Recombinant DNA (rDNA) Technology

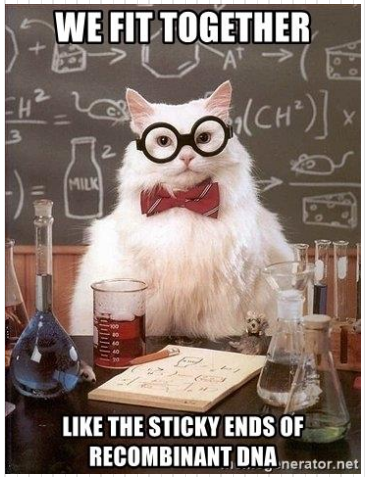
SLE254 Genetics and Genomics

Chapter 19 Concepts of Genetics (11th edition)

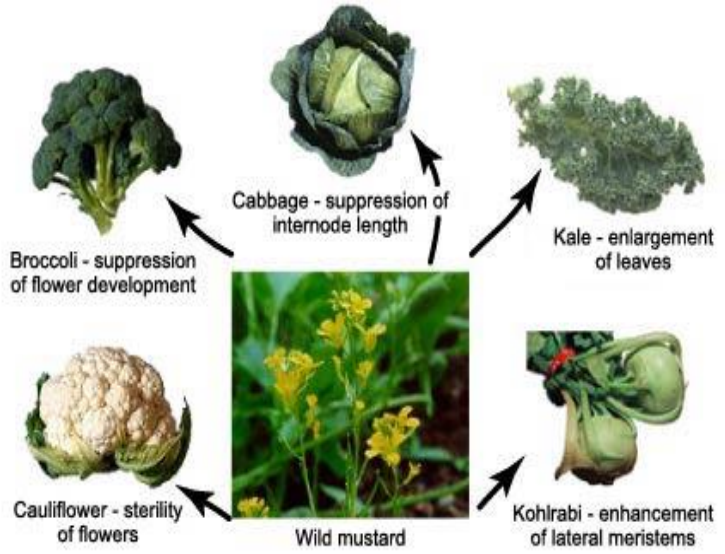
Pages 523-551

12th ed

Pages 493-521

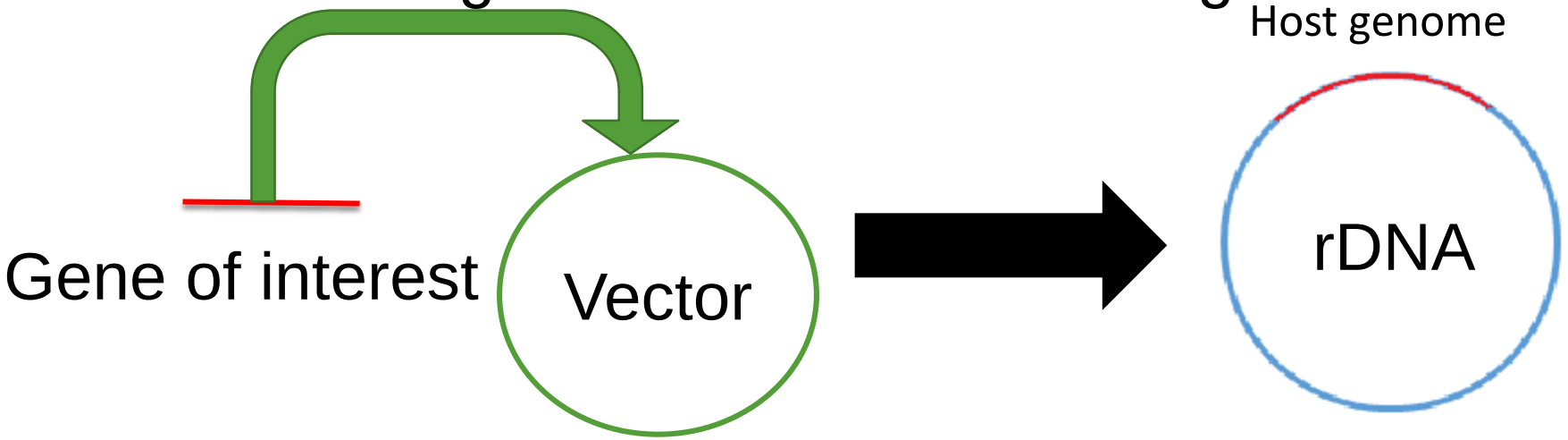


Genetic modification by means of **artificial selection** and **selective breeding** preceded genetic engineering



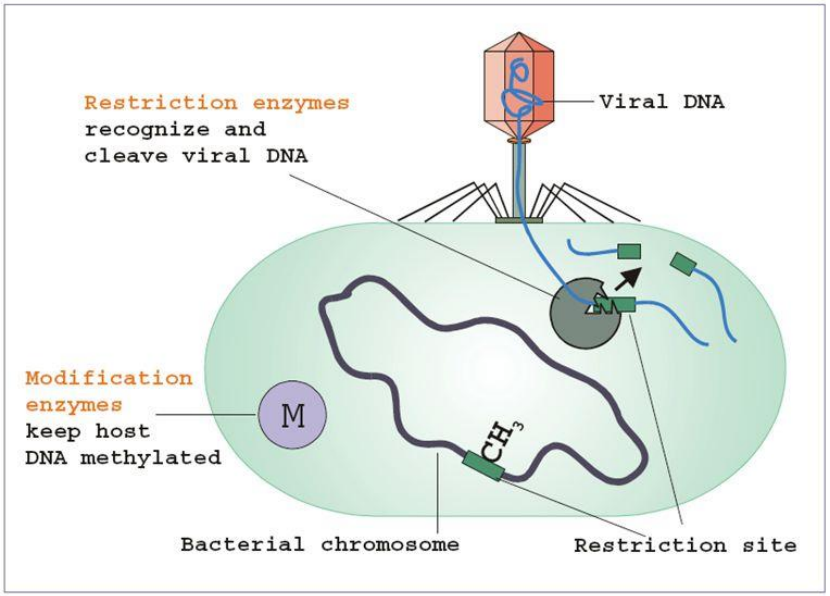
What does the “r” mean

- rDNA technology allows us to **artificially** combine DNA sequences from different sources
- Allows us to “clone” a sequence or gene of interest into vectors and allowing their transfer to a new genome to introduce the genetic trait

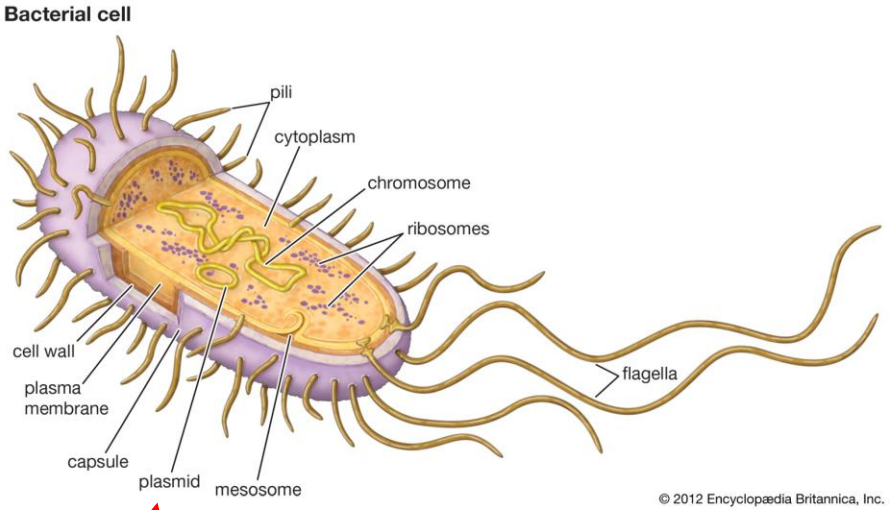


Restriction Enzymes

Bacterial defense against viral infection by restriction-modification complexes



Natural plasmids: free replicating circular DNA

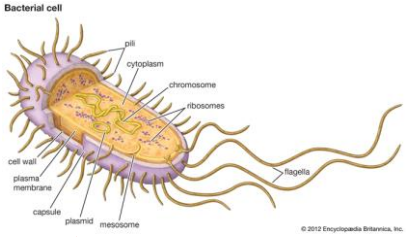


- **Restriction enzymes** exist naturally in bacteria to attack an chop viruses up into smaller and non harmful pieces
- Plasmids exists naturally in bacteria separate from the bacterial DNA: they self replicate, contain an origin of replication and usually important genes for the bacteria's survival such as antibiotic genes

https://slideplayer.com/slide/5670802/

rDNA technology relies on synthetic manipulations of these natural systems

Product No.	Name	Compatibility
E6783	pFLAG-CMV™-3 Expression Vector	Bacteria, mammalian cells
E7658	p3XFLAG-CMV™-10 Expression Vector	Bacteria, mammalian cells
E7283	p3XFLAG-myc-CMV™-26 Expression Vector	Bacteria, mammalian cells
E9033	pFLAG-Myc-CMV™-22 Expression Vector	Bacteria, mammalian cells
E6908	pFLAG-CMV™-5.1 Expression Vector	Bacteria, mammalian cells
E9408	p3xFLAG-Myc-CMV™-25 Expression Vector	Bacteria, mammalian cells
FLMAS	Mammalian Amino-terminal FLAG Stable Expression Kit	Bacteria, mammalian cells
E8158	pFLAG-ATS™ Expression Vector	Bacteria
E8408	pFLAG-CTC™ Expression Vector	Bacteria
D3404	pUC19 plasmid DNA from *E. coli* RRI	Bacteria
D4154	pUC18 plasmid DNA from *E. coli* RRI	Bacteria



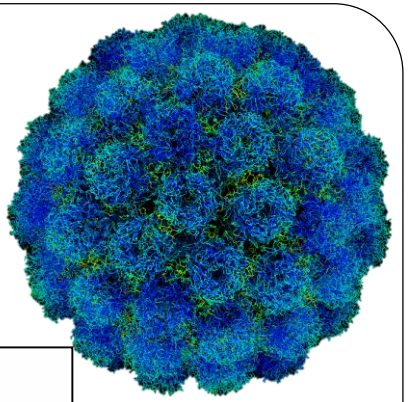
Plasmid vector

https://www.sigmaaldrich.com/technical-documents/articles/biology/blue-white-screening.html

rDNA technology: Cloning DNA

1. LacZ mutant *E.coli* (bacteria)
2. vector (genetically altered plasmid)

How it all began…



Proc. Nat. Acad. Sci. USA
Vol. 68, No. 12, pp. 2913–2917, December 1971

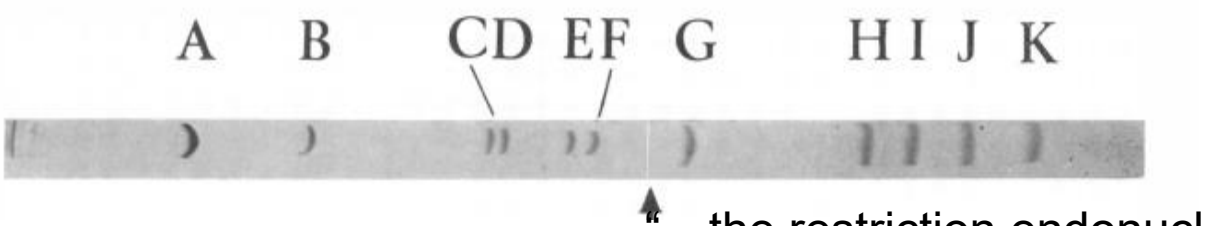
Specific Cleavage of Simian Virus 40 DNA by Restriction Endonuclease of Hemophilus Influenzae*

(gel electrophoresis/electron microscopy/DNA mapping/DNA fragments/tumor virus)

KATHLEEN DANNA AND DANIEL NATHANS

Department of Microbiology, The Johns Hopkins University School of Medicine, Baltimore, Maryland 21205

Communicated by Albert L. Lehninger, September 22, 1971

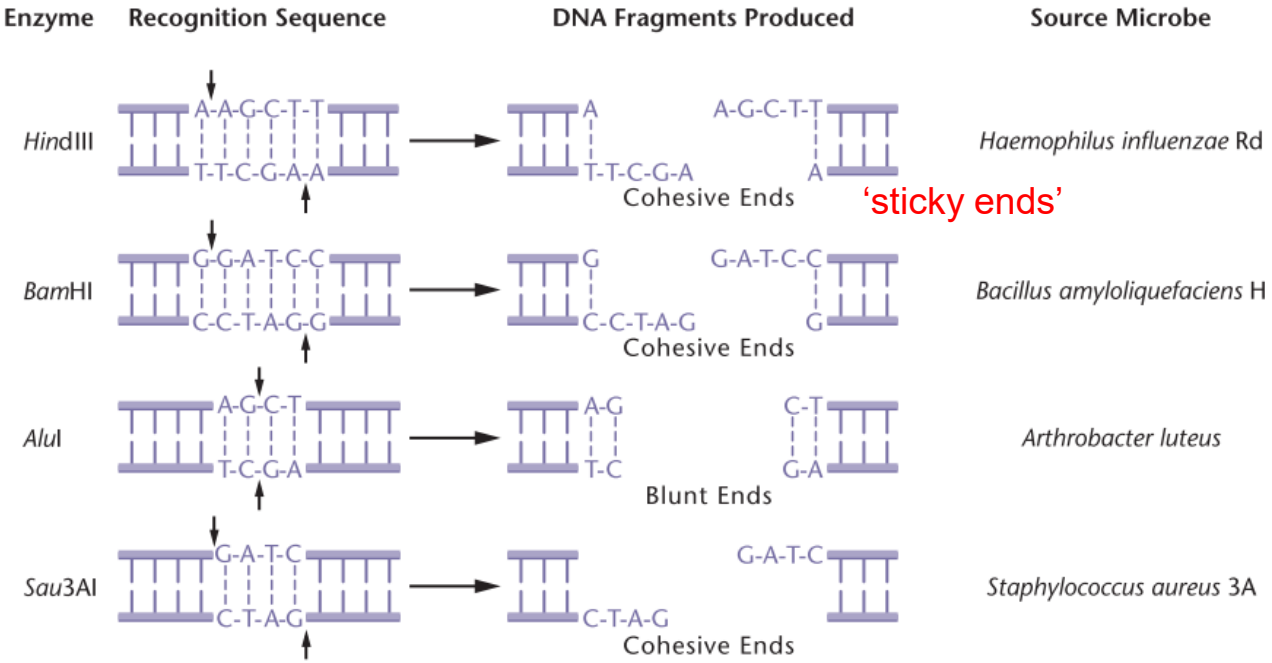


"…the restriction endonuclease should prove of general usefulness in the analysis of DNA, much as highly specific proteolytic enzymes have been used in the analysis of proteins."

