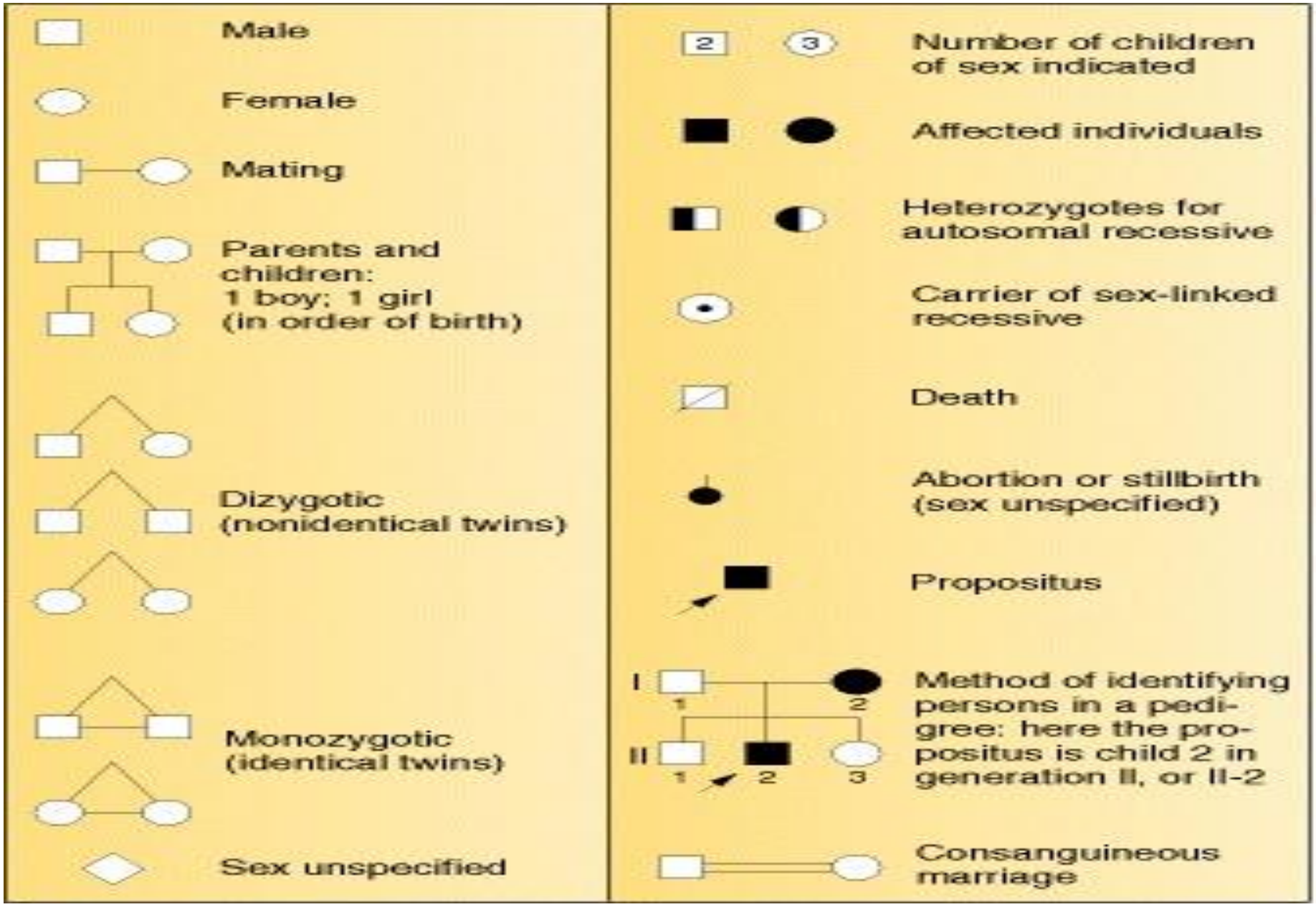
Chapter 5
Genetic Testing

Prepared by
Dr. Mohammed Laqqan

Introduction

- Genetic diseases often depend on the genotype of an individual at a single locus.
- Traits inherited in this manner are said to follow a Mendelian inheritance pattern.
- The mode of inheritance of a trait within a family can help suggest a diagnosis.
- **Pedigree Analysis**
- Pedigree analysis is a method of recording family information to trace the passage of a gene through generations.
- The pattern of inheritance one finds in a family depends on whether the mutant gene is located on an autosome, the X chromosome, or mitochondrial DNA. It also depends on whether the phenotype is dominant or recessive.

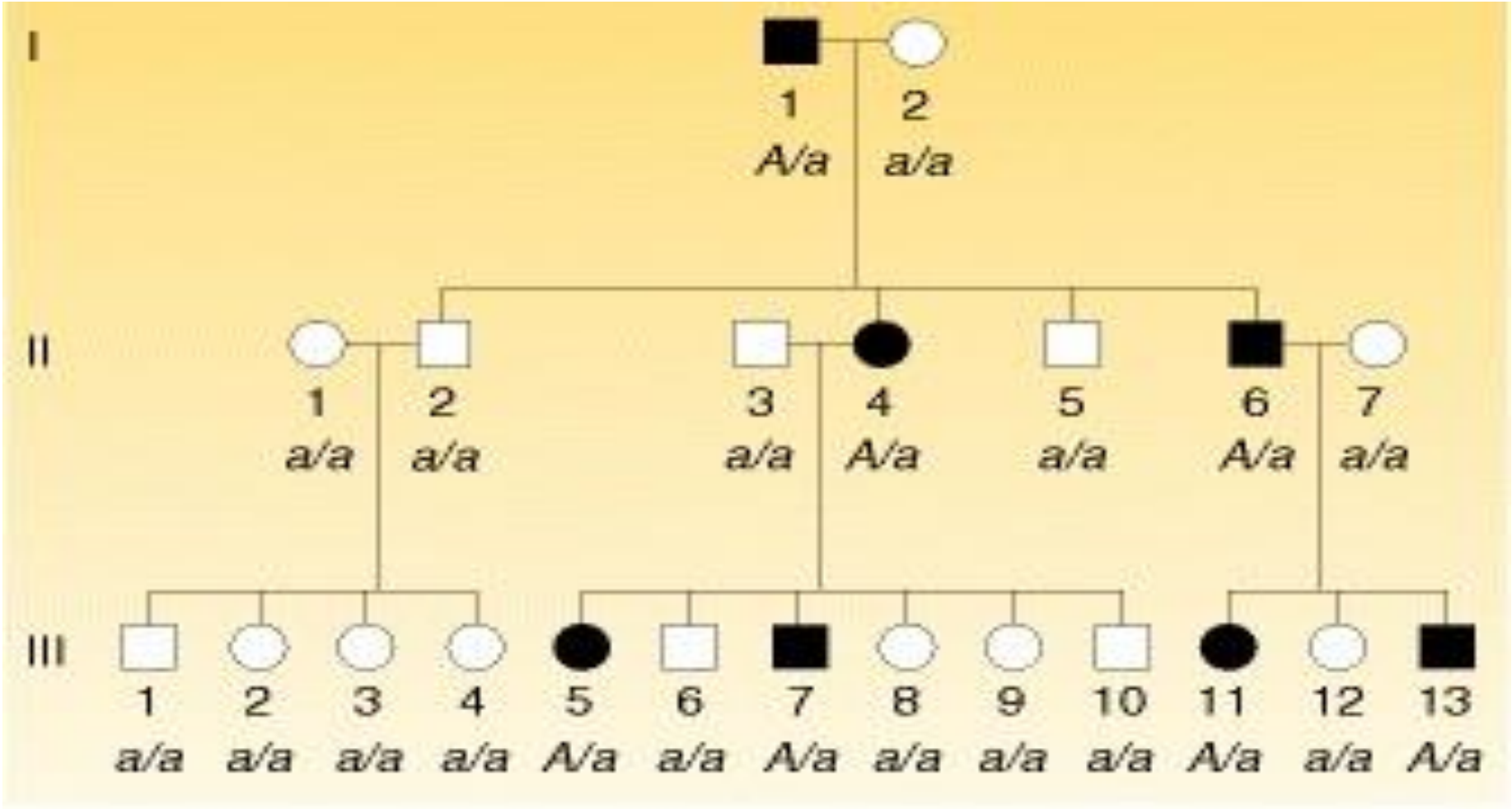

Male
Female
Mating
Parents and children: 1 boy; 1 girl (in order of birth)
Dizygotic (nonidentical twins)
Monozygotic (identical twins)
Sex unspecified
2
3
Number of children of sex indicated
Affected individuals
Heterozygotes for autosomal recessive
Carrier of sex-linked recessive
Death
Abortion or stillbirth (sex unspecified)
Propositus
I
1
2
II
1
2
3
Method of identifying persons in a pedigree: here the propositus is child 2 in generation II, or II-2
Consanguineous marriage

Autosomal Dominant

- In autosomal dominant disorders, the normal allele is recessive (d) and the abnormal allele is dominant (D).
- The phenotype appears in every generation because generally the abnormal allele carried by an individual must have come from a parent in the previous generation.
- Affected fathers and mothers transmit the phenotype to both sons and daughters.
- Individuals bearing one copy of the rare allele (D/d) are much more common than those bearing two copies (D/D), so most affected people are heterozygotes.
- Abnormal alleles can arise de novo by mutation. This is relatively rare, but must be kept in mind as a possibility.

Parental Mating	Offspring	Risk to Offspring
Affected by unaffected D/d ×d/d	1/2 D/d, 1/2 d/d	1/2 affected 1/2 unaffected
Affected by affected D/d ×D/d	1/4 D/D, 1/2 D/d, 1/4 d/d	If strictly dominant: 3/4 affected 1/4 unaffected
		If incompletely dominant: 1/2 affected similarly to the parents 1/4 affected more severely than the parents 1/4 unaffected

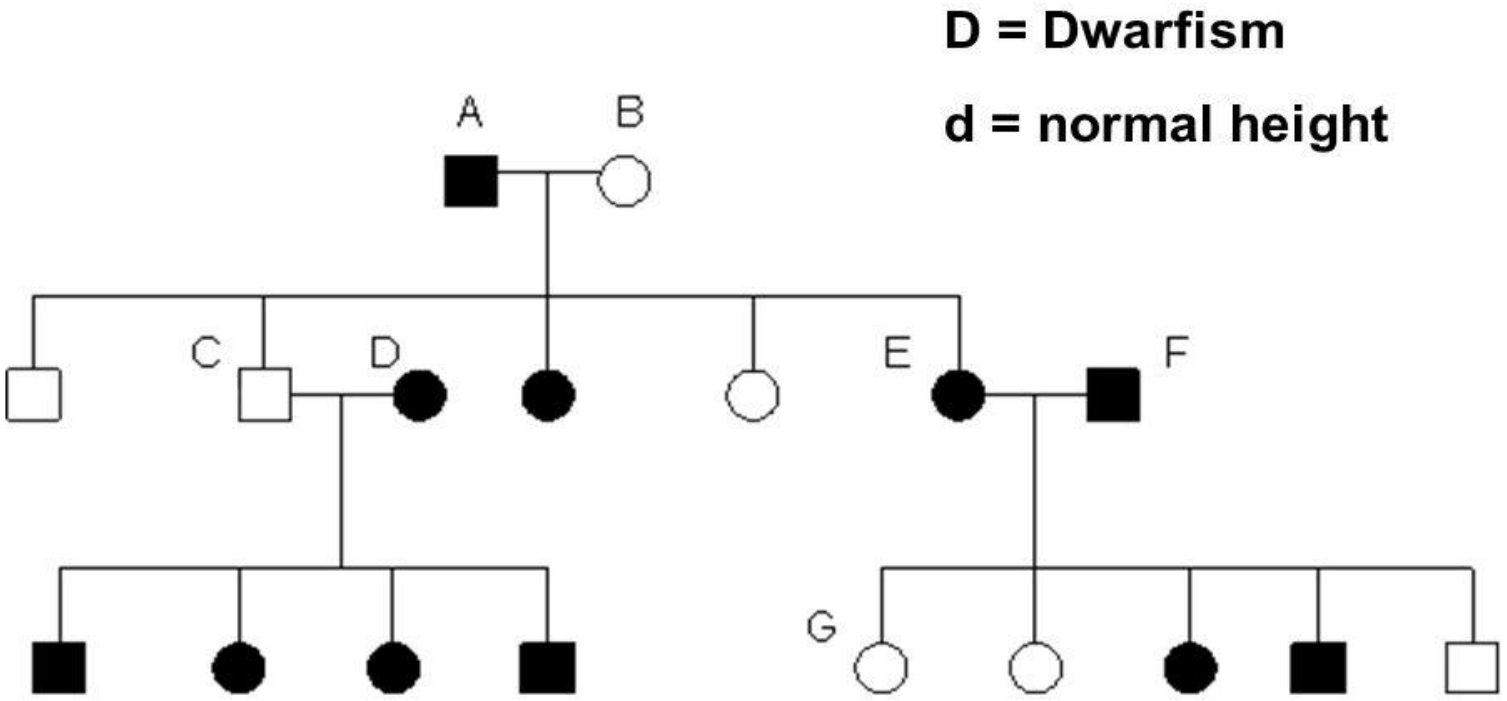
A typical pedigree for a dominant disorder



Example - Achondroplasia

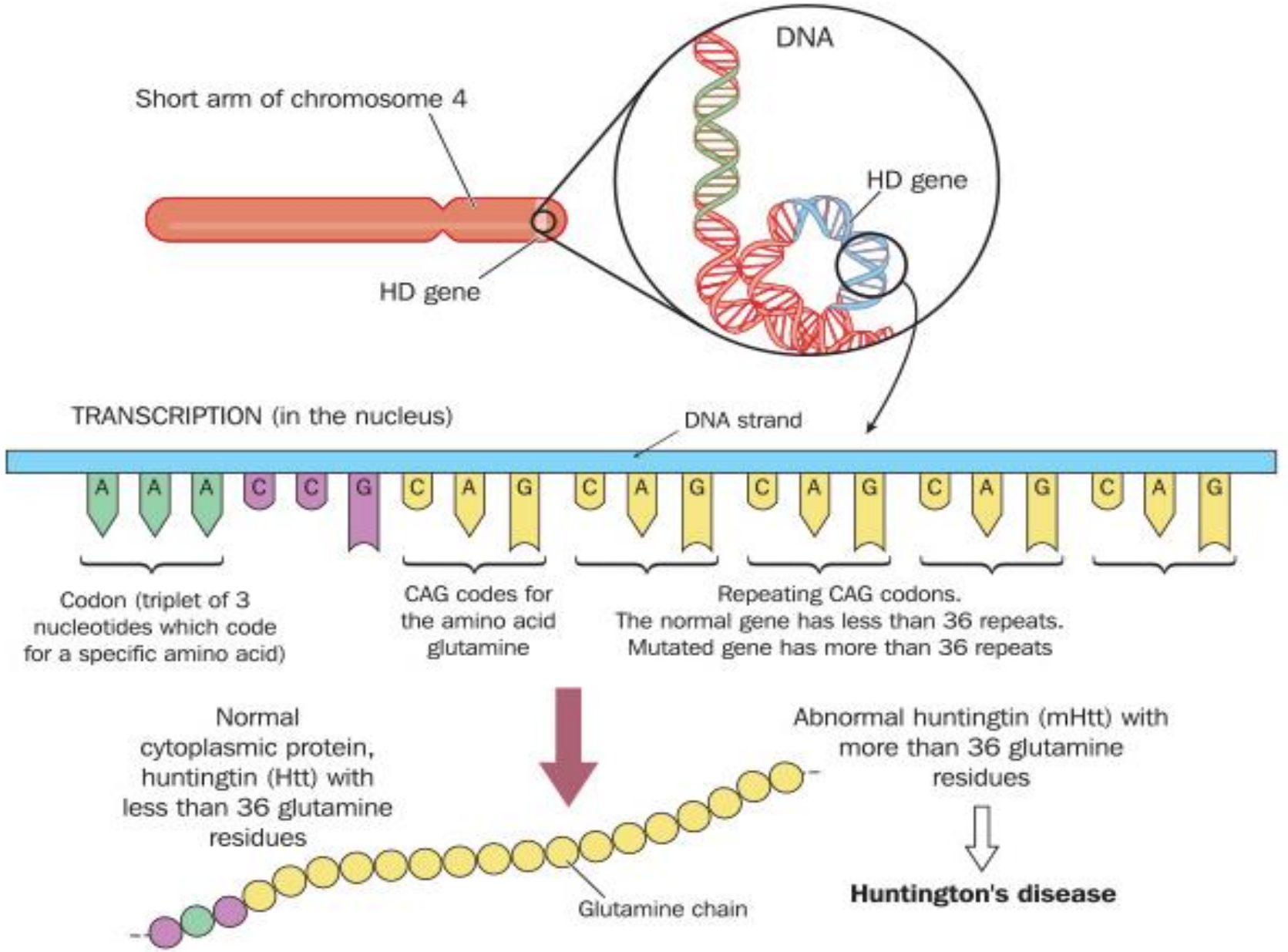
- **Dwarfism** is defined as a condition of short stature as an adult.
- In more than **80 percent** of cases, achondroplasia isn't inherited, and these cases are caused by spontaneous mutations in the ***FGFR3*** gene (**fibroblast growth factor receptor 3**).
- These proteins play a role in several important cellular processes, including regulation of cell growth and division, determination of cell type, formation of blood vessels, wound healing, and embryo development.

- The exact change that happens to the amino acid change that causes achondroplasia is that the amino acid **glycine** is replaced with **Arginine**.
- Infants born with homozygous achondroplasia are usually stillborn or die within a few months of being born.



