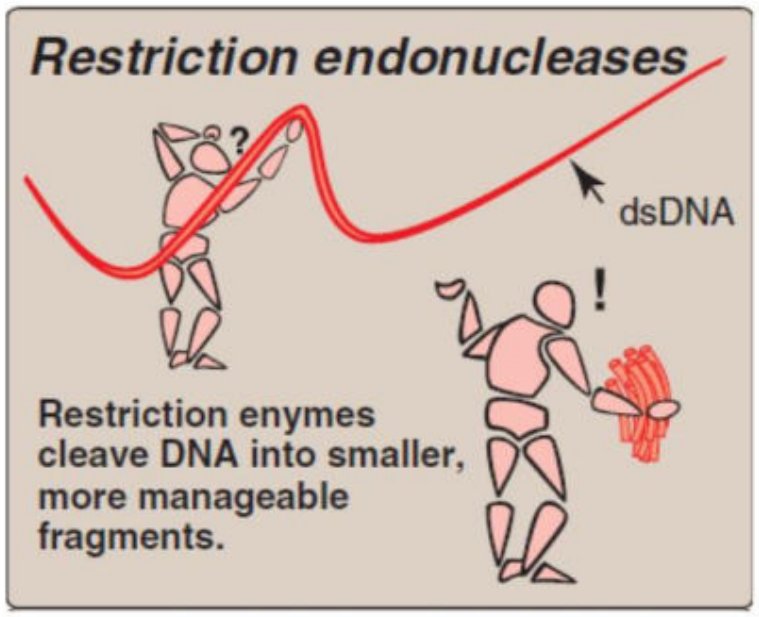
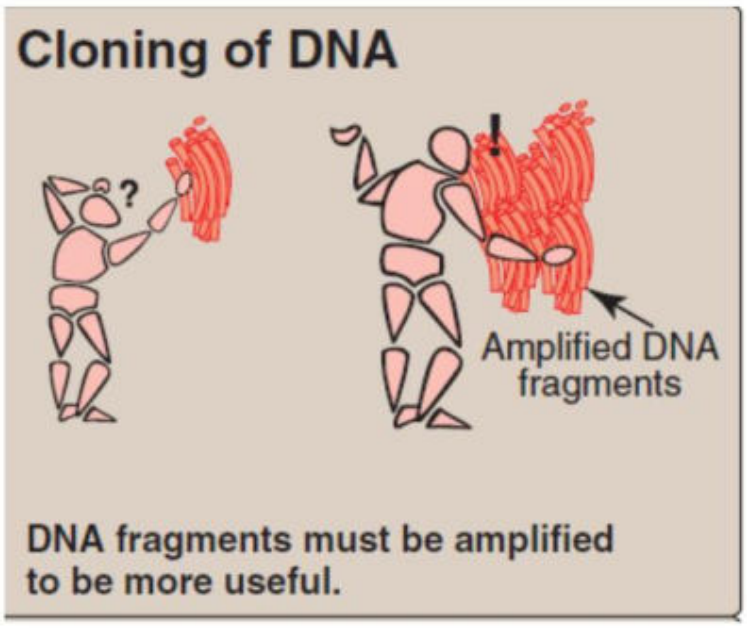
RECOMBINANT DNA TECHNOLOGY

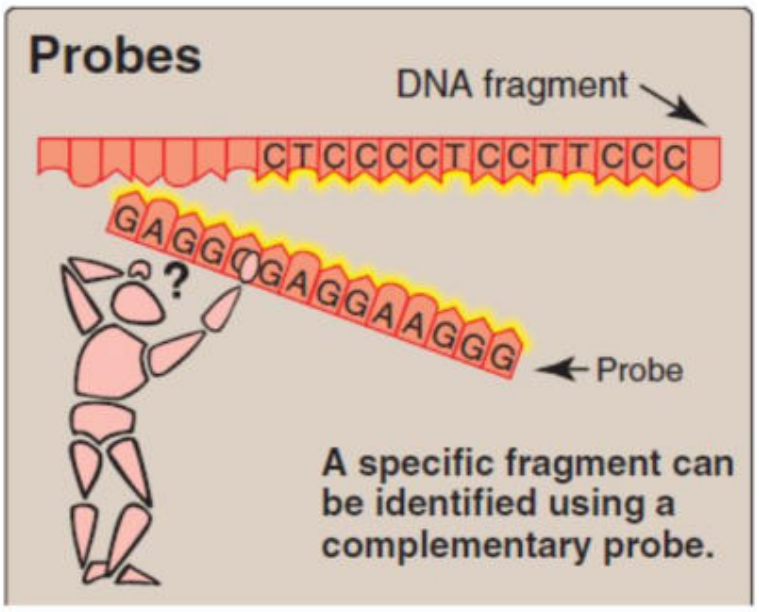
Objectives

- Recombinant DNA
- Probes
- Restriction map
- Gene cloning
- Gene library
- Cloning vectors
- RFLP
- DNA Fingerprinting
- DNA foot printing
- Genomic imprinting





Three techniques that facilitate analysis of human DNA



Steps of Genetic Engineering

- Cutting DNA at a specific site
- Joining of two DNA fragments to create a novel DNA
- Cloning or amplification of available DNA
- Expression of a DNA to obtain its product
- Sequencing of a DNA molecule
- Synthesis of an oligonucleotide

Application of Recombinant Technology

- Understanding of diseases: Sickle cell anaemia, Thalassemia
- Diagnosis of diseases: AIDS, Hepatitis
- Treatment of Diseases: Human Insulin
- Prevention of diseases: Hepatitis vaccines
- Gene therapy: SCID

Recombinant DNA

Two DNA fragments of interest: Even from different source or species

Cohesive or sticky ends with complementary sequences

Treated with same RE

in some cases blunt ends are joined by

Homopolymer tailing

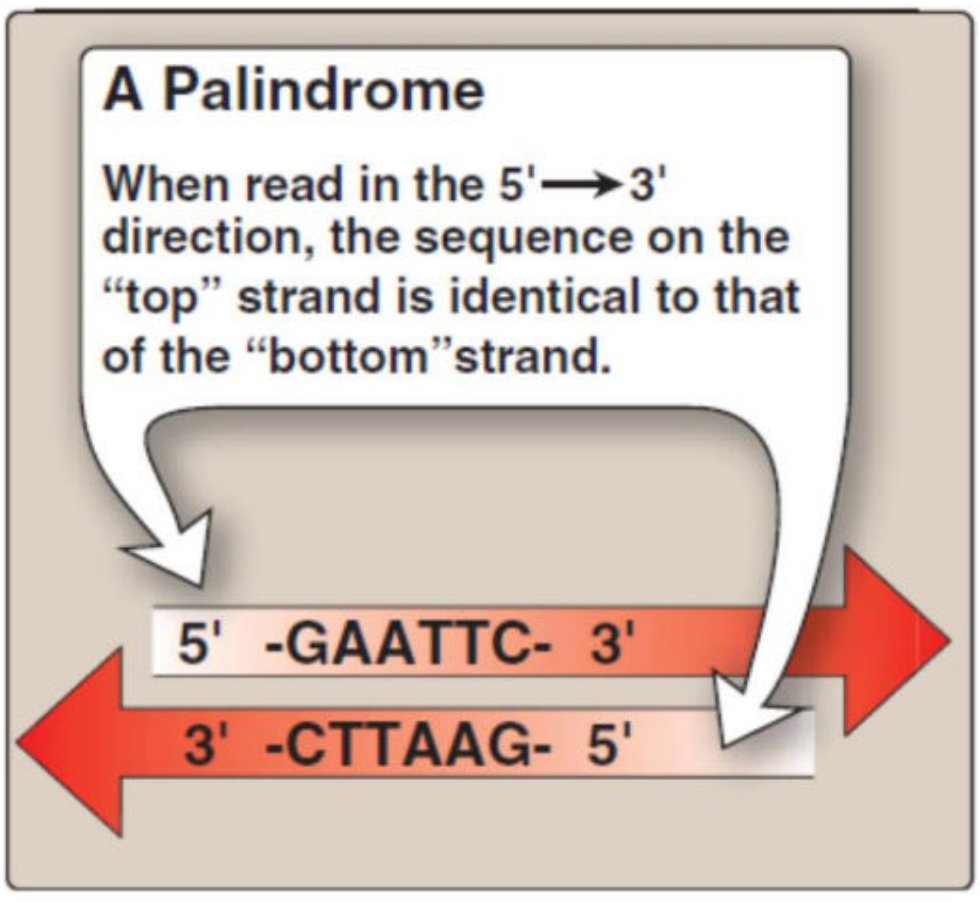
A small synthetic duplex oligonucleotide having RE sites attached

5‘ ends of the linker DNA are phosphorylated by polynucleotide kinase

Different Enzymes used in DNA Recombinant technology

Restriction Endonuclease

Enzyme	Functions
DNA ligase	joins of ends
DNA Pol I	Synthesis of double stranded DNA
DNAse I	Produces nicks in sDNA
Exonuclease III	Removes nt from 3‘ end
λ exonuclease	Removes from 5‘ end
Polynucleotide kinase	Phosphorylates 5‘ OH group
Alk phosphatase	Removes 5‘ PO4
S1 nuclease	Degrades sDNA



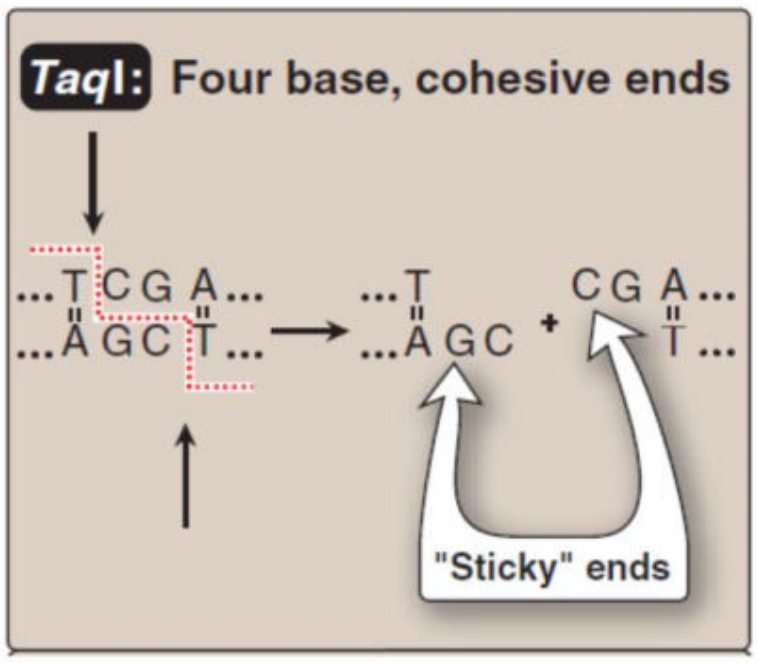
Recognition sequence of *restriction endonuclease EcoRI shows* two-fold rotational symmetry

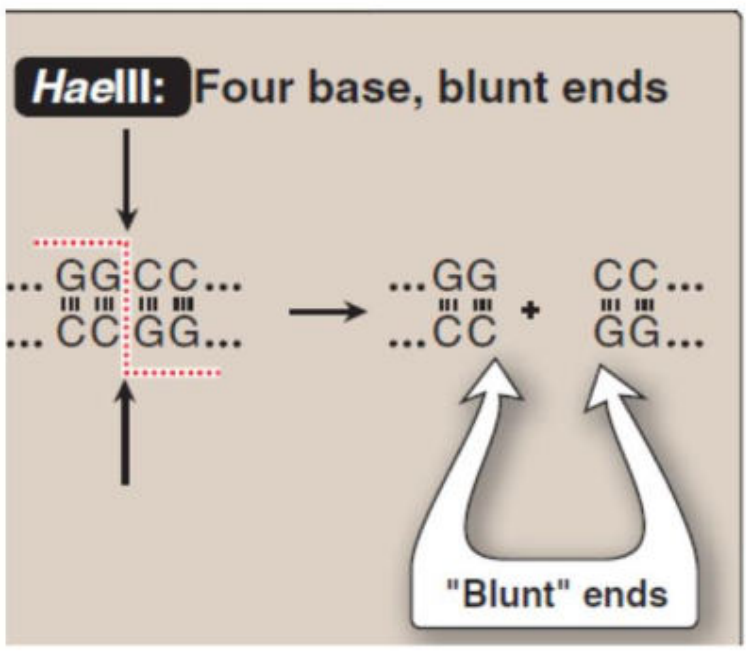
Nomenclature of
Restriction endonuclease

Why they are named so

What are sticky ends and
blunt ends

Restriction sites frequency

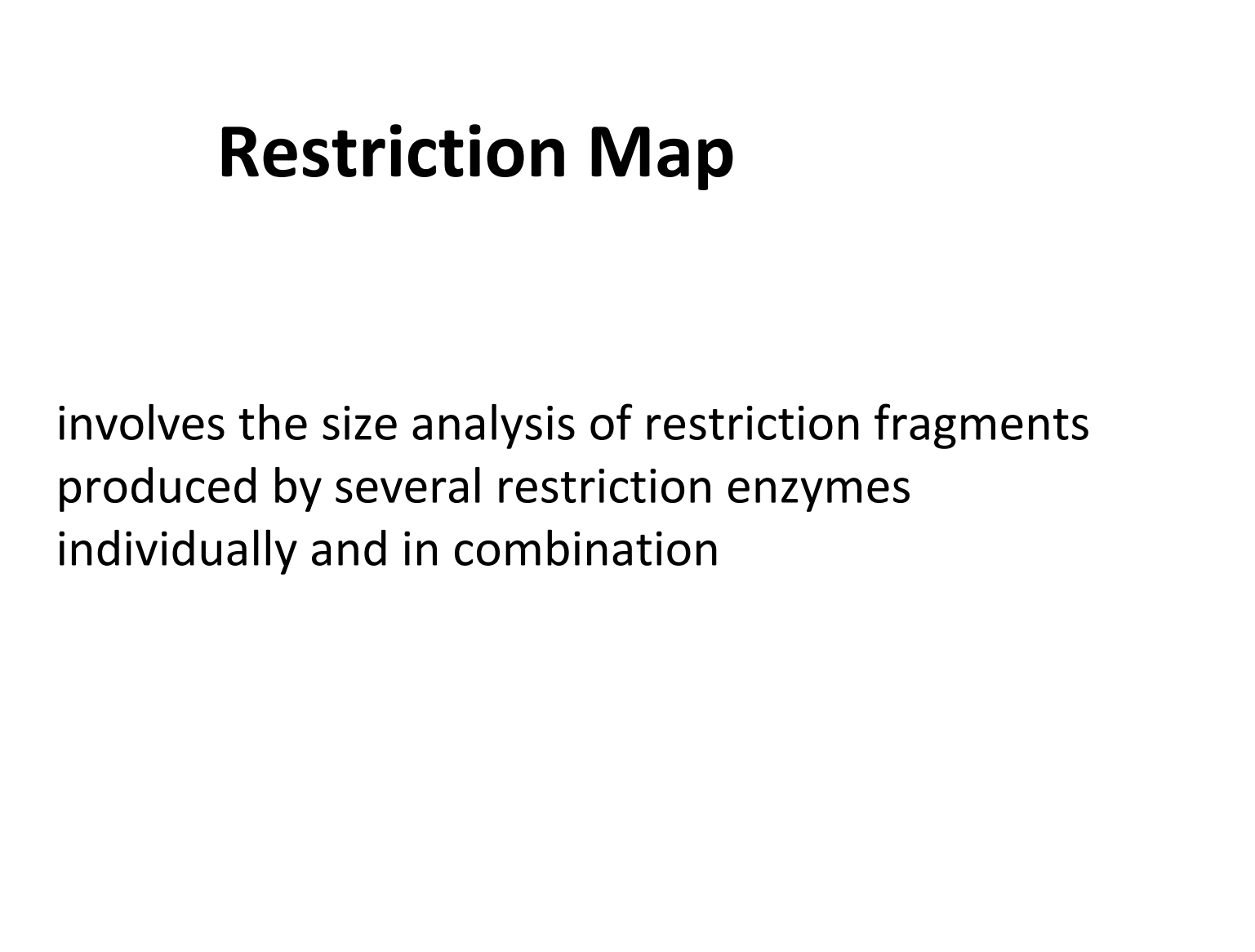




TaqI and Hae I I I (Haemophilus aegyptius) restriction endonucleases

Restriction Map

involves the size analysis of restriction fragments produced by several restriction enzymes individually and in combination



