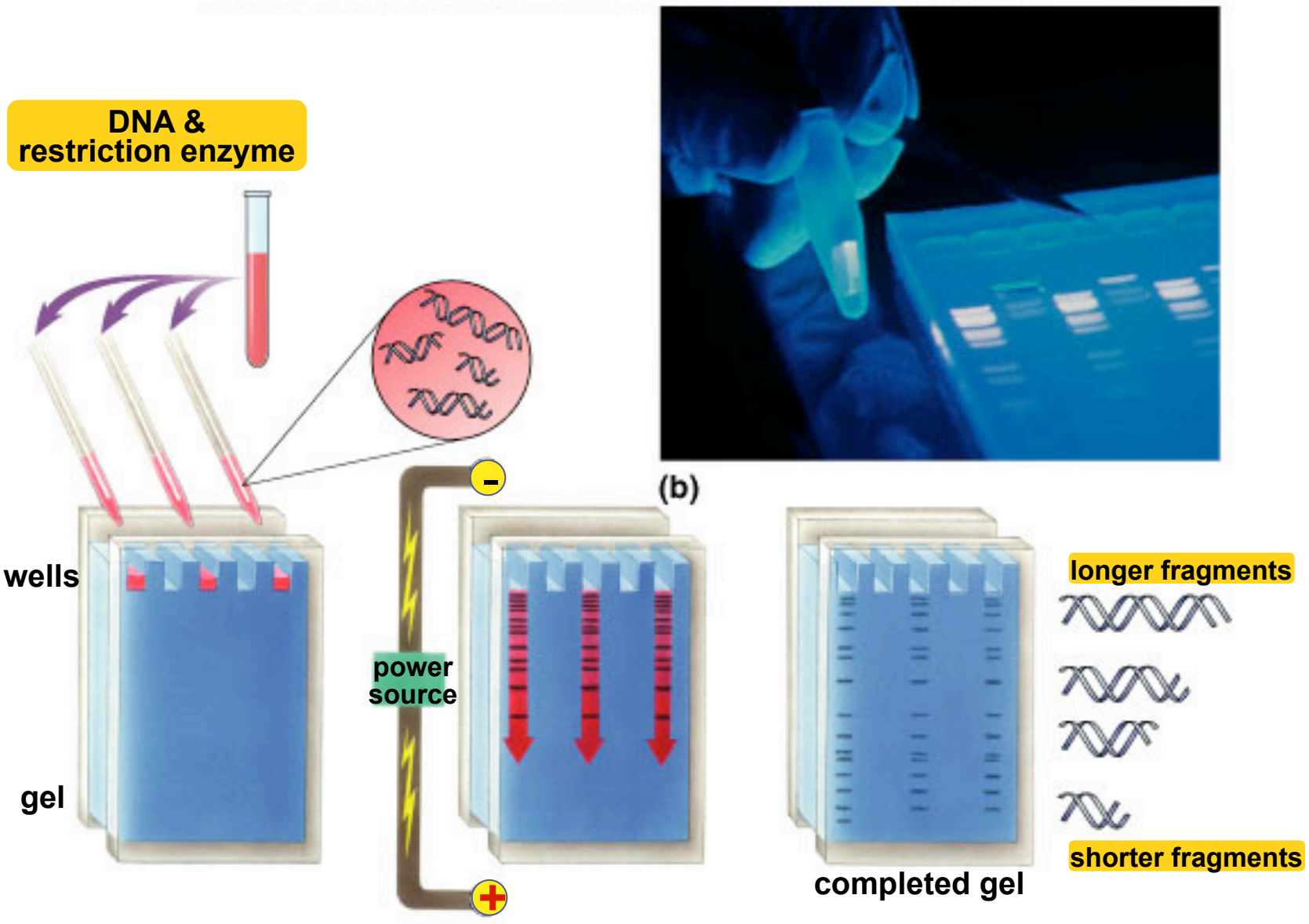
Gel electrophoresis - separates macromolecules on the basis of their **RATE** of movement through a polymeric gel in an electric field.

- **DNA moves in an electrical field…**
  - so how does that help you compare DNA fragments?
    - size of DNA fragment affects how far & fast it travels
      - small pieces travel farther
      - large pieces travel slower & lag behind
    - A mixture of restriction fragments (cut with restriction enzymes) or PCR amplified DNA fragments separate into bands
      - Each band contains thousands of molecules of the same length
      - The thickness of the band depends on the number of fragments of that particular size



Smaller pieces move through the polymer's pores faster

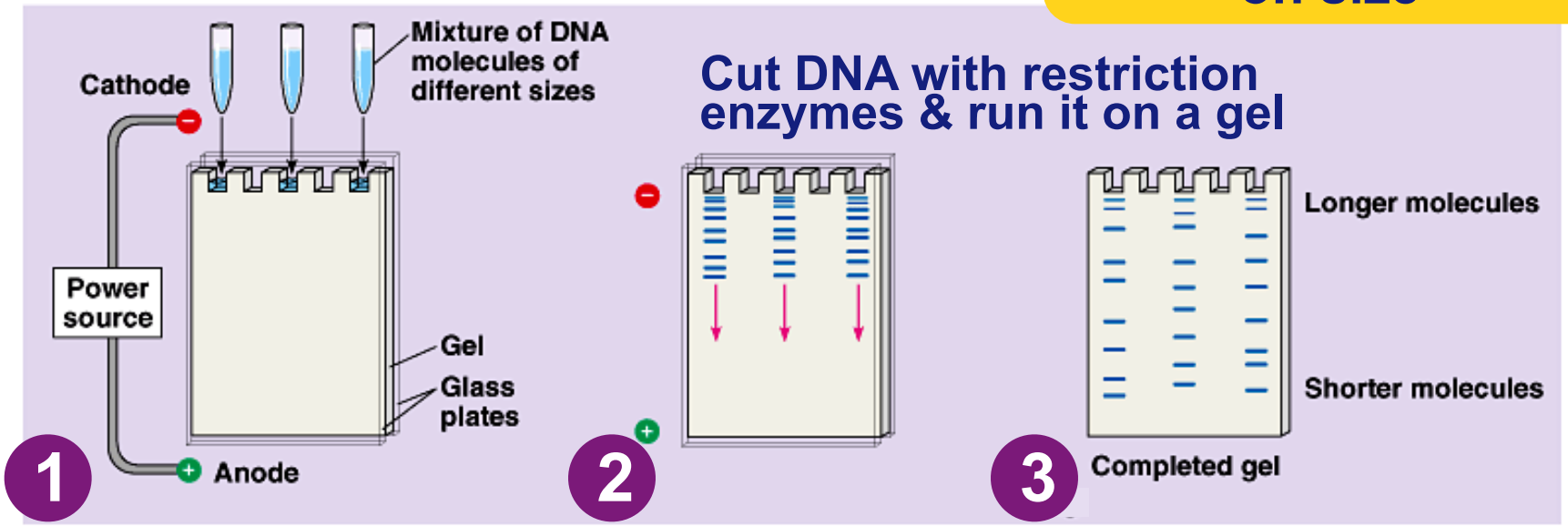
Gel Electrophoresis

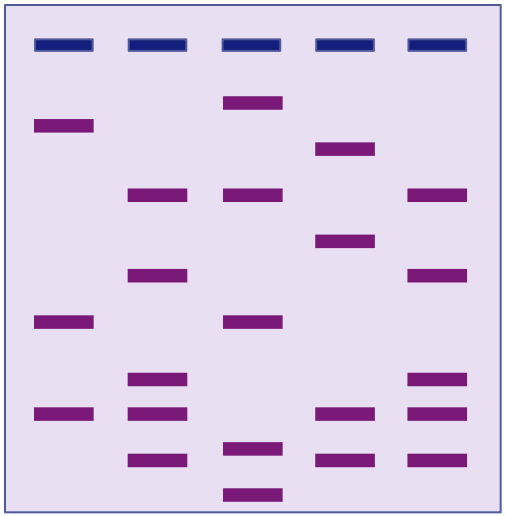


AP

Running a gel

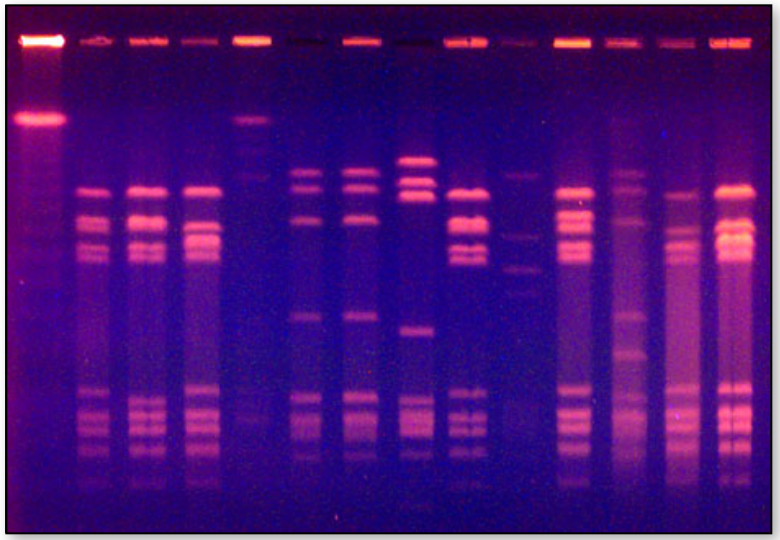
fragments of DNA separate out based on size