Example - Huntington's disease

- **Huntington's disease** is an inherited disease (autosomal dominant) that causes the progressive breakdown (degeneration) of nerve cells in the brain, leading to convulsions (spasms), emotional problems, and loss of thinking ability (cognition) and premature death.
- The movement disorders associated with Huntington's disease can include both involuntary movement problems and impairments in voluntary movements.
- Huntington disease is caused by a mutation in the gene that makes the protein called **huntingtin**.


DNA
Short arm of chromosome 4
HD gene
HD gene
TRANSCRIPTION (in the nucleus)
DNA strand
A A A C C G C A G C A G C A G C A G C A G
Codon (triplet of 3 nucleotides which code for a specific amino acid)
CAG codes for the amino acid glutamine
Repeating CAG codons.
The normal gene has less than 36 repeats.
Mutated gene has more than 36 repeats
Normal cytoplasmic protein, huntingtin (Htt) with less than 36 glutamine residues
Abnormal huntingtin (mHtt) with more than 36 glutamine residues
Glutamine chain
Huntington's disease

Example - Marfan syndrome

- Marfan syndrome is an inherited disorder that affects connective tissue.
- Marfan syndrome most commonly affects the heart, eyes, blood vessels and skeleton.
- People with Marfan syndrome are usually tall and thin with disproportionately long arms, legs, fingers, and toes.
- The damage caused by Marfan syndrome can be mild or severe.
- If your aorta is affected, the condition can become life-threatening.
- The incidence of Marfan syndrome is approximately 1 in 5,000 worldwide.

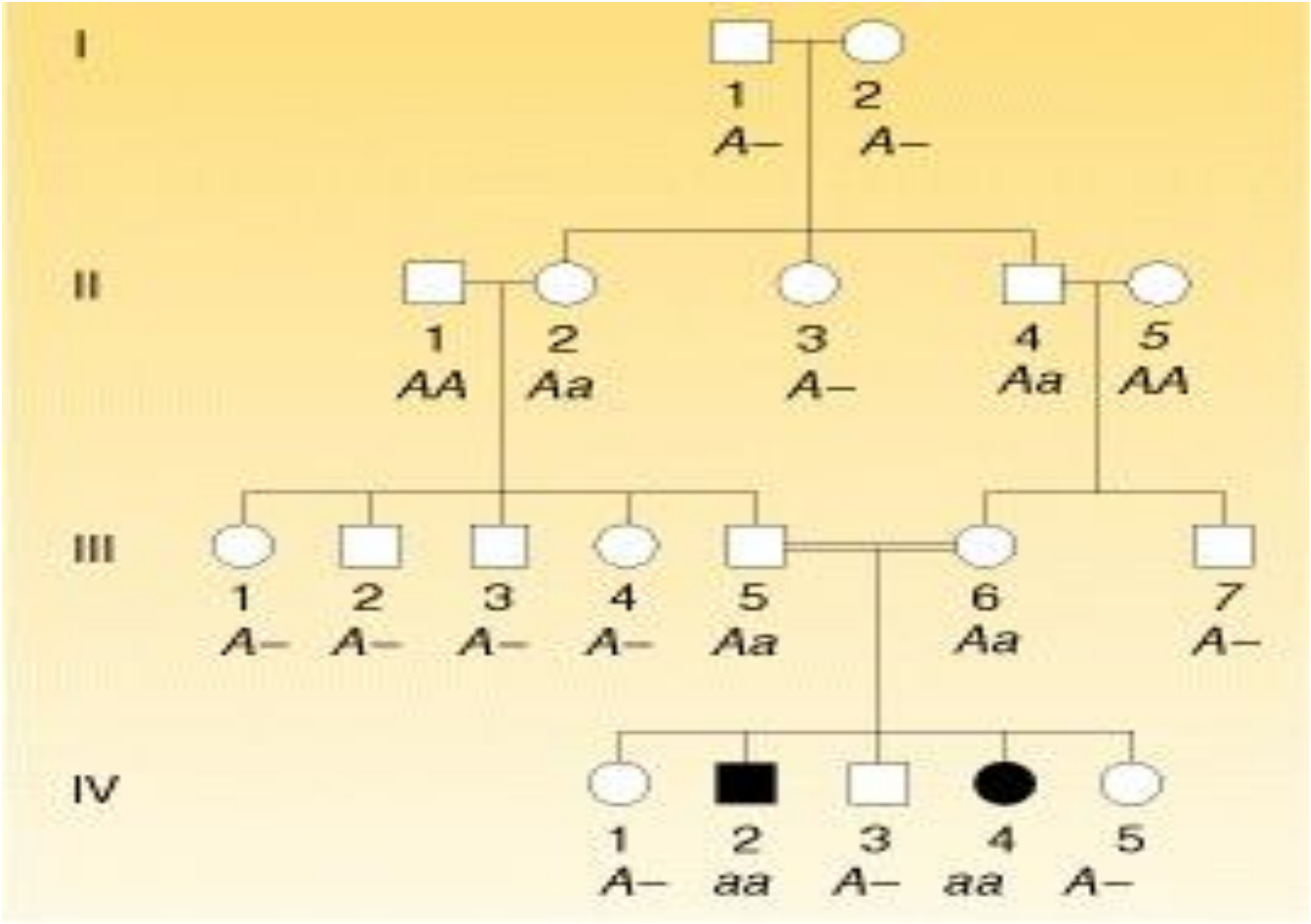
Causes

- Mutations in the ***FBN1*** gene cause Marfan syndrome.
- The ***FBN1*** gene provides instructions for making a protein called fibrillin-1.
- Fibrillin-1 attaches to other fibrillin-1 proteins and other molecules to form threadlike filaments called microfibrils.
- Microfibrils become part of the fibers that provide strength and flexibility to connective tissue.
- Additionally, microfibrils bind to molecules called growth factors and release them at various times to control the growth and repair of tissues and organs throughout the body.

- A mutation in the FBN1 gene can reduce the amount of functional fibrillin-1 that is available to form microfibrils, which leads to decreased microfibril formation.
- As a result, microfibrils cannot bind to growth factors, so excess growth factors are available and elasticity in many tissues is decreased, leading to overgrowth and instability of tissues in Marfan syndrome

Autosomal Recessive

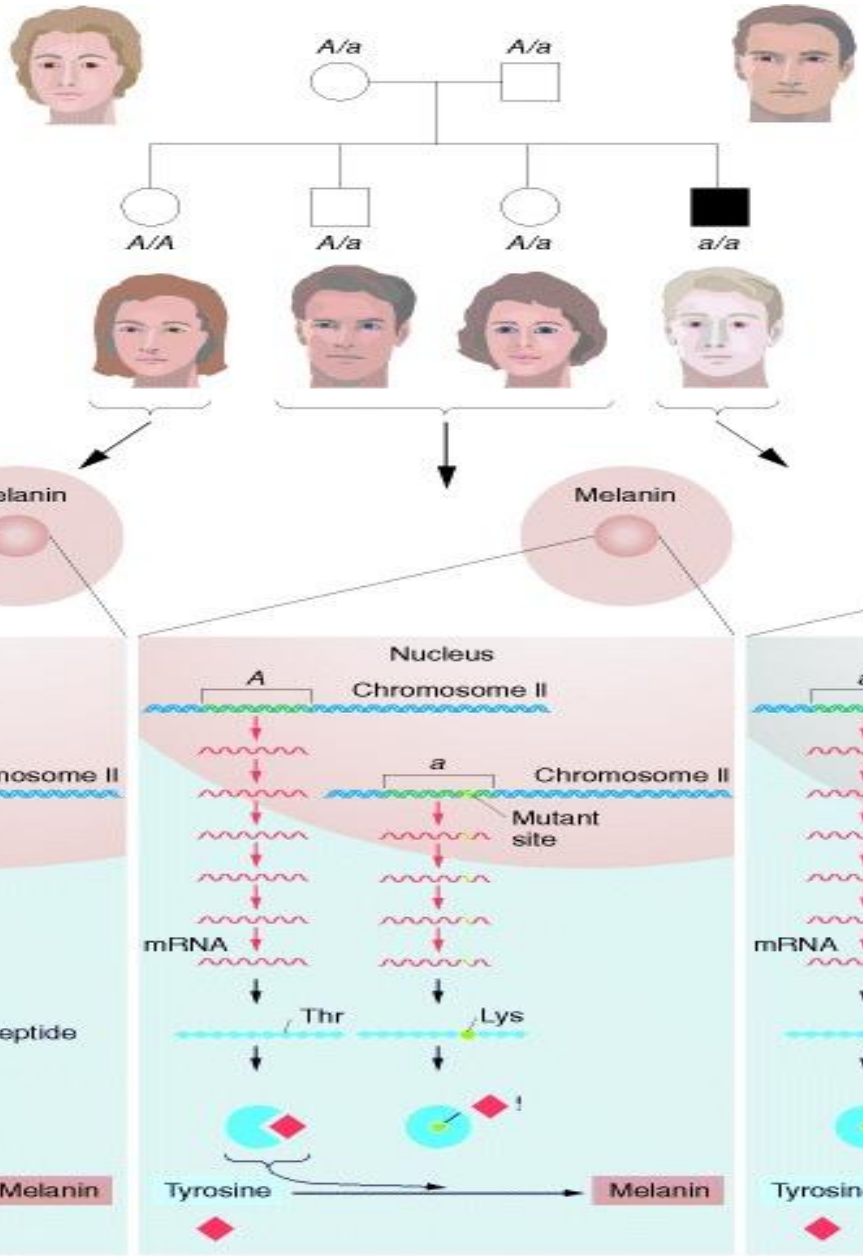
- Neither of the parents may have the disorder.
- An autosomal recessive disorder means two copies of an abnormal gene must be present in order for the disease or trait to develop (homozygous) (rr).
- If you are born to parents who carry the same autosomal recessive change (mutation), you have a 1 in 4 chance of inheriting the abnormal gene from both parents and developing the disease.
- **The disorder skips generations.**


I
1
A–
2
A–
II
1
AA
2
Aa
3
A–
4
Aa
5
AA
III
1
A–
2
A–
3
A–
4
A–
5
Aa
6
Aa
7
A–
IV
1
A–
2
aa
3
A–
4
aa
5
A–

- If the cases are born to parents who both carry the same autosomal recessive gene, cases have a 1 in 4 chance of inheriting the abnormal gene from both parents and developing the disease. Cases have a 50% (1 in 2) chance of inheriting one abnormal gene. This would make you a carrier.
- In other words, for a child born to a couple who both carry the gene (but do not have signs of disease), the expected outcome for each pregnancy is:
  - A 25% chance that the child is born with two normal genes (normal)
  - A 50% chance that the child is born with one normal and one abnormal gene (carrier, without disease).
  - A 25% chance that the child is born with two abnormal genes (at risk for the disease)

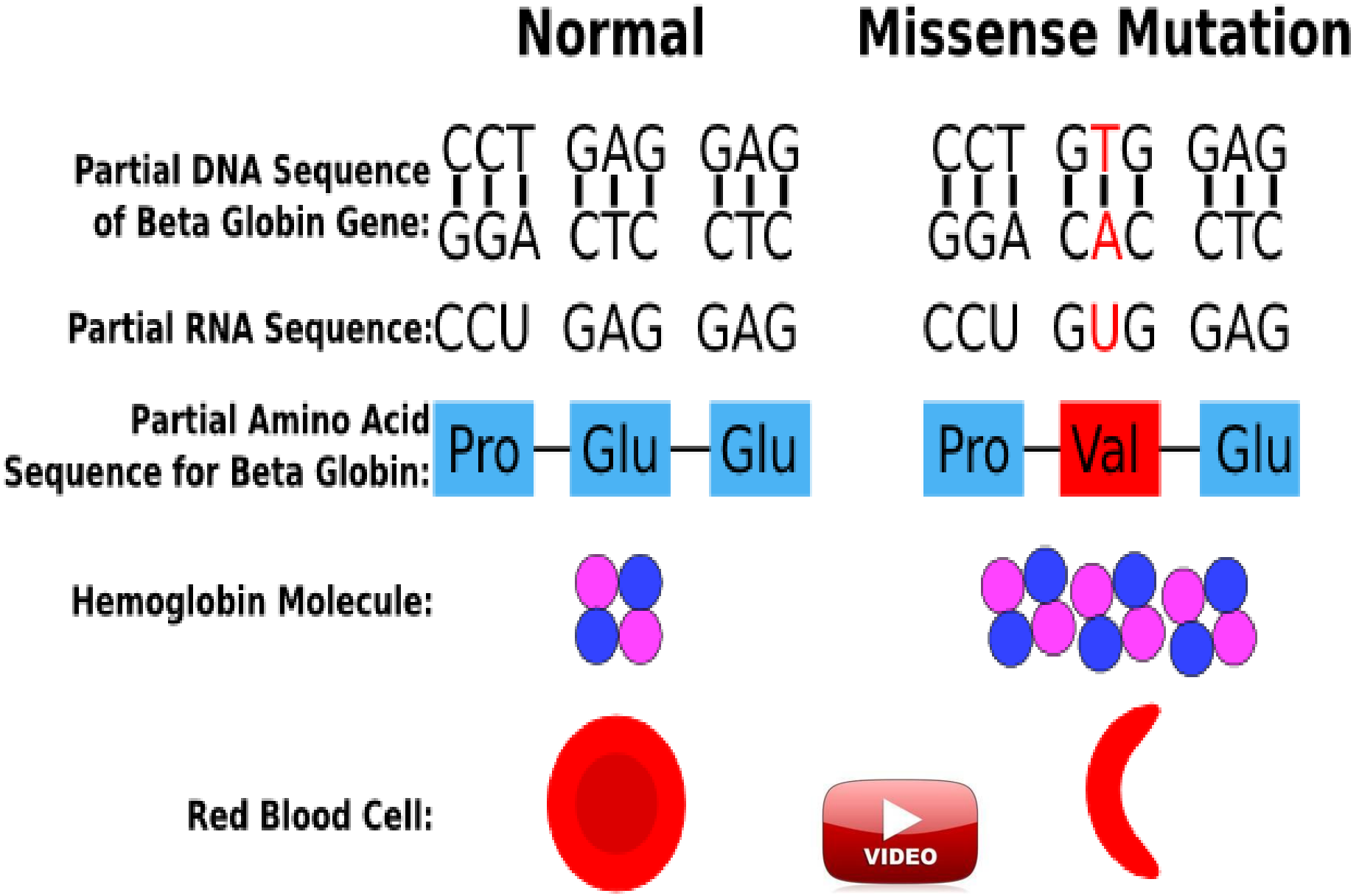
Example - Albinism

- **Albinism** is a rare group of genetic disorders that cause the skin, hair, or eyes to have little or no color.
- Albinism results from a mutation in one of several genes.
- The defect may result in the absence of melanin production, or a reduced amount of melanin production.
- Most commonly, the mutations interfere with the enzyme tyrosinase (**Tyrosine 3-monooxygenase**).
- This enzyme synthesizes melanin from the amino acid tyrosine.
- Most types of albinism are inherited as autosomal recessive. The exception is X-linked ocular albinism.