Probe

15-20 nt long oligont used to search a particular DNA fragment

Chemically synthesized DNA or RNA pieces

Generally labelled with radioactive material or a fluoroscent label

Construction of a probe

From Genetic database

homologous gene in other species : heterologous gene probe

mRNA

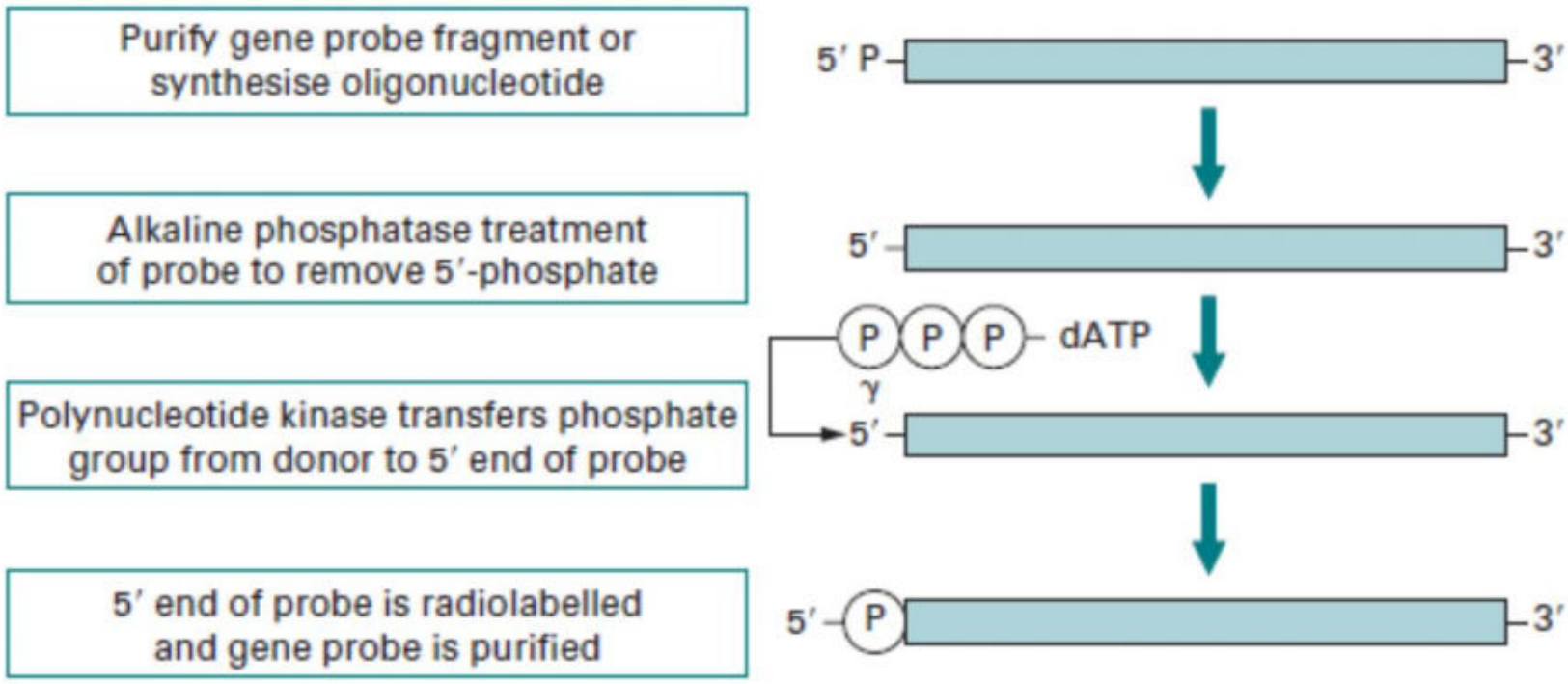
protein----- rich in Trp and Met

Labelling of a Probe

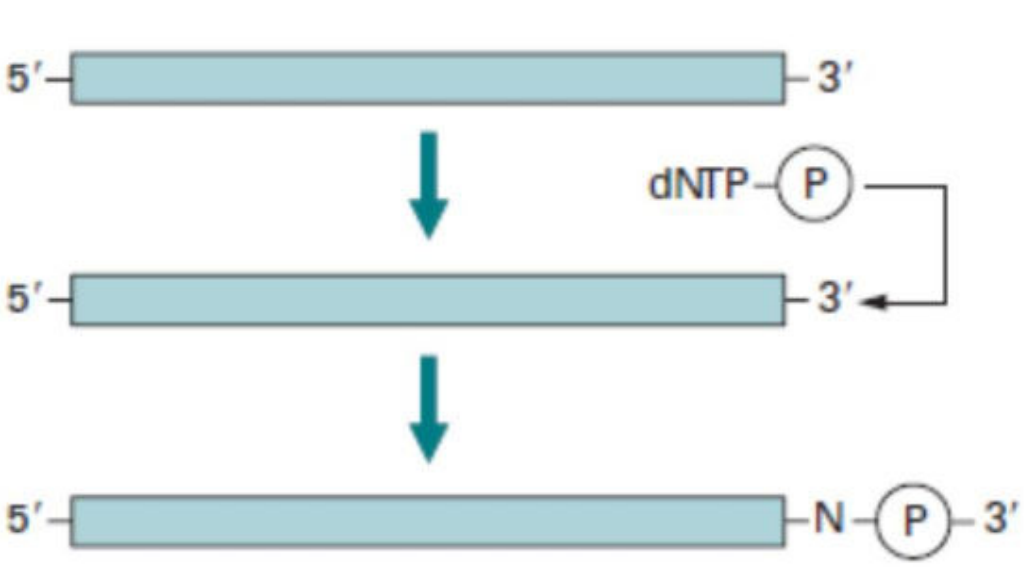
End labelling: ^{32}P

Random labelling: During synthesis: Usually GTP:
Fluorescent labelled

End-labelling of a gene probe at the 5’ end with alkaline phosphatase and polynucleotide kinase



End-labelling of a gene probe at the 3’ end using terminal transferase



Synthesise oligonucleotide or purify gene probe fragment

Transfer labelled dNTP to the 3′ end using terminal transferase

3′ end of probe is radiolabelled and gene probe is purified

Cloning

Process of producing large number of identical copies from one single original DNA molecule or fragment

Importance

To study gene: purified form and in sufficient amount

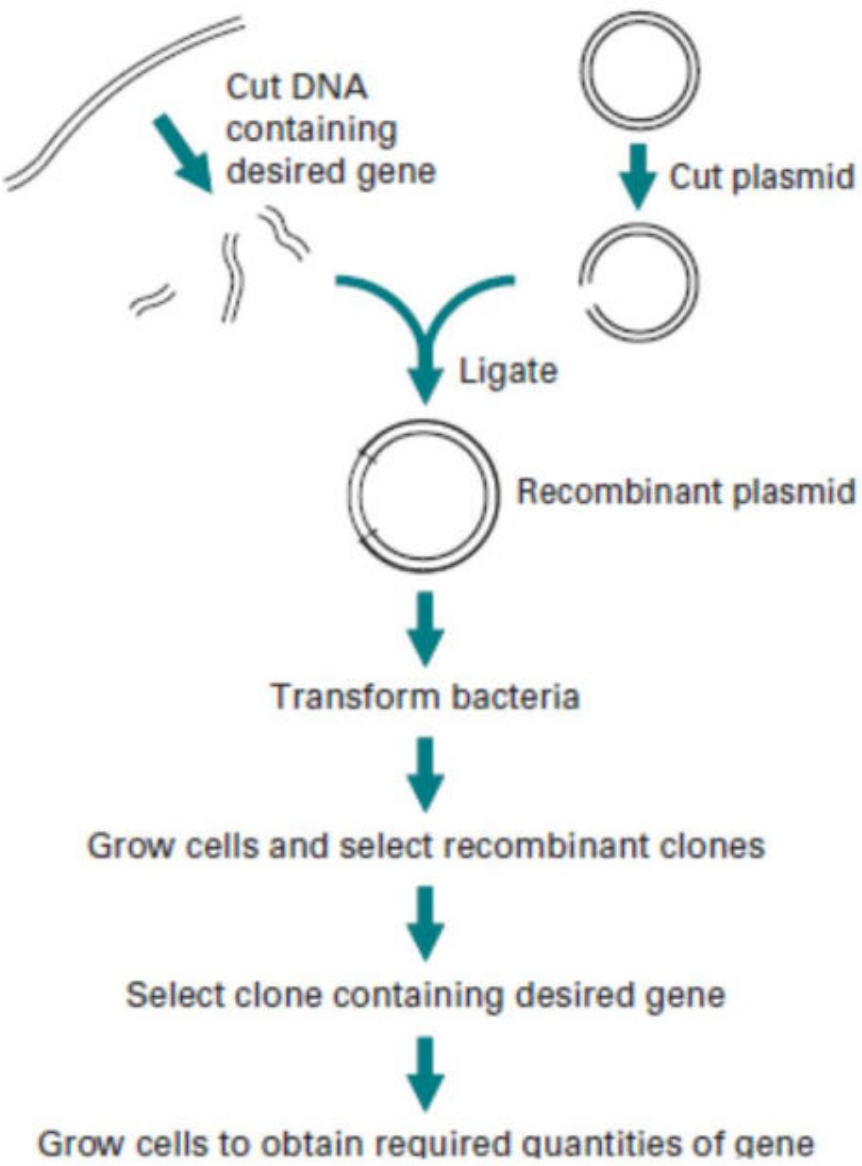
To study sequencing, expression in different tissues under different conditions

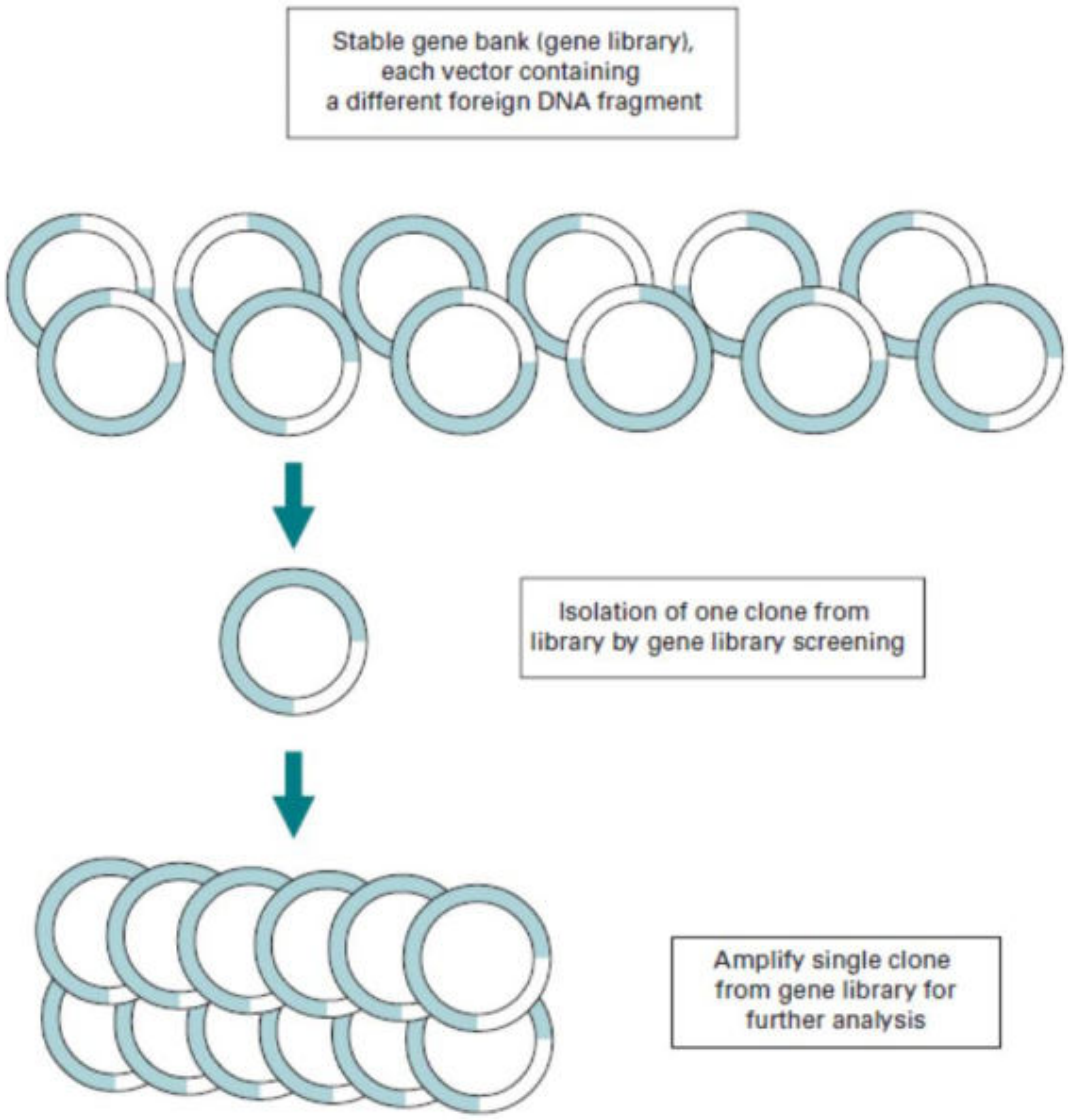
Methods of amplification

Cell based

Cell free

Outline of gene cloning





Gene Library

- All of the DNA extracted from an organism and digested with a restriction enzyme will result in a collection of clones. This collection of clones is known as a gene library