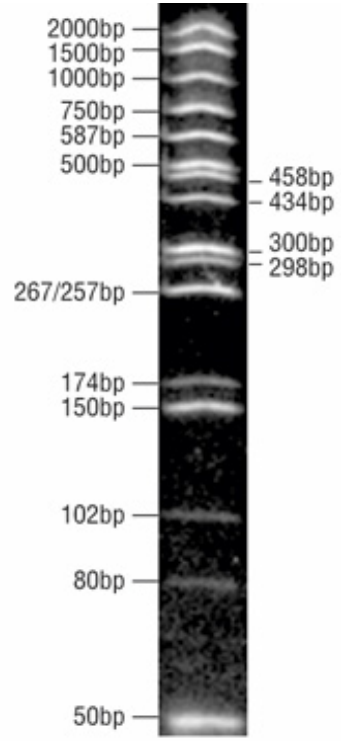
Must Stain DNA to see it

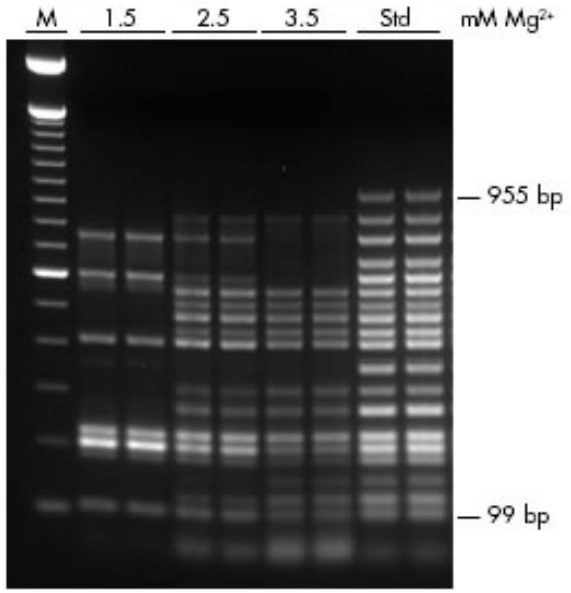
- ethidium bromide binds to DNA
- fluoresces under UV light
- So we can see the DNA and where DNA moved and compare the sizes of DNA fragments



Estimating the size of your DNA

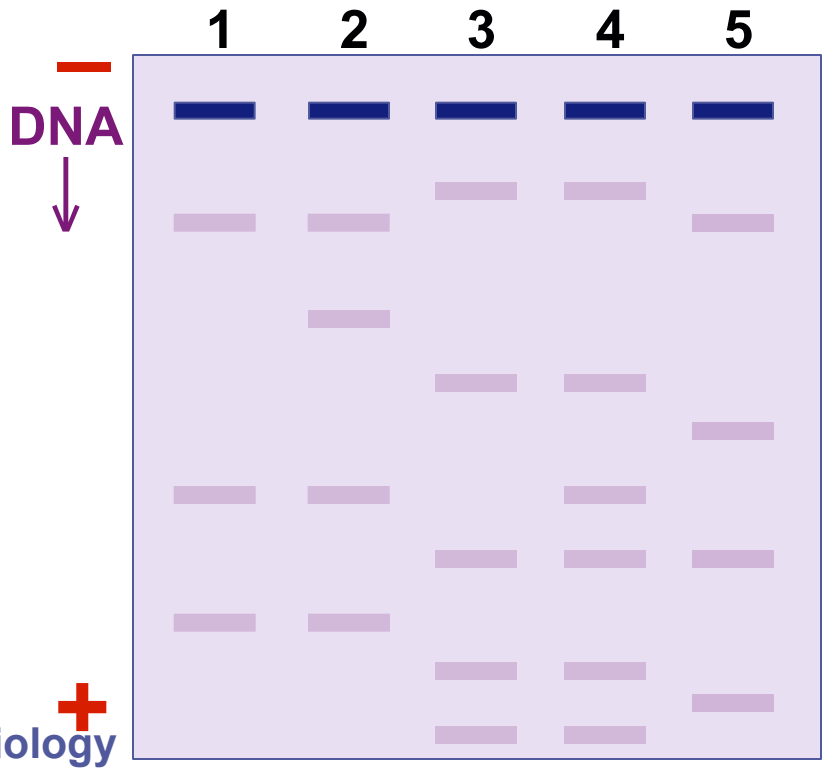
- To estimate the size of the separated fragments:
  - Run in one well a LADDER = a marker
    - A mixture of DNA of known sizes
      - Compare the bands with your bands so you can estimate the size of your DNA fragments

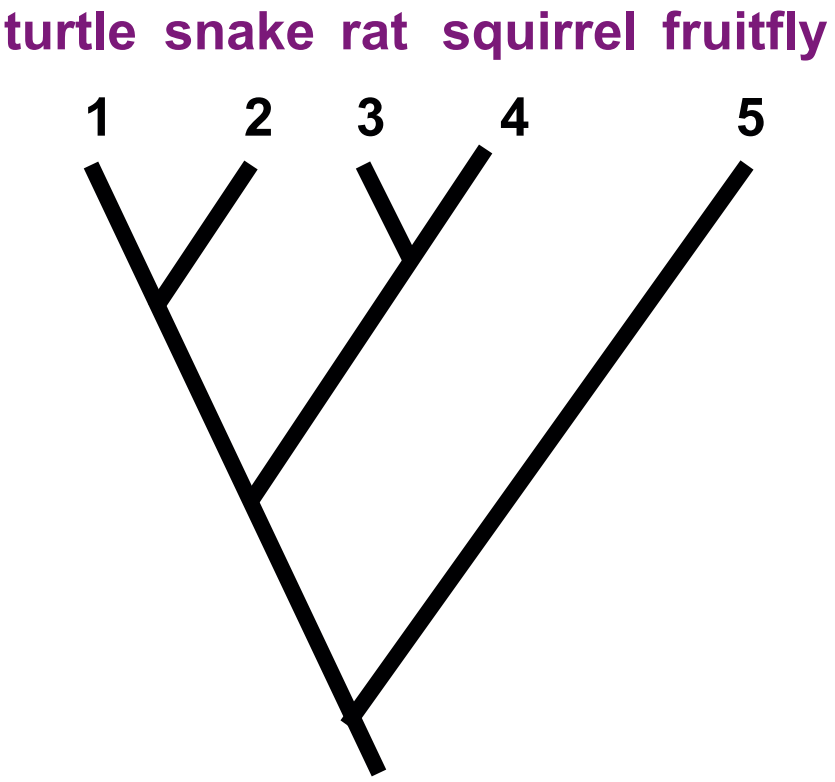




Uses: Studying Evolutionary Relationships

- Comparing DNA samples from different organisms to measure evolutionary relationships
  - Are the fragments of the same DNA location in each organism the same or does one have more or fewer nucleotides
    - The more differences between two samples, the further back their common ancestor in time

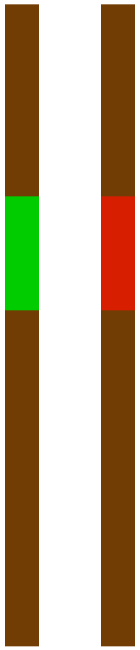




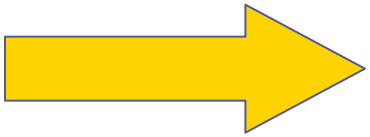
Uses: Medical diagnostic

- **Fetal are adult testing for diseased alleles**
  - **Comparing normal allele to disease allele**
    - **Changes in sequence may introduce or delete a restriction site**
      - **Digestion of each allele with a restriction enzymes will lead to different sized fragments depending on the allele**
    - **Changes in sequence may alter the length of the allele in PCR- amplified DNA copies**

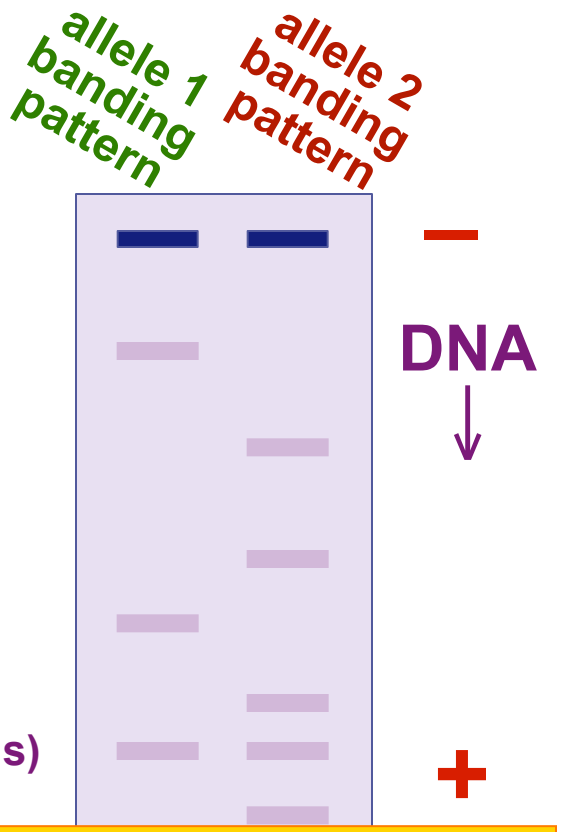
chromosome with normal allele 1



chromosome with disease-causing allele 2



Example: test for Huntington's disease (sickle cell, cystic fibrosis)



Each lane in a gel contains one person's digested or amplified DNA fragments

- **A heterozygote will show bands of both alleles in their lane.**
- **A homozygote will show bands of one or the other allele in their lane.**

Uses: Forensics (Crime Investigation)

- **Comparing DNA sample from crime scene with suspects (S) & victim (V) DNA**
  - The DNA fragments can be amplified sections of chromosomal DNA from PCR **or** the result of digesting chromosomal DNA with restriction enzymes.