A/a
A/a
A/A
A/a
A/a
a/a
Melanocytes in skin
Melanin
Melanin
Nucleus
A
Chromosome II
Multiple mRNAs
A
Chromosome II
Thr
Thr
Polypeptide
Tyrosinase enzyme
Active site
Tyrosine
Melanin
Nucleus
A
Chromosome II
a
Chromosome II
Mutant site
mRNA
Thr
Lys
!
Tyrosine
Melanin
Nucleus
a
Chromosome II
a
Chromosome II
mRNA
Lys
Lys
!
!
Tyrosine

Example - Sickle cell disease

- **Sickle cell anemia** is an inherited form of anemia, a condition in which there aren't enough healthy red blood cells to carry adequate oxygen throughout your body.
- **Sickle-cell anemia** is caused by a point mutation in the **HBB** gene, causing the hydrophilic amino acid glutamic acid to be replaced with the hydrophobic amino acid valine at the sixth position.
- The β-globin gene is found on the short arm of chromosome 11
- The sickle-shaped cells are not flexible and cannot change shape easily. Many of them burst apart as they move through your blood vessels

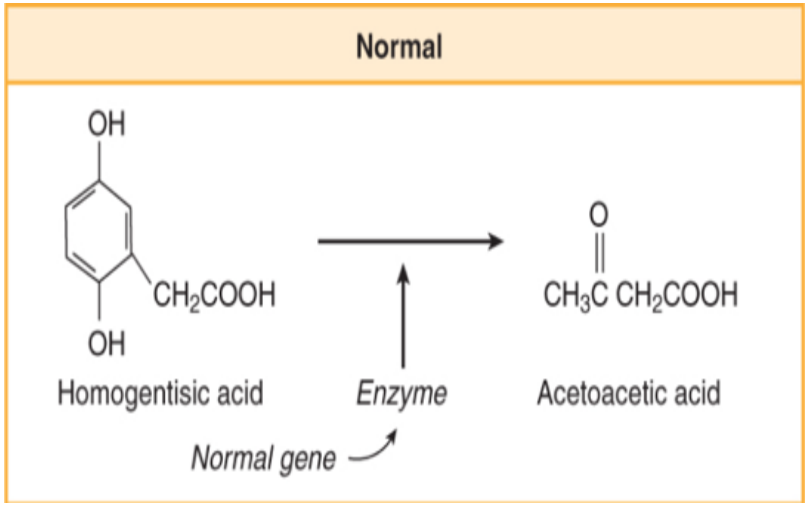

Normal
Missense Mutation
Partial DNA Sequence of Beta Globin Gene:
CCT GAG GAG
GGA CTC CTC
CCT GTG GAG
GGA CAC CTC
Partial RNA Sequence:
CCU GAG GAG
CCU GUG GAG
Partial Amino Acid Sequence for Beta Globin:
Pro Glu Glu
Pro Val Glu
Hemoglobin Molecule:
Red Blood Cell:
VIDEO

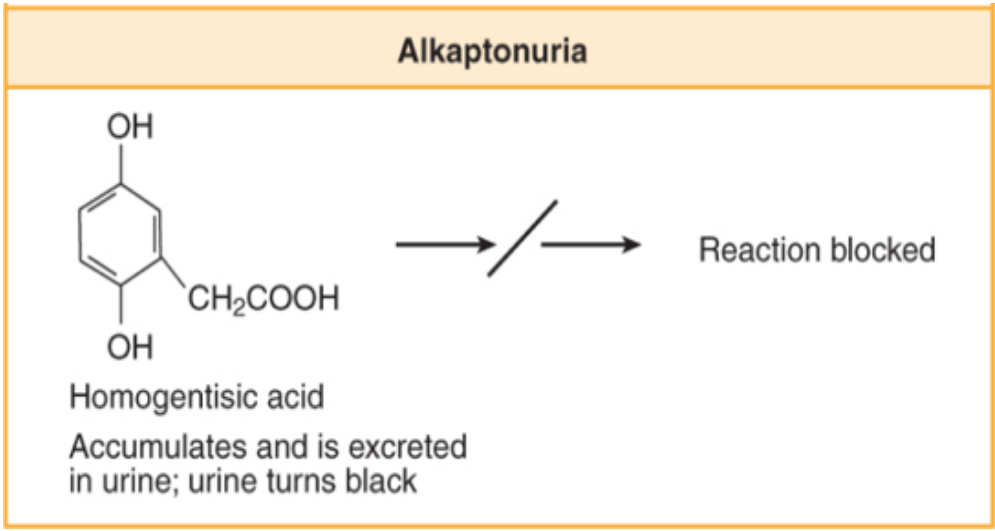
Alkaptonuria

- Alkaptonuria is a rare genetic metabolic disorder characterized by the accumulation of homogentisic acid in the body.
- Affected individuals lack enough functional levels of an enzyme required to breakdown homogentisic acid.
- Affected individuals may have dark urine or urine that turns black when exposed to air.
- Alkaptonuria is caused by mutation of the homogentisate 1,2-dioxygenase (*HGD*) gene. The *HGD* gene contains instructions for creating (encoding) an enzyme known as homogentisate 1,2-dioxygenase.

- This enzyme is essential for the breakdown of homogentisic acid.
- Mutations of the *HGD* gene result in deficient levels of functional homogentisate 1,2-dioxygenase, which, in turn, leads to excess levels of homogentisic acid.
- Although homogentisic acid is rapidly cleared from the body by the kidneys, it also slowly accumulates in the various tissues of the body, especially connective tissue such as cartilage.
- Over time (rarely before adulthood), it eventually changes the color of affected tissue to a slate blue or black.
- Long-term, chronic accumulation of homogentisic acid eventually weakens and damages affected tissue and leads to many of the characteristic symptoms of alkaptonuria.

- Alkaptonuria affects males and females in equal numbers, although symptoms tend to develop sooner and become more severe in males.
- More than 1,000 affected individuals have been reported in the medical literature.
- In the United States it is estimated to occur in 1 in 250,000-1,000,000 live births.

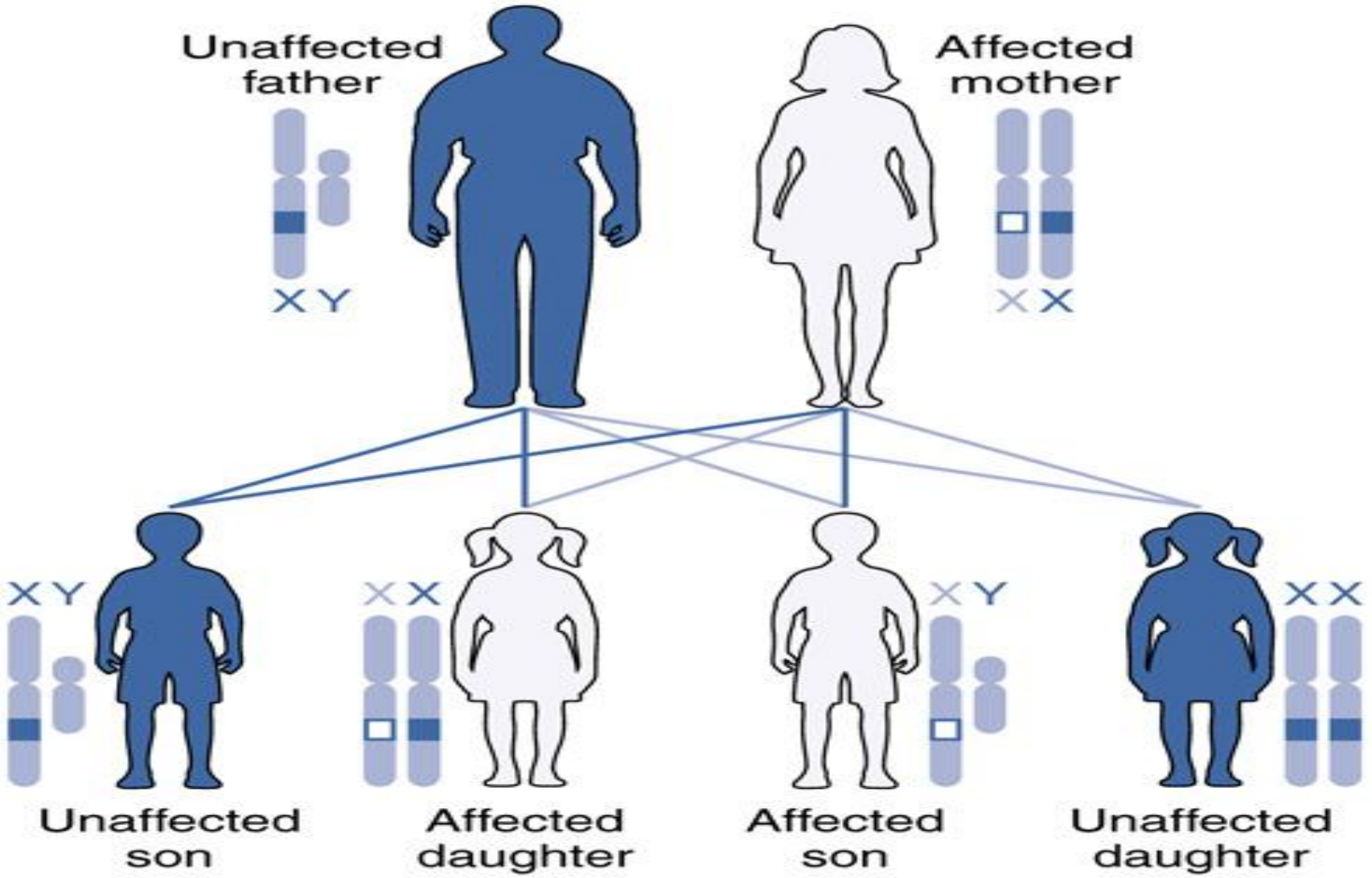




X-Linked Dominant

- A single copy of the mutation is enough to cause the disease in both males and females, but with a greater incidence in females due to the greater number of X chromosomes.
- In some conditions, the absence of a functional gene results in the death of affected males.
- The pedigree mirroring that of autosomal dominance.
- The only difference is that a positive father will give the condition to all of his daughters, but not his sons,
- Whereas a positive female will transmit the trait to half of her sons and half of her daughters.

X-linked Dominant Inheritance

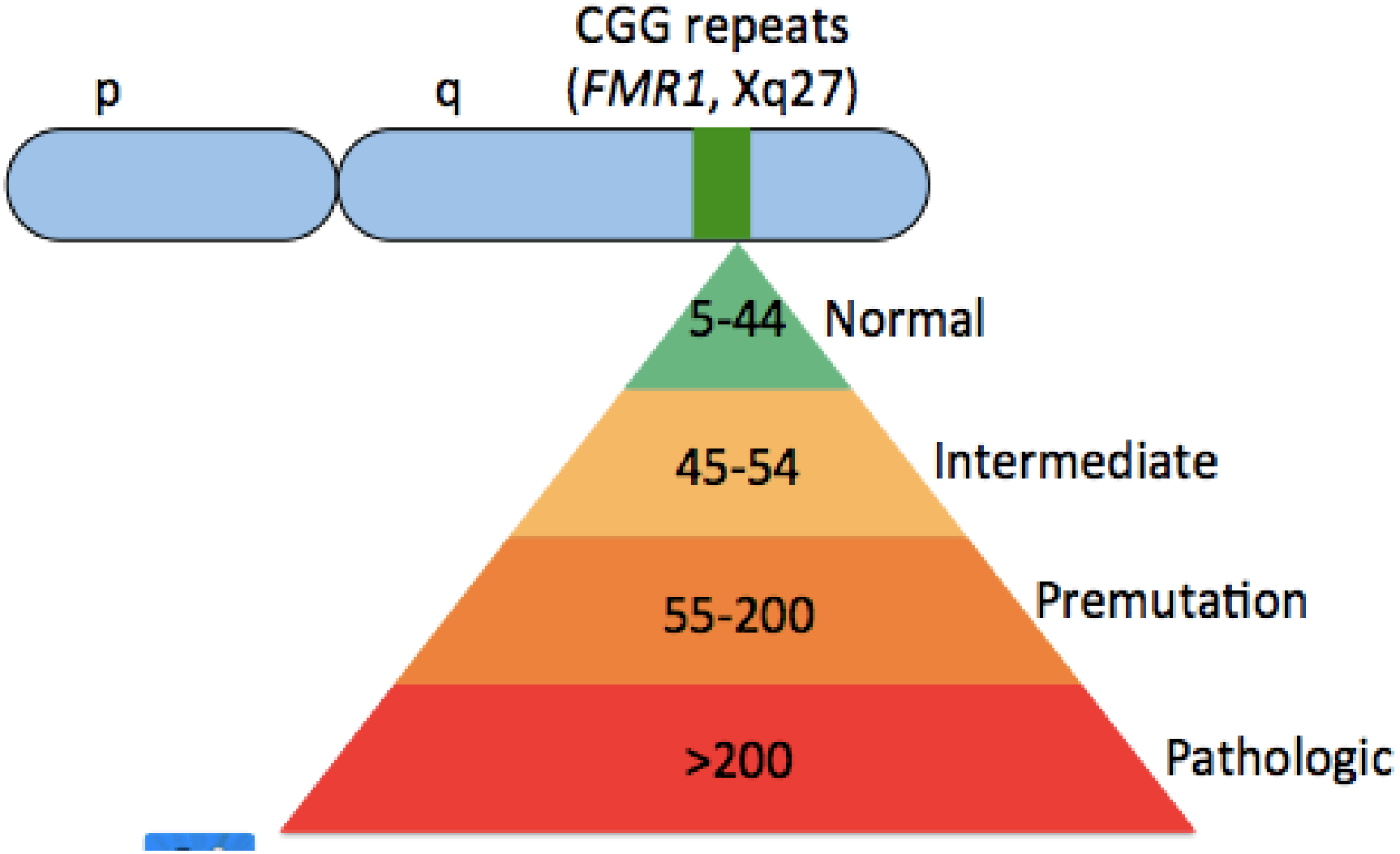


Example - Fragile X syndrome

- Fragile X syndrome is a genetic condition that causes a range of developmental problems including learning disabilities and cognitive impairment.
- Most males with fragile X syndrome have mild to moderate mental disability, while about one-third of affected females are intellectually disabled.
- Mutations in the **FMR1 gene** (fragile X mental retardation 1) cause fragile X syndrome.
- The **FMR1** gene provides instructions for making a protein called **FMRP**. This protein helps regulate the production of other proteins and plays a role in the development of synapses.

- Nearly all cases of fragile X syndrome are caused by a mutation in which a DNA segment, known as the CGG triplet repeat, is expanded within the *FMR1* gene.
- Normally, this DNA segment is repeated from 5 to about 40 times.
- In people with fragile X syndrome, however, the CGG segment is repeated more than 200 times.
- The abnormally expanded CGG segment turns off (silences) the FMR1 gene, which prevents the gene from producing FMRP.
- Loss or a shortage (deficiency) of this protein disrupts nervous system functions and leads to the signs and symptoms of fragile X syndrome.

Fragile X syndrome



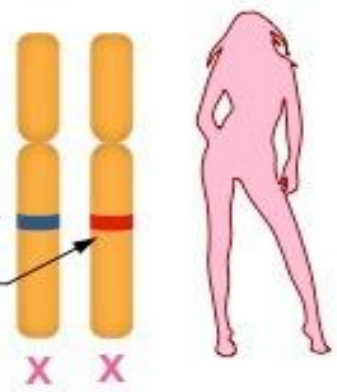
X-Linked Recessive

- X-linked recessive conditions mainly affect males.
- Men have one X and one Y chromosome. This means that any mutations in their singular X chromosome will generate the condition.
- Because females carry two X chromosomes, if one gene damaged, the other can often make up the shortfall.
- Females can still carry and pass on the gene.
- If the mother has an X-linked mutation, each daughter will have a 1 in 2 chance of becoming a carrier and each **son** will have a 1 in 2 chance of developing **mutation**.

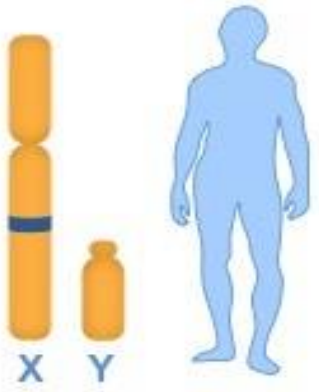
Parents:

X-linked gene

- *Normal allele*
- *Defective allele*

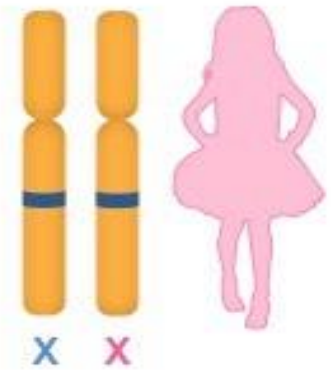


Normal - *carrier*

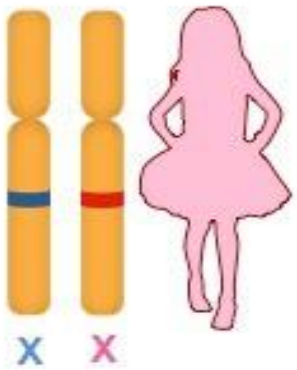


Normal

Possible offspring:

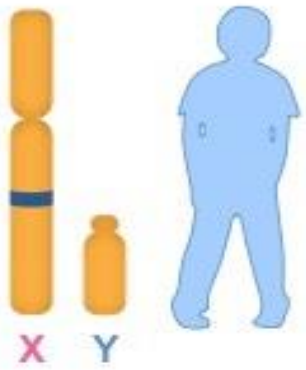


Normal

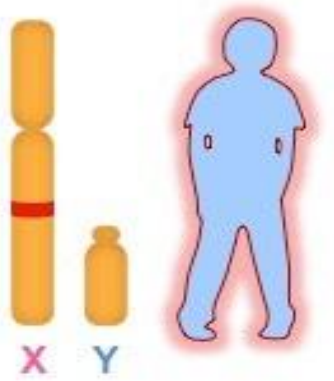


Normal - *carrier*

Inherits alleles from both parents, recessive trait can be masked (carriers)



Normal



Affected

Inherits allele from mother only, recessive trait **cannot** be masked

Example - Hemophilia

- Hemophilia is an inherited bleeding disorder in which a person lacks or has low levels of certain proteins called "clotting factors" and the blood doesn't clot properly as a result. This leads to excessive bleeding.
- There are 13 types of clotting factors, and these work with platelets to help the blood clot, hemophilia caused by a defect in the gene that determines how the body makes factors VIII, IX, or XI.
- These genes are located on the X chromosome, making hemophilia an X-linked recessive disease.
- Hemophilia A (also known as classic hemophilia or factor VIII deficiency) and hemophilia B (also known as factor IX deficiency).

Example - Duchenne Muscular Dystrophy

- Duchenne Muscular Dystrophy (DMD) is a genetic disorder characterized by progressive muscle degeneration and weakness.
- DMD is caused by an absence of **dystrophin**, a protein that helps keep muscle cells intact.
- Symptom onset is in early childhood, usually between ages 3 and 5 years.
- Over time, children with Duchenne will develop problems walking and breathing, and eventually the muscles that help them breathe and the heart will stop working.
- The disease primarily affects boys, but in rare cases it can affect girls.