Type of gene library

Genomic library: Total chromosomal DNA of organism

cDNA library: represents the mRNA from a cell or tissue at a specific point in time

Type of gene library depends on final application of DNA

Goal: Production of new or modified proteins or determination of tissue specific expression and timing pattern

cDNA library

Goal: To understand the control of protein production for a particular gene

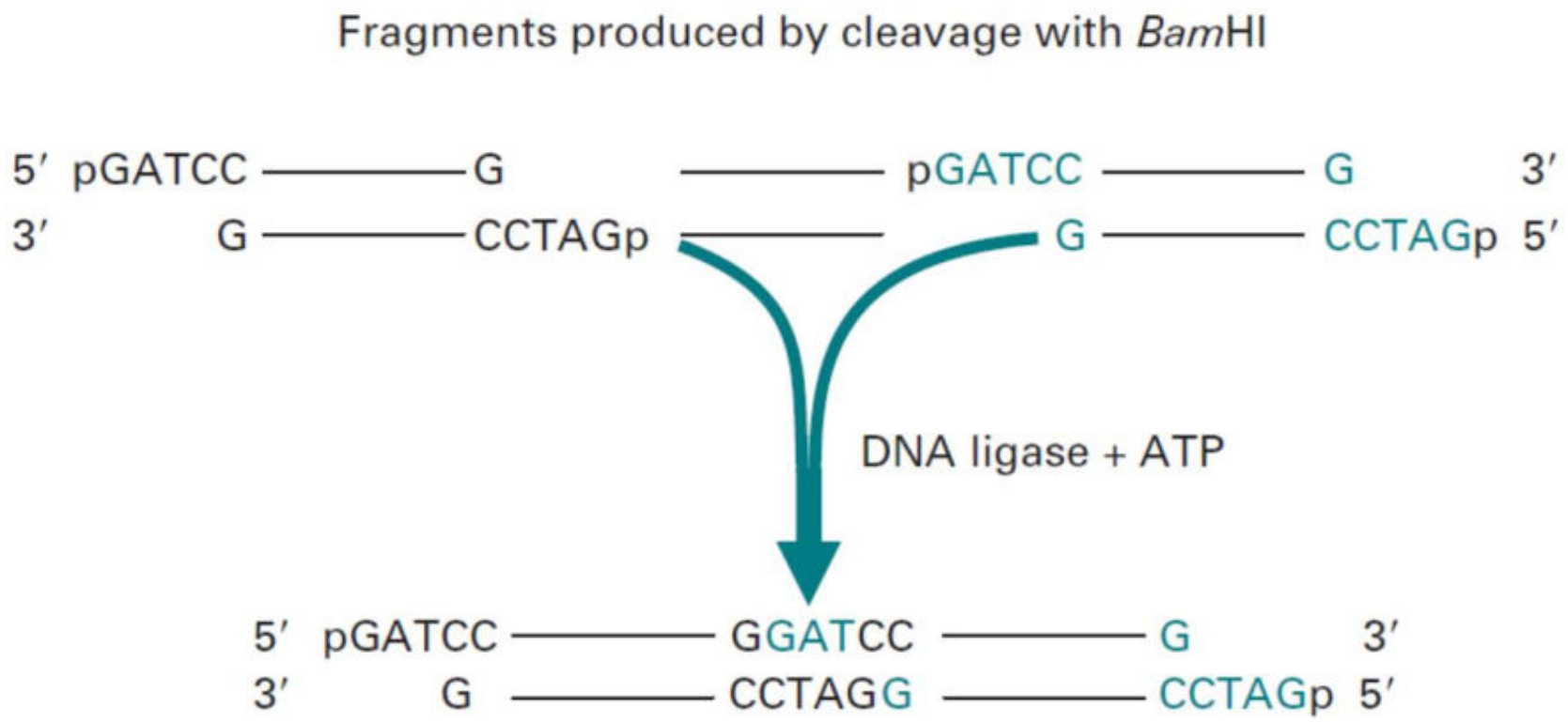
Genomic library

Gene Library

- 1. Construction of gene library
  - Digesting genomic DNA molecules
    - Choice of Enzyme?
  - Ligating DNA molecules
    - Carried out at 10o C to lower the kinetic energy and to reduce the chances of sticky ends parting
- 2. Cloning vectors
- 3. Screening Gene Library

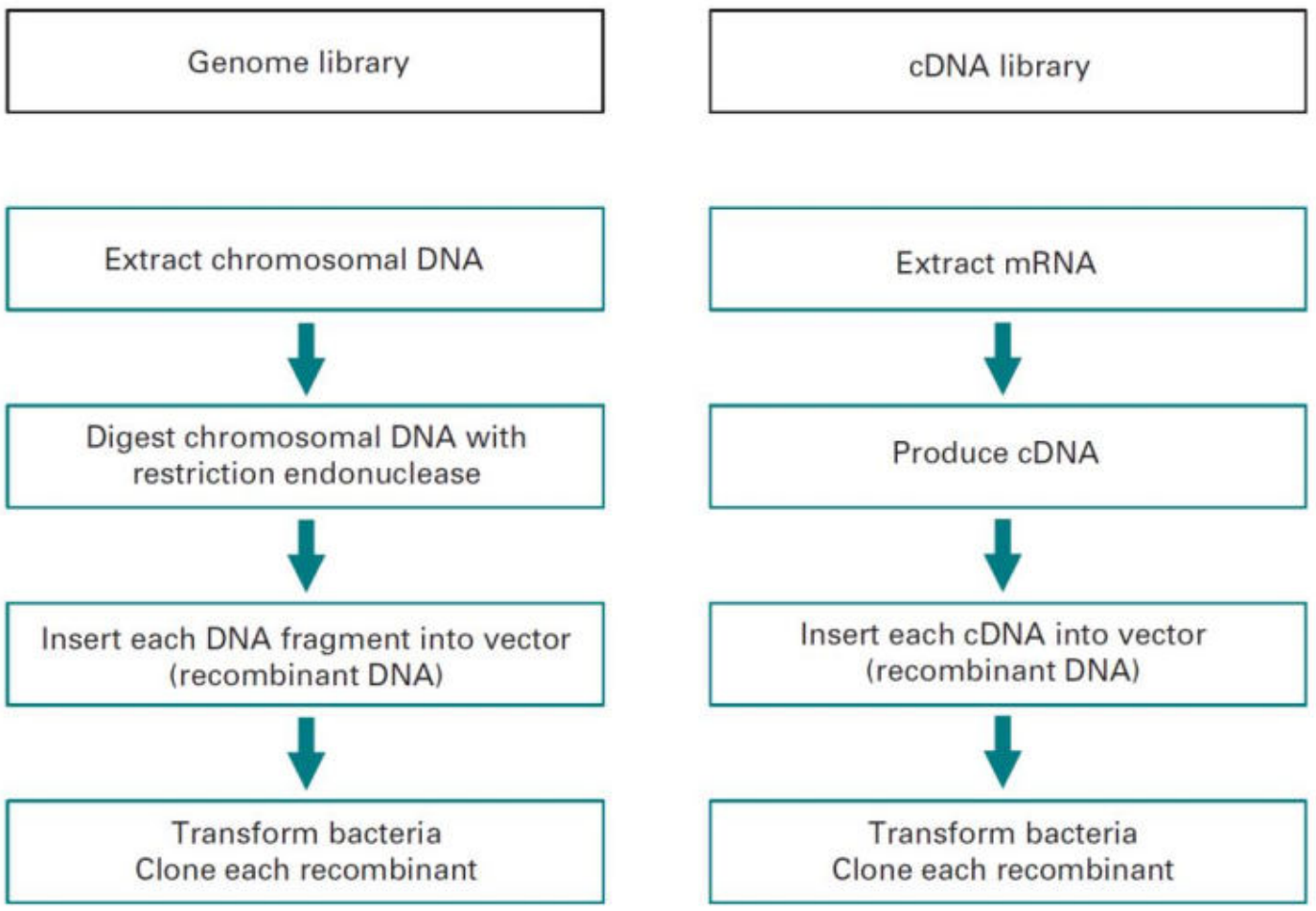
Numbers of clones required for representation of DNA in a genome library

Species	Genome size (kb)	No. of clones required	
		17 kb fragments	35 kb fragments
Bacteria (*E. coli*)	4 000	700	340
Yeast	20 000	3 500	1 700
Fruit fly	165 000	29 000	14 500
Man	3 000 000	535 000	258 250
Maize	15 000 000	2 700 000	1 350 000



Ligation molecules with cohesive ends. Complementary cohesive ends base-pair, forming a temporary link between two DNA fragments. This association of fragments is stabilised by the formation of 3′ to 5′ phosphodiester linkages between cohesive ends, a reaction catalysed by DNA ligase.

Comparison of general steps in the construction of genomic and cDNA library



Cloning Vector

. DNA elements that may be stably maintained and propagated in a host organism for which the vector has replication functions.

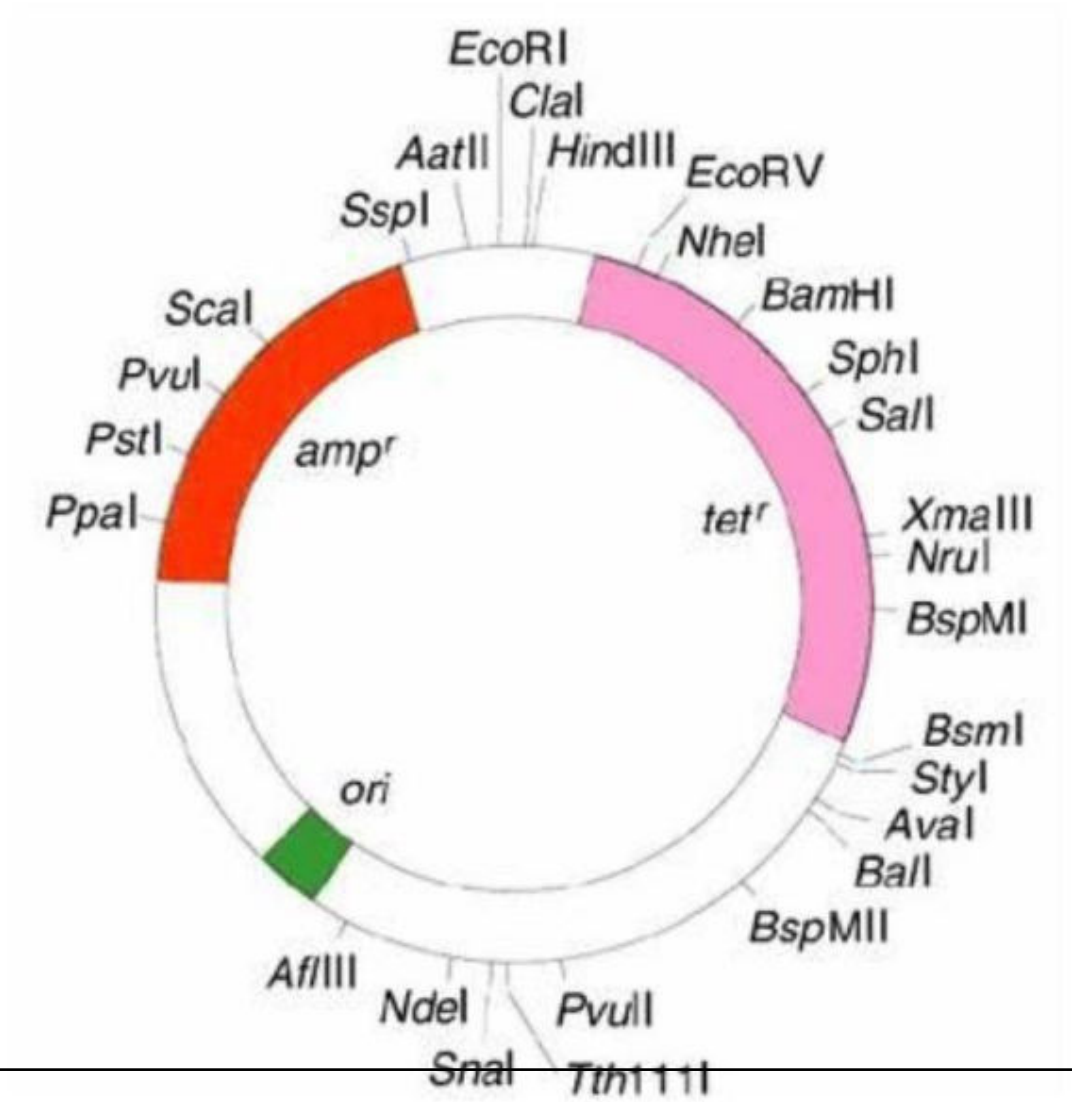
. Host organism is a bacterium such as E. coli

. vector with a replication origin in E. coli will replicate (together with any incorporated DNA) efficiently