Critical tools for rDNA: Restriction Enzymes

- Specific bacterial **endonucleases** evolved as a defence mechanism against **phage**- **natural enzymes**
- Cut within DNA at a particular **restriction site** by identifying a **recognition sequence**

First identified and commercially used



Critical tools for rDNA: DNA vectors

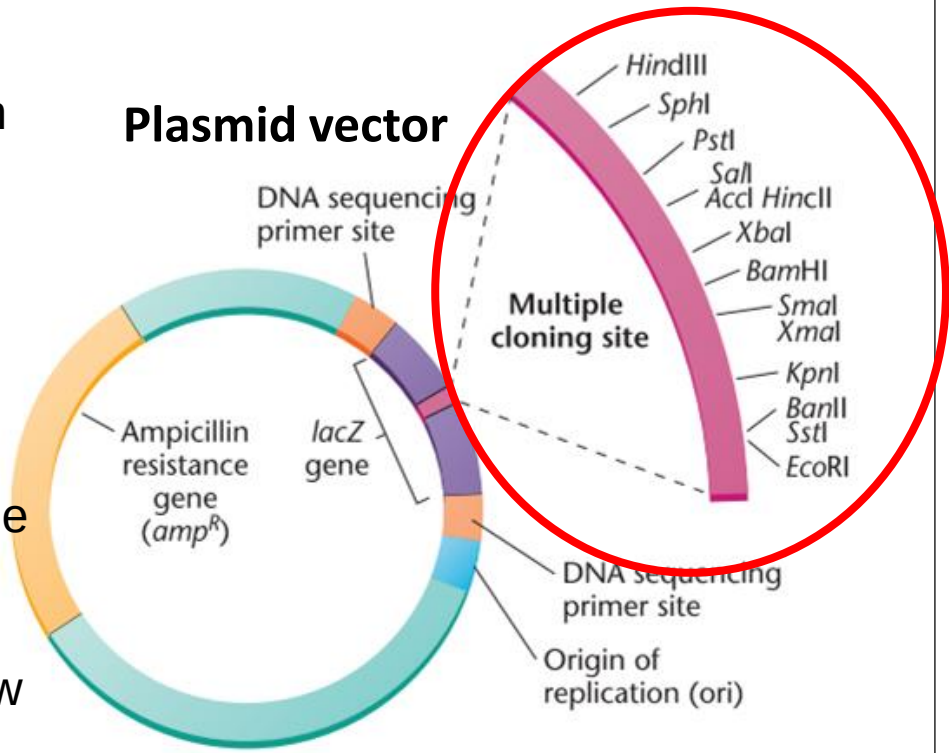
- **Vector: DNA molecules that can be used to artificially incorporate and replicate the target sequence of interest into another cell- bacteria**

- A vector contains several restriction sites that allow insertion of the DNA fragments to be cloned.

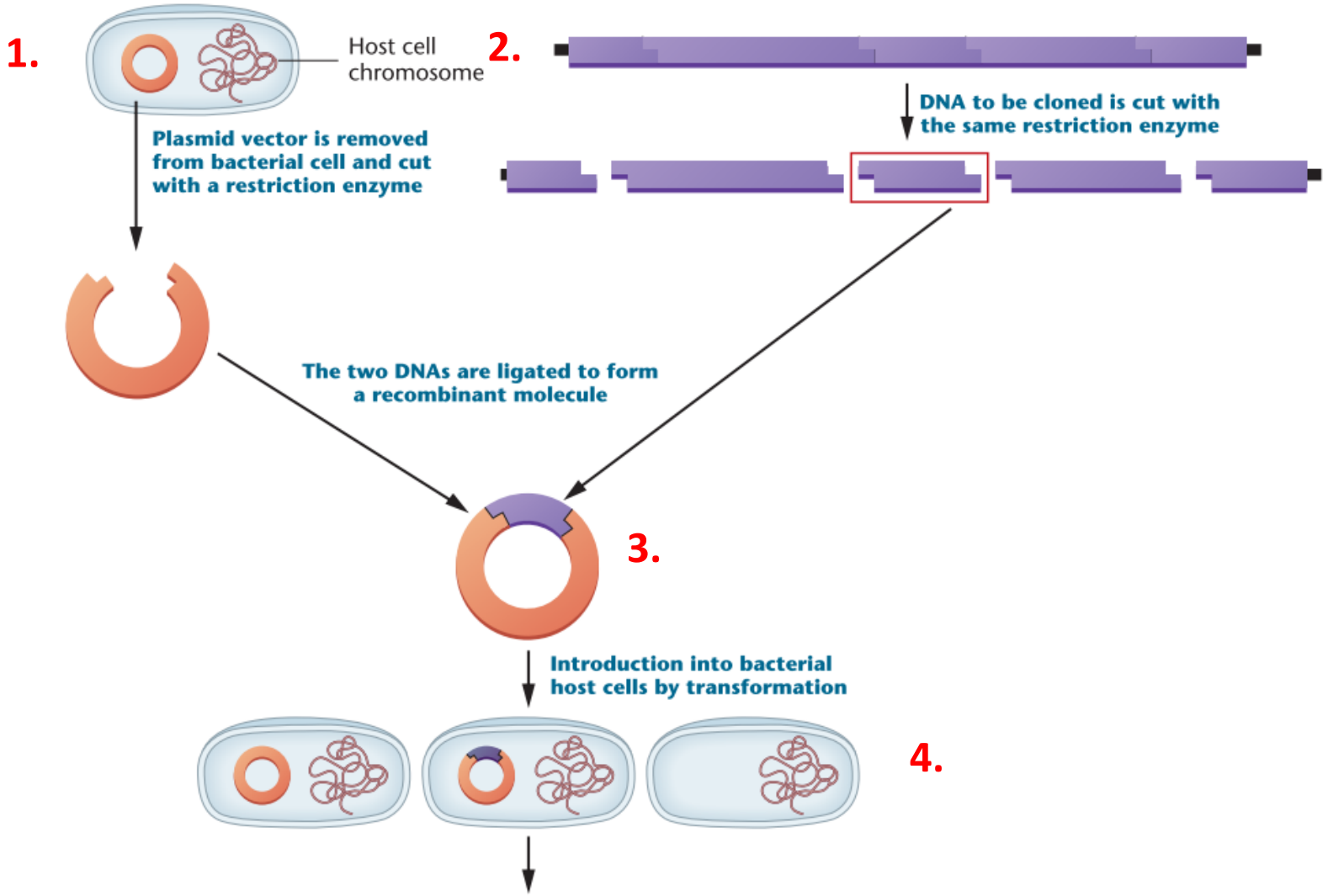
- Vectors must be capable of replicating in host cells to allow for independent replication of the vector DNA and any DNA fragment it carries.

- To distinguish host cells that have taken up vectors from host cells that have not, the vector carry a selectable marker gene (antibiotic resistance gene)

- Incorporate specific sequences that allow for sequencing inserted DNA



How to create a GMO:



Competent Cells

- Bacterial cells that are made **competent** are more likely to take up plasmid containing the **foreign DNA**
- Cells are made competent in different ways based on the transformation protocol to be utilised later

Transformation protocols

1. Heat shock method

- Utilises initial cooling on ice followed by a incubation at high temperature (37-42 °C)
- Cells are made competent with $CaCl_2$

2. Electroporation method

- Utilises a high voltage to **permeabilised the membrane** to allow uptake of foreign DNA
- Cells are prepared in log phase with diminished salt concentration (10% glycerol)

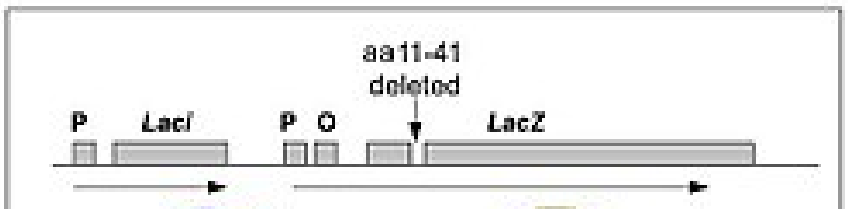
Blue white screening relies on several factors:

1. Identifying recombinant bacteria (mutant *E. coli*)

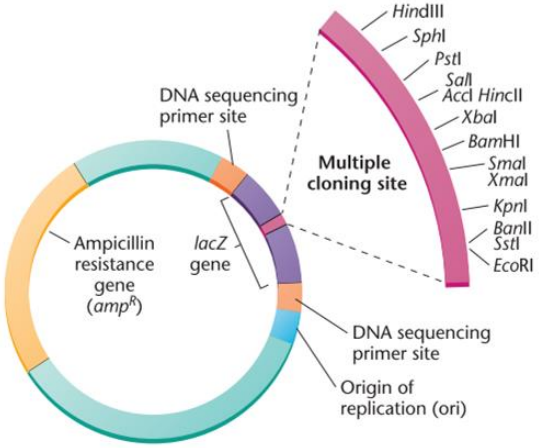
-this relies on the activity of the *E. coli* β-galactosidase enzyme

2. Disrupting the LacZ gene

***E. Coli* lacZΔM15 deletion mutation cells**



ARTIFICAL PLASMID WITH SECTION OF LACZ



Selection: Enriching transformed cells

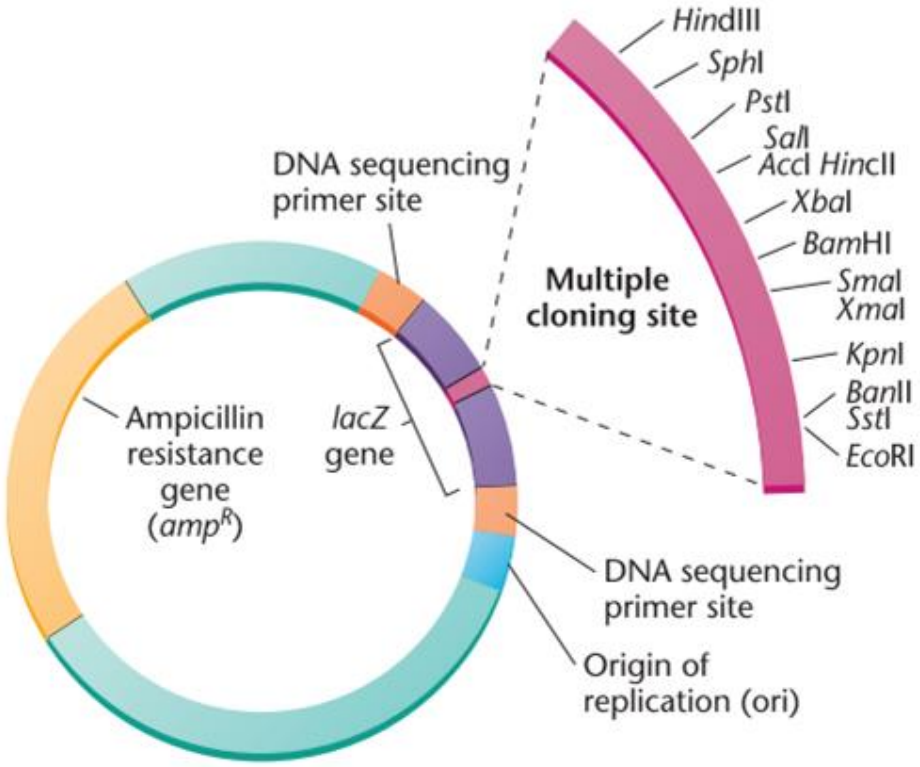
- **Antibiotic selection marker: Ampicillin, Kanamycin**

  Ampicillin-resistance gene (amp^r) encodes β-lactamase, which inactivates the antibiotic ampicillin.