Genetic Profile
(Creating "DNA fingerprints")

- **Genetic Markers** = DNA sequences that vary in the population in the coding and especially noncoding DNA
  - Recall 99% of DNA is non-coding
  - DNA sequences at all these locations for every person is unique (except identical twins).
  - We can analyze multiple markers in a persons DNA and establish a person's genetic profile and compare their profile to other DNA or samples of DNA found elsewhere
    - Ex: Comparing blood samples on defendant's clothing to determine if it belongs to victim
      - comparing DNA banding pattern between different individuals

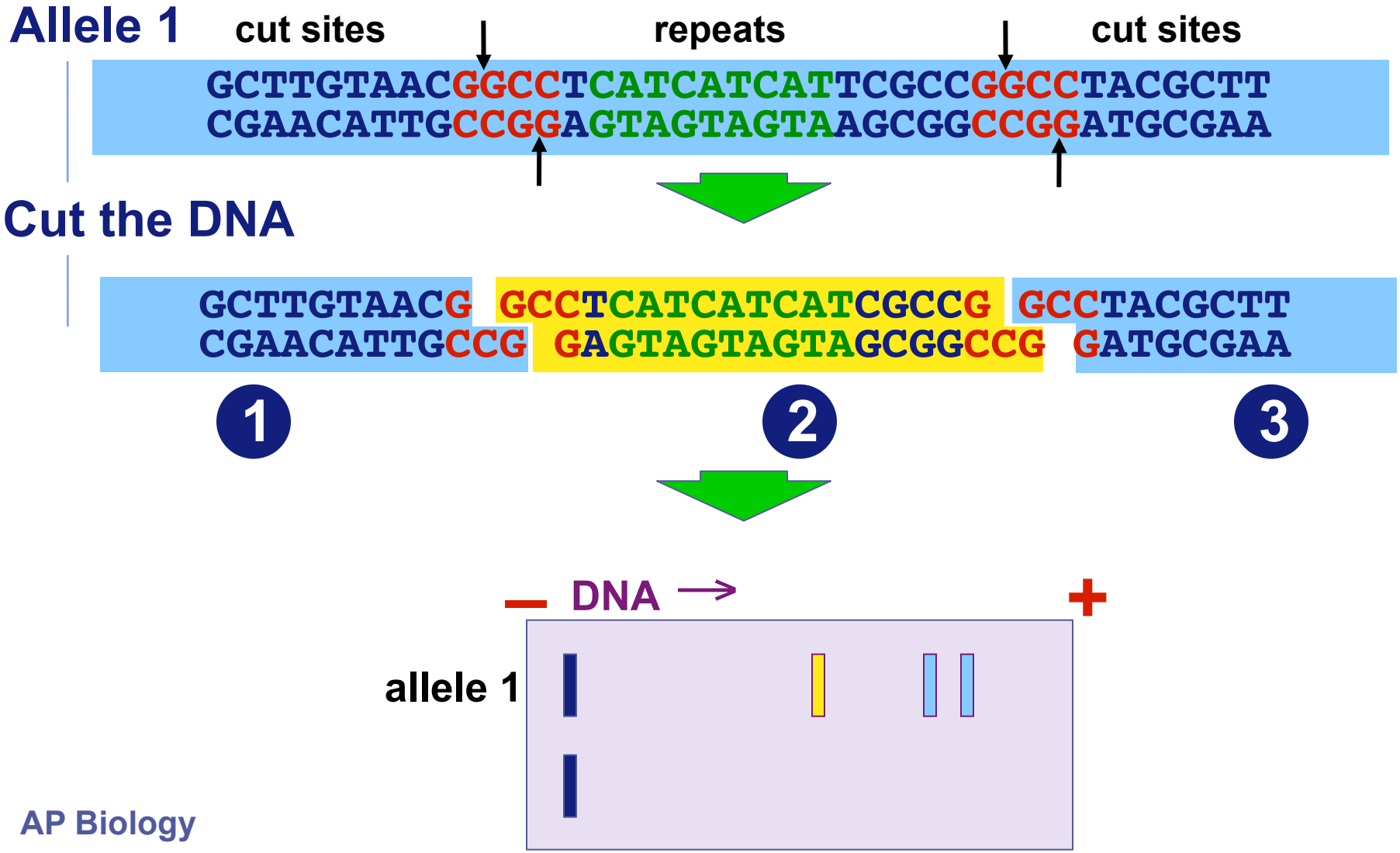
Use Differences at the DNA Level

- **Why is each person's genetic profile (DNA banding pattern) different if we amplify multiple sections of your DNA?**
  - amplify with PCR sections of non-coding DNA with are highly variable *(polymorphic)* from person to person
  - these regions contain sections with repeated nucleotide patterns
    - each person may have different number of repeats so will generate different length fragments using PCR
    - many sites on our 23 chromosomes with different repeat patterns can be used as GENETIC MARKERS
  - One types: Short Tandem Repeats (STRs)
    - Tandem repeated units of 2 to 5 base sequences in specific noncoding regions on our genome
      - Highly variable lengths from person to person (polymorphism)
      - CATCATCATCAT, GCCGCCGCCGCCGCCGCC

GCTTGTAACGGCCTCATCATCATTCGCCGGCCTACGCTT
CGAACATTGCCGGAGTAGTAGTAAGCGGCCGGATGCGAA

GCTTGTAACGGCATCATCATCATCATCATCCGGCCTACGCTT
CGAACATTGCCGTAGTAGTAGTAGTAGTAGGCCGGATGCGAA

Banding Patters of DNA fingerprints also differ based on where you have a restriction site compared to someone else or the length or your restriction fragment one produced



Analyzing Differences between people

Allele 1 cut sites cut sites

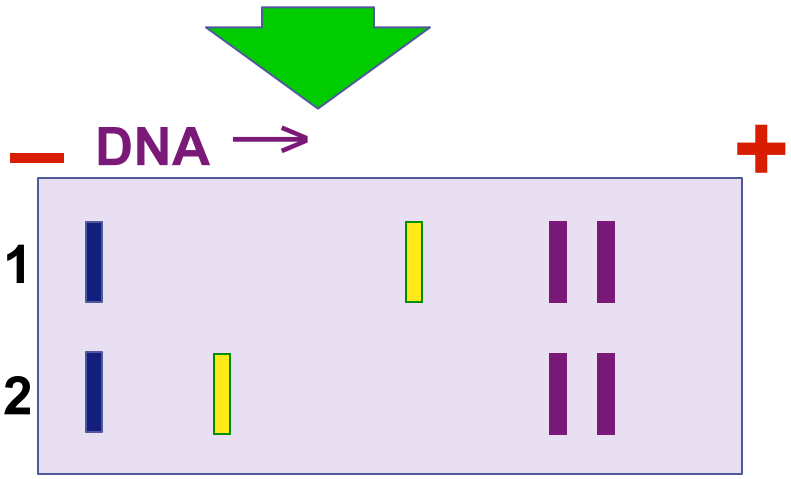
GCTTGTAACG GCCTCATCATCATTCGCCG GCCTACGCTT
CGAACATTGCCG GAGTAGTAGTAAGCGGCCG GATGCGAA

Allele 2: more repeats

GCTTGTAACG GCCTCATCATCATCATCATCATCCG GCCTACGCTT
CGAACATTGCCG GAGTAGTAGTAGTAGTAGTAGGCCG GATGCGAA

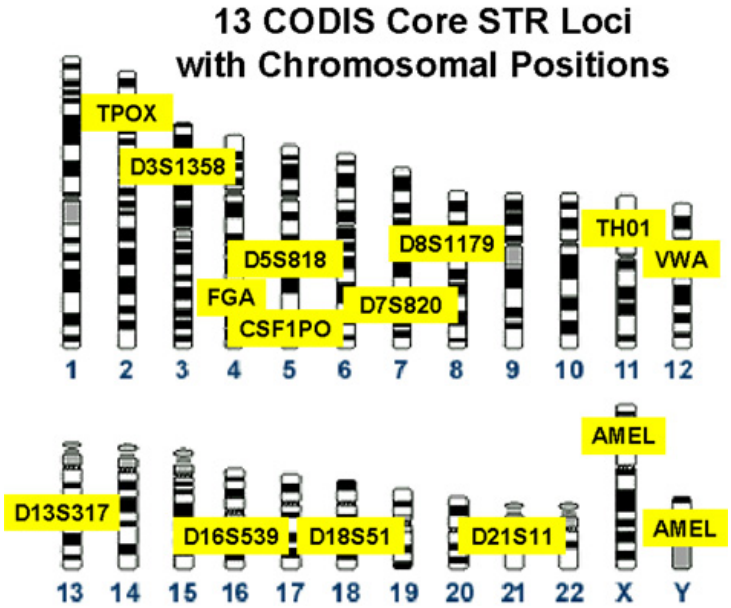
1 2 3

DNA fingerprint

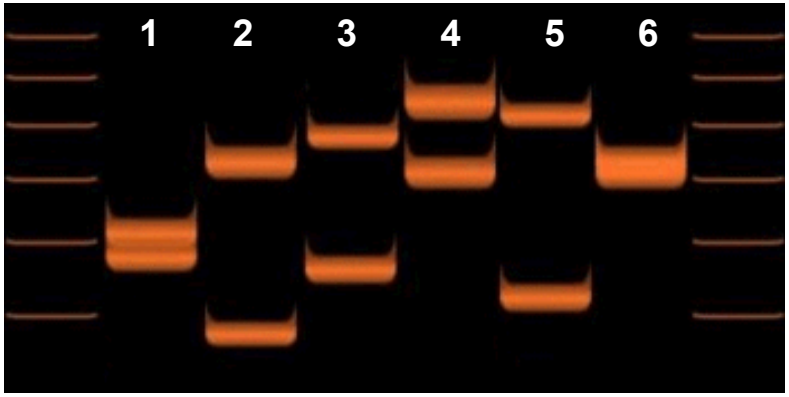


Analyzing Differences between people

- Can also use PCR instead of using restriction enzymes to amplify (make many copies of) specific STR loci
  - Construct primers that flank multiple STRs in the DNA
  - Run PCR to amplify and make millions of copies of multiple STRs or many region of DNA at once
  - Run the amplified DNA from different individuals on a gel to separate out one or more STRs of each person
  - Compare Samples
- In Forensic uses and paternity testing, we often test 13 or 15 STRs of a person to get a full Genetic Profile