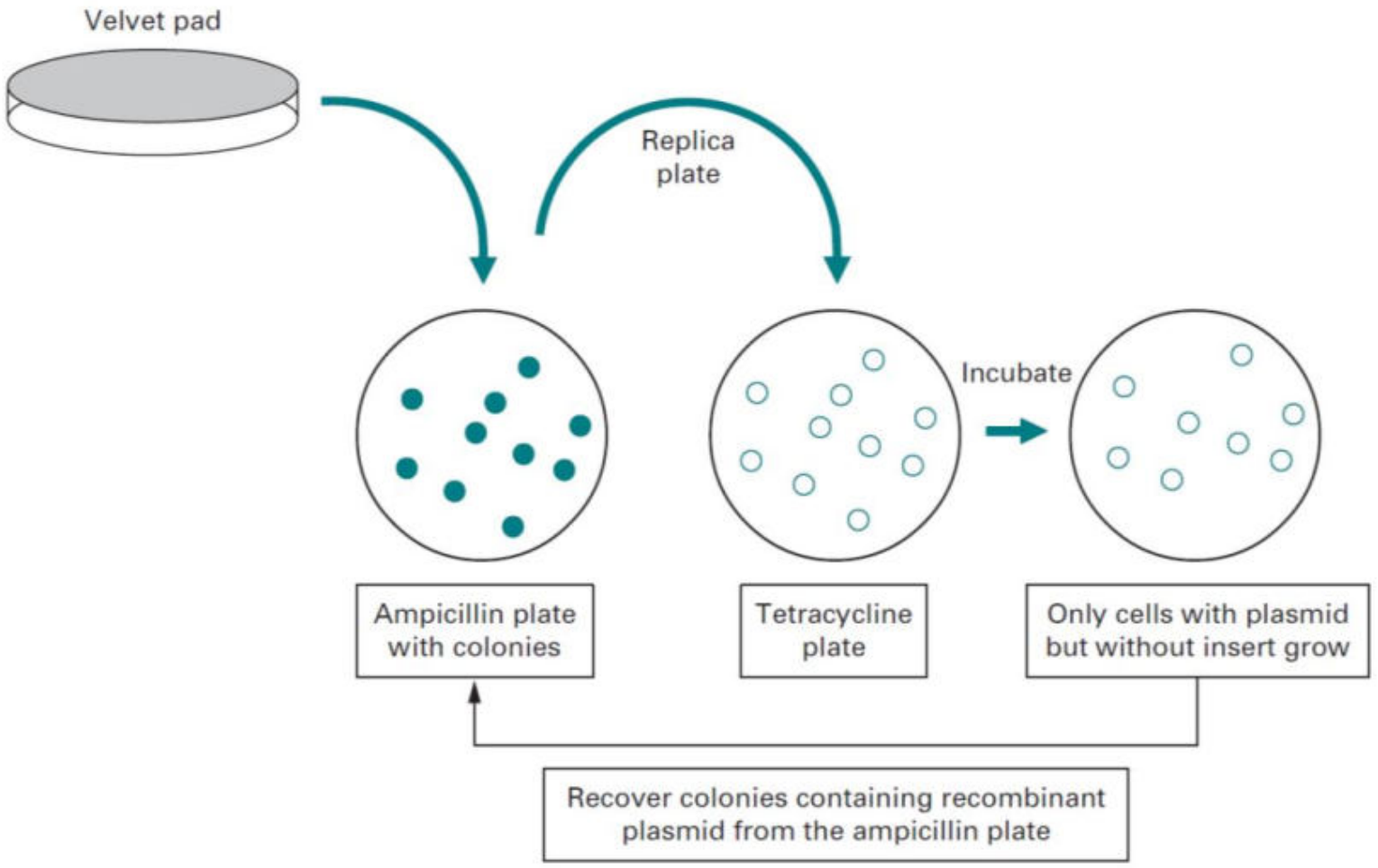
Comparison of vectors generally available for cloning

Vector	Host cell	Vector structure	Insert range(kb)
M13	E coli	Circular virus	1-4
Plasmid	E coli	Circular plasmid	1-5
Phageλ	E coli	Linear virus	2-25
Cosmids	E coli	Circular plasmid	35-45
BACs	E coli	Circular plasmid	50-300
YACs	S. Cerevisiae	Linear chromosome	100-2000

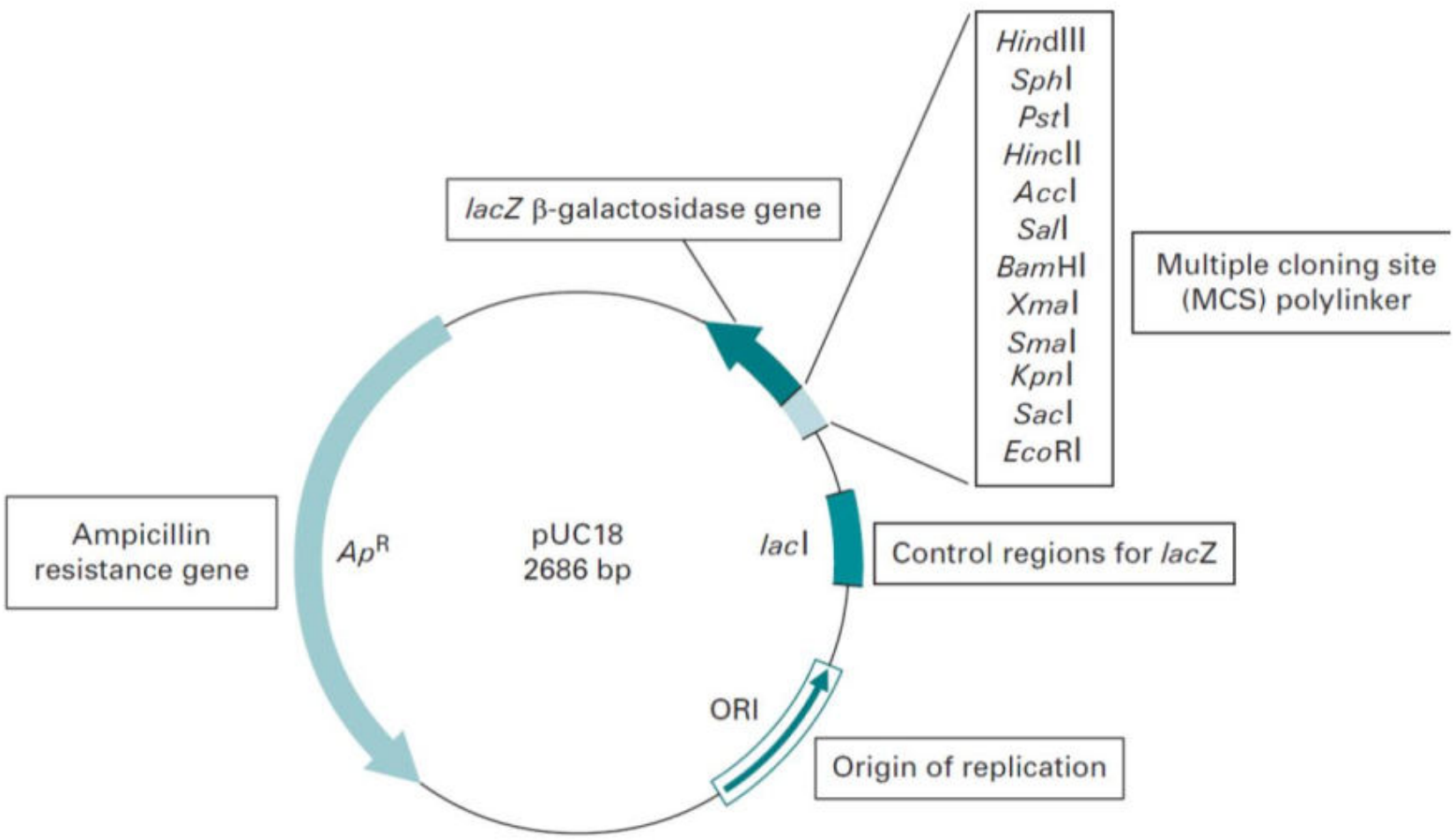
Structure of E.Coli plasmid cloning vector **pBR322**



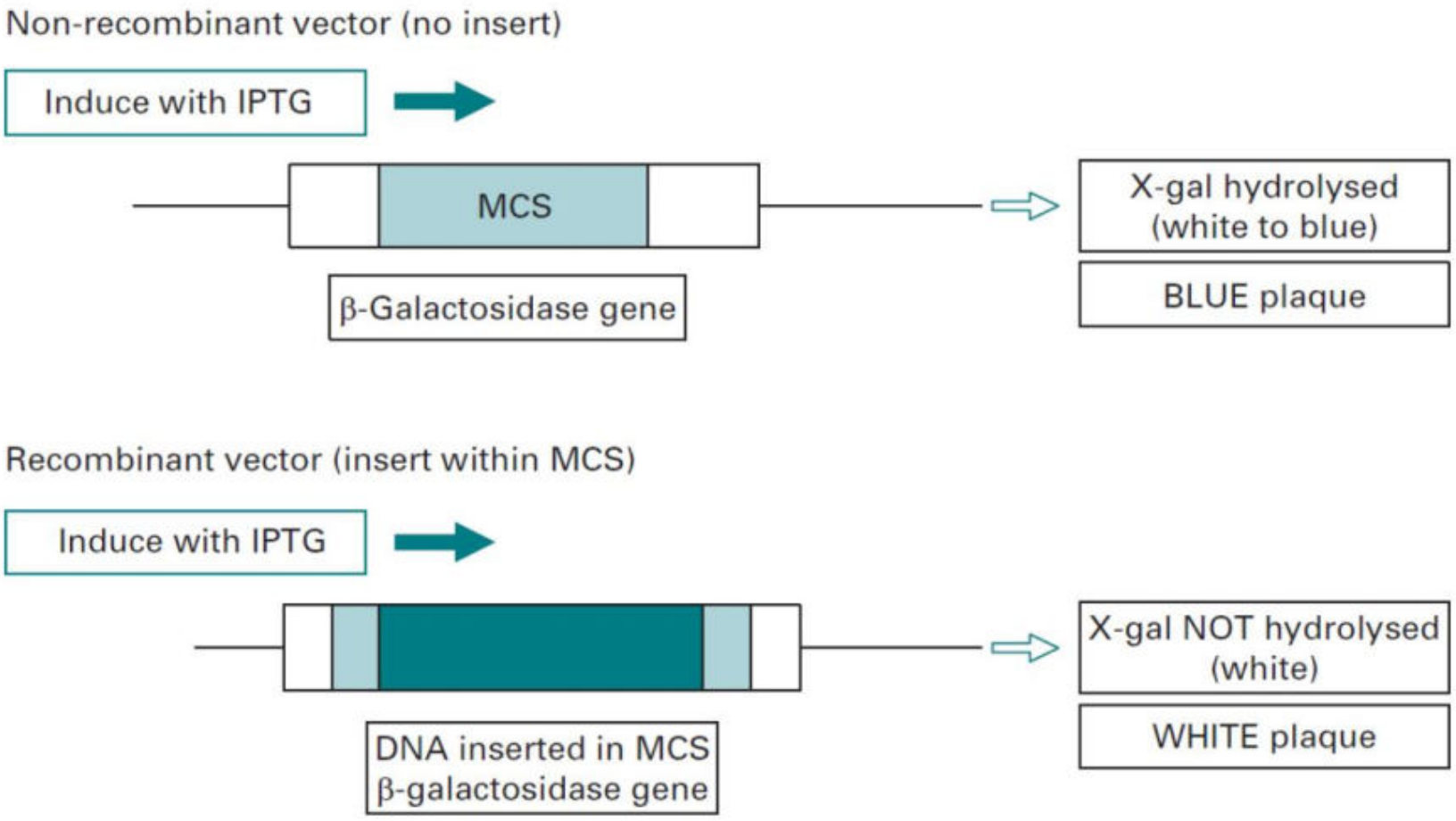
Replica plating to detect recombinant plasmids



Map and important features of pUC18

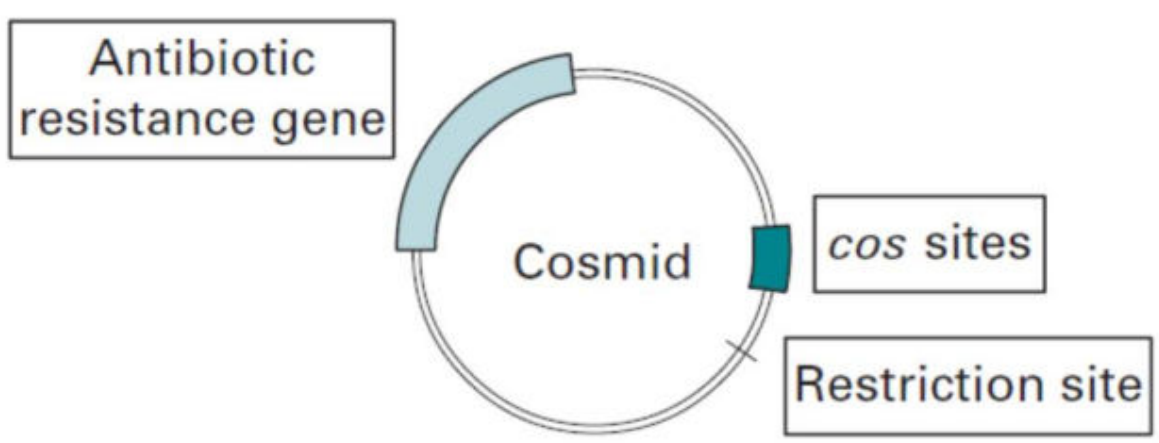


Principle of blue/white selection for the detection of recombinant vectors.



Cosmid vectors

Cosmid vectors incorporate the cos sites from phage l and also the essential features of a plasmid, such as the plasmid origin of replication, a gene for drug resistance, and several unique restriction sites for insertion of the DNA to be cloned.



Expression vector

- Insertion of a strong promoter.
- Insertion of a strong termination codon.
- Adjustment of distance between promoter and cloned gene.
- Insertion of transcription termination sequence.
- Insertion of a strong translation initiation sequence.

The inserted sequence must be placed so that its reading frame is in phase with the regulatory sequence

Shuttle vectors

- Shuttle vectors have origins of replication for yeast and bacteria such as E. coli. This means that constructs may be prepared rapidly in the bacteria and delivered into yeast for expression studies.