  E. coli that take up the plasmid can be easily selected from those cells that do not by growing them in an ampicillin-containing medium.

- Blue-White Screening

LacZ:
β-galactosidase enzyme that metabolizes lactose- **the cloned DNA will be inserted in this gene and disrupt the function**

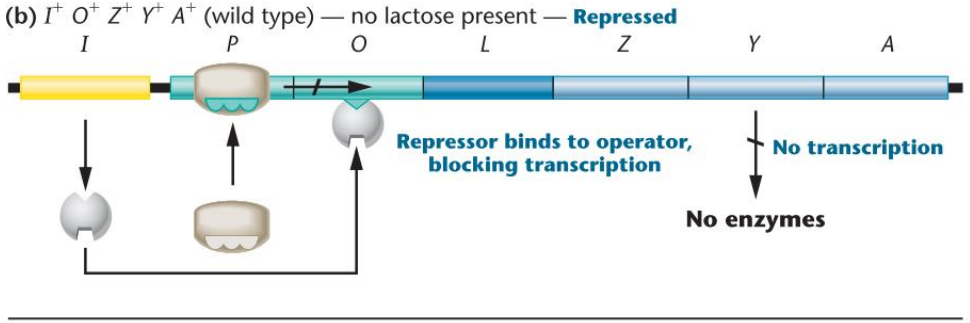


The Lac operon

Lac Z codes for β-galactosidase enzyme which cleaves lactose into it's disaccharides galactose and glucose

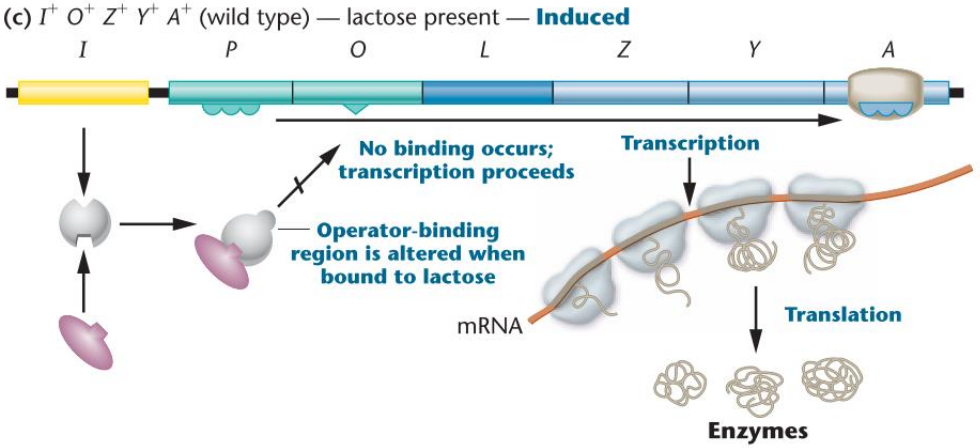
Alone- the mutant ***E. Coli*** **lacZΔM15 cannot produce** β-galactosidase

Remember LacZ operon



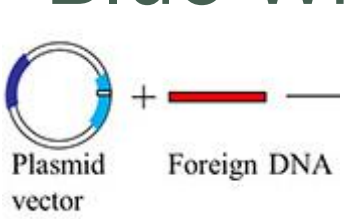
Background

- Presence of lactose triggers the lacZ operon in *E. coli.*



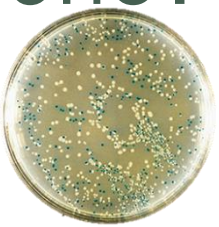
Negatively controlled gene that is induced by the environment and the removal of a repressor = *negative inducible operon*

Blue-White Screening- how is it done?



Transformatic

Culture with X-Gal+IPTG



Screening

Background:

- Presence of lactose in the medium triggers the lacZ operon in *E. coli*

- Operon activity leads to the production of β-galactosidase enzyme that metabolizes the lactose

- **The *E. coli* are competent cells containing lacZΔM15 deletion mutation (truncated β-galactosidase produced)**

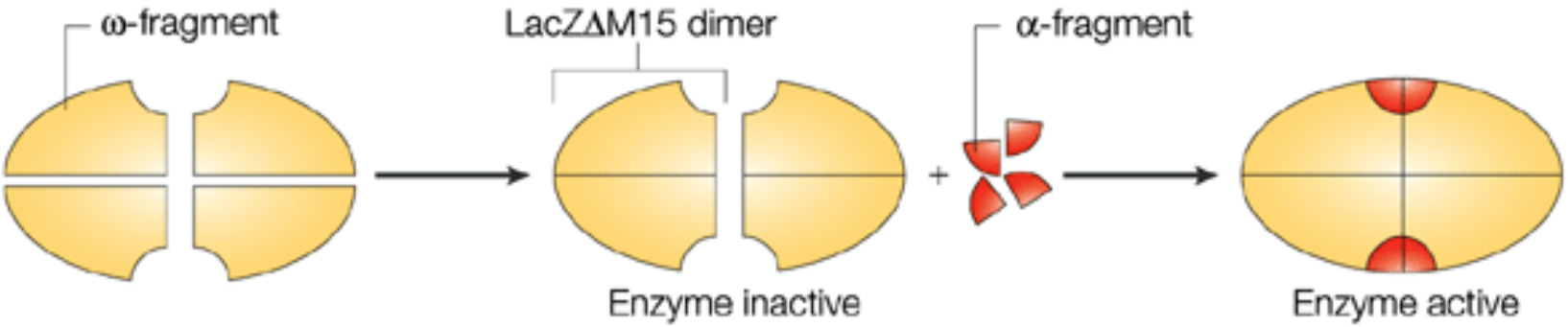
- Plasmid vectors carry a short segment of lacZ gene (only the first 146 amino acids of β-galactosidase)

- **When the plasmid vector is taken up by such cells, due to α-complementation process, a functional β-galatosidase enzyme is produced**

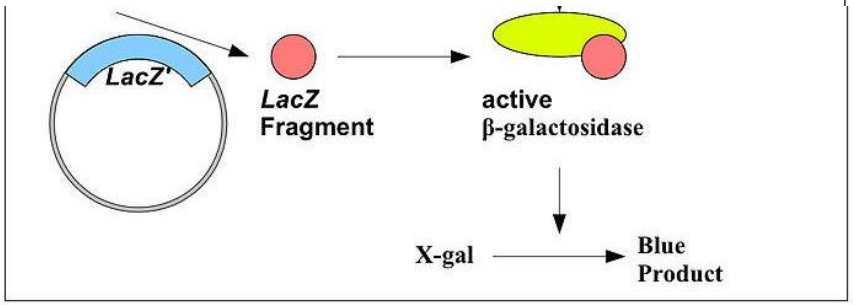
What is α-complementation?

- **The *E. coli* are competent cells containing lacZΔM15 deletion mutation (truncated β-galactosidase produced)**

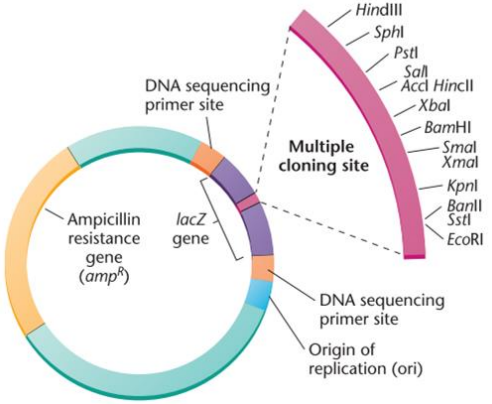
The ***lacZα*** sequence is inserted into the **plasmid**



Result of the peptide produced by the *E. coli* mutant-
It cannot form a dimer and active enzyme



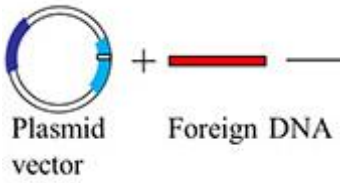
Blue-White Screening



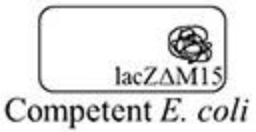
Background cont.:

- Multiple cloning sites (MCS) are present in the lacZ sequence of the plasmid
- Foreign DNA can be inserted by nicking the MCS by restriction enzymes
- Foreign DNA interrupts the lacZ gene in the plasmid vector = no β-galactosidase fragment produced
- Plasmid with foreign DNA taken up by the host *E. coli*
- α-complementation does not occur = no functional β-galactosidase enzyme is produced
- If **foreign DNA is not inserted** or **inserted outside of the lacZ/MCS**, the lacZ gene will function in the plasmid vector and its product will complement the lacZ deletion mutation in the host *E. coli* producing a functional β-galactosidase enzyme.

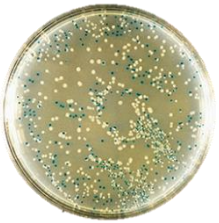
Blue-White Screening



Transformation



Culture with X-Gal+IPTG

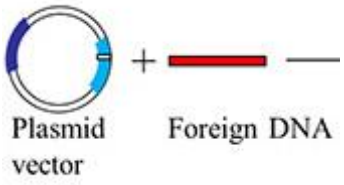


Screening

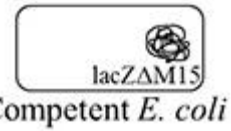
Screening

- A chromogenic substrate known as X-gal is added to the agar plate
- When β-galactosidase is produced X-gal is hydrolyzed, resulting in an insoluble blue pigment
- Colonies that haven't taken up the foreign DNA (non-recombinant cells) and hence produce β-galactosidase, appear blue in colour
- Colonies that contain the foreign DNA (recombinant), and hence do not produce β-galactosidase, appear white
- The desired recombinant colonies can be easily picked and cultured