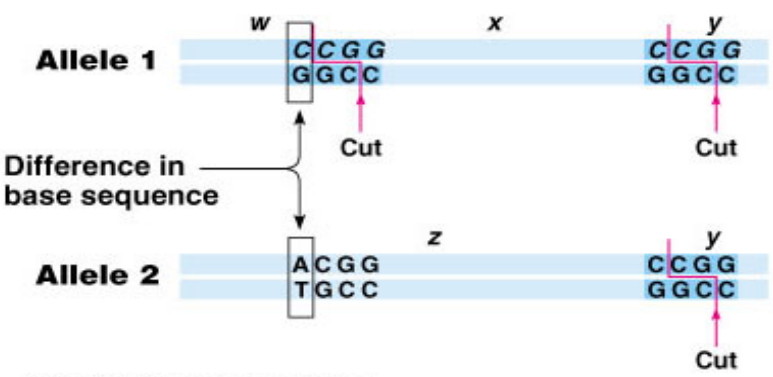


Ex: Amplified STR fragments from two STR loci (in 6 different individuals

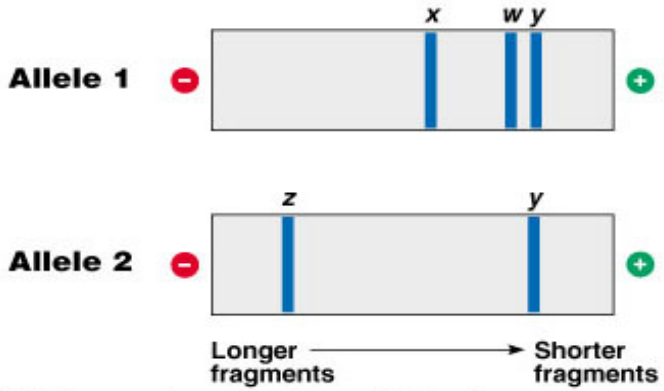


SNPs & RFLPs

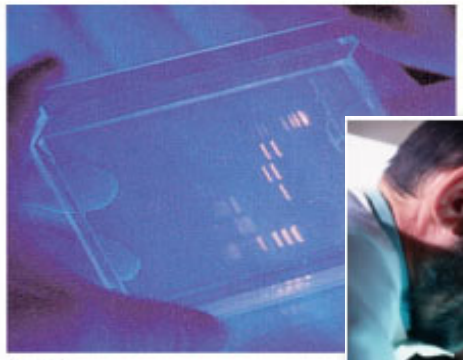
- **Single Nucleotide Polymorphism (SNP pronounced‘snips’)**
  - occur every 100 - 300 bases in humans.
  - They are single base-pair variations
  - May **alter the sequence recognized by a restriction enzyme**
    - Changes the length of restriction fragments formed by digestion
    - This polymorphism (variation) in humans is called **Restriction Fragment Length Polymorphism (RFLP)**



(a) DNA from two alleles

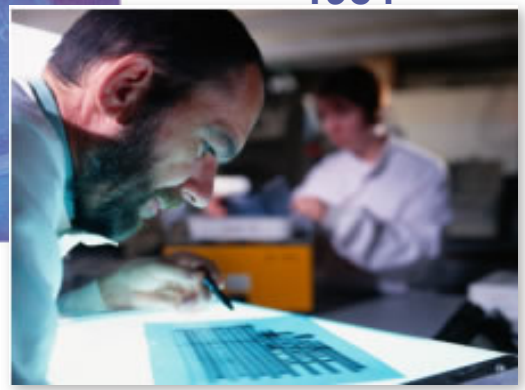


(b) Electrophoresis of restriction fragments



(c) Completed gel

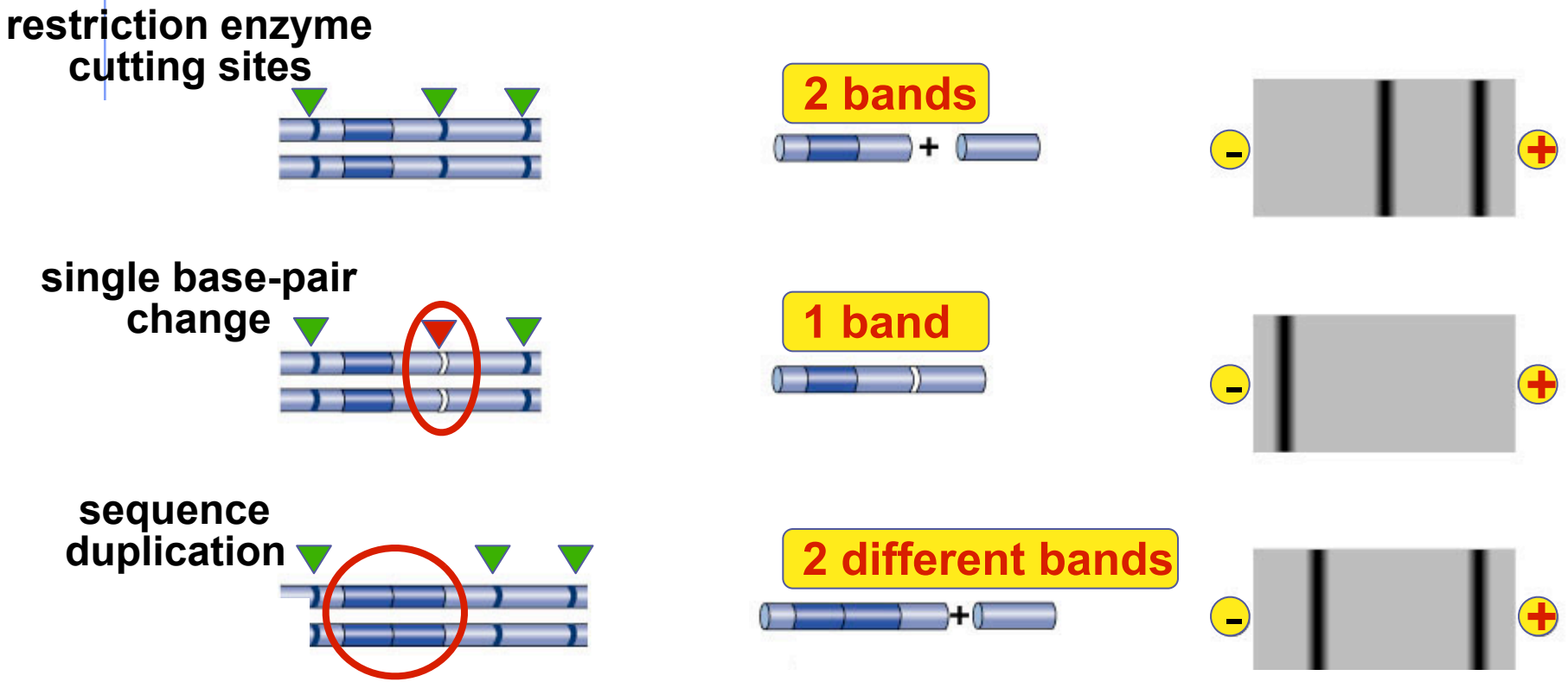
Alec Jeffries
1984



- change in DNA sequence affects restriction enzyme “cut” site
- creates different fragment sizes & different band pattern between different alleles