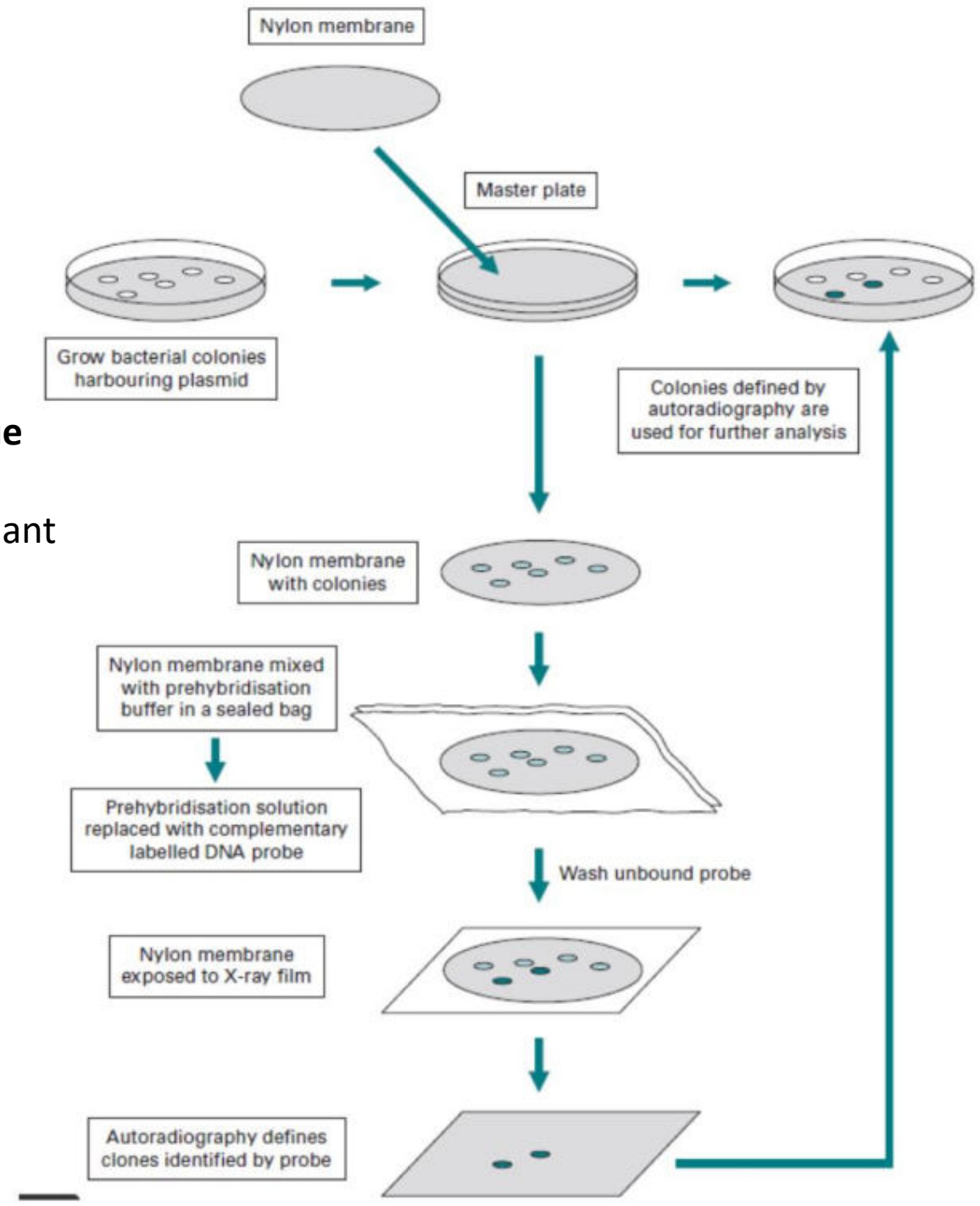
Delivery of vectors into Eukaryotes

- Transfection:
  - to deliver recombinant molecules into animal cells

- 1. making the membrane permeable with divalent cations / use of polyethylene glycol (PEG)
- 2. electroporation: subjected to pulses of a high-voltage gradient
- 3. lipofection: DNA is encapsulated by a core of lipid-coated particles

SCREENING GENE LIBRARIES

Colony hybridisation technique for locating specific bacterial colonies harbouring recombinant plasmid vectors containing desired DNA fragments**.**



Application of gene cloning

Molecular analysis of disease
- Normal gene variation—Polymorphism
- Gene variation causing disease—Beta globin gene
- Detection of point mutation---Sickle cell disease
- Detection of deletion /insertion/rearrangement—Beta globin gene

Prenatal diagnosis

Preimplantation diagnosis: done in IVF

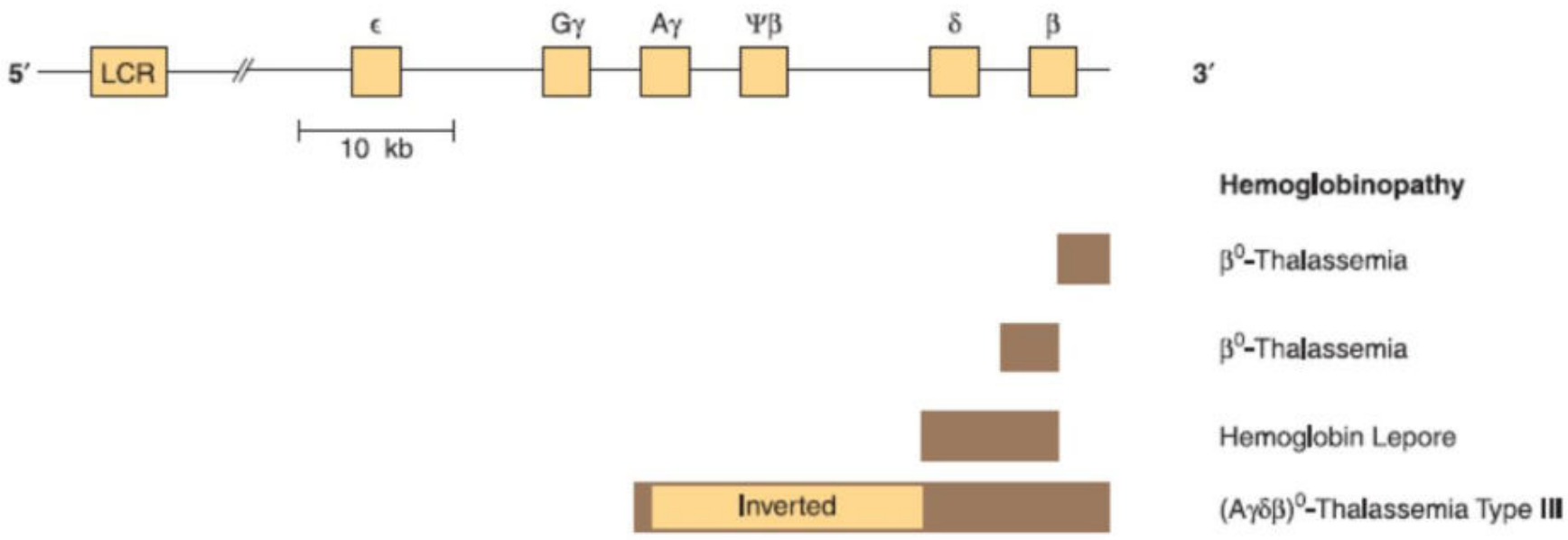
Disease linkage analysis—Microsatellite repeats in families

Forensic medicine

Structural Alterations of the a-Globin Gene

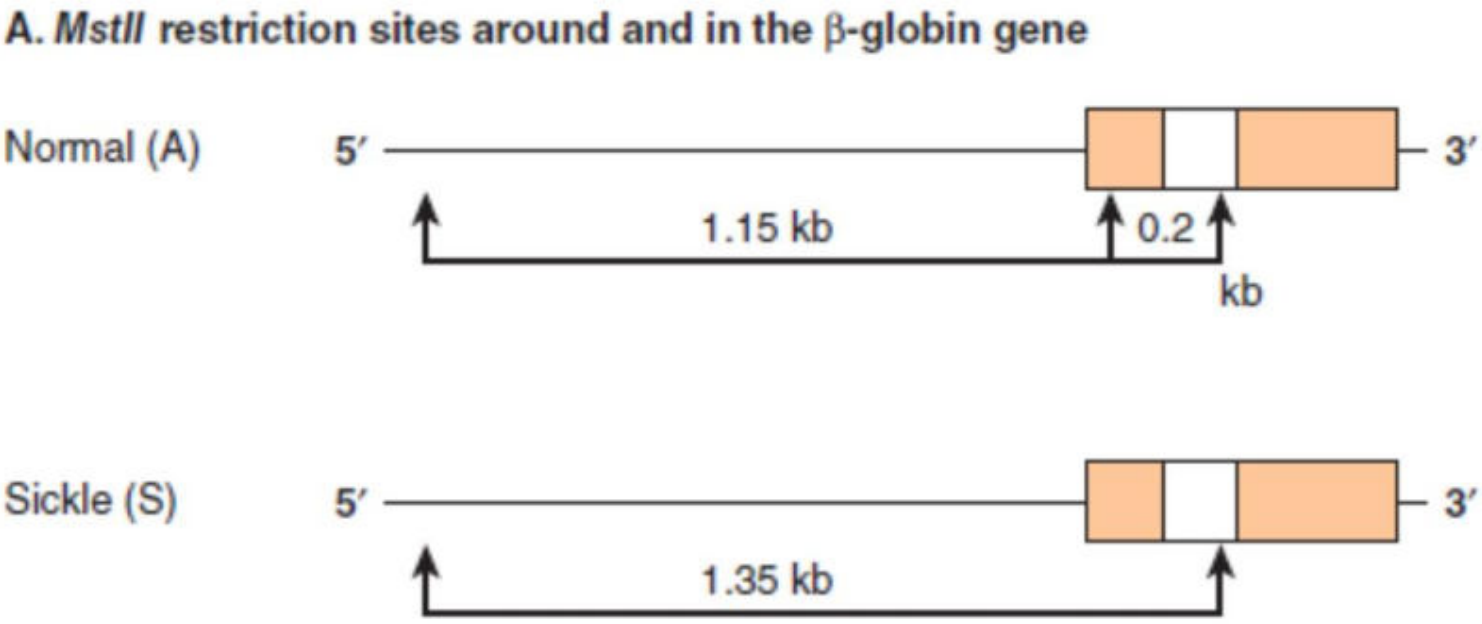
Alteration	Function Affected	Disease
Point mutations	Protein folding	Sickle cell disease
	Transcriptional control	β-Thalassemia
	Frameshift and nonsense mutations	β-Thalassemia
	RNA processing	β-Thalassemia
Deletion	mRNA production	β^0-Thalassemia
		Hemoglobin Lepore
Rearrangement	mRNA production	β-Thalassemia type III

Schematic representation of the β-globin gene cluster and of the lesions in some genetic disorders.

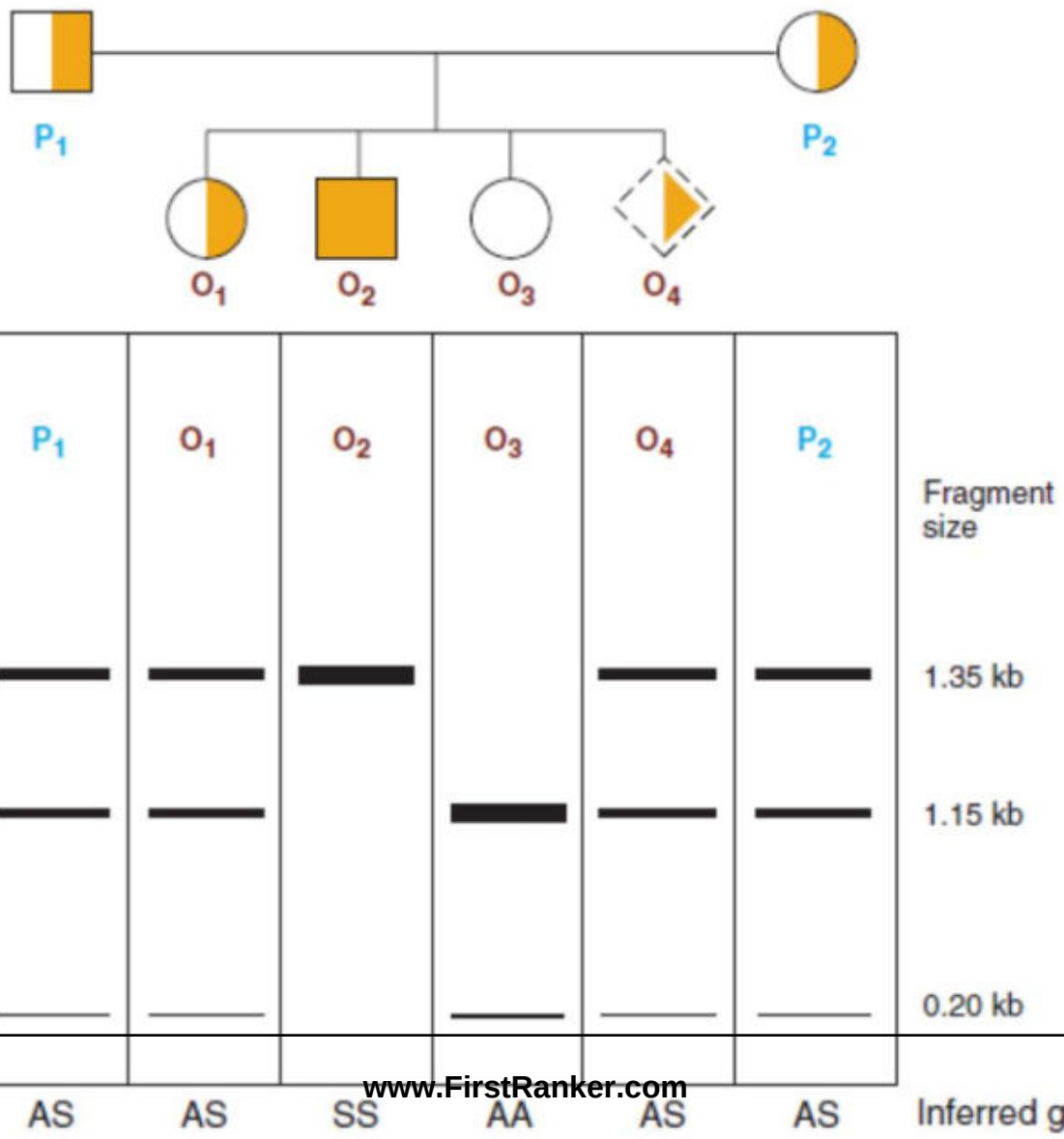


Sickle cell disease

C C T G A G G	Coding strand	C C T G T G G	Coding strand
G G A C Ⓣ C C	Template strand	G G A C Ⓐ C C	Template strand



Pedigree analysis of Sickle cell disease

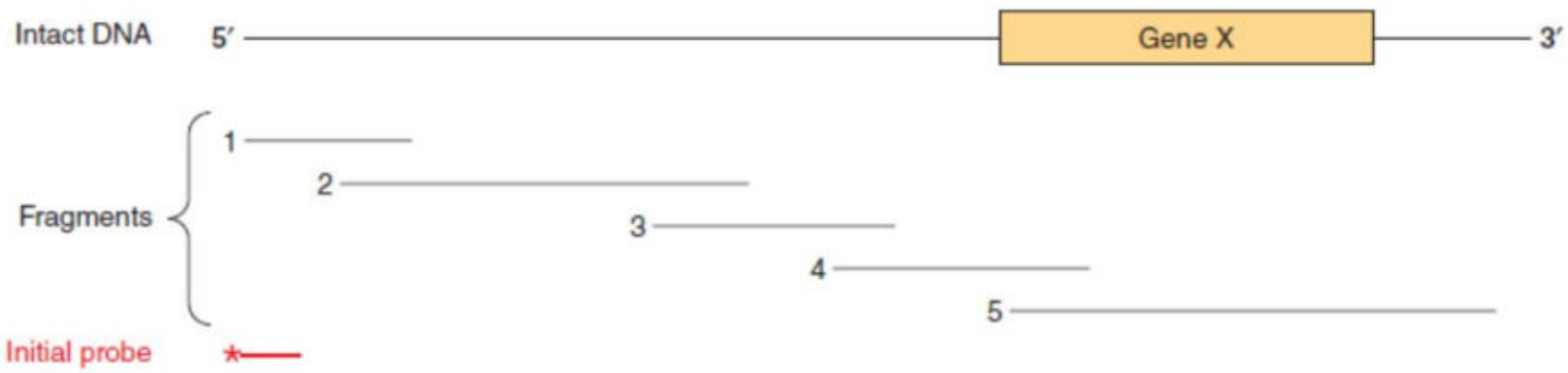


Restriction Fragment Length Polymorphism and SNPs

major use of SNPs/RFLPs is in the definition of inherited diseases in which the functional deficit is unknown