Blue-White Screening



Transformatic

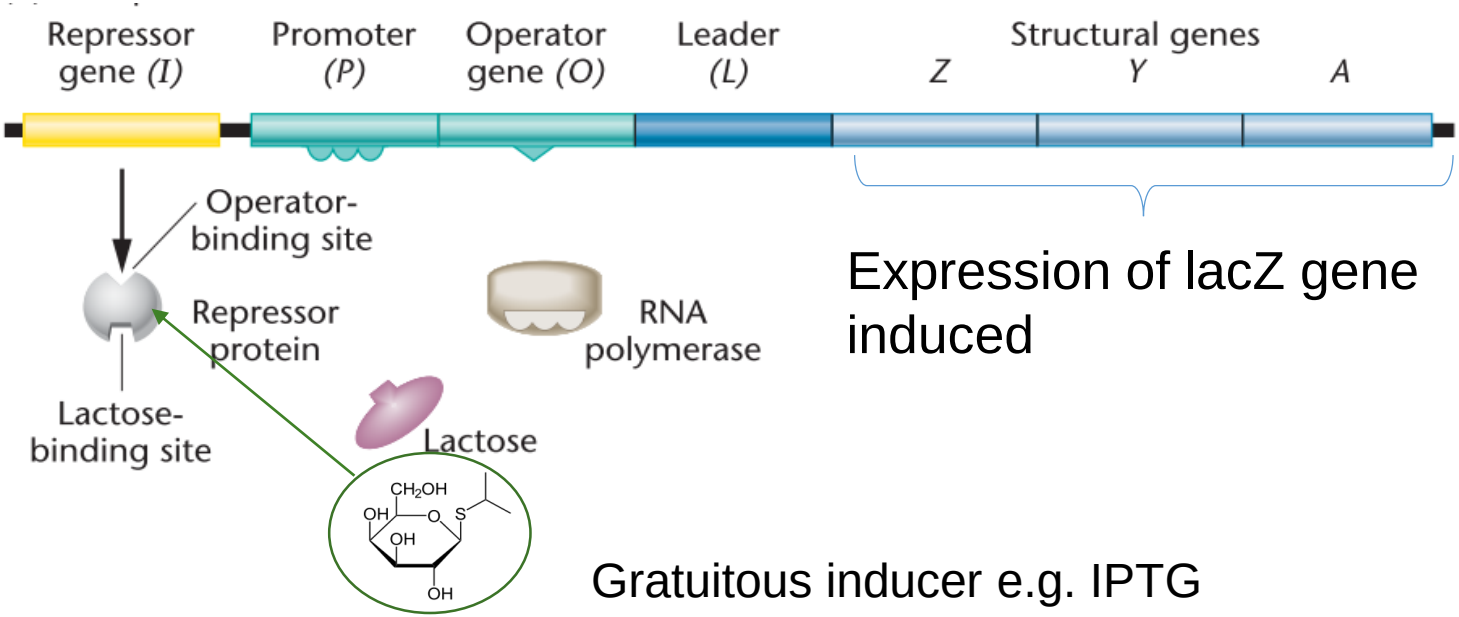


Culture with X-Gal+IPTG

Screening

Screening

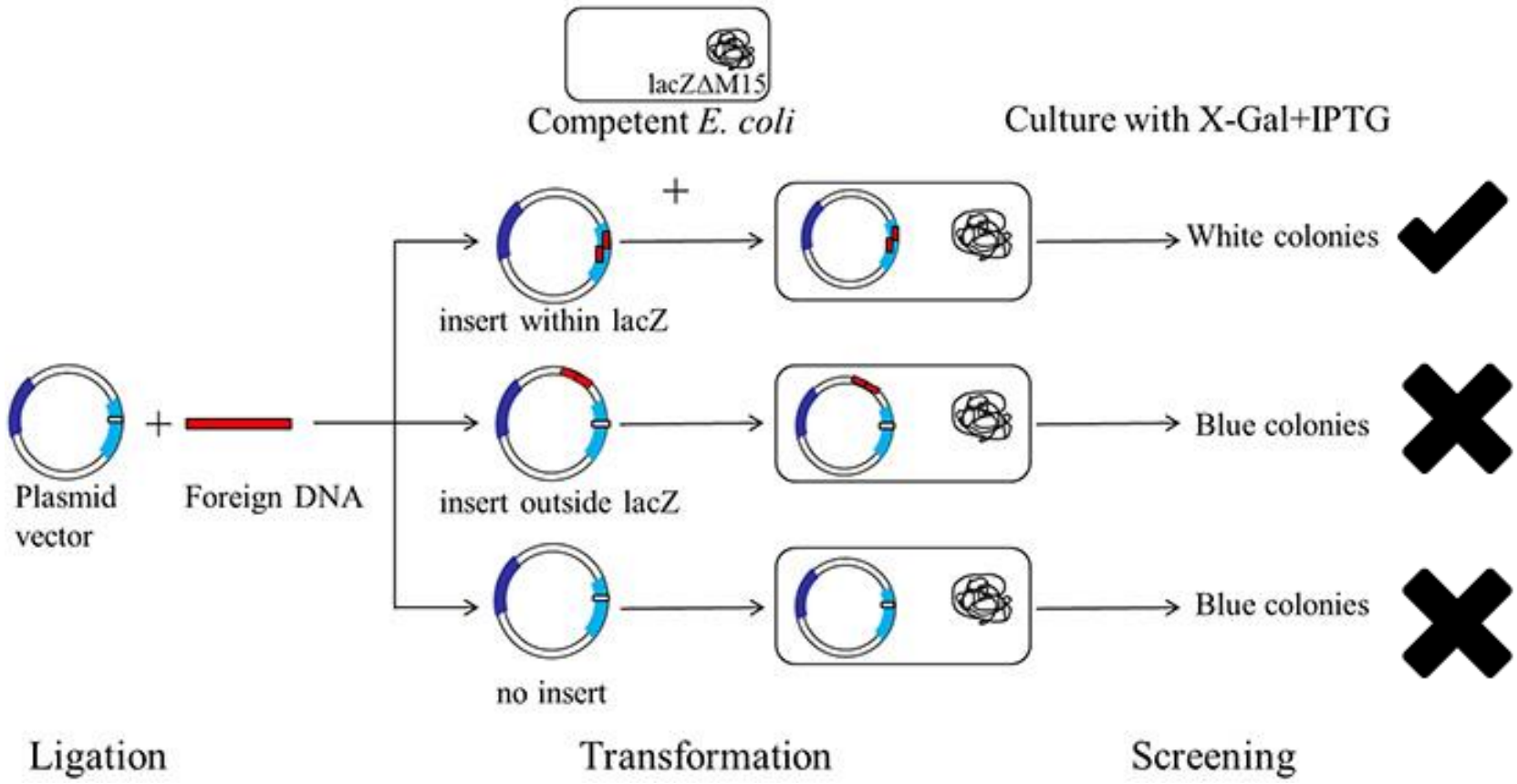
- Gratuitous inducer (IPTG) is used with X-gal for blue-white screening
- IPTG is a non-metabolizable analogue that induces the expression of lacZ gene
- IPTG is **not a substrate for β-galactosidase but only an inducer**



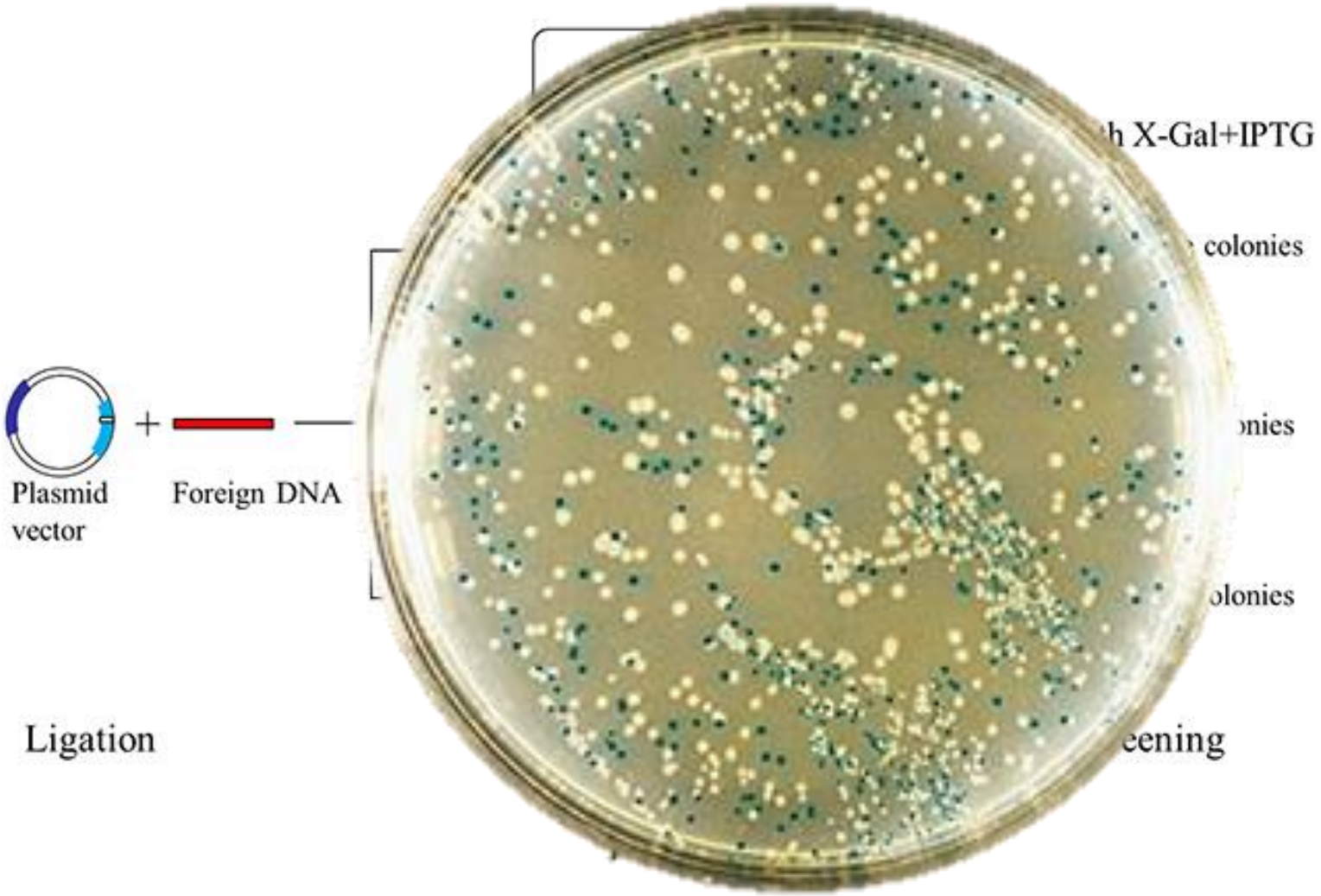
Expression of lacZ gene induced

Gratuitous inducer e.g. IPTG

Summary: Blue-White Screening



Blue-White Screening



Types of cloning vectors

- Bacterial plasmids
- Phage vector systems: Λ phage
- Bacterial Artificial Chromosomes (BACs) and Yeast Artificial Chromosomes (YACs)

Mammalian expression Vectors

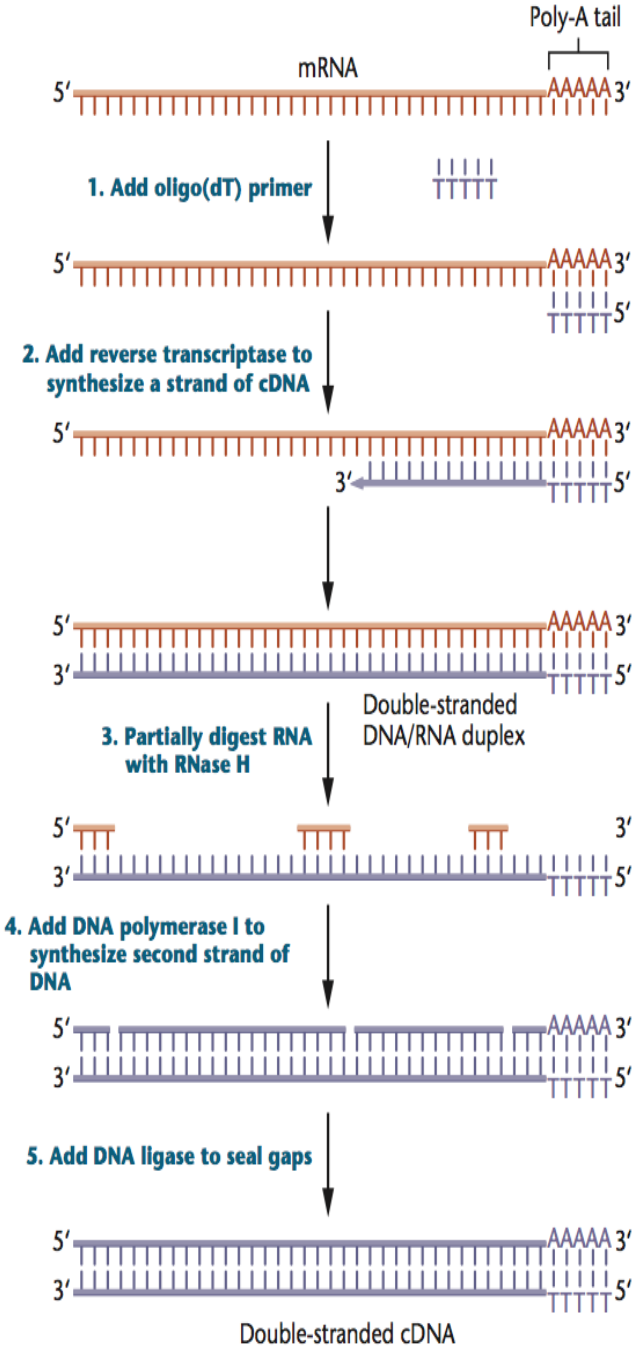
- **Similar to bacterial transformation**
- Designed for transient or stable **expression of target gene**
- How? Contains viral Ori and promotor sequences (eg. CMV or SV40)
- Have different antibiotic selection markers (geniticin, hygromycin, blasticidin or puromycin)

DNA Libraries

A collection of DNA fragments that have been cloned into vectors and from which interest of DNA can be isolated.