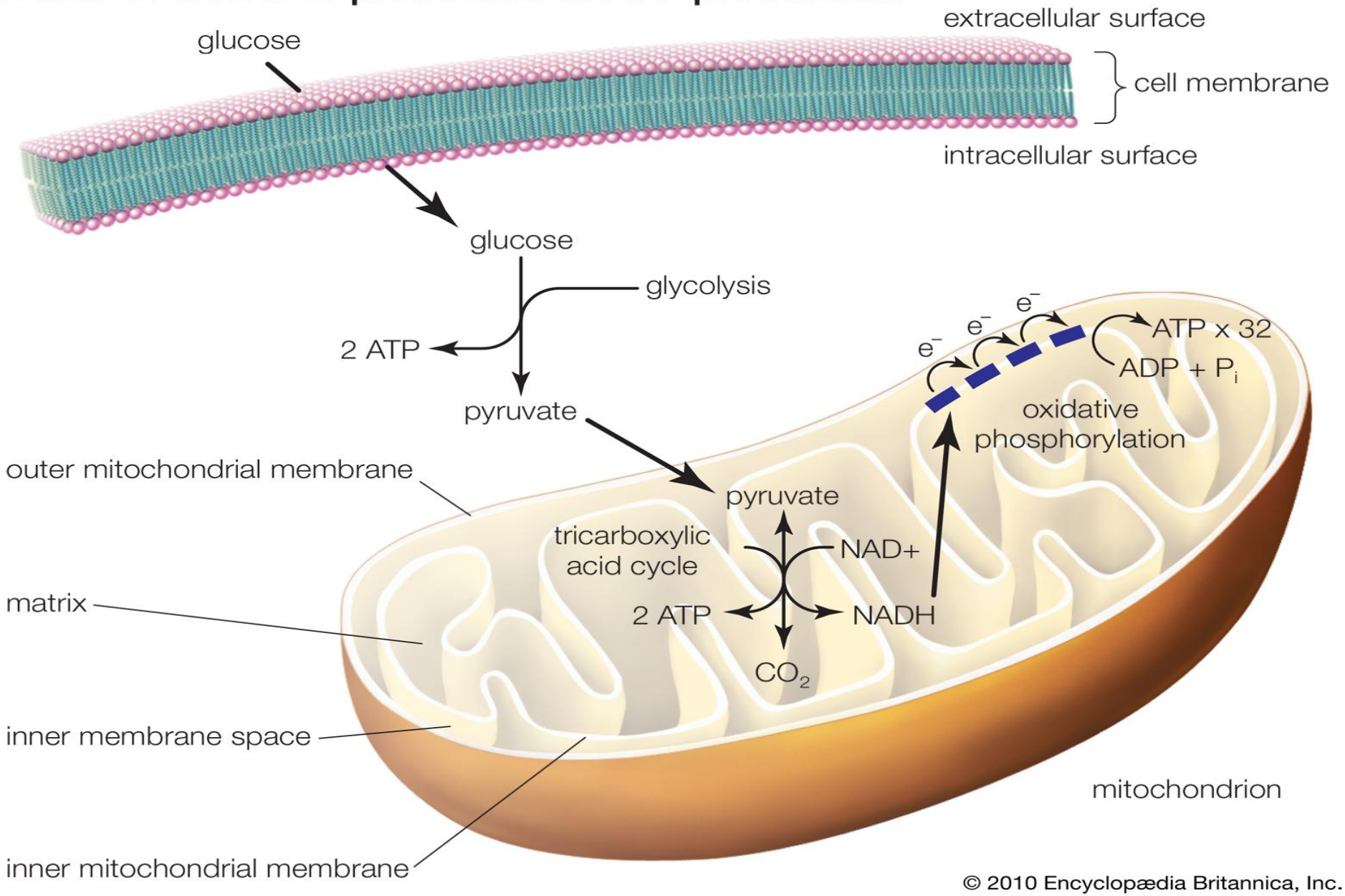
Red-Green Color Blindness

- A form of colorblindness in which red and green are perceived as identical.
- This is the most common type of colorblindness.
- It is inherited in an X-linked recessive manner and affects 6% of males.
- It is also known as deutan colorblindness and Daltonism.
- Mutations in the *OPN1LW*, *OPN1MW*, and *OPN1SW* genes cause the forms of color vision deficiency described above.
- The proteins produced from these genes play essential roles in color vision. They are found in the retina, which is the light-sensitive tissue at the back of the eye.

Introduction

- In fact, mitochondria enable cells to produce 15 times more ATP than they could otherwise, and complex animals, like humans, need large amounts of energy in order to survive.
- Mitochondria produce about 90% of the chemical energy that cells need to survive.
- The number of mitochondria present in a cell depends upon the metabolic requirements of that cell, and may range from a single large mitochondrion to thousands of the organelles.
- Mitochondria have their own DNA (mtDNA) which encodes 13, that encode 13 proteins, 22 tRNAs, and 2 rRNAs.

Basic overview of processes of ATP production



© 2010 Encyclopædia Britannica, Inc.

Mitochondrial vs. Nuclear DNA

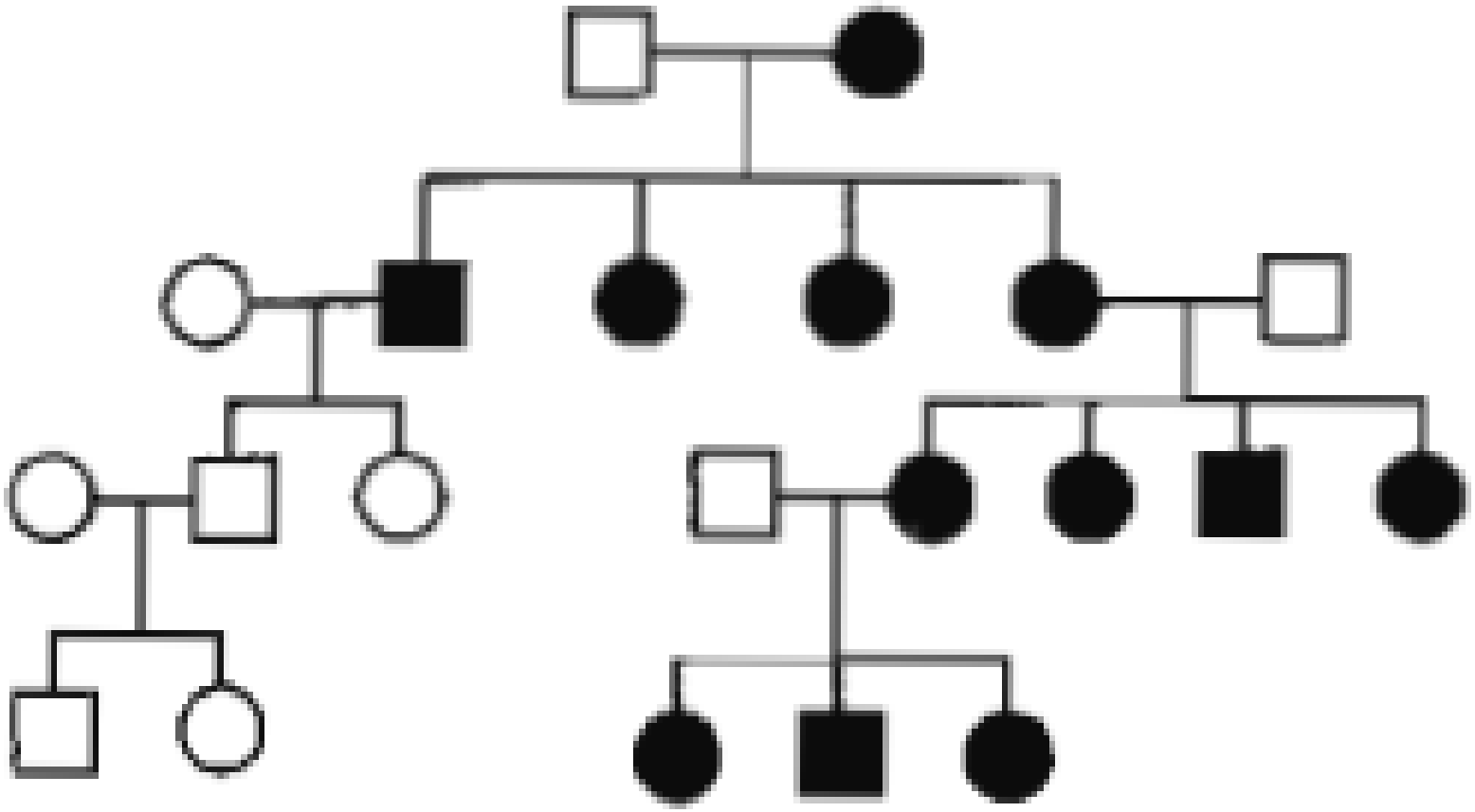
Mitochondrial	Nuclear DNA
The mitochondrial genome is circular	The nuclear genome is linear
Mitochondrial DNA is short	Nuclear DNA is long
The mitochondrial genome is built of 16,569 DNA base pairs	The nuclear genome is made of 3.3 billion DNA base pairs.
The mitochondrial genome contains 37 genes.	The nuclear DNA contains 20,000 to 25,000 genes.
3% of the mitochondrial genome is noncoding DNA	93% of the nuclear genome is noncoding DNA
Mitochondrial DNA is inherited from the mother only.	Nuclear DNA is inherited from the mother and father

- The cells in the body, especially in organs such as the brain, heart, muscle, kidneys and liver, cannot function normally unless they are receiving a constant supply of energy.
- The genes found within the mitochondria contain the information that codes for the production of some important enzymes.
- A variation in a gene that creates a defect is called a pathogenic variant or mutation.
- A mitochondrial DNA mutation can result in biochemical problems due to absence of enzymes involved in the respiratory chain, or enzymes that are impaired and do not work properly.
- Mitochondrial DNA (mtDNA) is highly susceptible to mutations, largely because it does not possess the robust DNA repair mechanisms common to nuclear DNA.

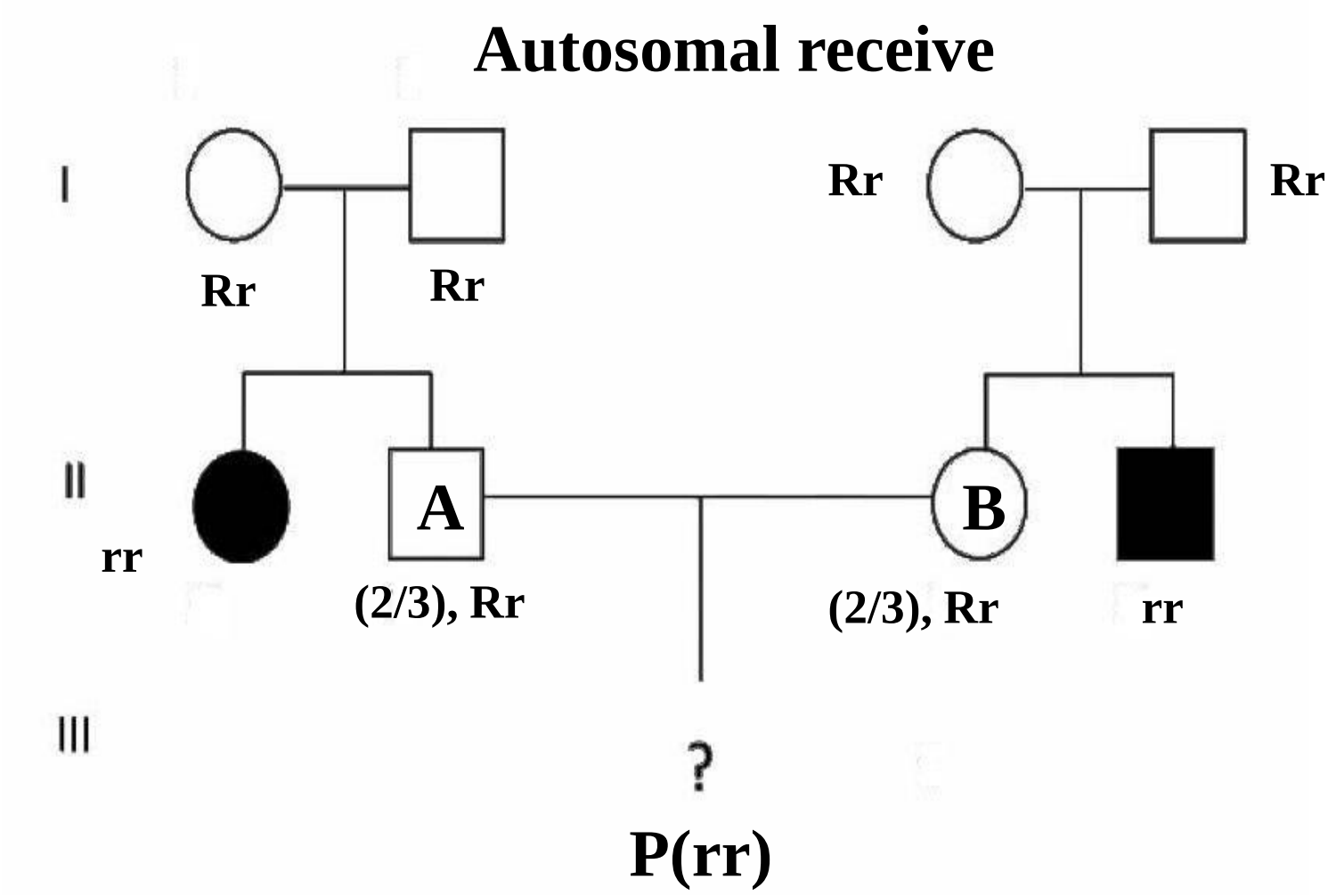
Mitochondrial Inheritance

- Mitochondrial traits are inherited in a non-mendelian fashion because they are carried on mitochondrial DNA.
- The disease is inherited only maternally, since only the mother contributes mitochondrial DNA to the progeny.
- Both males and females can be affected by the disease.
- All offspring of an affected female are affected, whereas there is no inheritance of the disease from an affected male.
- Mitochondrial diseases are often expressed as neuropathies and myopathies because brain and muscle are highly dependent on oxidative phosphorylation.
- Mitochondrial genes code for some of the components of the electron transport chain and oxidative phosphorylation, as well as some mitochondrial tRNA molecules.