SNPs/RFLPs can be used to establish linkage groups, which in turn, by the process of **chromosome walking,**
will eventually define the disease locus

The technique of chromosome walking



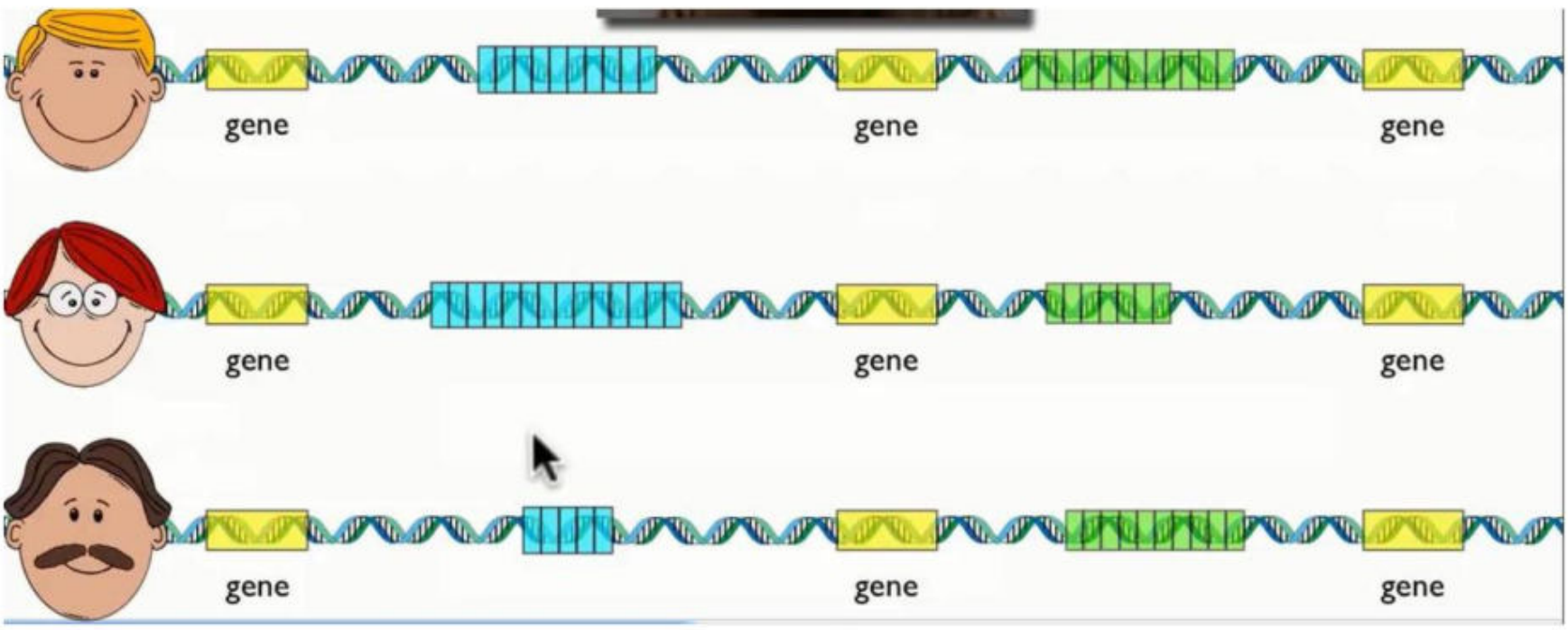
Microsatellite repeat variation in some diseases

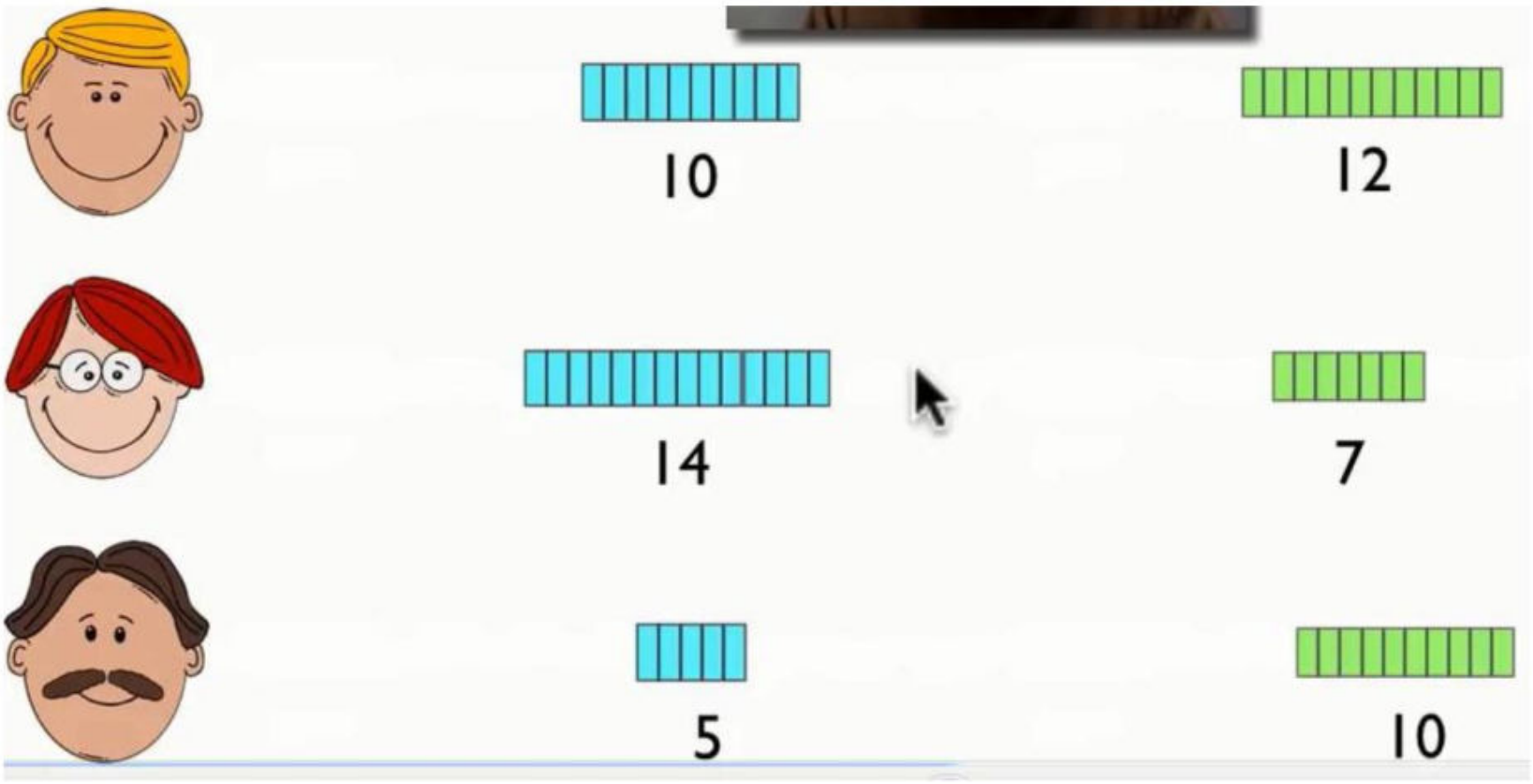
Disease	Repeat	Normal length of repeats	Mutation length
Fragile X syndrome	(CGG)n	6-54	200-1000
Fredriech ataxia	(GAA)n	7-22	7200
Myotonic dystrophy	(CTG)n	50-35	50-4000
Spino cerebellar ataxia	(CAG)n	19-36	43-81