- **Genomic libraries:**
- - large fragments of DNA in bacteriophages or bacterial artificial chromosomes

- **cDNA libraries (complimentary DNA):**
- **cloned, reverse-transcribed mRNA** (no DNA sequences corresponding to genomic regions without introns)
- much smaller fragments cloned into plasmid vectors with bacterial uptake and selection



Polymerase Chain Reaction (PCR)

- TEMPLATE gDNA/cDNA/RNA
- PRIMERS: Specific bits of DNA to aid specific amplification of DNA segments
- Thermostable DNA polymerase- Taq/Pfu/Vent/Deep Vent
- DNA polymerase buffer (to keep optimal activity of enzyme)
- A means to carry out thermal cycling- Thermocycler

Polymerase Chain Reaction

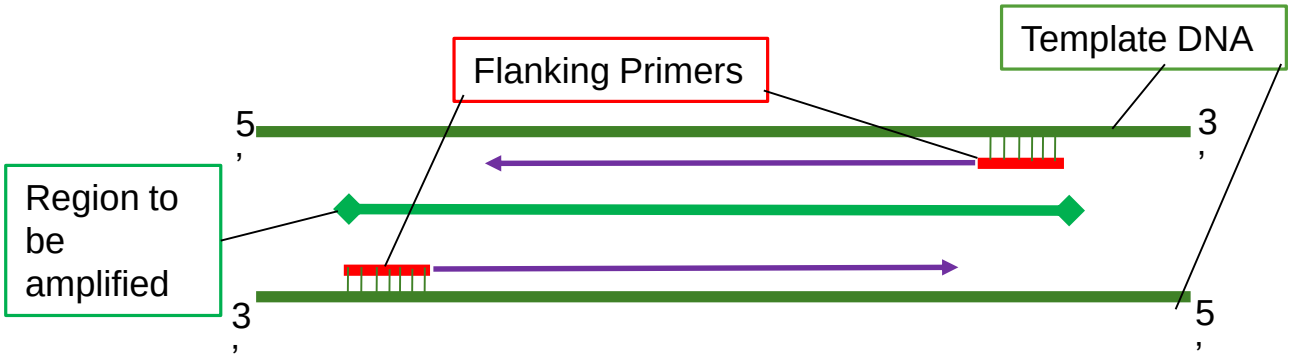
- DNA replication carried out in vitro
- Uses a pair of primers that are specific (complementary) to the DNA which is to be amplified.
- Each primer flanks the ends of the DNA which is to be amplified
- PCR utilises a thermostable DNA polymerase (Taq – from Thermos aquaticus)

1. Denature at 95°C
2. Anneal at 55-65°C
3. Extension at 72°C

Repeat 30+ Times

Each Reaction contains:

Template DNA
Buffer
Forward Primer
Reverse Primer
$MgCl_2$
dNTPs
Taq
H_2O



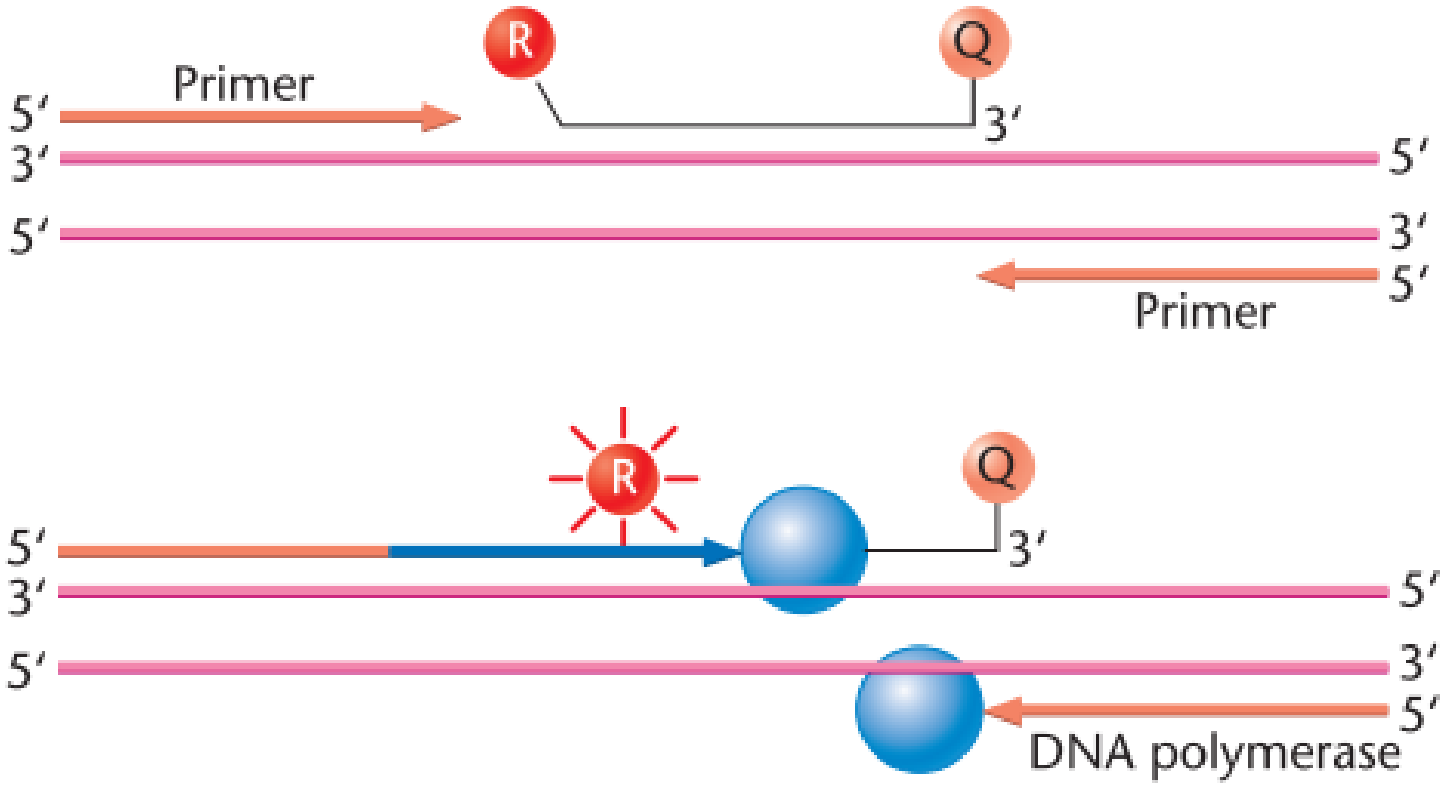
Polymerase Chain Reaction (PCR)

Applications

- Diagnostics for infectious disease: bacteria or viruses (HIV, HPV) etc.
- Diagnosing genetic disorders: by synthesizing allele-specific probes
- Forensic applications- Short Tandem Repeats (STRs), VNTRs etc profiling
- Reverse transcription PCR (RT-PCR)
- Quantitative real-time PCR (qPCR)

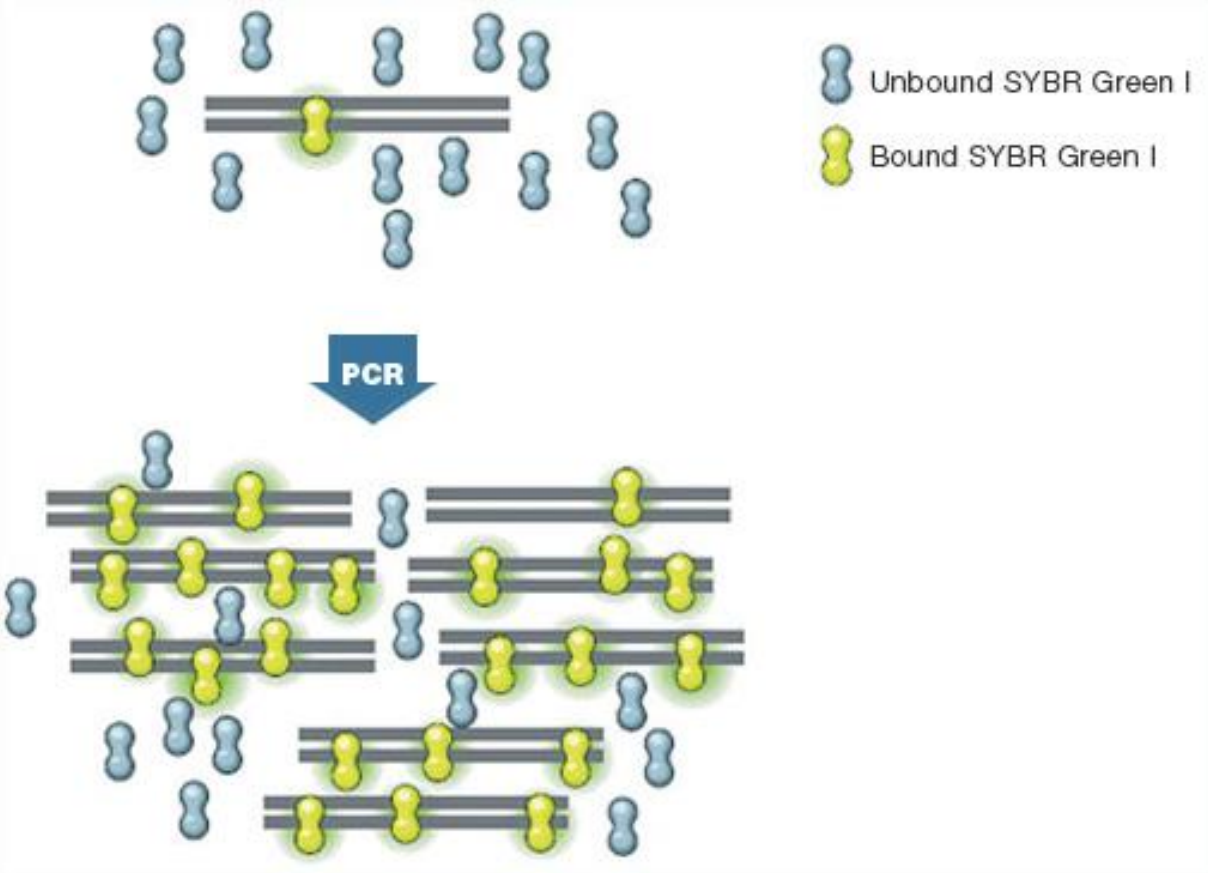
Quantitative real time PCR- **qPCR**

- TaqMan Probe

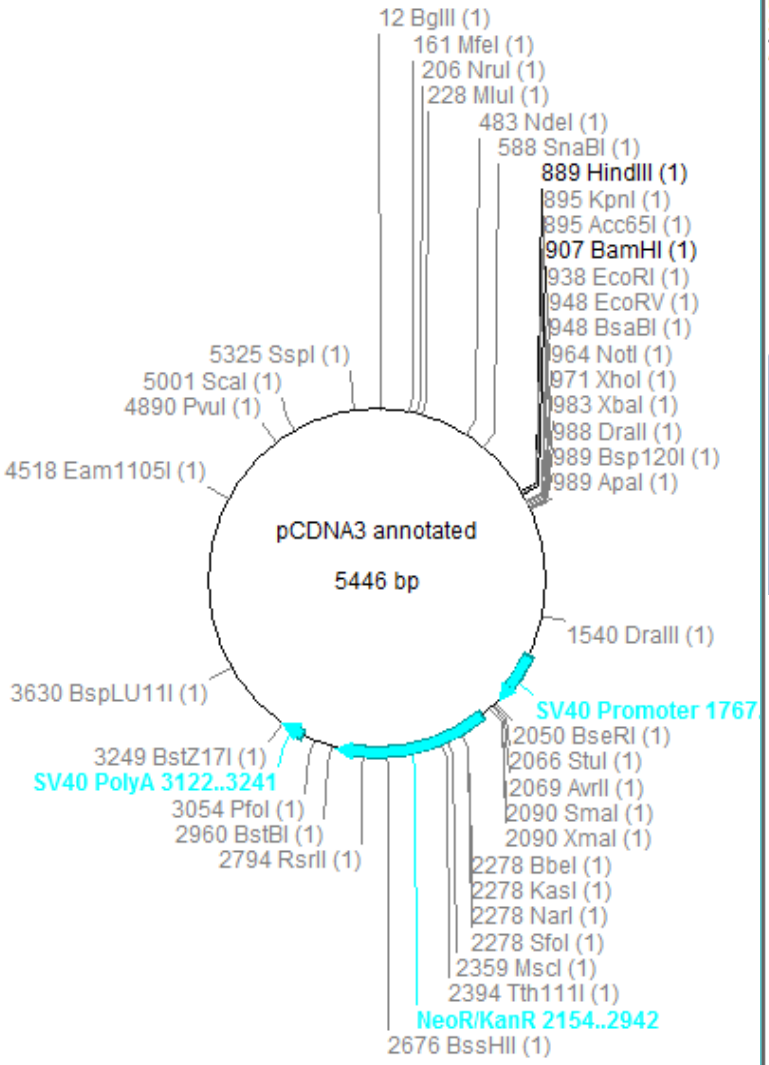


Quantitative real time PCR- **qPCR**

- SYBR Green Dye



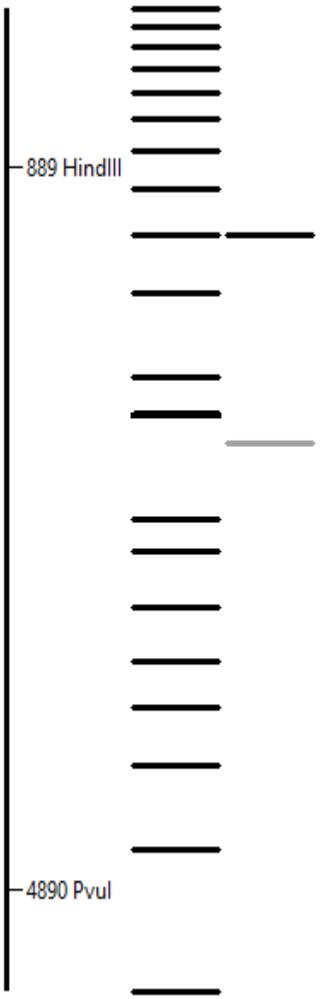
Analysing DNA : Restriction mapping



pCDNA3 annotated.ape

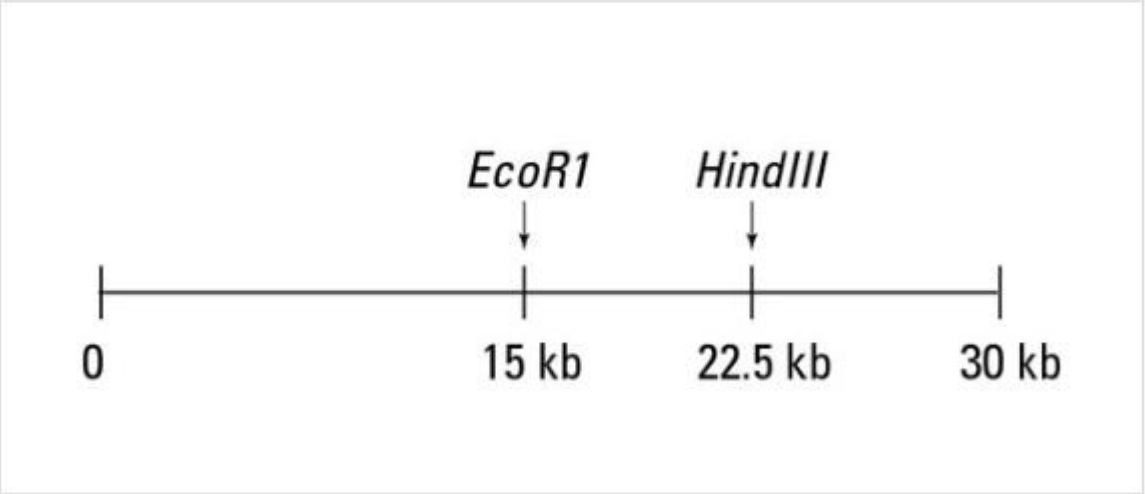
Size	site1		site2		Mass %
4001	HindIII	889	PvuI	4890	73
1445	PvuI	4890	HindIII	889	27