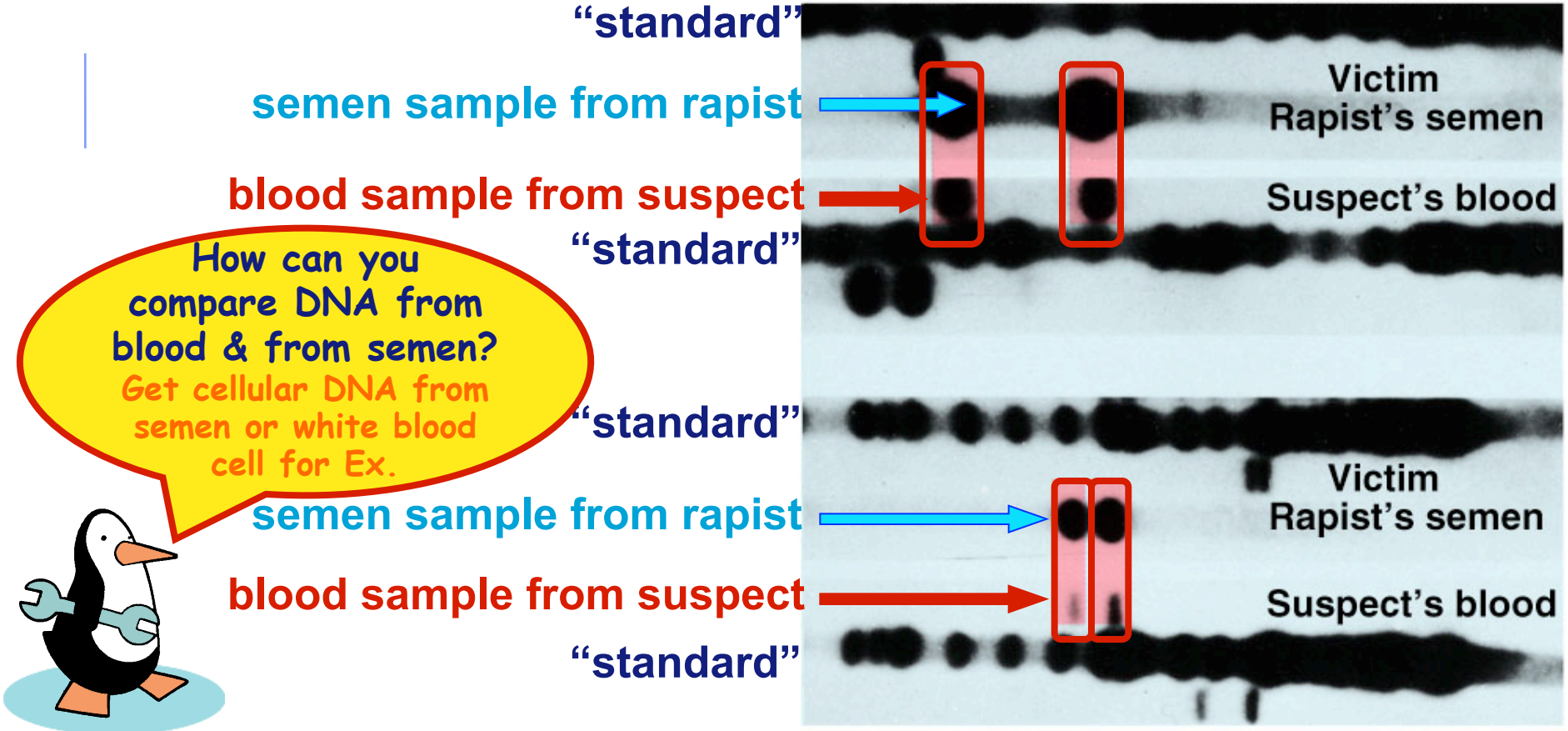
Polymorphisms in populations

- Differences between individuals at the DNA level
  - many differences accumulate in non-coding DNA
    - *these differences lead to PCR amplified fragments or restriction digests fragments of different sizes*



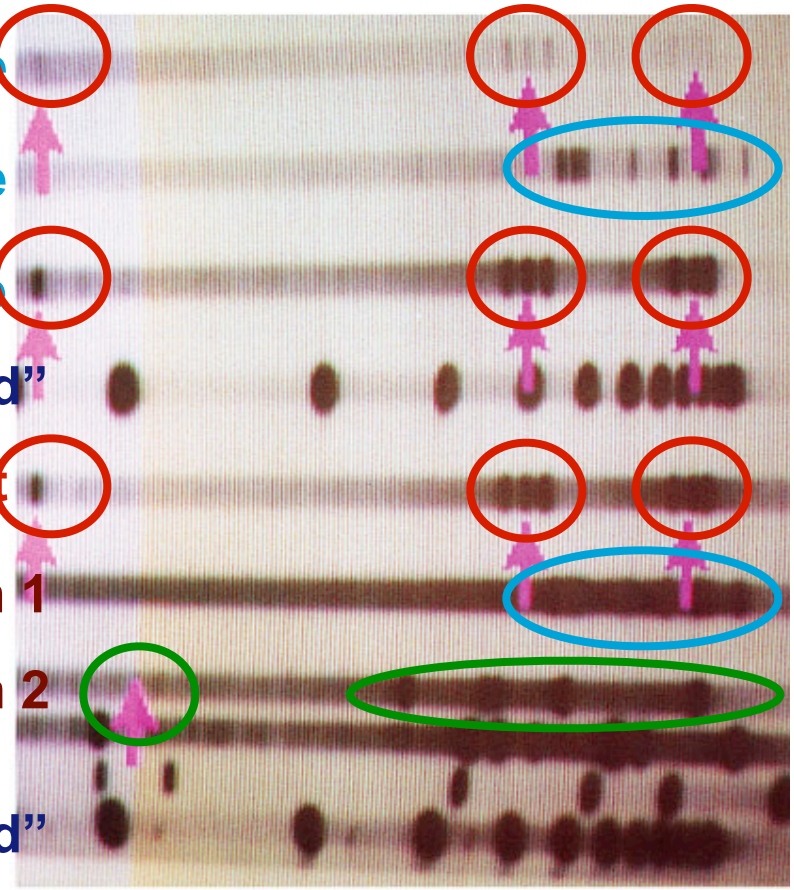
RFLP / electrophoresis use in forensics

- 1st case successfully using DNA evidence
  - 1987 rape case convicting Tommie Lee Andrews
    - In May of 1986, a man entered the Orlando apartment of Nancy Hodge and raped her at knifepoint. During the succeeding months, he raped more women, taking care to keep them from seeing him. In six months, he had raped more than twenty-three women. His fingerprints were found and matched to those from an window screen when reported as a prowler by another woman and he was arrested. 115 years for serial rape.



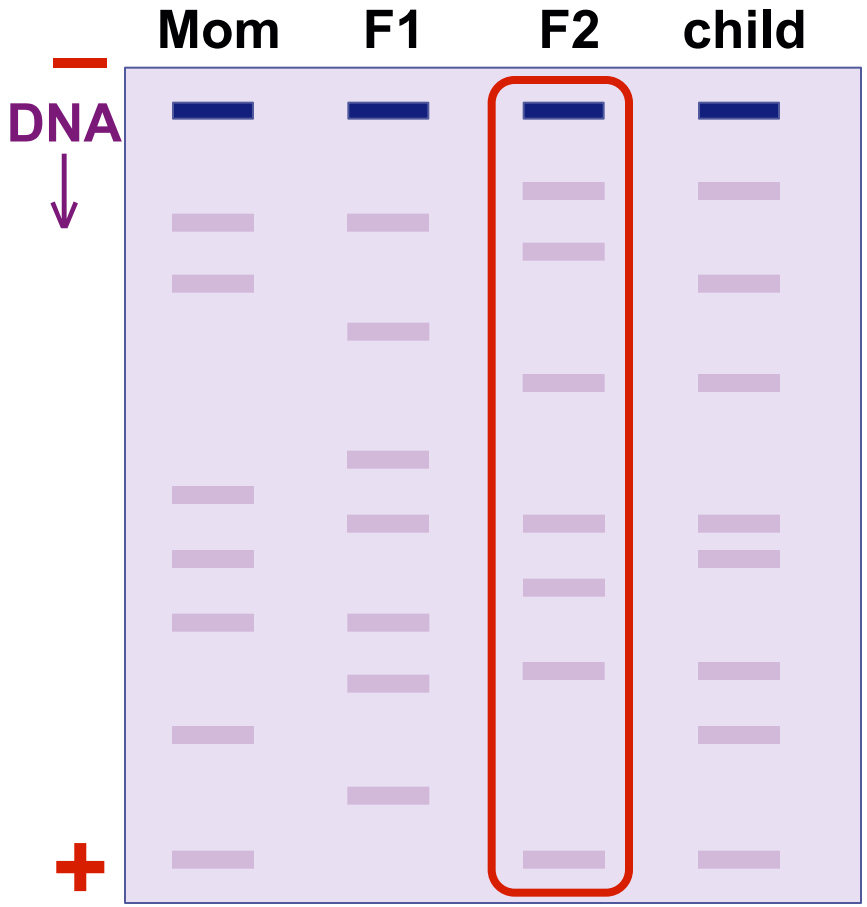
Electrophoresis use in forensics

- Evidence from murder trial
  - Do you think suspect is guilty?