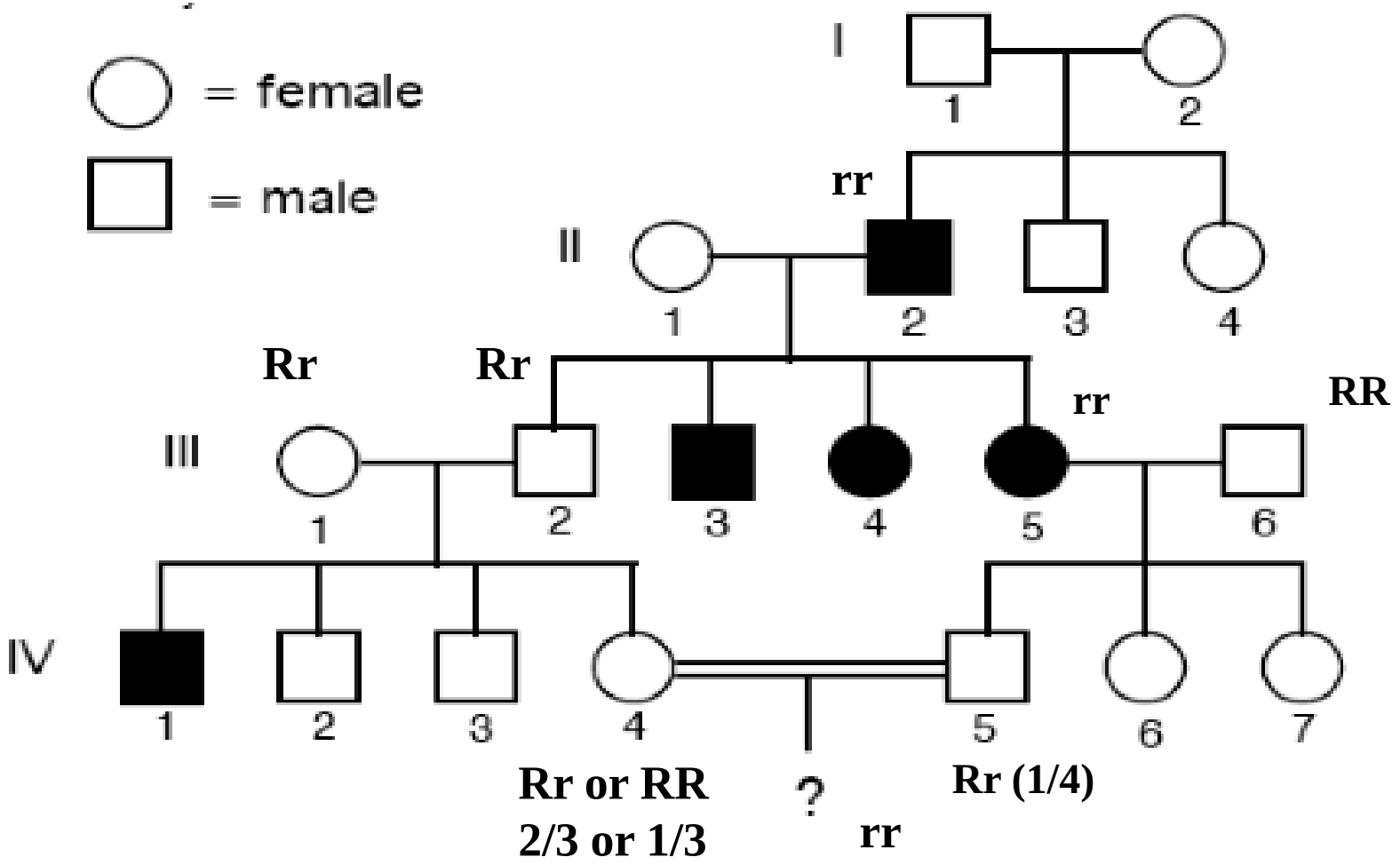
Mitochondrial Inheritance Pattern



Calculating Risks in Pedigree Analysis

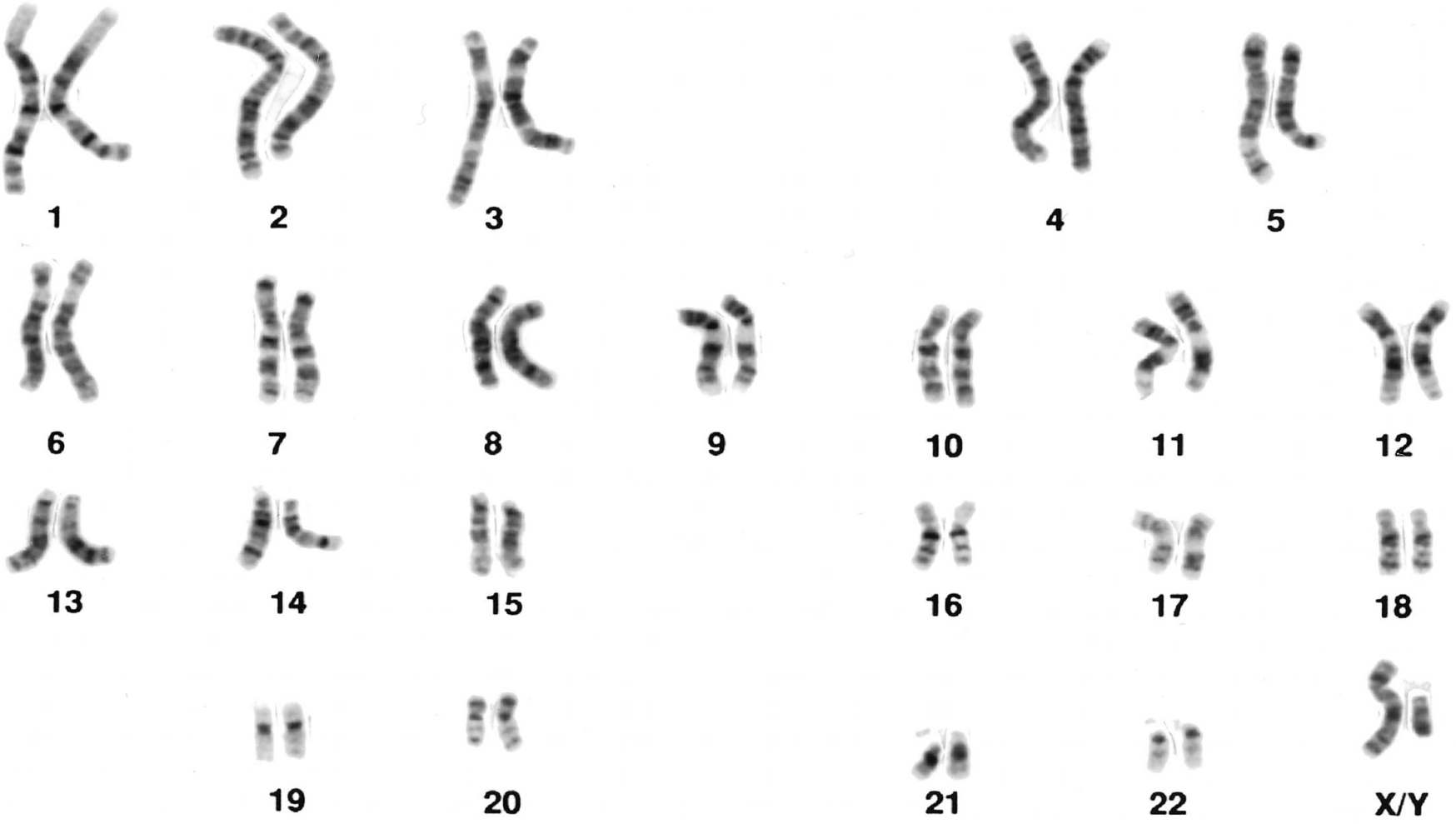


Autosomal Recessive

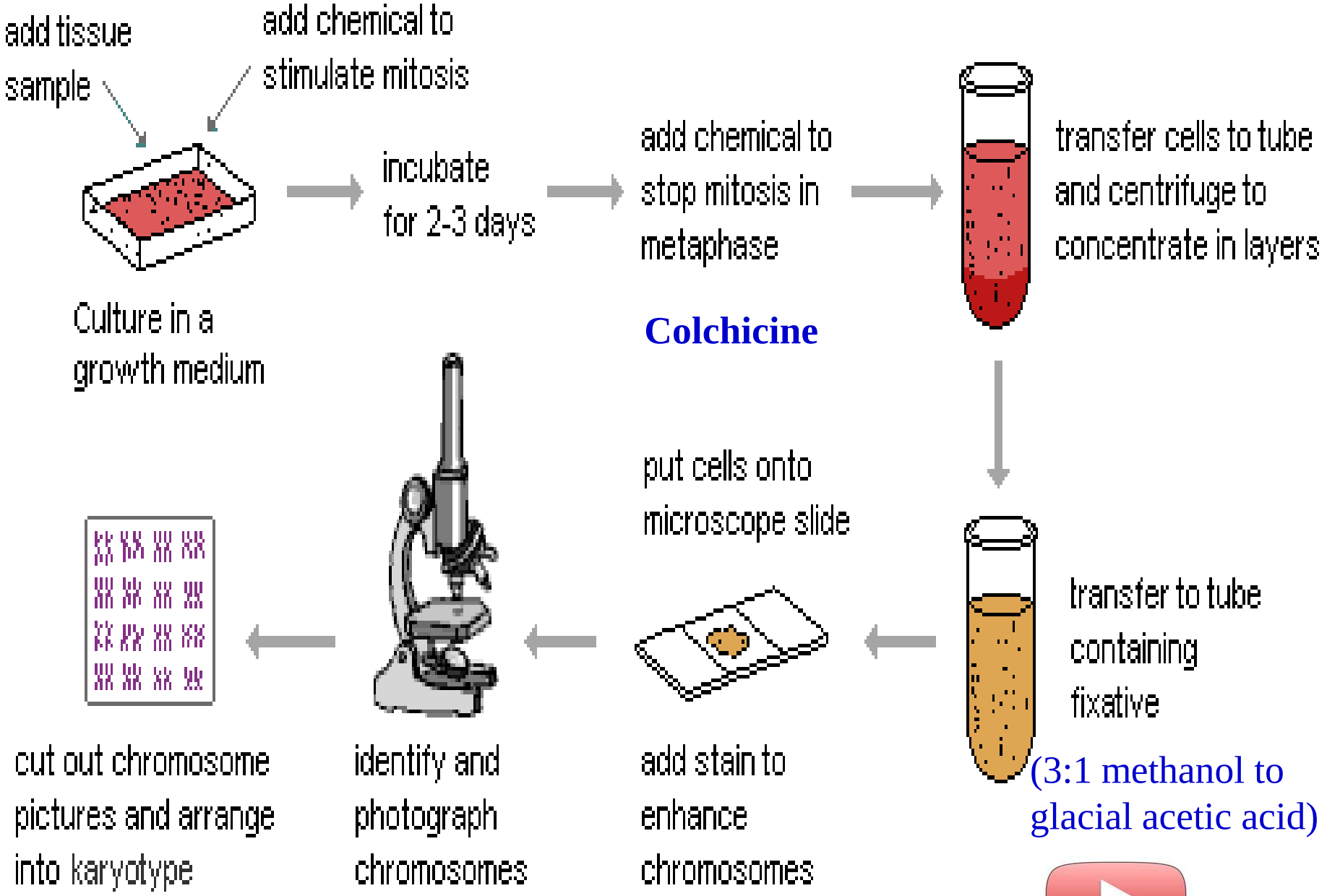


Karyotyping

- Karyotyping is a valuable research tool used to determine the chromosome complement within somatic or cultured cells.
- Many morphological and physiological problems can be traced to the change in the karyotype.
- Karyotyping is often used for
  - ✓ The parental diagnosis,
  - ✓ Detection of variations in the chromosome number and structure, aberrations, and anomalies,
- which are the common cause of many congenital defects and spontaneous abortions.



- The normal **human karyotypes** contain 22 pairs of autosomal chromosomes and one pair of sex chromosomes.
- Normal **karyotypes** for females contain two X chromosomes and are denoted 46,XX; males have both an X and a Y chromosome denoted 46,XY.

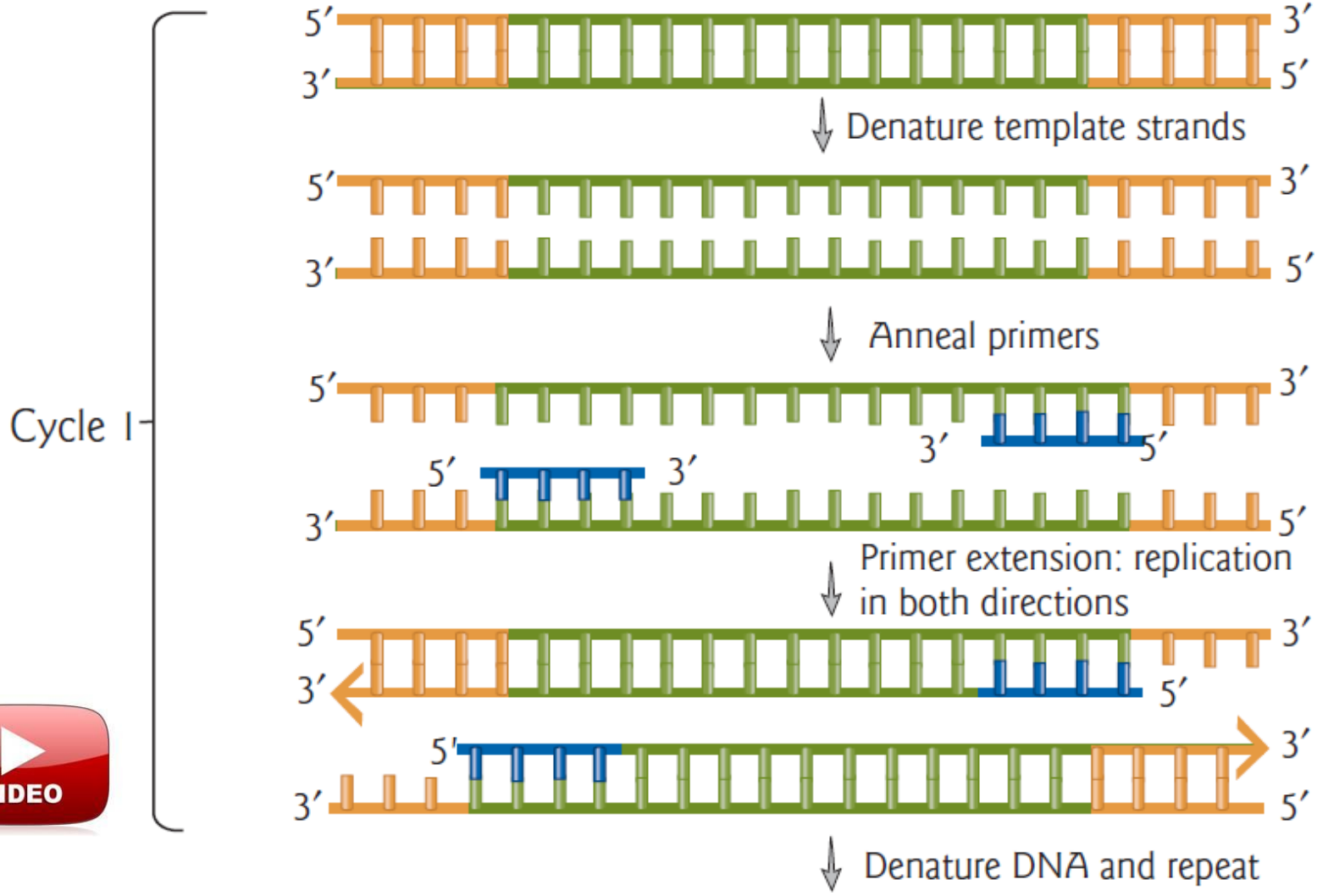

add tissue sample
add chemical to stimulate mitosis
Culture in a growth medium
incubate for 2-3 days
add chemical to stop mitosis in metaphase
Colchicine
transfer cells to tube and centrifuge to concentrate in layers
transfer to tube containing fixative
(3:1 methanol to glacial acetic acid)
put cells onto microscope slide
add stain to enhance chromosomes
identify and photograph chromosomes
cut out chromosome pictures and arrange into karyotype
VIDEO

Chromosomal Banding Patterns

- Most of chromosomes, at the prophase and the metaphase, are characterized by **a banding pattern**.
- The most common type of staining done by cytogeneticists today uses a chemical called **Giemsa.**
- **Banding** can be used to identify chromosomal abnormalities, such as translocations, because there is a **unique** pattern of light and **dark** bands for each chromosome.
- **Why they appear unstained or colored lightly?**
- Giemsa stains the proteins of the chromatin.
- During digestion with **Trypsin** (enzyme) , protein present in G-C rich region will be digested whereas protein which are present in A-T rich region will not. So it take Giemsa stain.

PCR For Genetic Testing

- PCR is a technique in which a short sequence of DNA can be amplified more than a million fold within a few hours.
- The DNA to be amplified is flanked by two short oligonucleotide primers (F and R) that hybridize to opposite strands of the target sequence.
- Synthesis of the DNA target sequence is achieved by the addition of ***Taq* polymerase**, which is heat stable, and the four deoxynucleotides.
- The basic steps are:
  - Denaturation (96 °C),
  - Annealing (55- 65 °C),
  - Extension (72°C)



VIDEO

This cycle repeats 25-35 times in a typical PCR reaction, which generally takes 2-4 hours, depending on the length of the DNA region being copied.

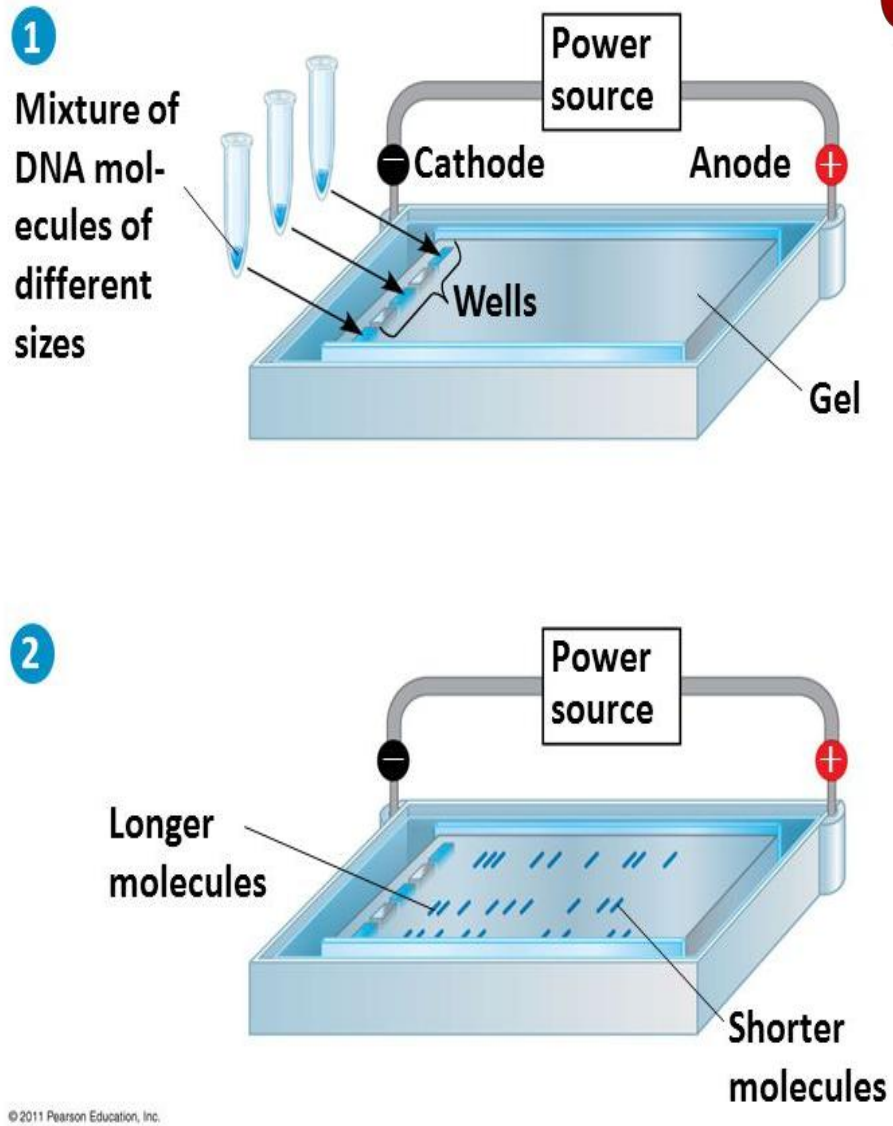
Electrophoresis

- Electrophoresis is the standard method for analyzing, identifying, and purifying fragments of DNA or RNA that differ in size, charge, or both.
- When charged molecules are placed in an electric field, they migrate toward the positive (anode, red) or negative (cathode, black) pole according to their charge.
- The gels used for electrophoresis are composed either of agarose or polyacrylamide.
- Agarose gels are used in a horizontal gel apparatus, while polyacrylamide gels are used in a vertical gel apparatus.