ApE
A plasmid Editor
by M. Wayne Davis



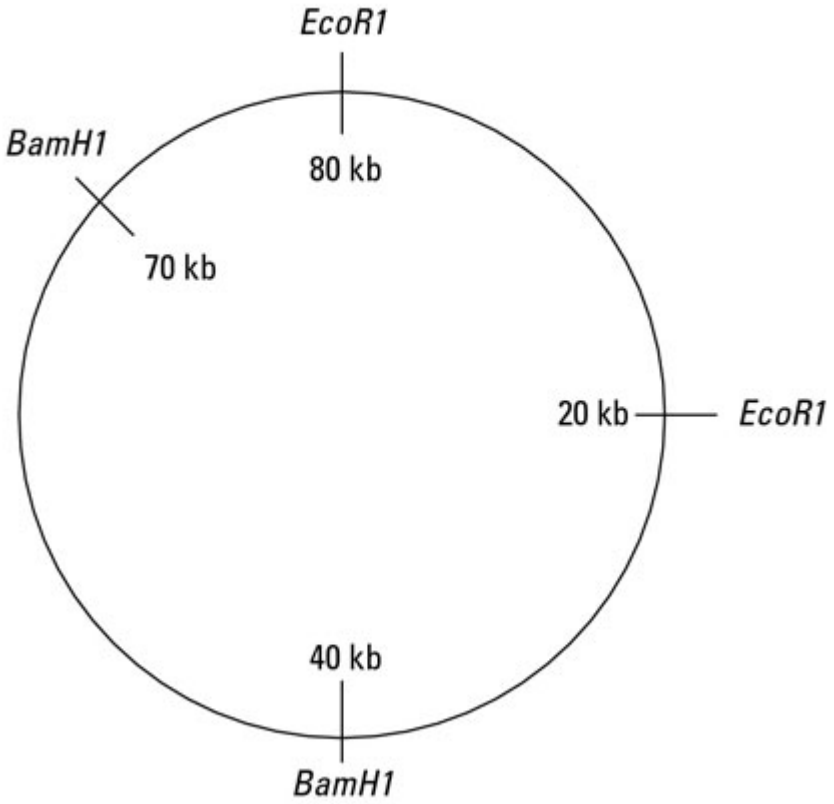
http://biologylabs.utah.edu/jorgensen/wayned/ape/

Examples: a small linear piece of DNA



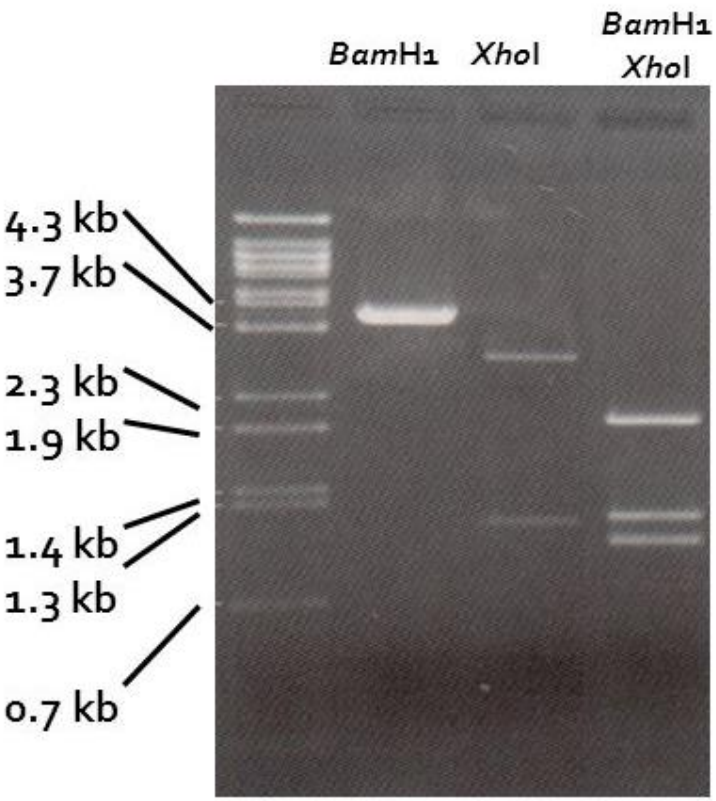
1. How many different sizes pieces of DNA would you get if you cut with EcoR1?
2. How many different size pieces would you get if you cut with HindIII?
3. What would happen if you cut the DNA with both enzymes?

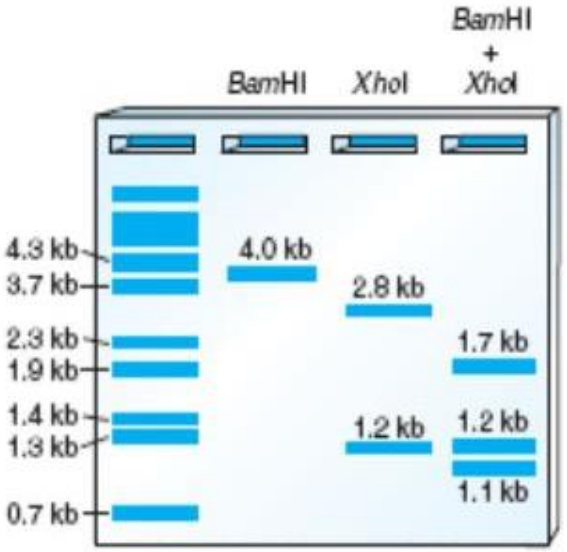
Examples: a circular plasmid

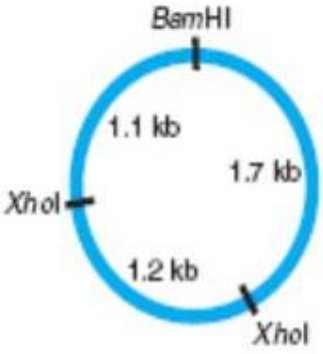


1. How many different sizes pieces of DNA would you get if you cut with EcoR1?
2. How many different size pieces would you get if you cut with BamH1?
3. What would happen if you cut the DNA with both enzymes?

Restriction mapping in a circular plasmid of 4kb (4000 bases)



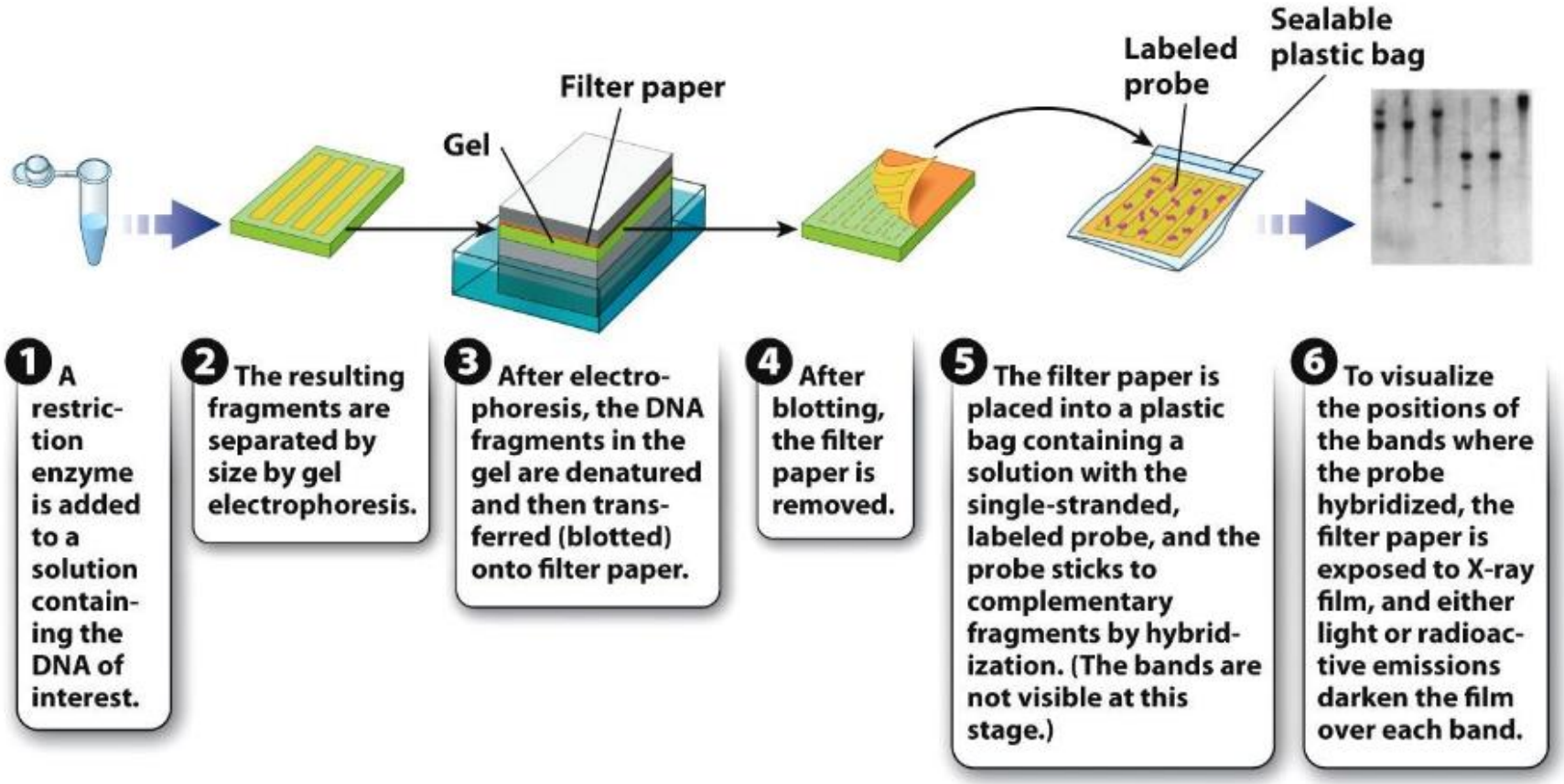




Analysing DNA : Nucleic acid blotting

- Southern Blot : For analysing DNA
- Northern Blot: For analysing RNA
- FISH (Fluorescence in situ hybridization)