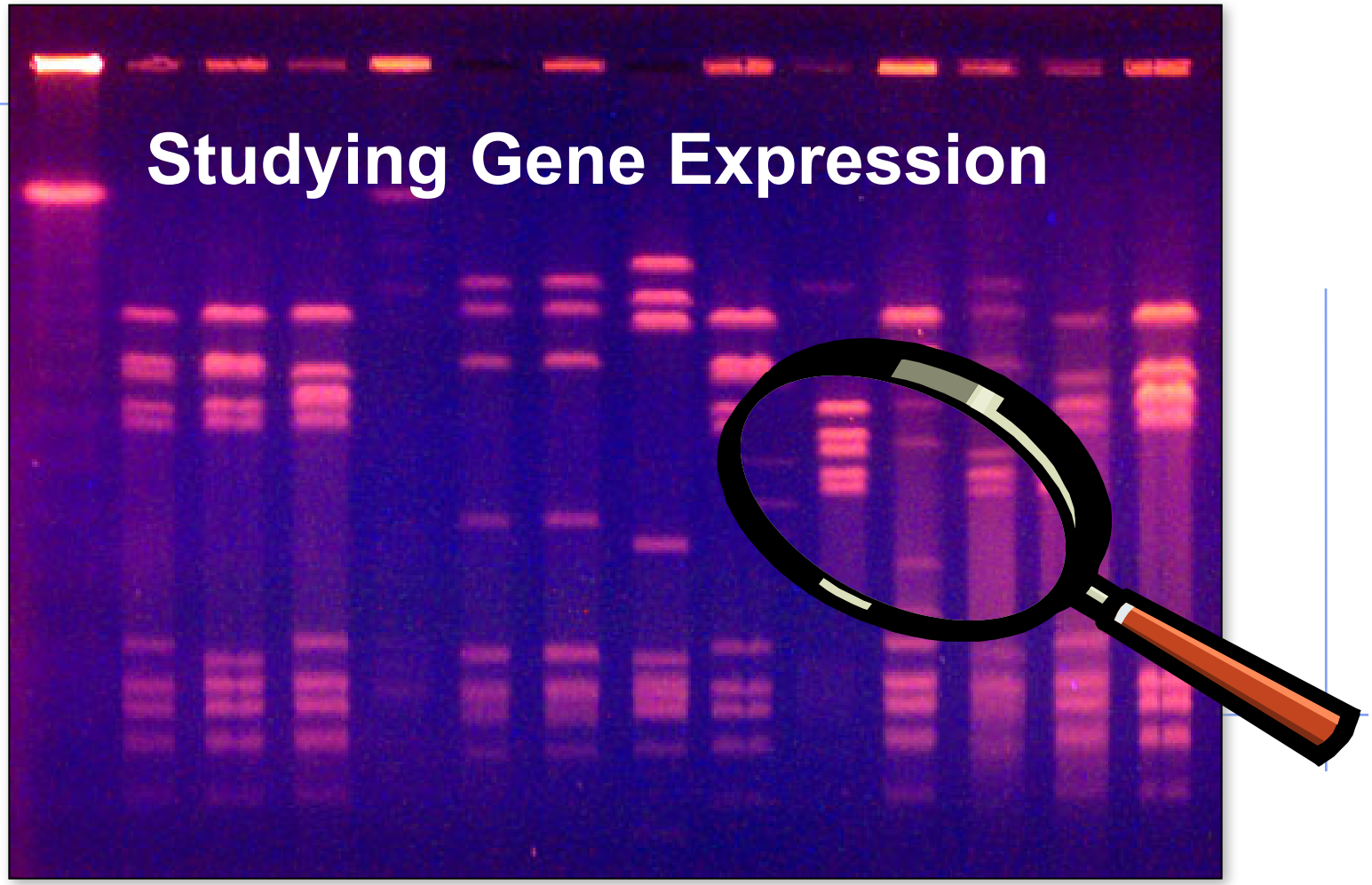


Uses: Paternity Testing

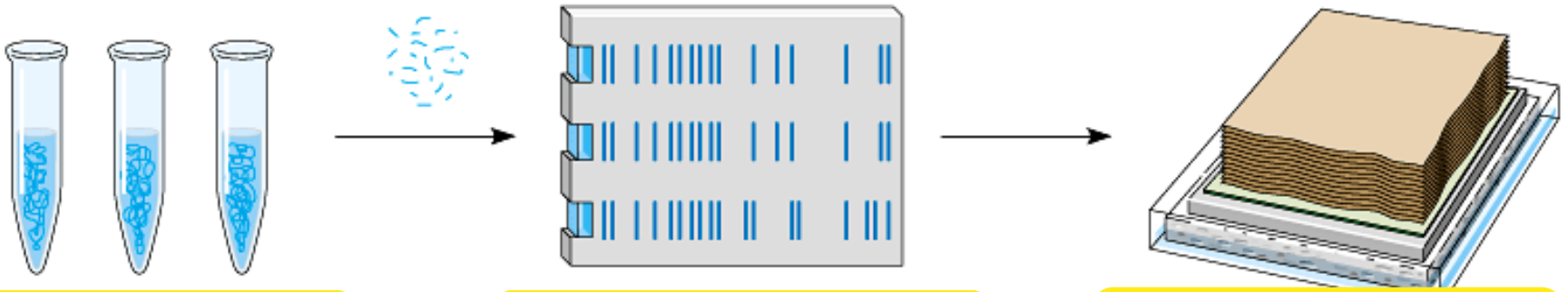
- **Who's the father?**
  - Since the child inherits one set of chromosomes from their biological mother and one from their biological father, the child's DNA should produce a gel banding patter after restriction digestion or multi-loci PCR that matches some of the bands of the mother and some of the bands of the father.



Studying Gene Expression



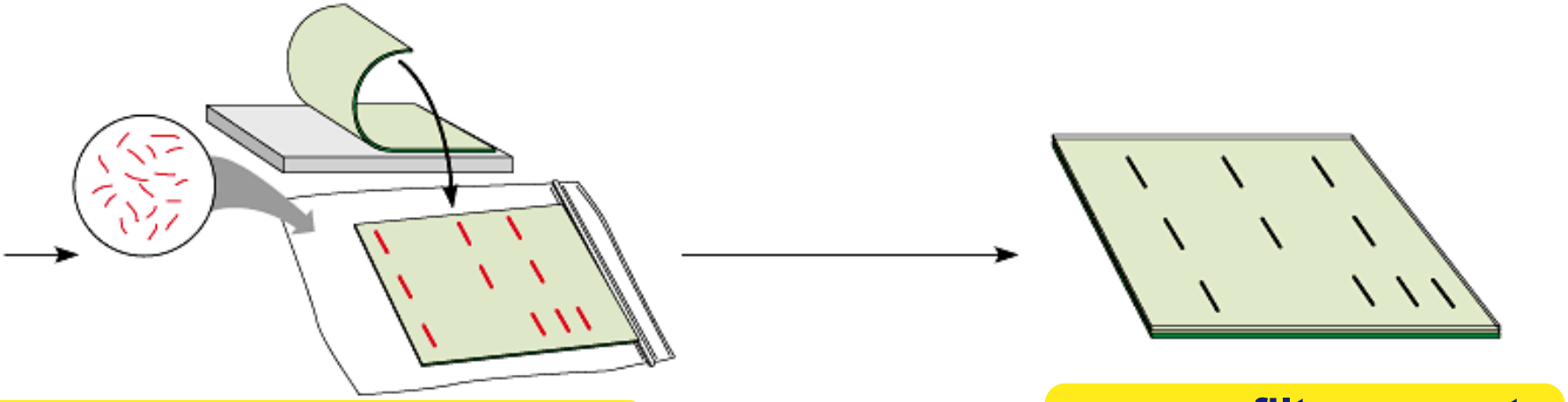
Combining electrophoresis & nucleic acid hybridization = Southern blotting