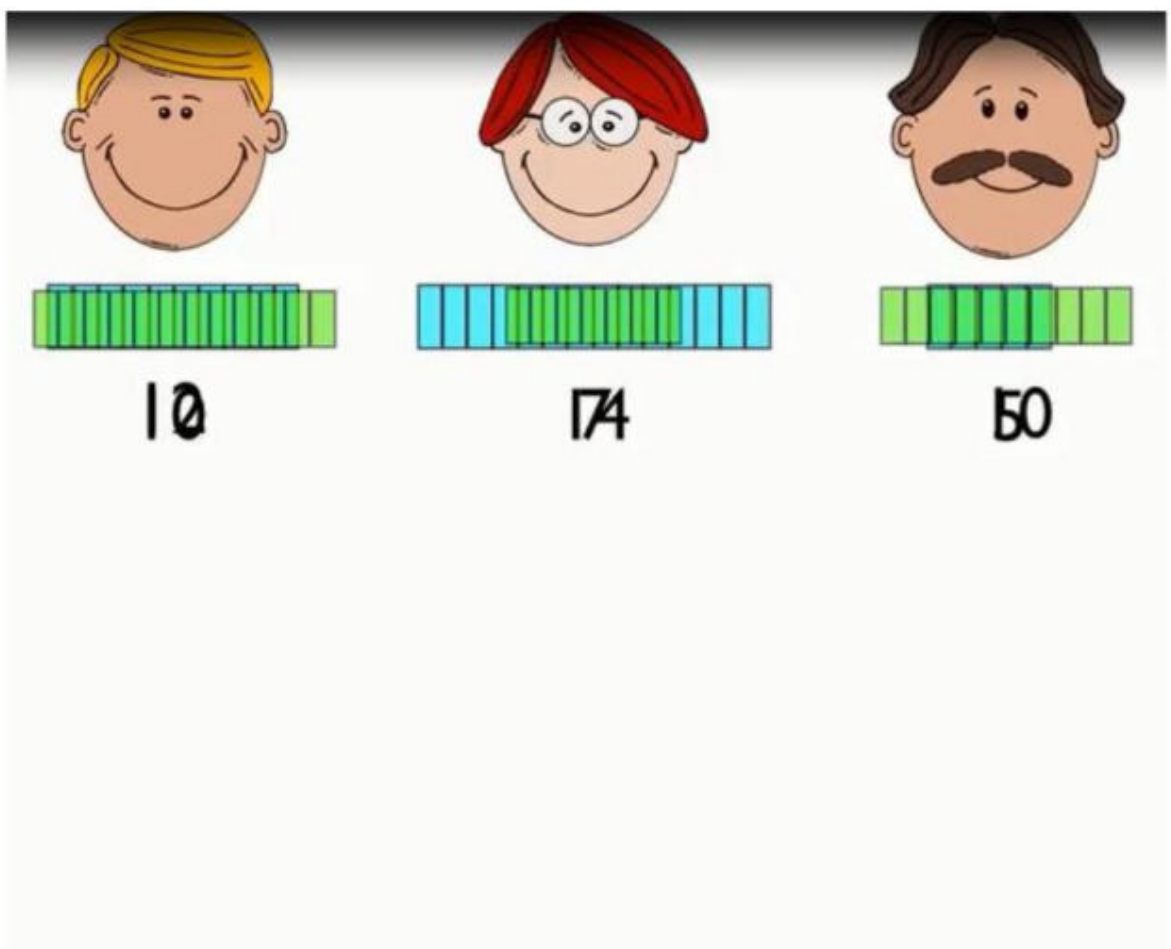
- **DNA Finger printing**

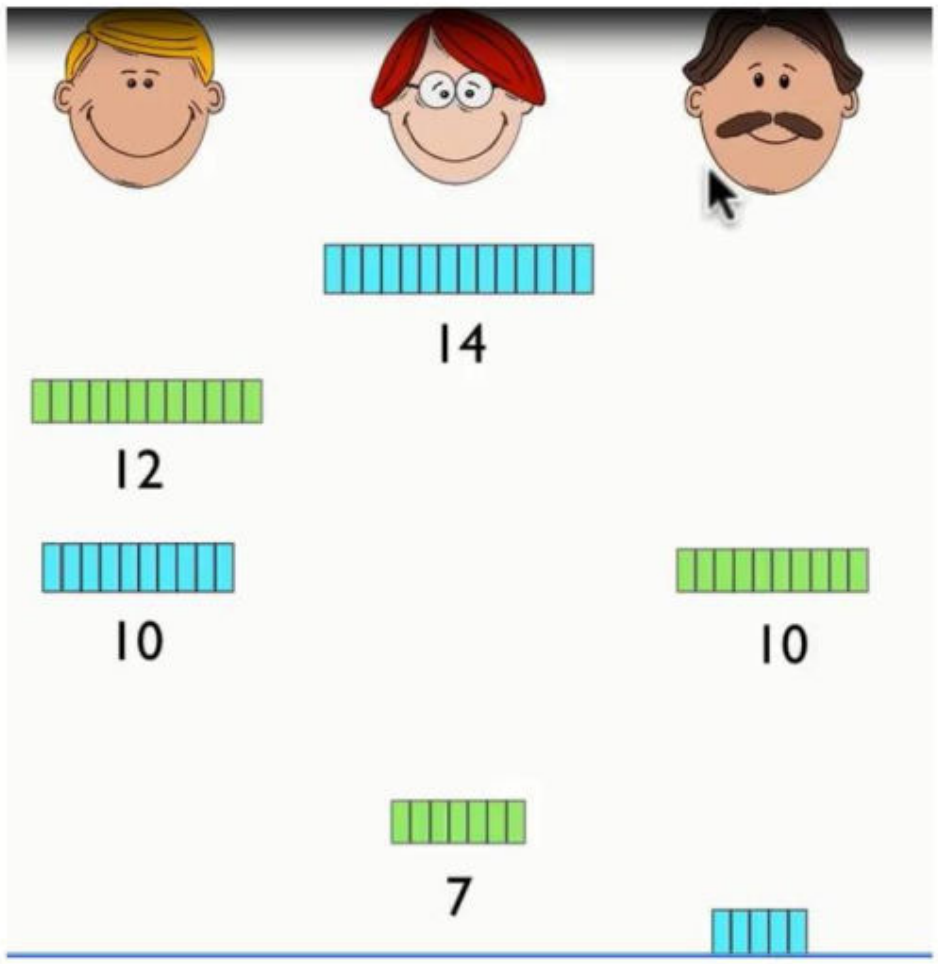
- Alec Jeffrey in 1984

- Each individual has unique sequences







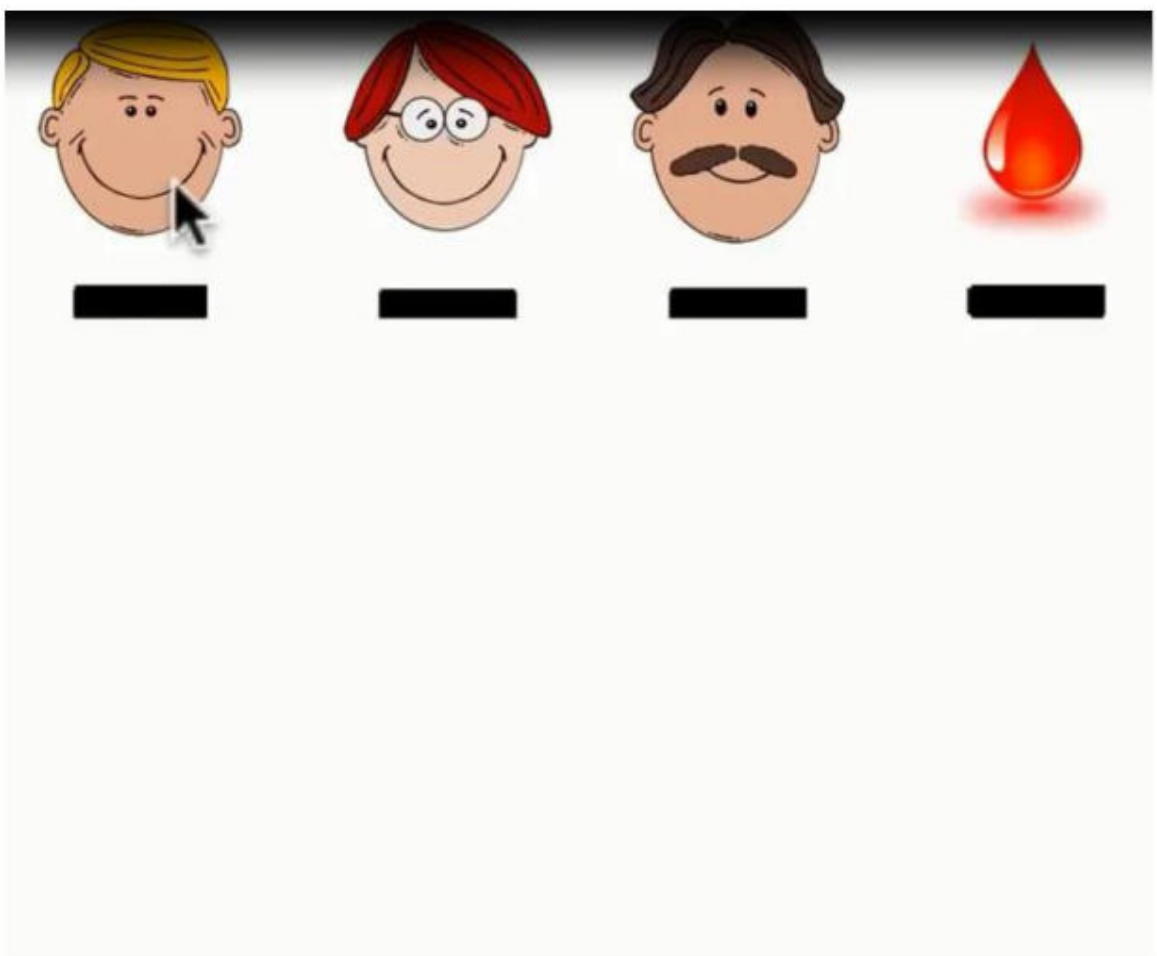


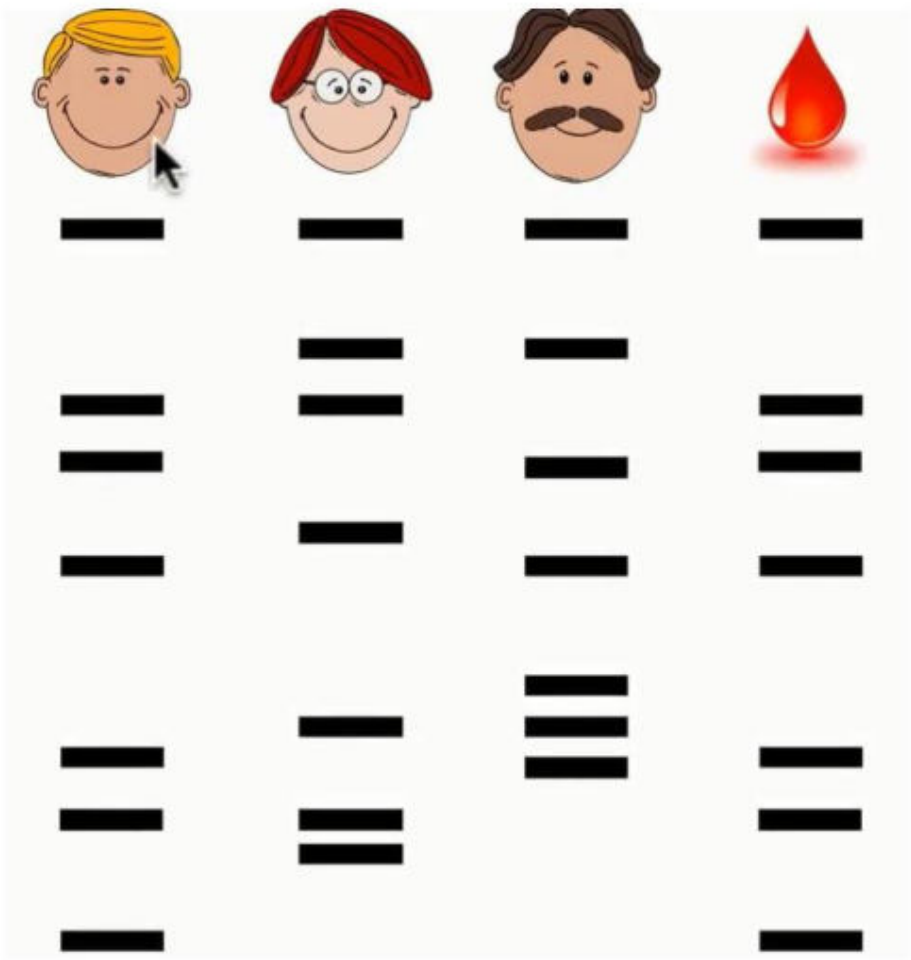
Purpose

- 1. Paternity dispute
- 2. Criminal identification

Method

- Isolation of DNA
- Digestion of DNA by RE
- Amplification
- Separation by gel electrophoresis
- Blotting
- Hybridisation with radiolabelled probe
- Autoradiography

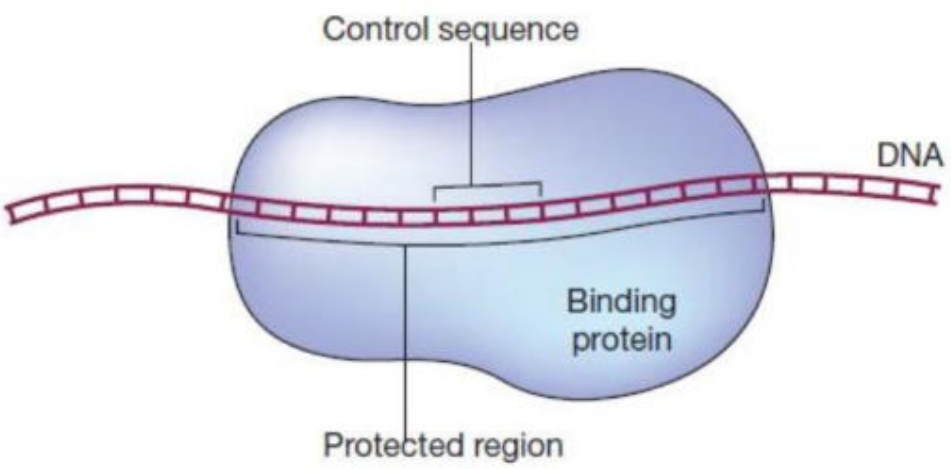




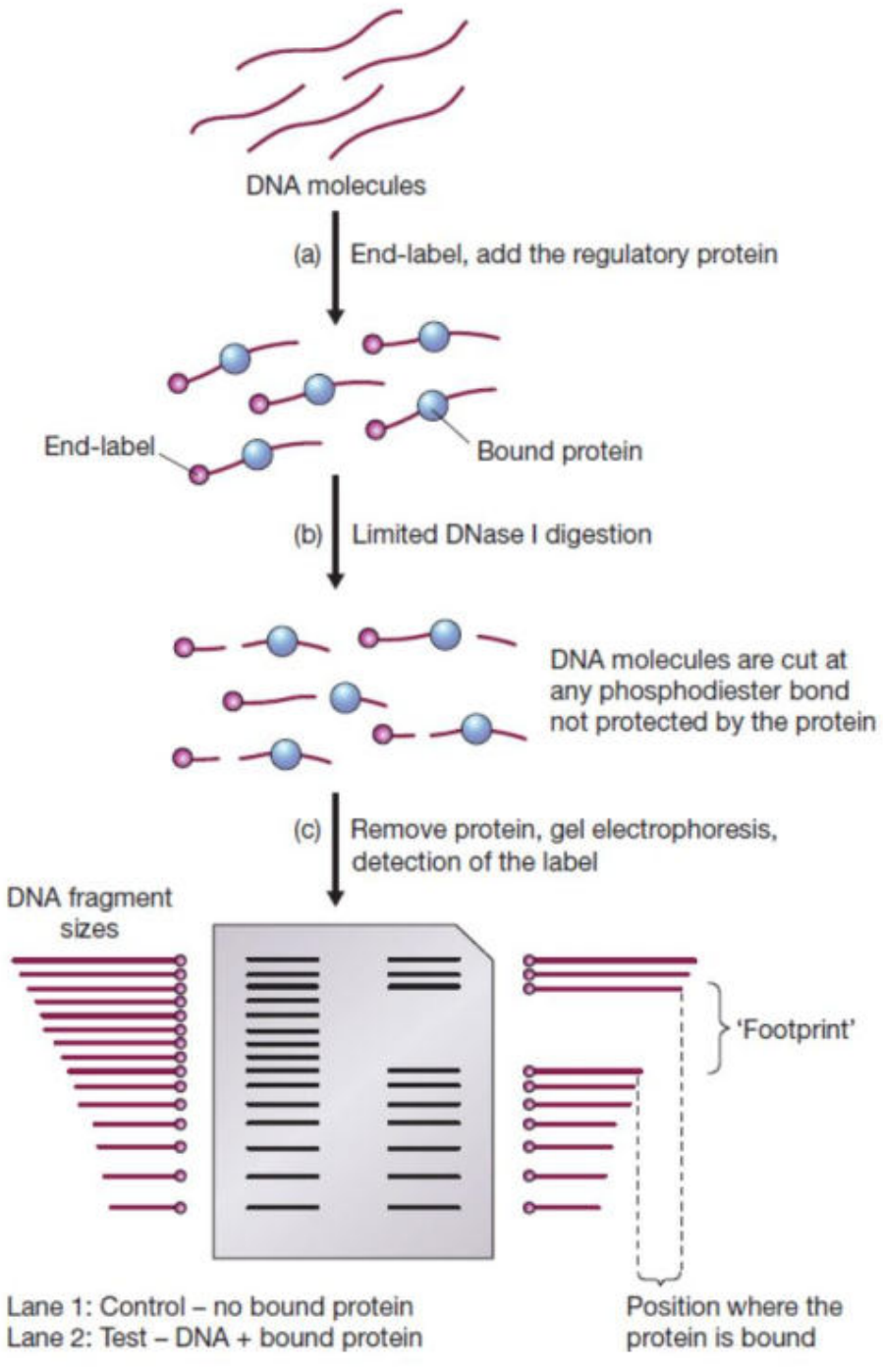
- ***DNA Footprinting***

Footprinting with DNase I

- **Footprinting enables a control region to be positioned** within a restriction fragment that has been identified by gel retardation.
- Footprinting works on the basis that the interaction with a regulatory protein protects the DNA in the region of a control sequence from the degradative action of an endonuclease such as deoxyribonuclease (DNase) I.
- This phenomenon can be used to locate the protein binding site on the DNA molecule.



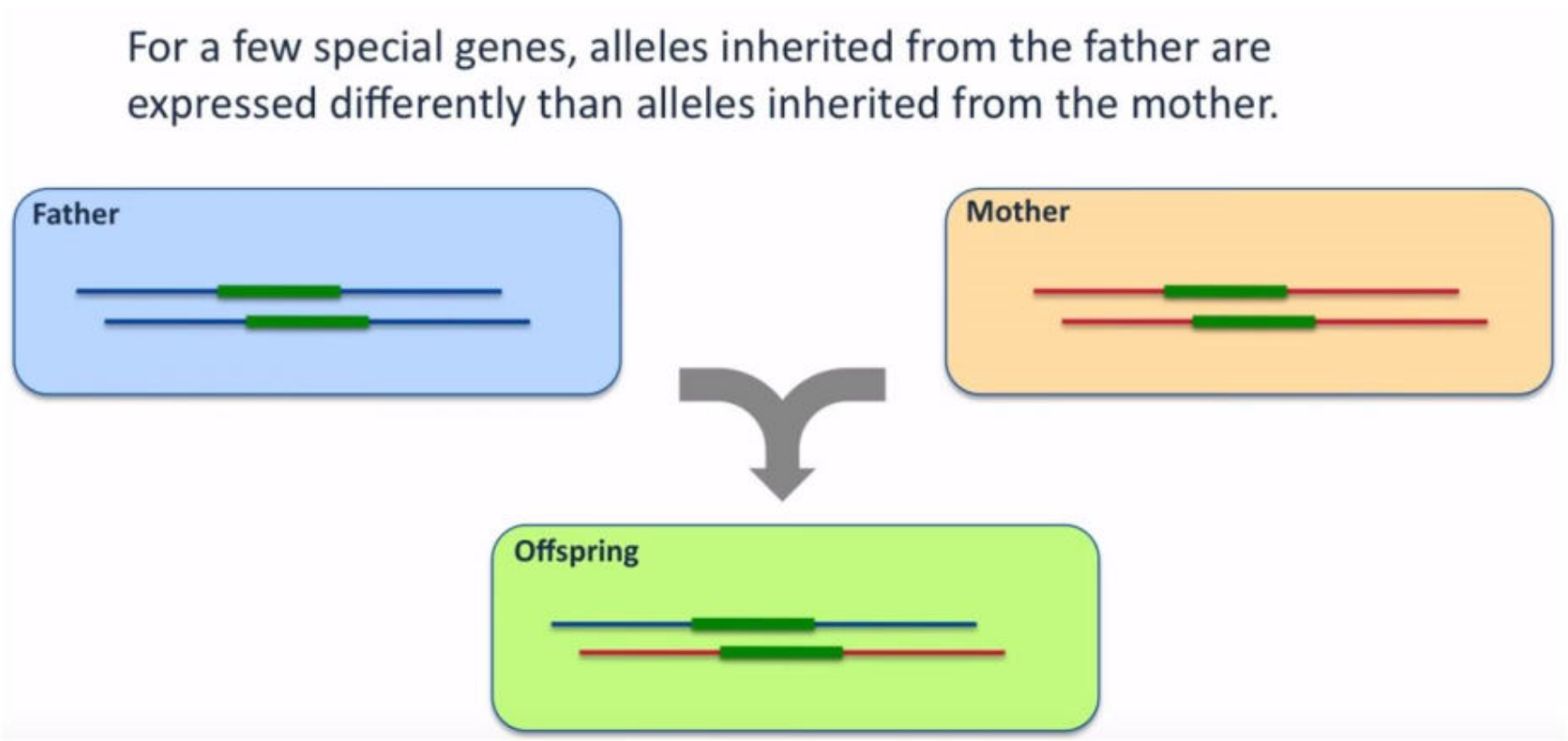
A bound protein can protect a region of DNA that is much longer than the control sequence.