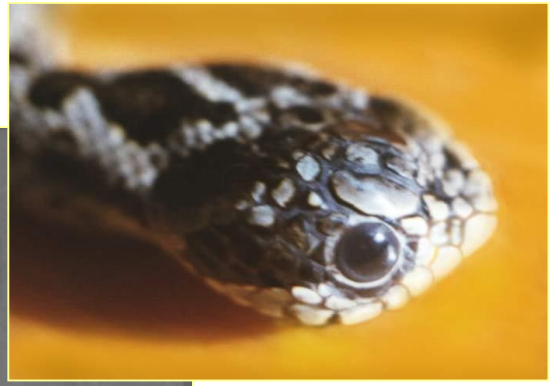
- Southern Blot : For analysing DNA

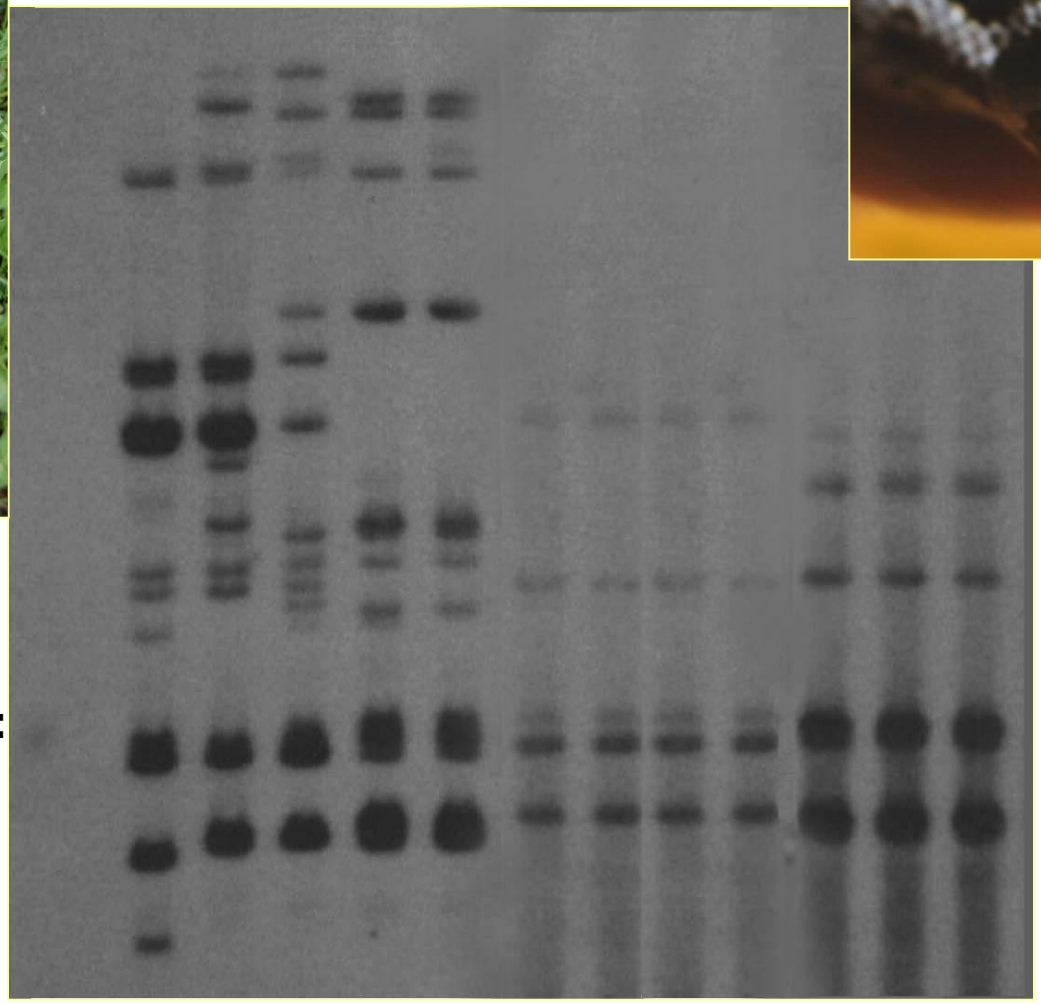


Genetic polymorphism of an outbred vs an inbred snake population

Outbred Ukrainian meadow vipers

Inbred Hungarian meadow vipers

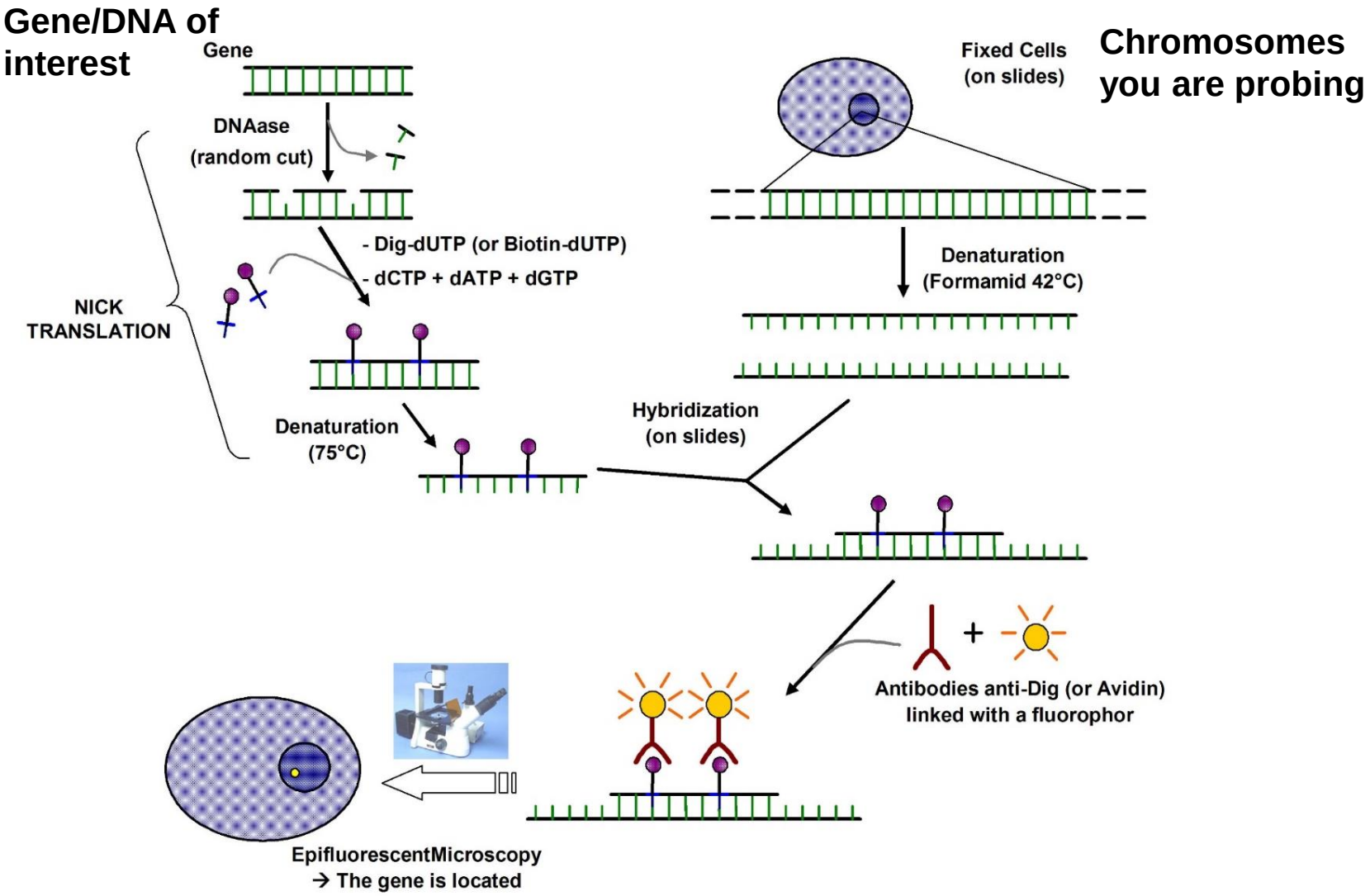




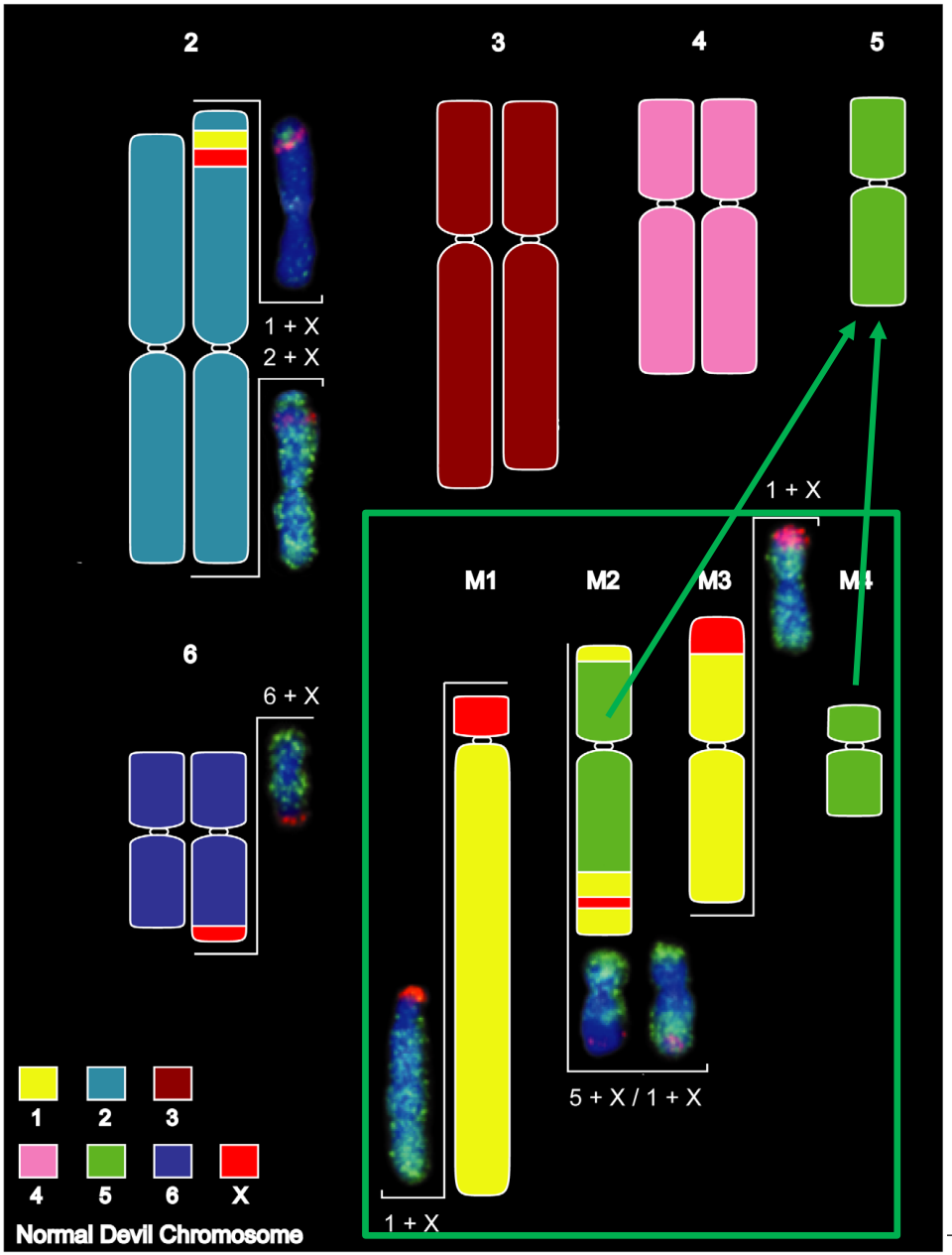
Many cutting sites: Genetic diversity

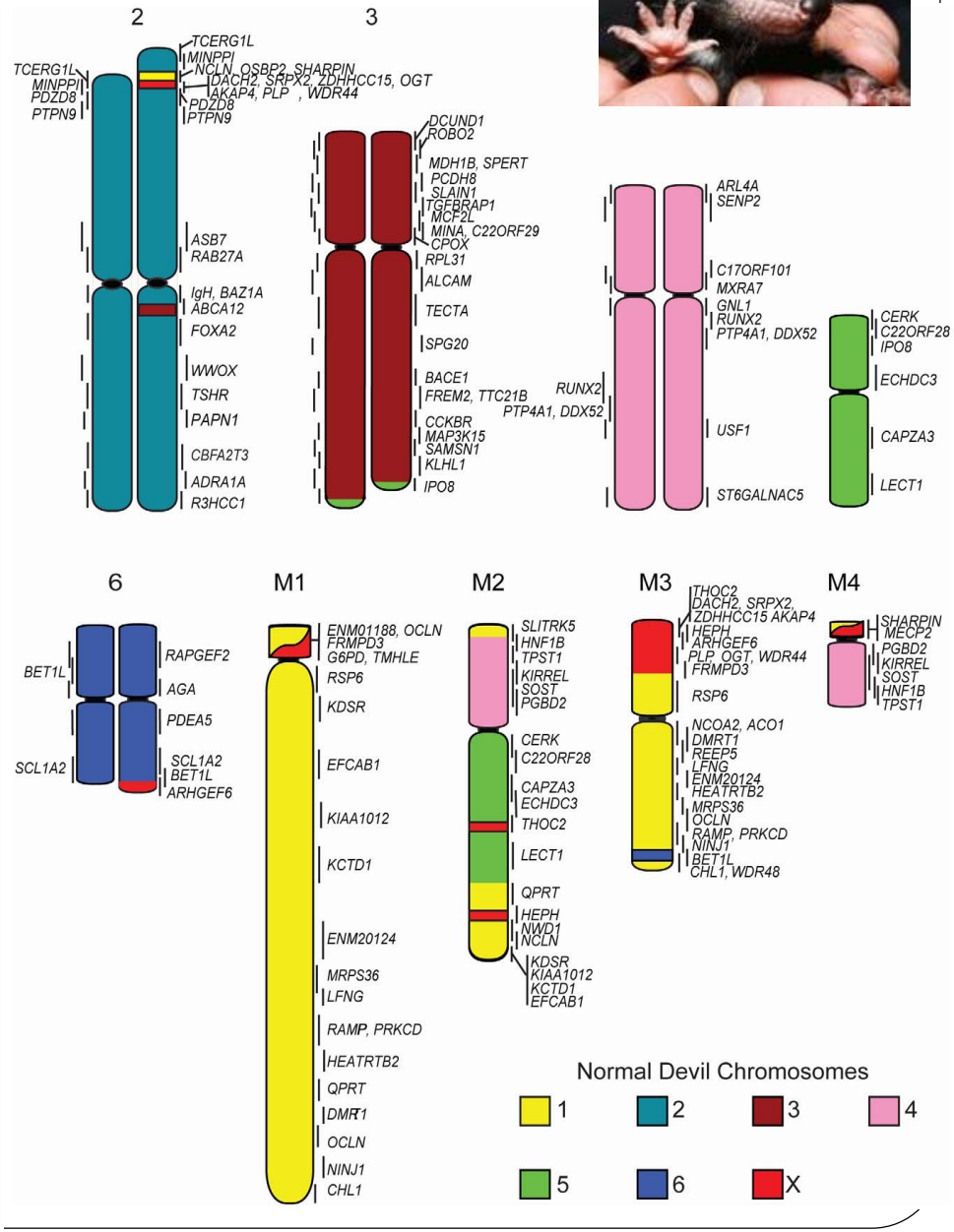
Fewer cutting sites: Loss of genetic diversity