restriction digest

gel electrophoresis

blot DNA off of gel onto filter paper

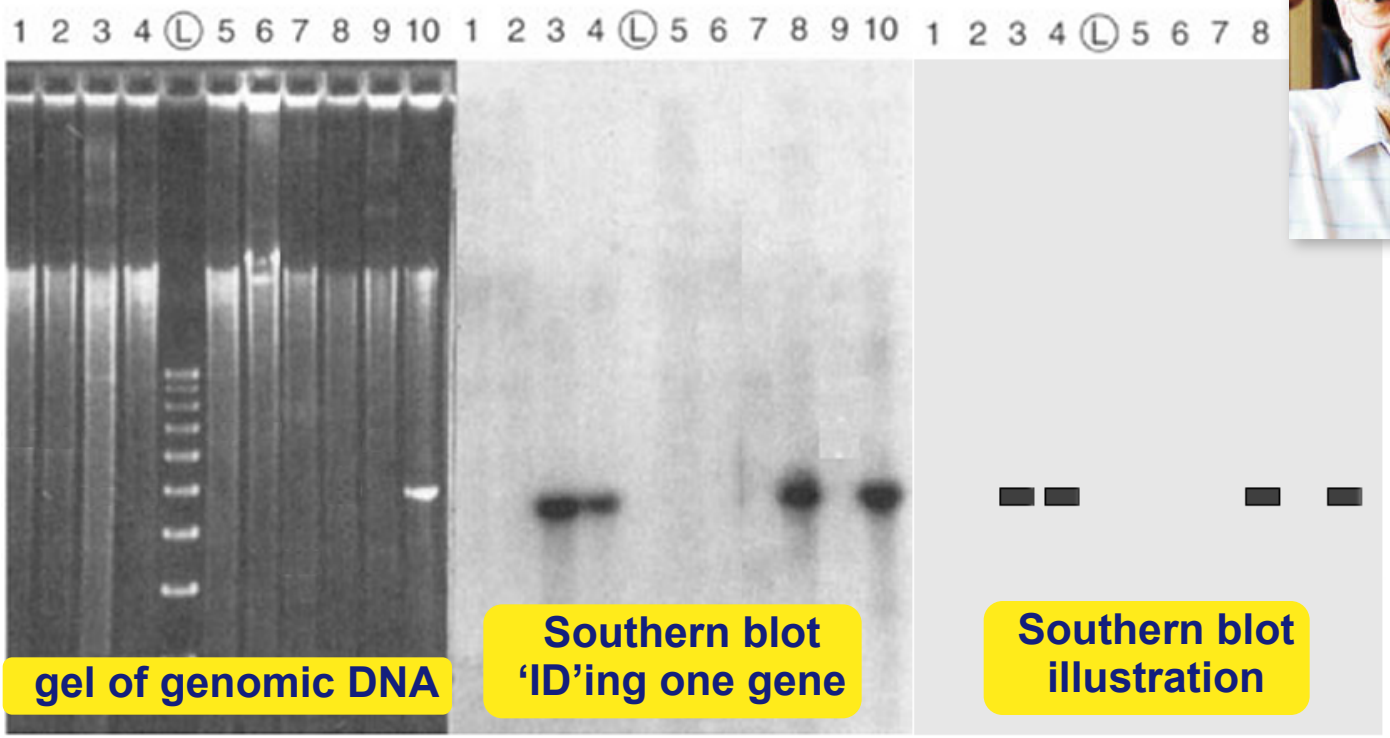


denature DNA fragments & wash filter with labeled probe

expose filter paper to X-ray film

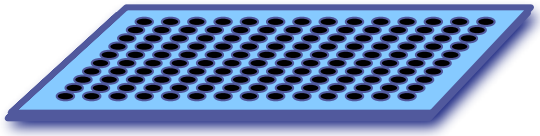
Southern blotting

Edwin Southern



- **Northern blot:** Run RNA on gel, transfer to filter paper & use a DNA probe.
  - Used to measures which genes are expressed because you grab mRNAs from a live cell to run on the gel
    - Represents only expressed genes
- **Western blot:** Run protein on gel, transfer to filter (labeled directly)

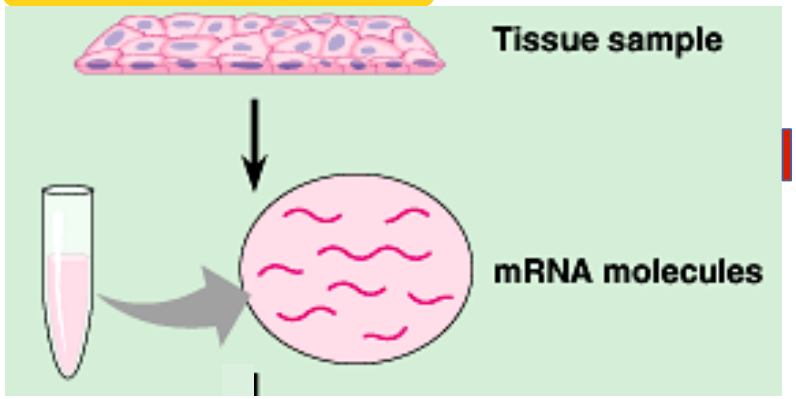
Microarrays



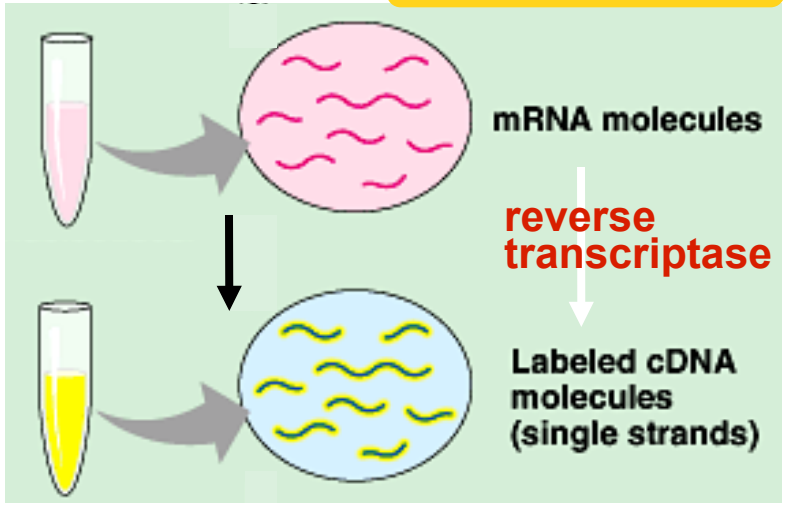
slide with spots of DNA
each spot = 1 gene

- **Used to test thousands of genes simultaneously to determine which ones are expressed in a particular tissue, under different conditions, during certain diseases, or at certain developmental stages.**
  - Create a microchip with a single-stranded samples of DNA genes from the organism
    - each spot represents many copies of one gene
  - Isolate mRNA from tissue
  - Convert mRNA into single stranded cDNA labeled with fluorescently labeled nucleotides.
  - Apply cDNA mix to microarray
  - cDNA hybridizes with complimentary DNA on the array
  - Rinse off excess DNA
  - Scan microarray for fluorescence.
  - Fluorescing spots represent genes being expressed at that moment in time

mRNA from cells

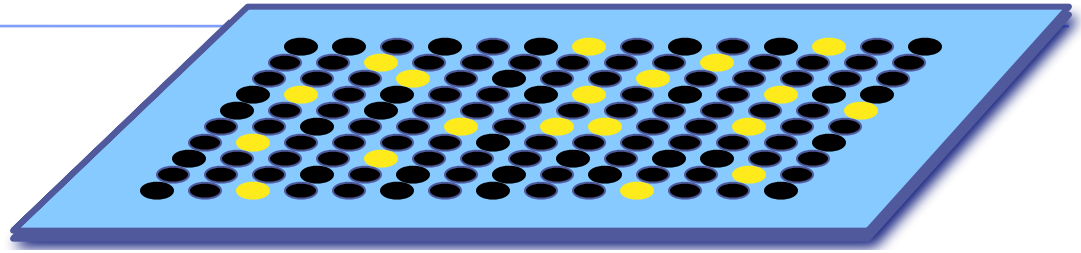


mRNA → cDNA



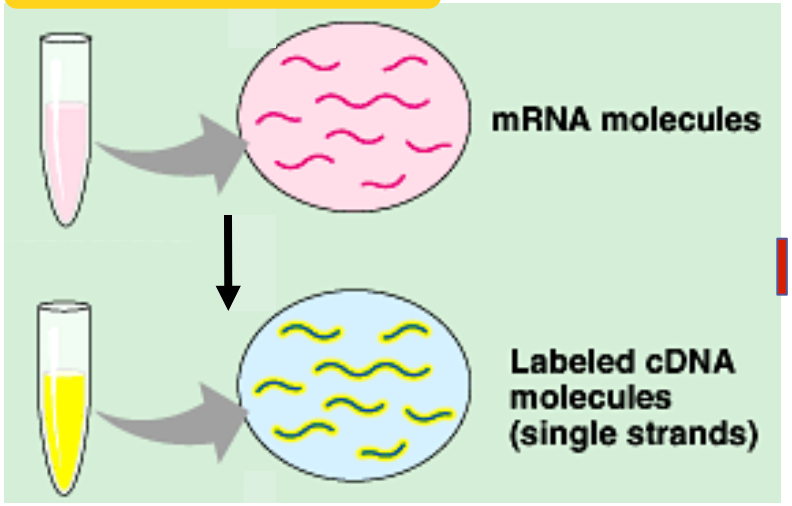
Microarrays

slide with spots of DNA
each spot = represents 1 gene

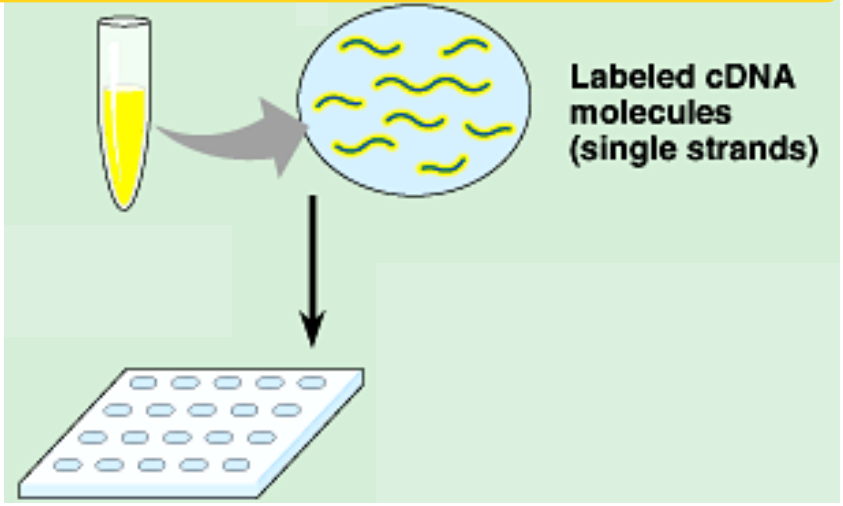


- Fluorescent labeled cDNA made from the mRNA found in the cell hybridizes with ssDNA on slide when the two pieces of nucleic acid are complimentary
  - each yellow spot = cDNA hydrogen bonding with ssDNA in that region o the microarray, representing a gene in the genome of the organism
  - so each yellow spot = highlights an expressed gene in that tissue