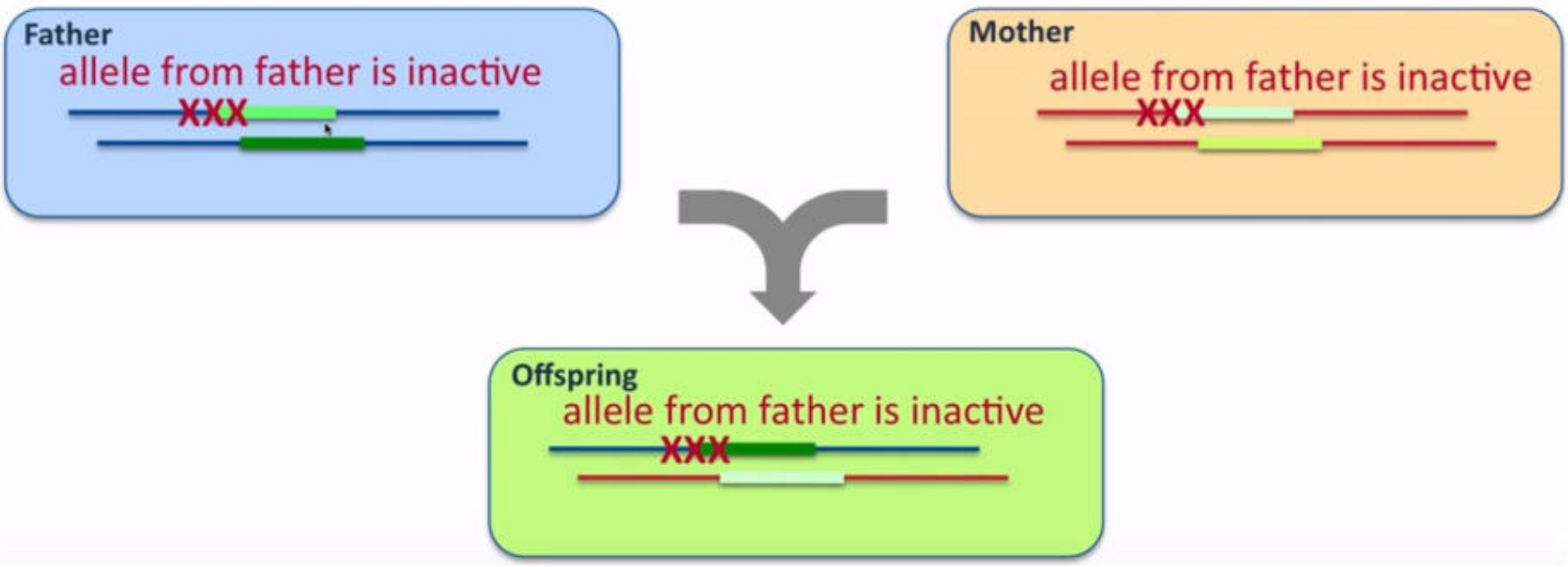
Genomic imprinting

What Mendel found out

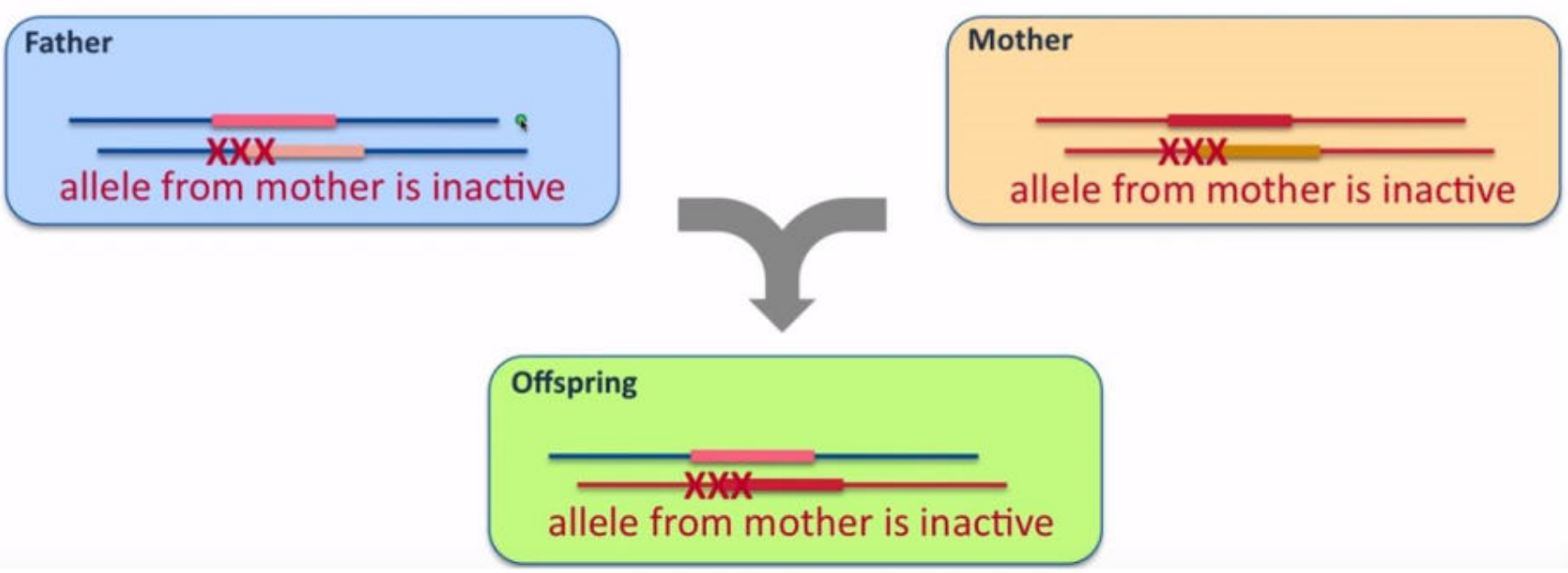
- Two parents make equal contribution to the character
- The effect of an allele is independent of whether it comes from the female or male gamete



The identity of the allele doesn't matter, just which parent it came from.



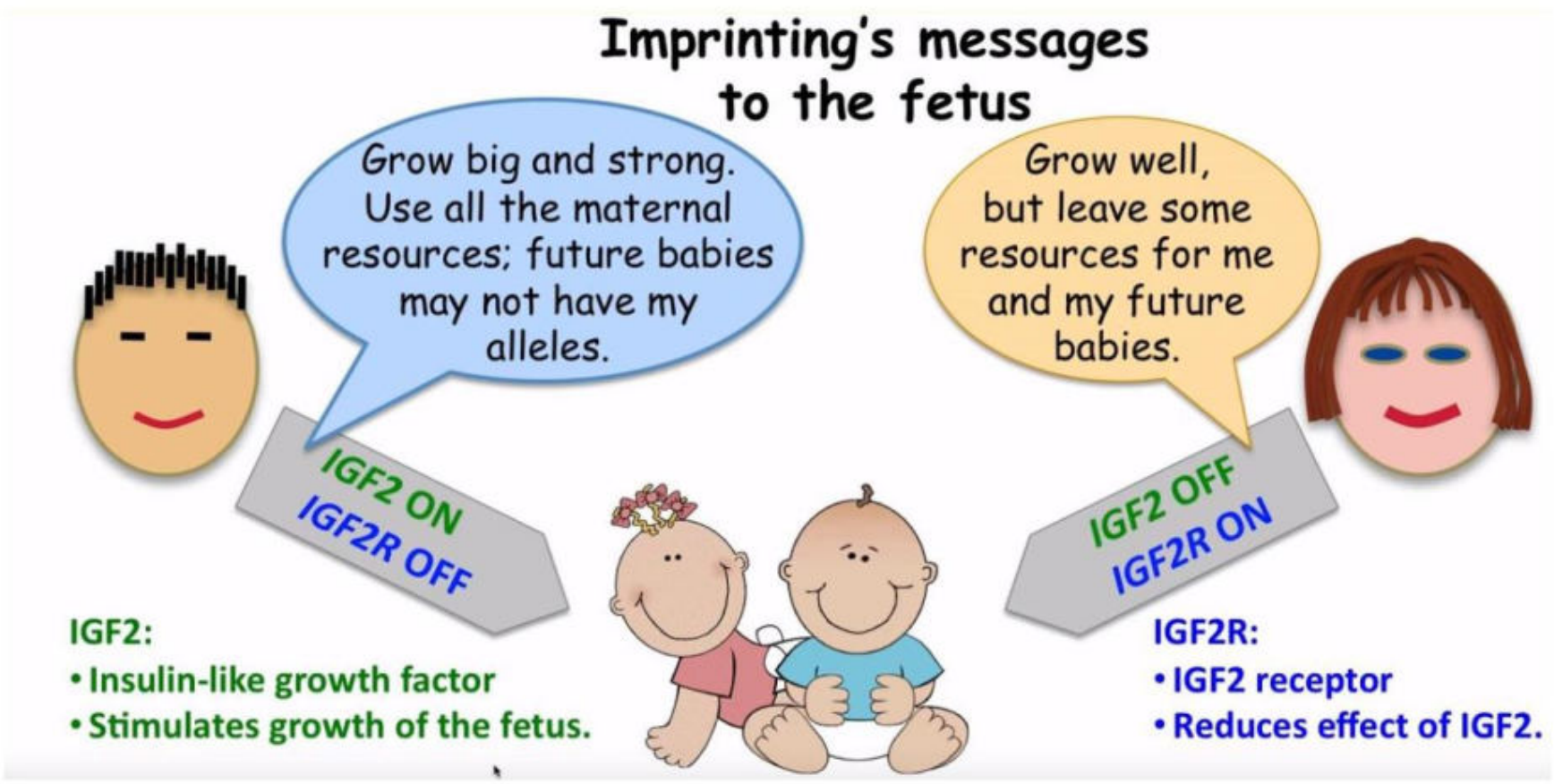
Here's an example of a different gene, where the ***maternal*** allele is always imprinted.



What is the function of genomic imprinting? (Why did it evolve?)

The best hypothesis:

Parental Conflict



Genomic imprinting usually uses DNA methylation:

When the germ line develops, all of the existing (inherited) imprinting methylation is erased.

Then new methylation is created:

A gene that's evolved to be paternally imprinted has a sequence-tag that says:

- If you're male, methylate this gene (turn it off) before you make gametes.

A gene that's evolved to be maternally imprinted has a sequence-tag that says:

- If you're female, methylate this gene (turn it off) before you make gametes.

Imprinting has evolved to a state where both parents' interests are roughly in balance.

But it causes problems if something goes wrong with normal inheritance:

a) Defective allele from the non-imprinted parent

b) Deletion from the non-imprinted parent

c) Both alleles inherited from one parent ('uniparental disomy').

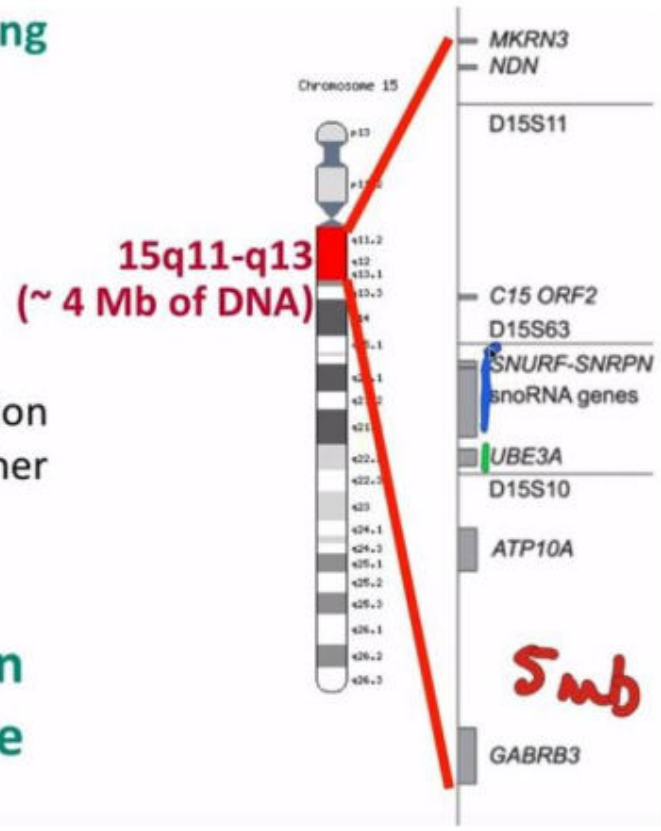
b) and c) are especially bad because imprinted genes are often clustered together on one chromosome.

Imprinting causes problems if something goes wrong with normal inheritance:

One segment of chromosome 15 includes genes that are paternally imprinted and genes that are maternally imprinted.

Inherit deletion from father → **Prader-Willi Syndrome**

Inherit deletion from mother → **Angelman Syndrome**



Summary

- Recombinent DNA is the joining of two fragments cut with same restriction endonuclease
- Probe is a 15-20 nt long oligonucleotide used to search a particular DNA fragment
- Cloning is a process of producing large number of identical copies from one single original DNA molecule or fragment
- Type of gene library depends on final application of DNA
- Delivery of vector in bacteria is called transformation and in animal cells is transfection
- RFLP is a technique used to identify the individual as well as to detect disease condition such as Sickle cell anaemia
- DNA finger printing is to identify the individual based on differential tandem repeat sequences
- Footprinting works on the basis that the interaction with a regulatory protein protects the DNA from the degradative action of an endonuclease such as deoxyribonuclease (DNase) I.
- Genomic imprinting usually uses DNA methylation (epigenetic regulatiion).