- FISH (Fluorescence in situ hybridization)



Chromosome paintings of DFTD chromosomes



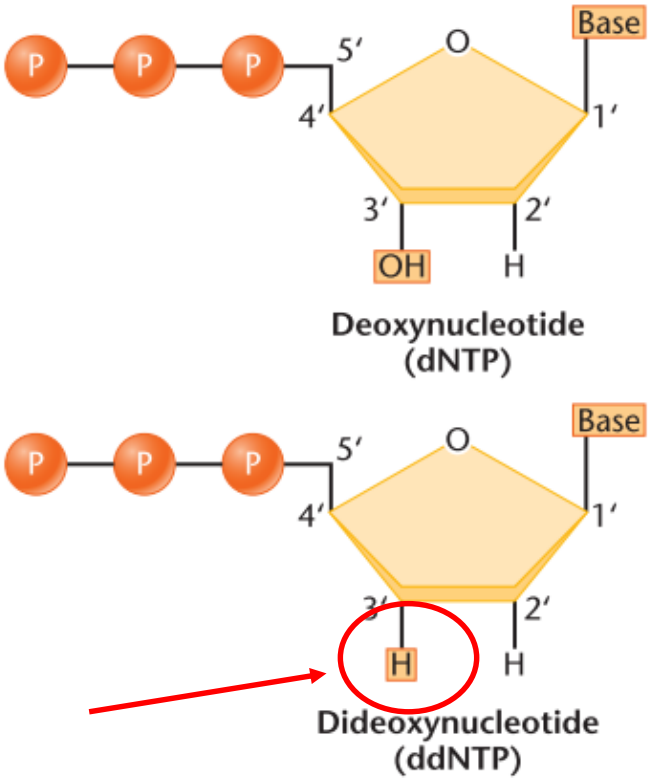


DNA Sequencing: Sanger's Method

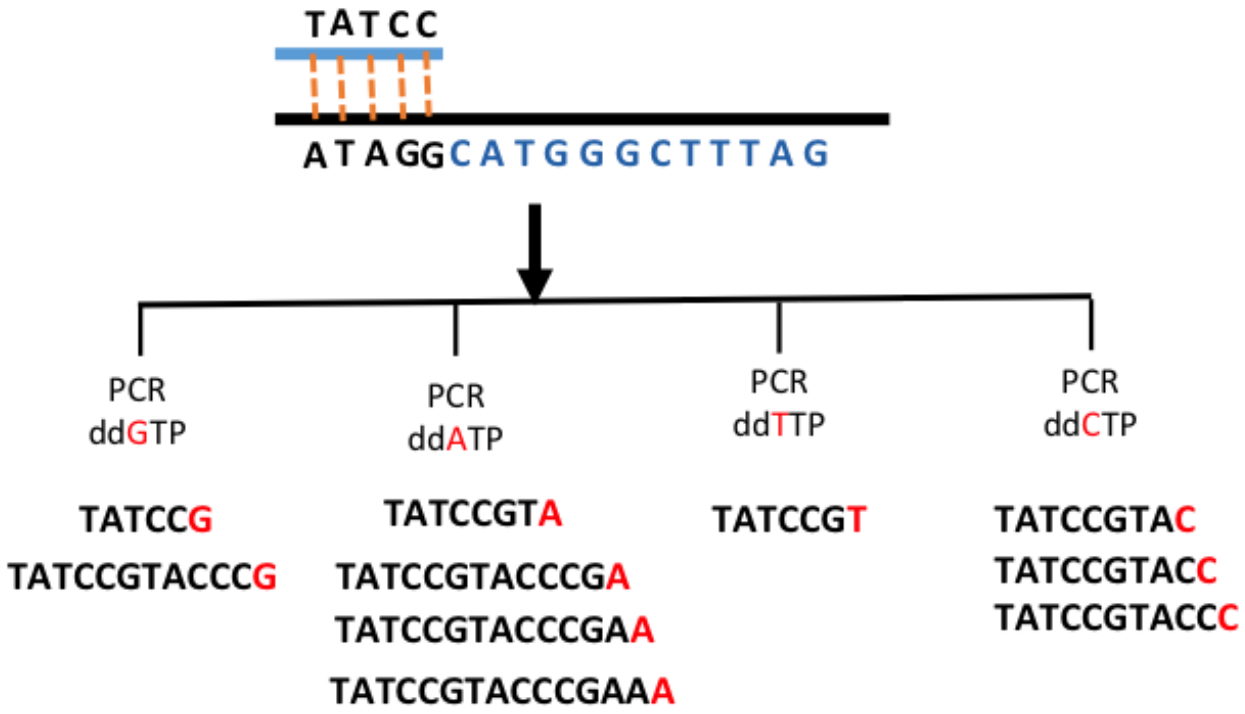
- Dideoxynucleotide (ddNTP) chain termination

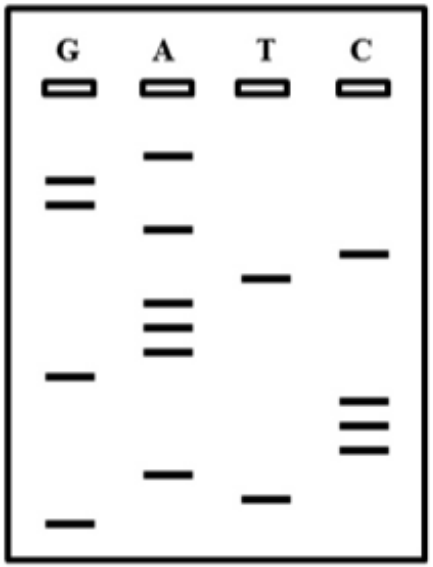
Requires:

- DNA template
- Primer
- DNA Polymerase
- dNTPs
- ddNTPs



DNA Sequencing: Sanger's Method

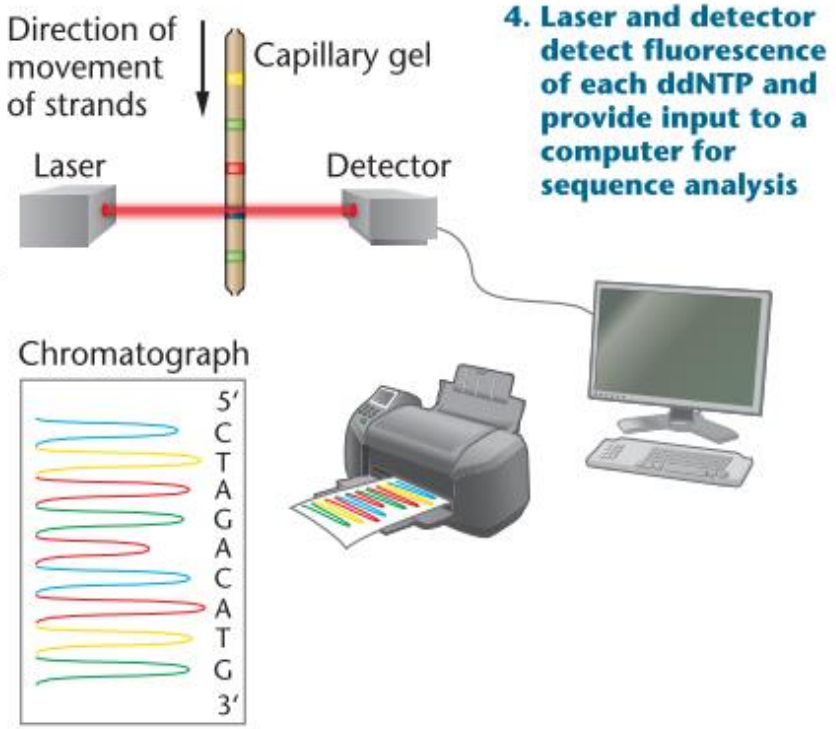




Automated Sanger's Method

Requires:

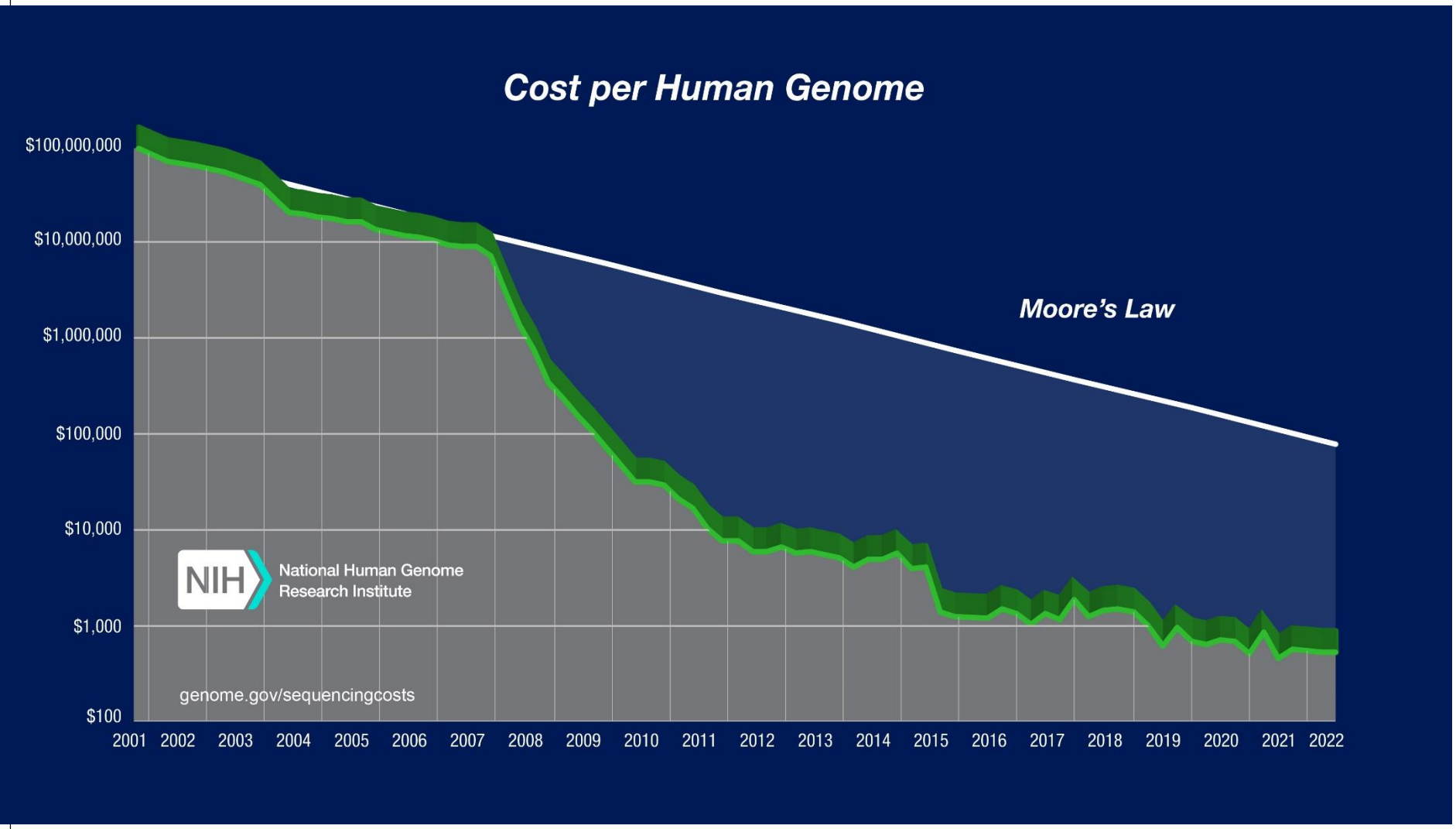
- Fluorescence labelled ddNTPs
- Capillary electrophoresis



DNA Sequencing: Present Day

- Technology that allows fast and cheap ways to sequence large amount of DNA
- Open further avenues for using genetic information in medicine
- Next Generations Sequencing (NGS): Pyrosequencing, Third Generation Sequencing, Sequencing by synthesis vs Long read sequencing

SLE340_ Bioinformatics and Genomics = learn more about sequencing



https://www.genome.gov/about-genomics/fact-sheets/Sequencing-Human-Genome-cost