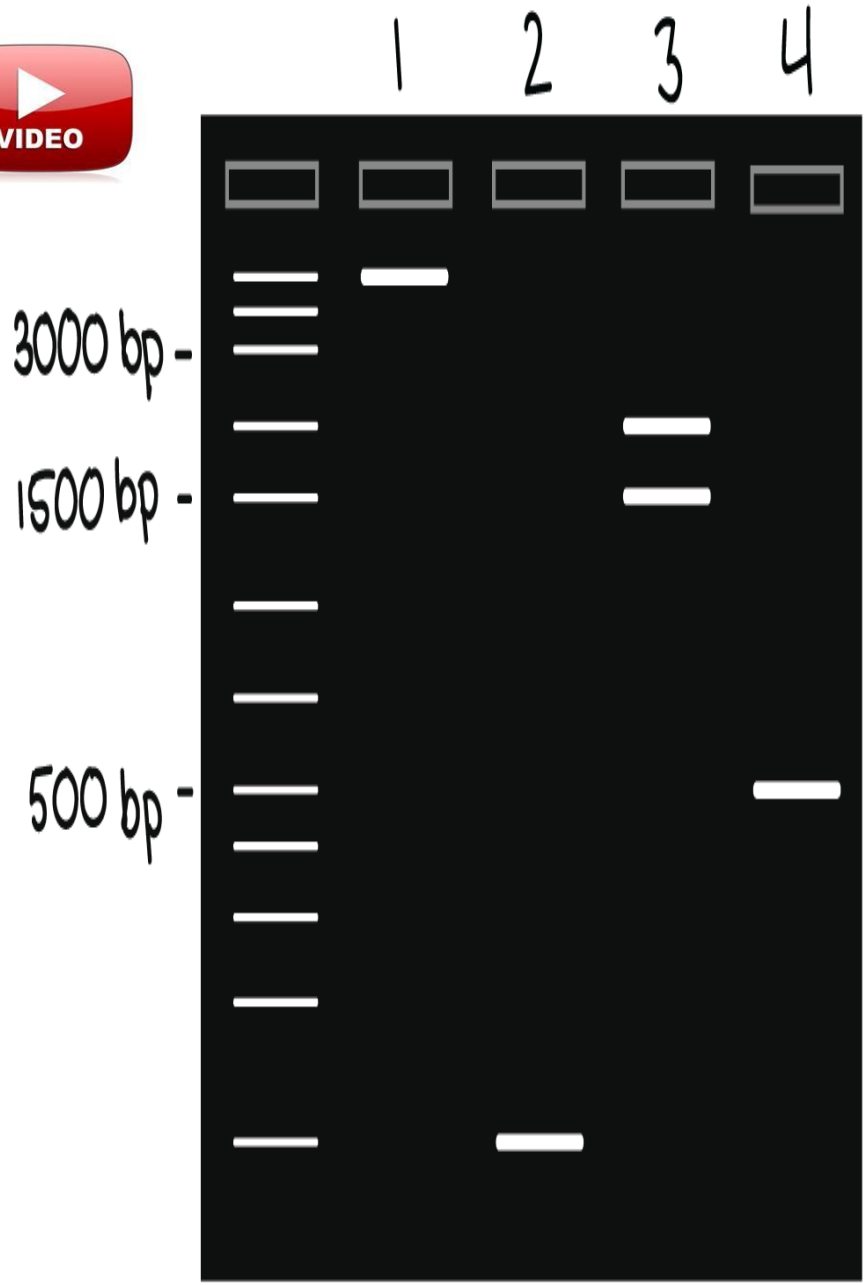
- Agarose gels are used for the analysis and preparation of fragments between 100 and 50,000 bp in size with moderate resolution.
- Polyacrylamide gels are used for the analysis and preparation of small molecules with single nucleotide resolution.
- Agarose is a polysaccharide extracted from seaweed.
- Agarose gels are prepared by mixing agarose powder with buffer solution, boiling in a microwave to melt, and pouring the gel into a mold where the agarose (generally 0.5–2.0%) solidifies into a slab.

- Pores between the agarose molecules act like a **sieve** that separates the molecules by size.
- In an electrophoretic gel, nucleic acids migrate through the pores; thus fragments separate by size with the smallest pieces moving the fastest and farthest through the gel.
- Because DNA by itself is not visible in the gel, the DNA is stained with a fluorescent dye such as Ethidium bromide (EtBr).
- Ethidium bromide intercalates between the bases causing DNA to fluoresce orange when the dye is illuminated by ultraviolet light.

TECHNIQUE

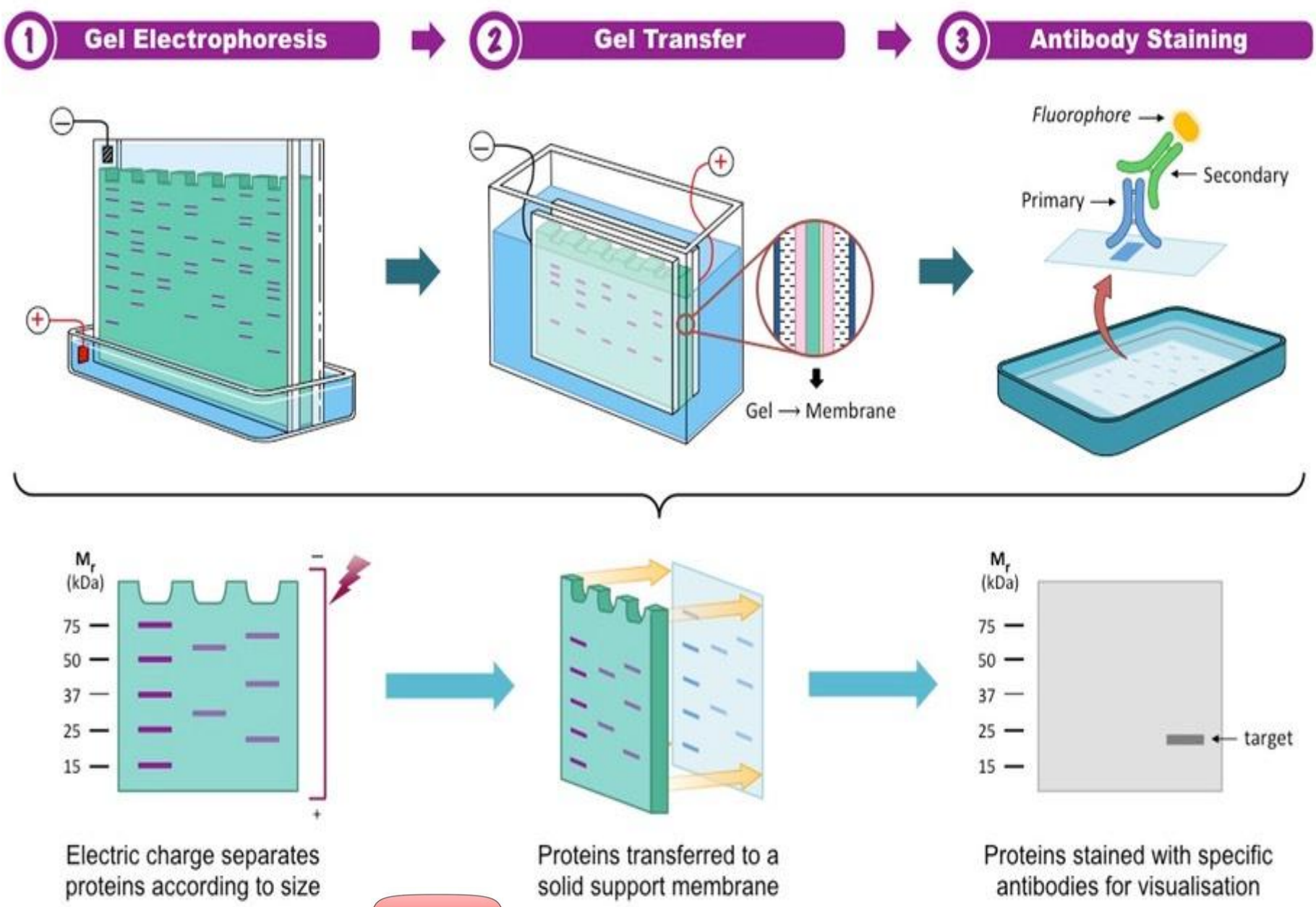


VIDEO



Polyacrylamide Gel Electrophoresis (PAGE)

- Polyacrylamide is a cross-linked polymer of acrylamide.
- The length of the polymer chains is dictated by the concentration of acrylamide used, which is typically between 3.5 and 20%.
- In the case of DNA, polyacrylamide is used for separating fragments of less than about 500 bp.
- In PAGE, the sodium dodecyl sulfate (SDS) is used to bind to proteins and give them a negative charge.
- Proteins are then separated electrophoretically according to their size using a gel matrix made of polyacrylamide in an electric field.
- Bands in polyacrylamide gels are usually detected by autoradiography, although silver staining can also be used.


1 Gel Electrophoresis
2 Gel Transfer
3 Antibody Staining
Fluorophore
Secondary
Primary
Gel → Membrane
Mr (kDa)
75
50
37
25
15
target
Electric charge separates proteins according to size
Proteins transferred to a solid support membrane
Proteins stained with specific antibodies for visualisation

VIDEO

Agarose vs. Polyacrylamide

Agarose	Polyacrylamide
Agarose is poured horizontally	Polyacrylamide is poured vertically
Agarose can be heated to aid the pouring process	Polyacrylamide cannot be re-heated and then poured.
The size of the bands are the same in agarose,	The size of the bands are various in polyacrylamide.
Staining can be done before pouring	Staining can be done after pouring
Non toxic	Potent neuro-toxic
Concentration 0.5-2%	Concentration 3.5-20%
Simple preparation	Complex perpetration
If the polyacrylamide molecule is exposed to air, it will not solidify in an even manner.	
The gaps between the gels of polyacrylamide are smaller than those between the gels of agarose	

Probes

- **Genetic Probe**: is a fragment of DNA or RNA of variable length (usually 100-1000 bases long) which is radioactively labelled used in DNA or RNA samples to detect the presence of target nucleotide sequences that are complementary to the sequence in the probe.
- The principle is that the probe will bind to any clone containing sequences similar to those found on the probe.
- This binding step is called hybridization.
- The labeled probe is first denatured (by heating or under alkaline conditions) into single DNA strands and then hybridized to the target DNA (Southern blotting) or RNA (northern blotting) immobilized on a membrane or in situ.

- There are three major types of probe:
  I. Oligonucleotide probes: which are synthesized chemically and end-labeled,
  II. DNA probes: are stretches of single-stranded DNA used to detect the presence of complementary nucleic acid sequences (target sequences) by hybridization. DNA probes are usually labelled, for example with radioisotopes, epitopes, biotin
  III. RNA probes: are made by *in vitro* transcription of template DNA by an RNA polymerase which initiates RNA synthesis at specific binding sites called promoters
- RNA probes and oligonucleotide probes are generally single-stranded.

Difference Between Probe and Primer Definition

Probe	Primer
A fragment of DNA or RNA used to detect the presence of a specific DNA fragment within a sample	A short strand of DNA or RNA that serves as the starting point for DNA synthesis.
Used to detect a specific DNA fragment in qPCR.	Used to initiate the DNA replication. It is also used in the initiation of the PCR.
A probe can range from 25-1000 base pairs.	A primer can range from 18-22 base pairs
Probes are hybridized with double-stranded DNA.	Primers are hybridized with single-stranded DNA.
Probes are generally labeled with a fluorophore for the detection.	Primers can be labeled based on the purpose.

Southern Blot

- This technique is used to identify specific biomolecules present in a sample.
- Southern blot technique used to detects specific DNA sequences and therefore is also called a DNA blot.
- Southern blots analyzing DNA have been very useful in identifying the genes involved in many genetic diseases.
- In Southern blotting, enzymes called restriction endonucleases are used to cut large DNA sequences in to smaller pieces.

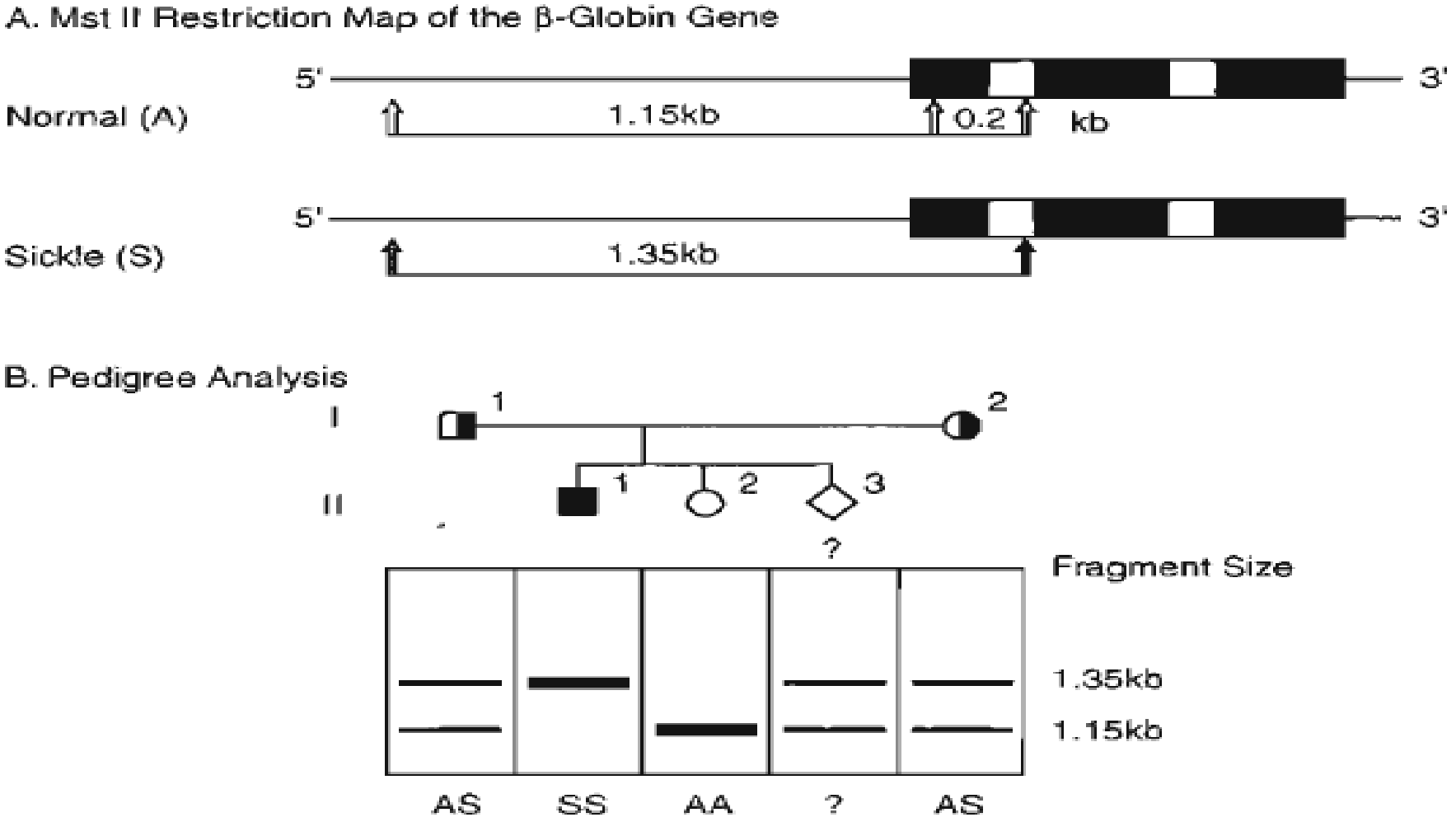
Southern Blot

- **Southern Blot- Principle:** In Southern blotting, DNA fragments are first separated using gel electrophoresis and then the separated molecules are transferred to a membrane surface.
- In the next step, hybridization analysis is carried out on the membrane using labeled probes complimentary to the target sequence to be identified, thus detecting the presence of DNA fragment of interest.