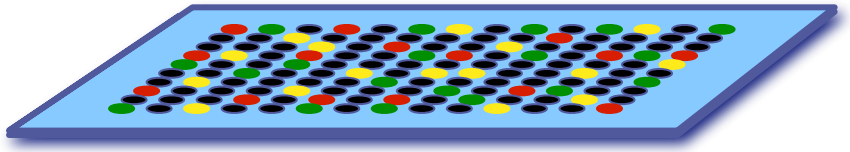
mRNA → cDNA



cDNA matched to genomic DNA

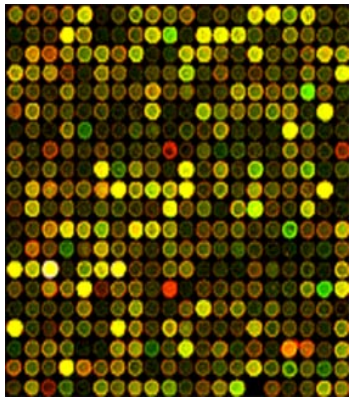


Application of Microarrays "DNA Chip"

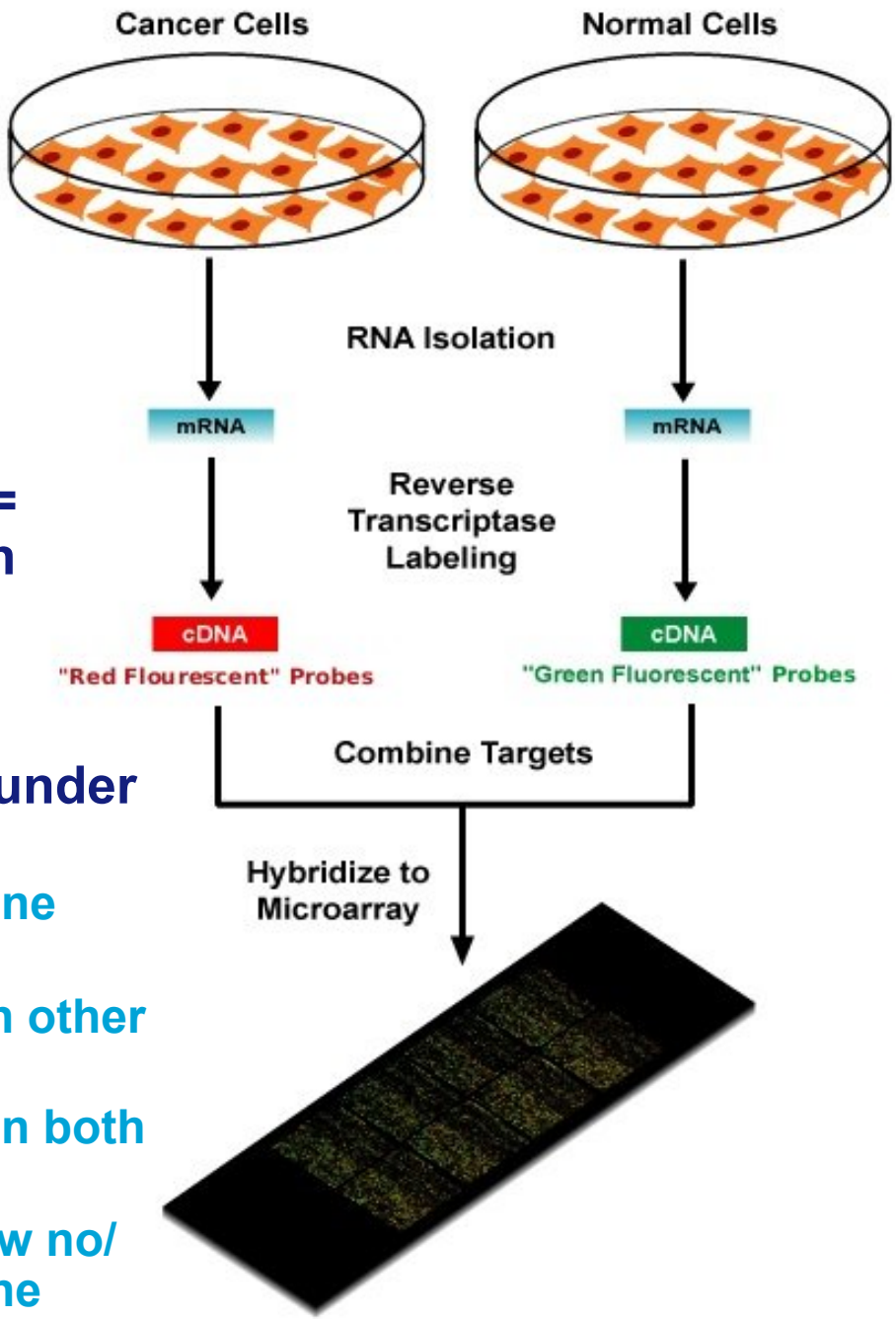


2-color fluorescent tagging

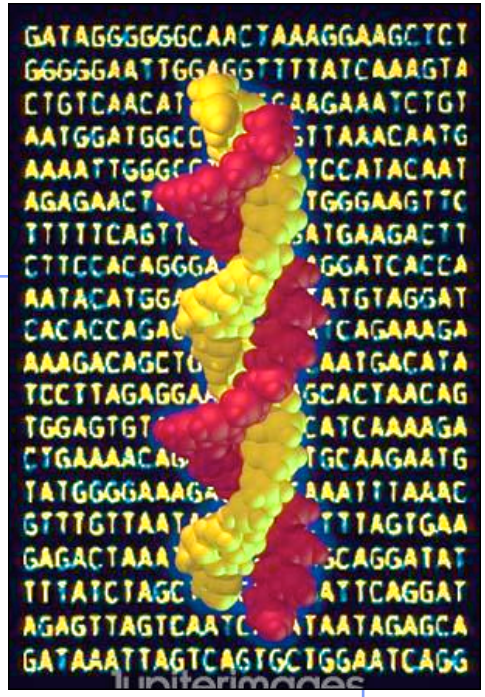
- **Comparing treatments or conditions = Measuring change in gene expression**
  - **sick vs. healthy organism/cells**
  - **before vs. after treatment with drug**
  - **different stages in development**
- **Color coding: label mRNA from cells under each condition with different color**



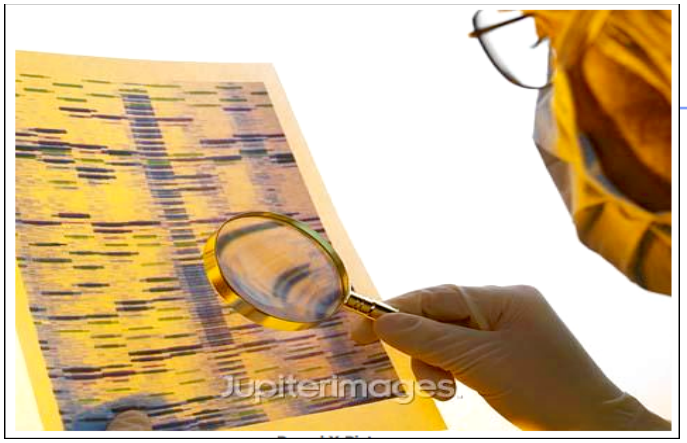
- **red = gene expressed in one sample**
- **green = gene expressed in other sample**
- **yellow = gene expressed in both samples**
- **black = both samples show no/ low expression of that gene**

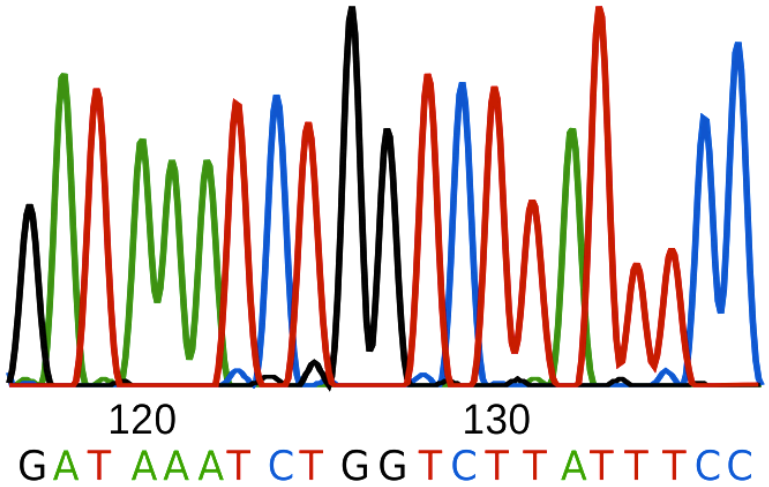


What is you want to figure out the nucleotide arrangement in a piece of DNA?



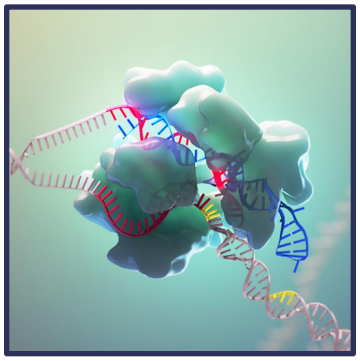
DNA Sequencing - Technique for determining the order of nucleotides in fragment strand of DNA.





The CRISPR-Cas9 Revolution

- CRISPR/Cas9 is a type of "immune system" discovered in bacteria.
- Scientists have adapted its components into a biotechnology tool for cutting and editing DNA.
  - Made up of a DNA-cutting (nuclease) enzyme (Cas9) and a programmable ssRNA molecule (guide RNA), CRISPR-Cas9 can be used to precisely target nearly any gene.
  - Review videos and tutorial to learn how it works:
    - https://youtu.be/2pp17E4E-O8
    - https://youtu.be/6tw_JVz_IEc
    - https://youtu.be/4YKFw2KZA5o
    - https://media.hhmi.org/biointeractive/click/CRISPR/



I'm a-glow!

Got any Questions?