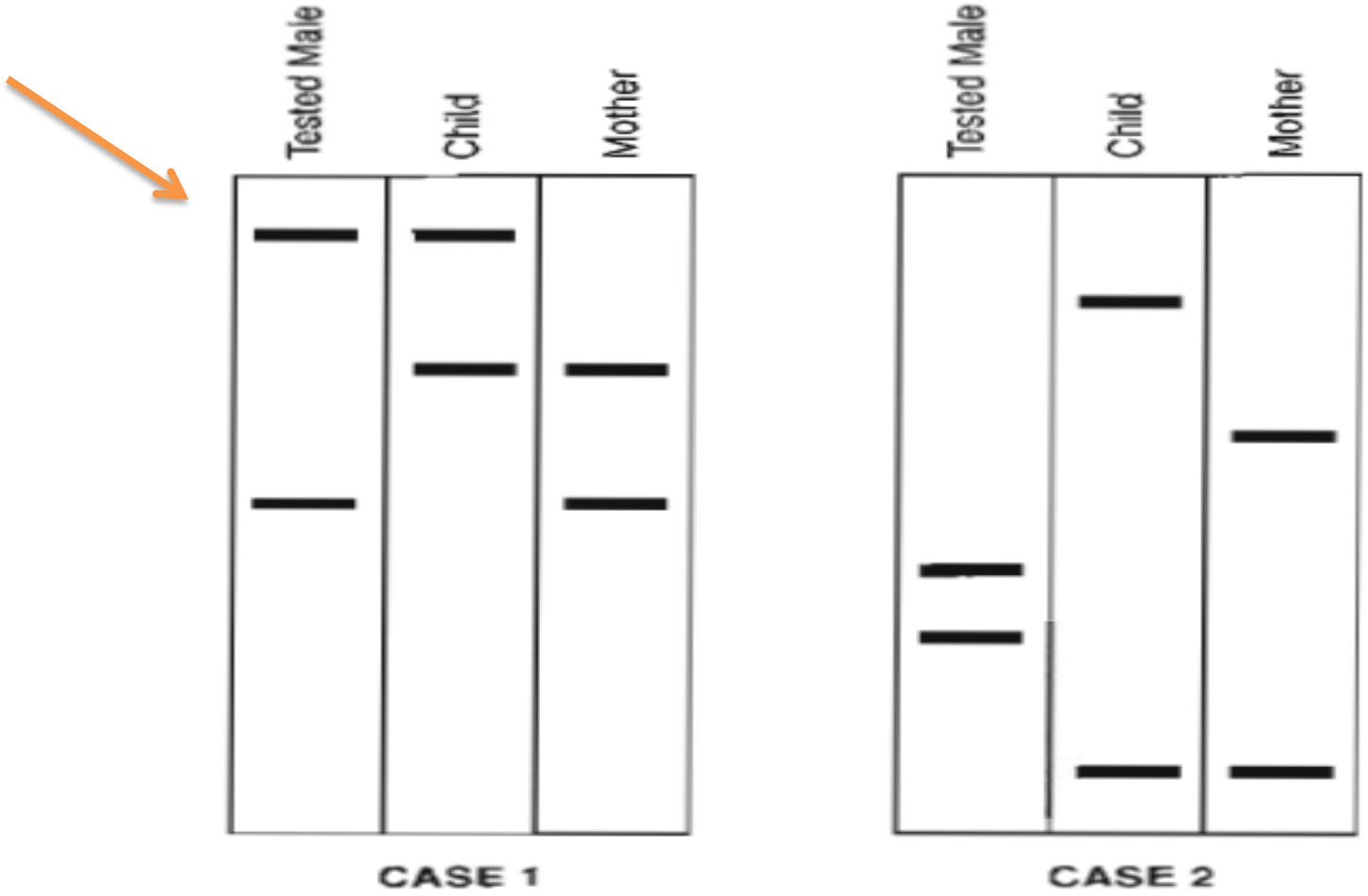
Southern Blot - Applications

1) Identification of a single gene in a pool of DNA fragments.
2) Analysis of genetic patterns of DNA
3) Detection of specific DNA sequences in a genome.
4) Study of gene deletions, duplications, and mutations that cause various diseases.
5) Detection of genetic diseases and cancers such as monoclonal leukemia and sickle cell mutations.
6) Detect the presence of a gene family in a genome.
7) DNA fingerprinting and forensic tests such as paternity testing and sex determination.

Prenatal diagnosis of Sickle cell disease



Paternity Testing



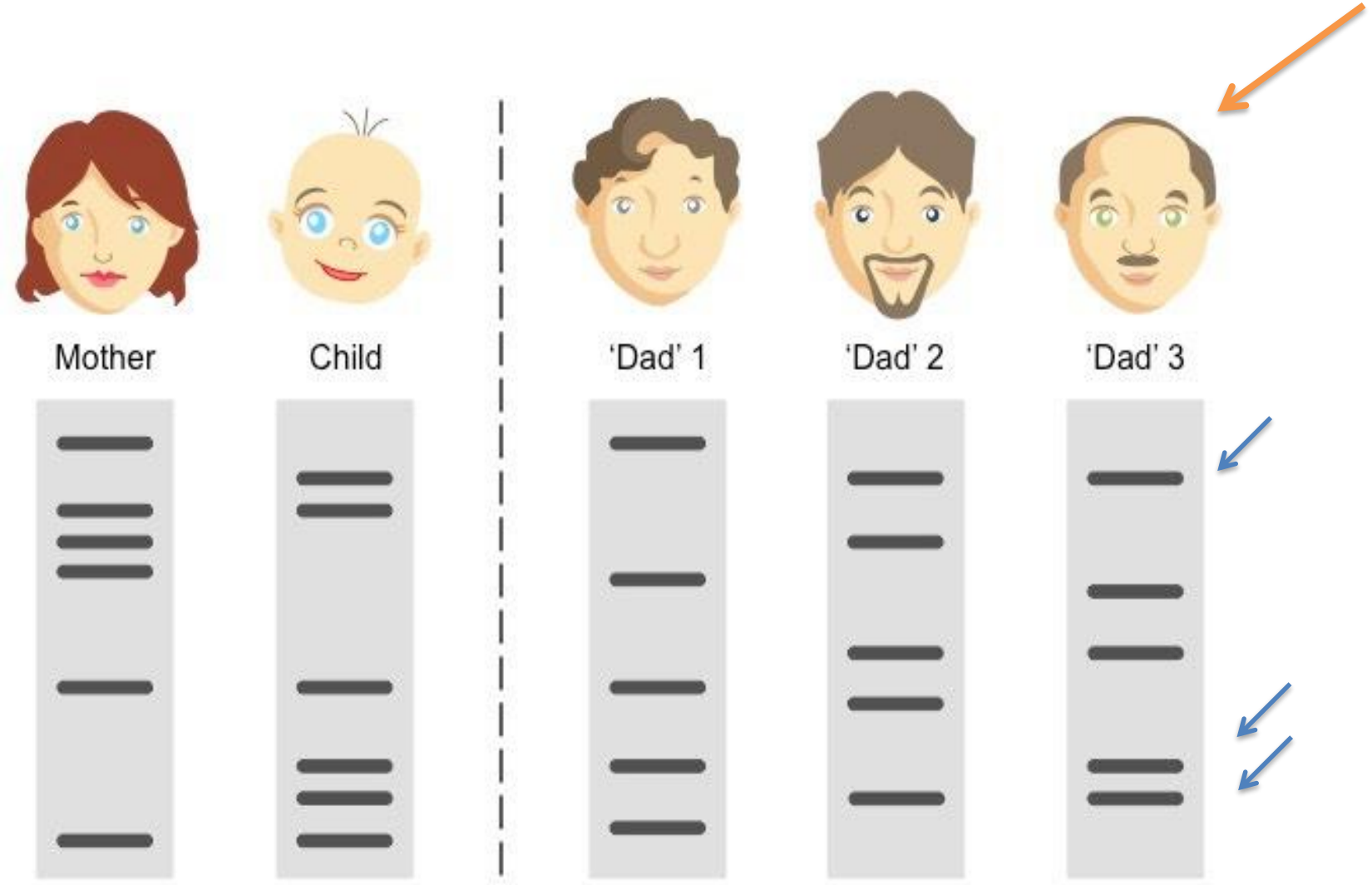
Question: Are the tested males in case 1 and case 2 the fathers of the children?

Approach:

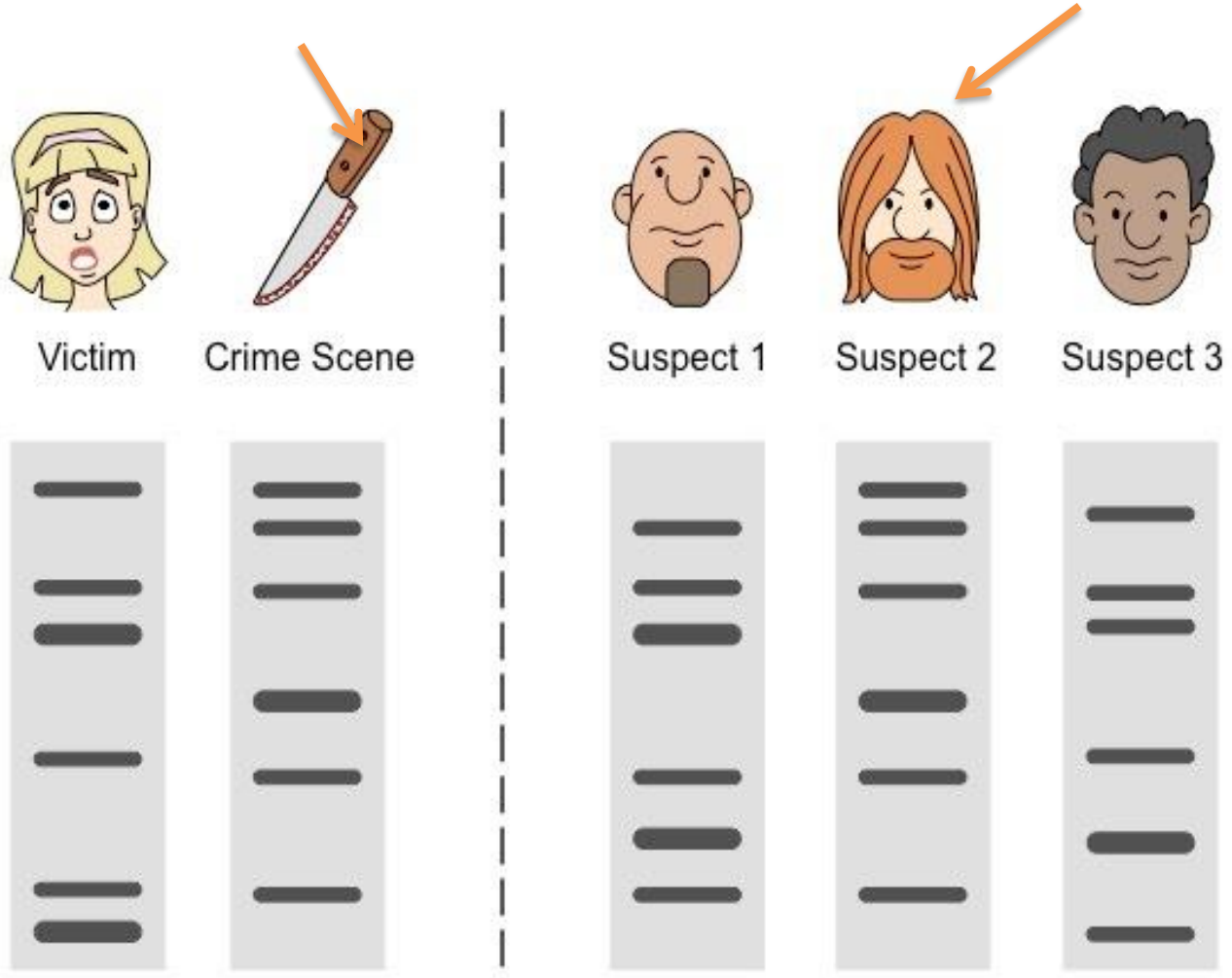
- Identify the child's band in common with the mother. The other band must be from the father.
- Does the tested male have a band matching the band from the father?

Draw conclusion.

- **Case 1:** The tested male in case 1 may be the father, as he shares a band with the child. We cannot be certain, however, because many other men in the population could have this same band. Matches are required at several different loci to indicate with high probability that he is the father.
- **Case 2:** The tested male in case 2 cannot be the father, as neither of his bands is shared with the child.



- Compare the profiles of three men with that of a mother and child to determine the biological father



- Compare the DNA collected from the crime scene to determine which of the three suspects was present

Tools for analyzing gene expression

Introduction

- In general, changes in cellular mRNA levels directly correlate to changes in their corresponding protein levels, although there are exceptions to this rule.
- Monitoring mRNA levels can be accomplished by using a number of different techniques, such as
  - ✓ Northern blotting,
  - ✓ *In situ* hybridization,
  - ✓ Reverse transcription–polymerase chain reaction (RT-PCR).
  - ✓ Ribonuclease (RNase) protection assays.

Reverse transcription-PCR (RT-PCR)

- In RT-PCR, complementary DNA (cDNA) is made by reverse transcribing of the RNA templates with the enzyme reverse transcriptase.
- This technique is used to qualitatively study gene transcription or expression, and can be combined with real time PCR (qPCR) to quantify RNA levels.
- RT-PCR is routinely used as a diagnostic test for the presence of RNA viruses, such as the agents causing acquired immune deficiency syndrome (AIDS), measles, mumps, and Coronavirus disease (COVID-19).

Reverse transcription-PCR (RT-PCR)

1. Rapid
2. Quantitative measurement
3. Lower contamination rate
4. Easy standardization

- **Real Time Reporters:** All real time PCR systems rely upon the detection and quantitation of a fluorescent reporter, the signal of which increases in direct proportion of the amount of PCR product in a reaction.
- **How SYBR Green works?** SYBR green binds to double stranded DNA and upon excitation emits light. Thus as PCR product accumulates the fluorescence increases.

Limitations of SYBER Green

Advantages

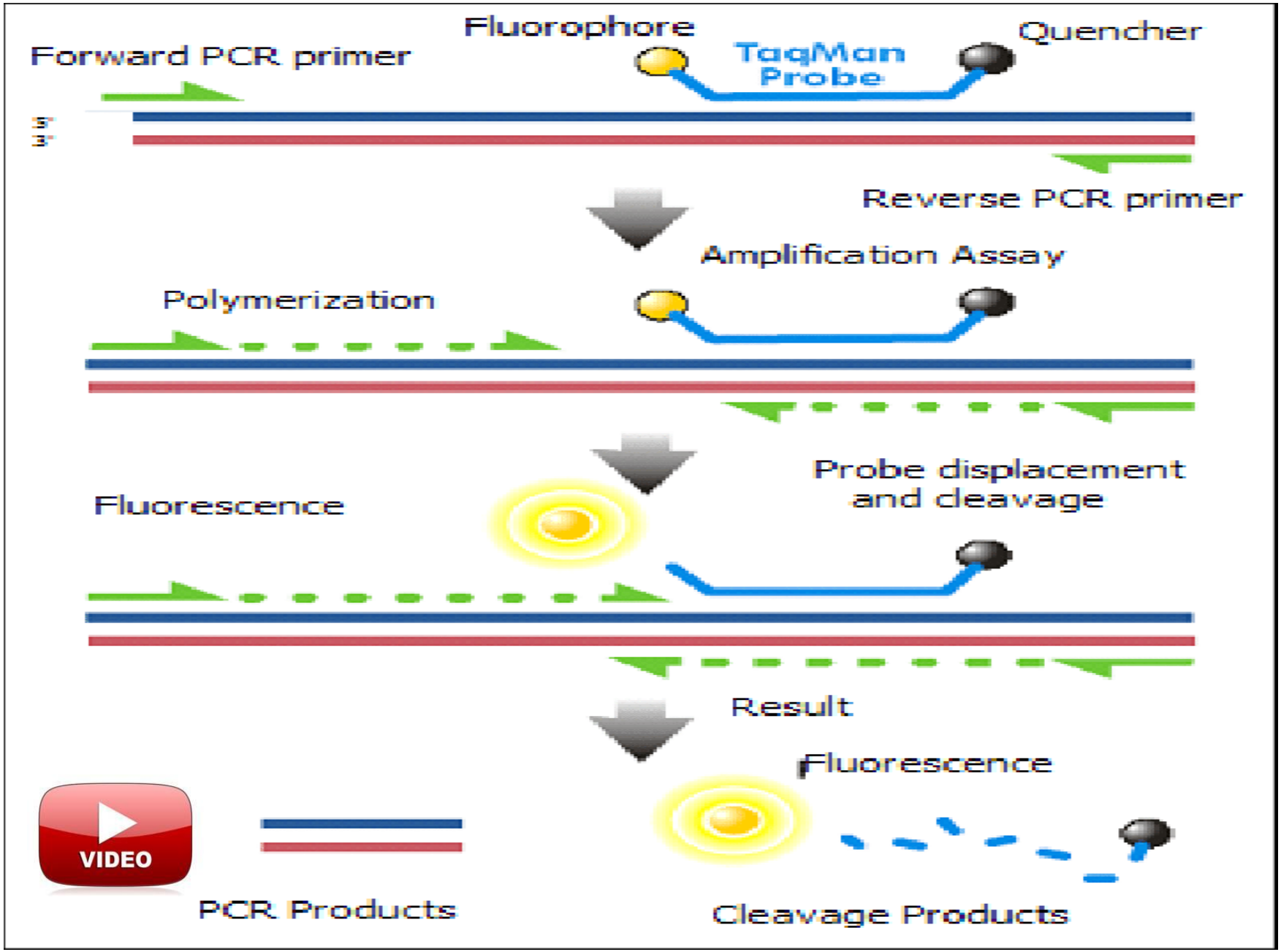
1. Inexpensive
2. Easy to Use
3. Sensitive

Disadvantages

1) SYBR green will bind to any double stranded DNA in a reaction, may result in an overestimation of the target concentration

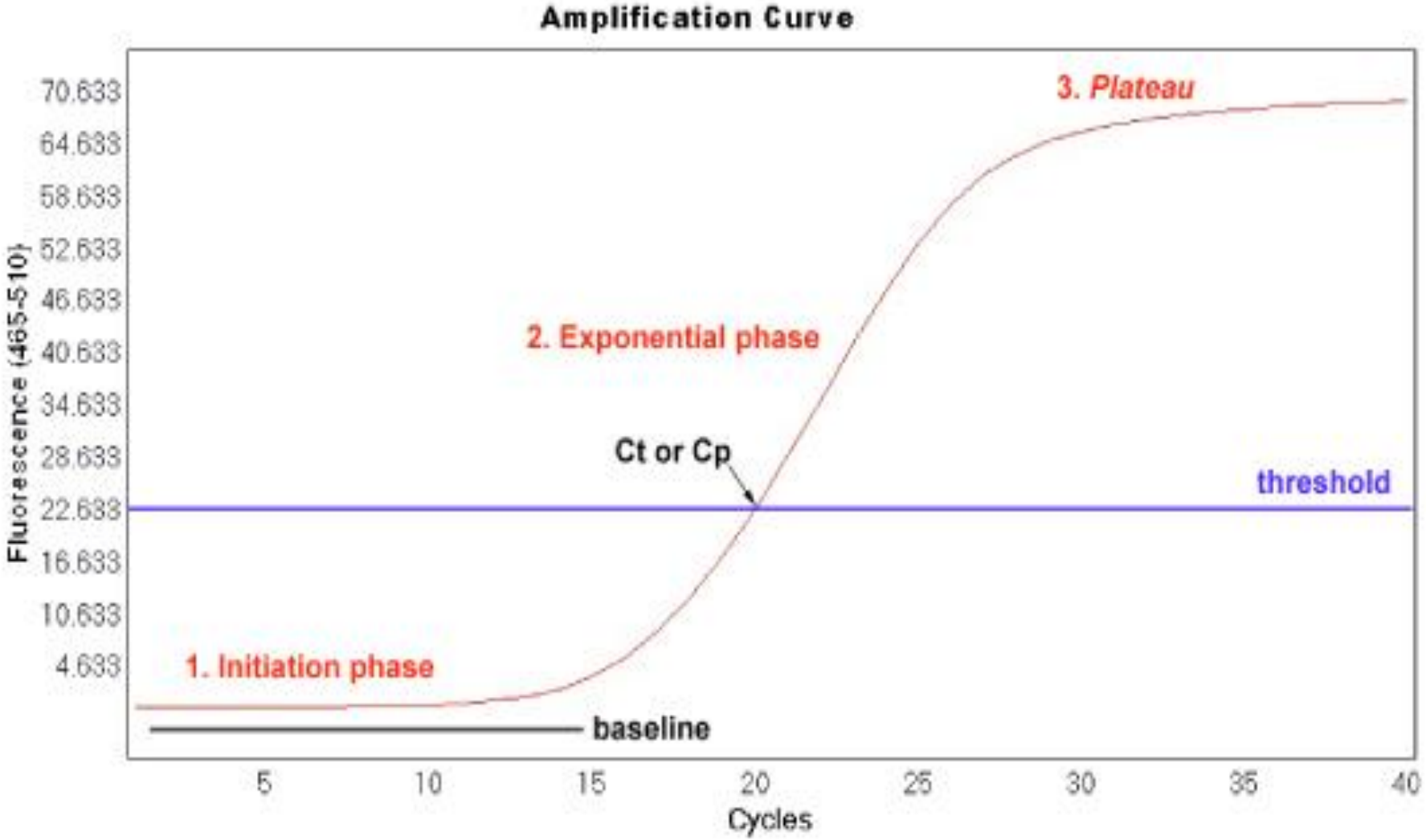
qPCR - TaqMan

- This is an alternatives to SYBR green.
- This technology depends on hybridization probes relying on fluorescence resonance energy transfer (FRET) and quantitation
- The probe sequence is intended to hybridize specifically in the DNA target region of interest between the two PCR primers.
- Typically the probe is designed to have a slightly higher annealing temperature compared to the PCR primers so that the probe will be hybridized when extension (polymerization) of the primers begins.
- The “reporter” (R) dye is attached at the 5′-end of the probe sequence while the “quencher” (Q) dye is synthesized on the 3′-end.


Fluorophore
Quencher
Forward PCR primer
TaqMan Probe
5'
3'
Reverse PCR primer
Amplification Assay
Polymerization
Probe displacement and cleavage
Fluorescence
Result
Fluorescence
VIDEO
PCR Products
Cleavage Products

SYBR Green and Taqman

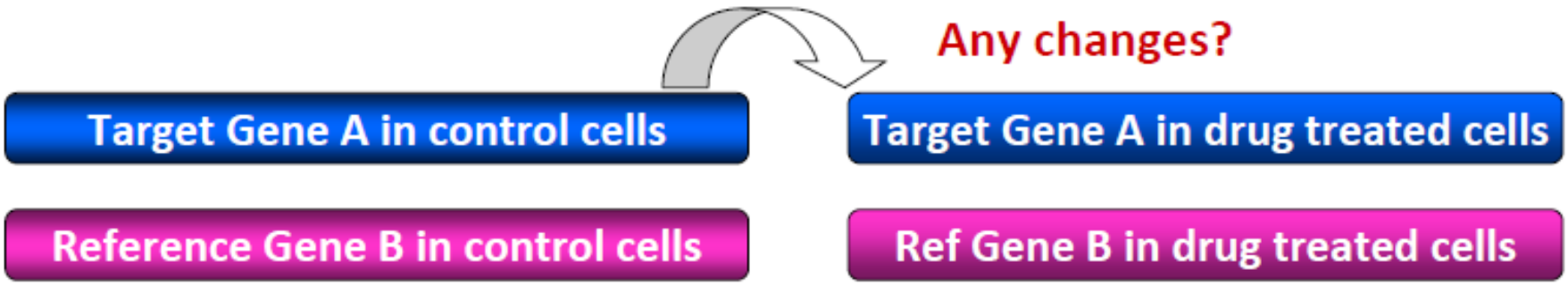
SYBR Green	Taqman probe
Advantages	
• Simple operation • Lower cost • More suitable to relative qPCR	• Stronger specificity • Perform multiplex qPCR • More suitable to absolute qPCR
Disadvantages	
• Slightly lower accuracy • Specificity lower than TaqMan probe method • Do not perform multiplex qPCR	• Higher cost • Complex operation



Typically, an amplification curve presents three different phases

- **The threshold line** is the level of detection or the point at which a reaction reaches a fluorescent intensity above background levels.
- **The C_q value** (cycle quantification value) or C_t (threshold cycle) is the PCR cycle number at which your sample's reaction curve intersects the threshold line.
- C_q values are inverse to the amount of target nucleic acid that is in your sample, and correlate to the number of target copies in your sample.
- Lower C_q values (typically below 29 cycles) indicate high amounts of target sequence.
- Higher C_q values (above 38 cycles) mean lower amounts of your target nucleic acid.

Comparative quantification



→ $\Delta Ct1 = Ct$ (Target A -treated) – Ct (Ref B-treated)

Ref: GAPDH

→ $\Delta Ct2 = Ct$ (Target A-control) – Ct (Ref B-control)

→ $\Delta\,\Delta\ Ct = \Delta Ct1$ (treated) – $\Delta Ct2$ (control)

Normalized target gene expression level = $2^{\Delta\Delta Ct}$

$2^{-(\Delta\Delta Ct)}$

Example

	Gene A (ΔCt)	Gene B (ΔCt)
Cases	4.59	4.75
Control	8.06	9.68
ΔΔCt	−3.47	−4.93
Regulation	UP	UP
Fold change	11.08	30.48

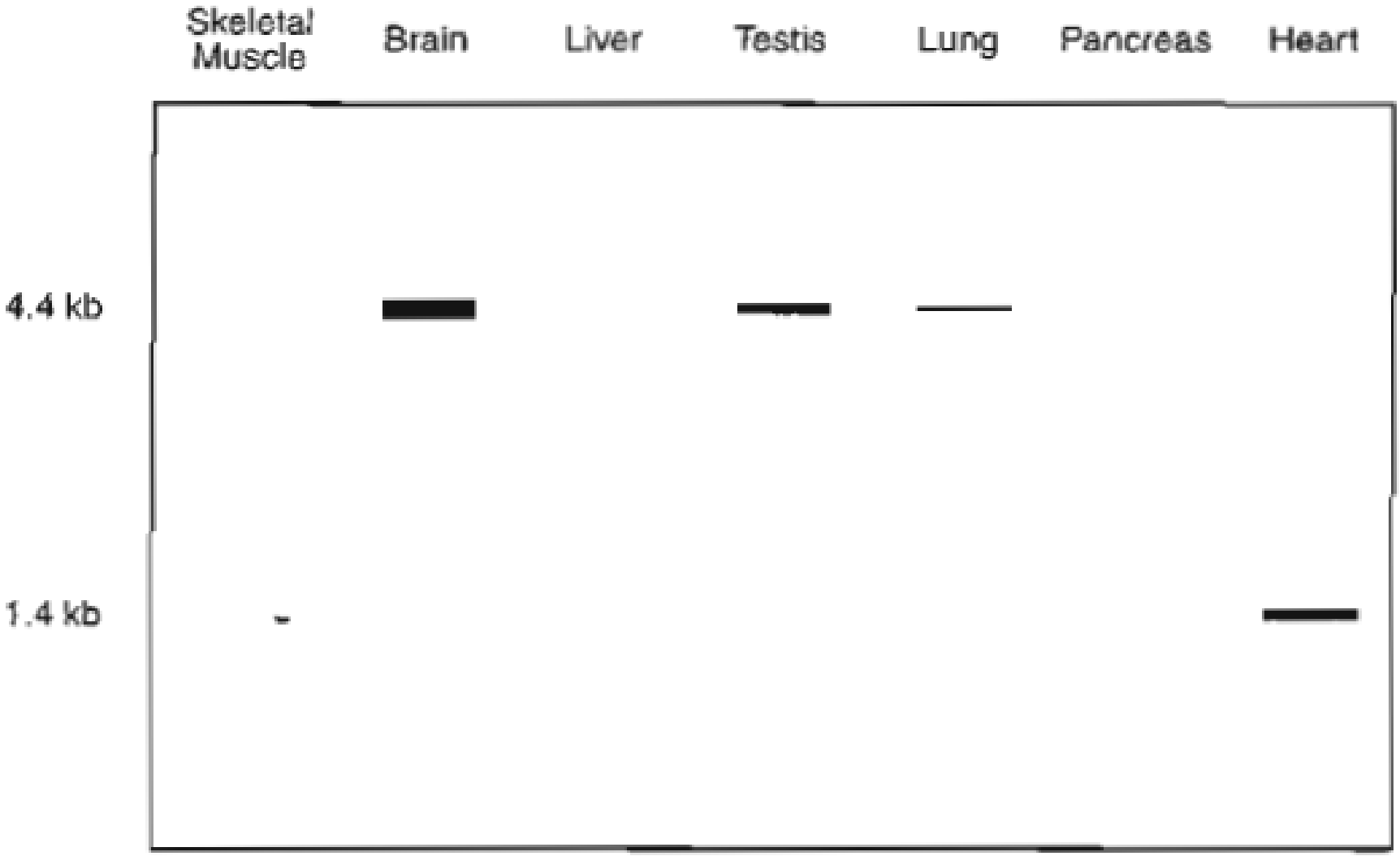
Northern blot

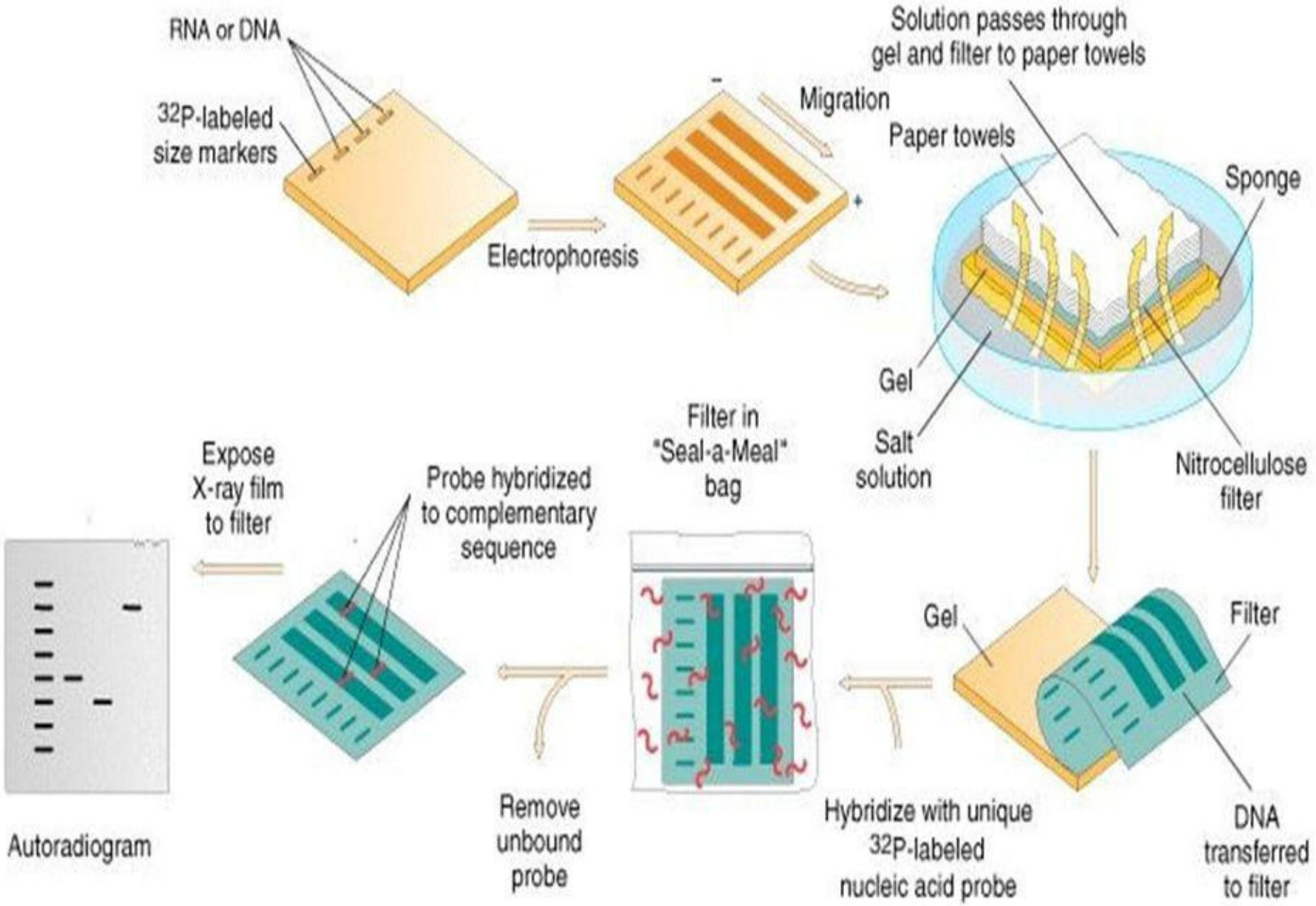
- A method similar to the DNA blotting and hybridization method can be used to probe RNA molecules.
- Because mRNAs are relatively short (typically less than 5 kb), there is no need for them to be digested with any enzymes prior to gel electrophoresis.
- Northern blot hybridization is used to measure the quantity and determine the size of specific transcribed RNAs. Thus, this method is useful for studying the expression of specific genes.
- For example, RNA can be isolated and analyzed from different tissues and from different developmental stages of an organism.

Northern blot

- One example, the goal is to determine which tissues express the *FMRI* gene involved in fragile-X syndrome.
- RNA samples from multiple tissues have been separated by electrophoresis, blotted, and probed with a ^{32}P-cDNA probe from the *FMRI* gene.
- The results are consistent with high-level expression (a 4.4-kb transcript) of this gene in brain and testis and lower level expression in the lung.
- In the heart, the gene is also expressed, but the transcripts are only 1.4kb long.

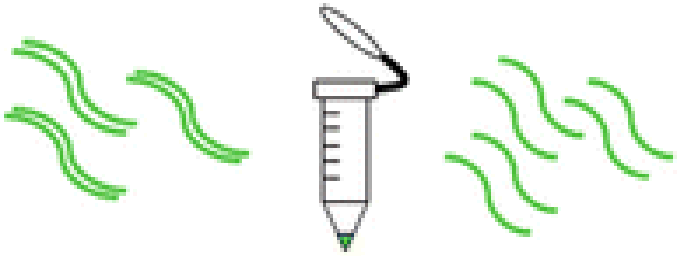
Northern Blot to Determine Pattern *of FMR1* Expression



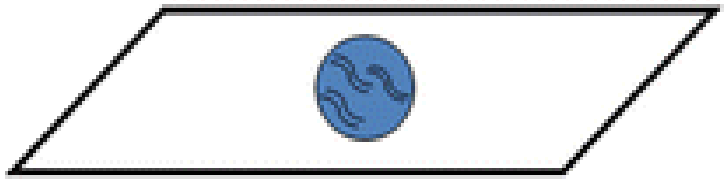

RNA or DNA
32P-labeled size markers
Electrophoresis
Migration
Solution passes through gel and filter to paper towels
Paper towels
Sponge
Gel
Salt solution
Nitrocellulose filter
Filter in "Seal-a-Meal" bag
Probe hybridized to complementary sequence
Expose X-ray film to filter
Autoradiogram
Remove unbound probe
Hybridize with unique 32P-labeled nucleic acid probe
Gel
Filter
DNA transferred to filter

In situ hybridization

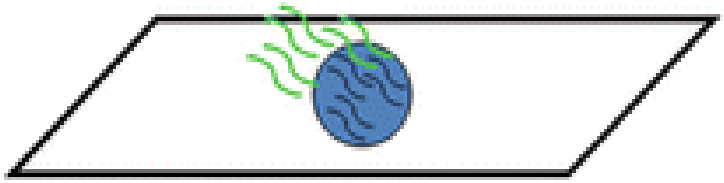
- The technique is based on the principle that double-stranded DNA denatures on heating to single-stranded DNA.
- On cooling, the single-stranded DNA re-anneals with its complementary sequence into double-stranded DNA.
- For radioactive labeling, hydrogen-3 (^{3}H) is often used because it has lower energy emissions than phosphorus-32 (^{32}P).
- Nonradioactive-detection methods use fluorescently labeled probes or probes tagged with an antigen.
- When a fluorescently labeled probe is used the technique is called fluorescent *in situ* hybridization (FISH).
- The samples are visualized by fluorescence microscopy.



Green fluorochrome-labeled probe is denatured



Tissue or cell component on glass slide is denatured to create single-stranded DNA

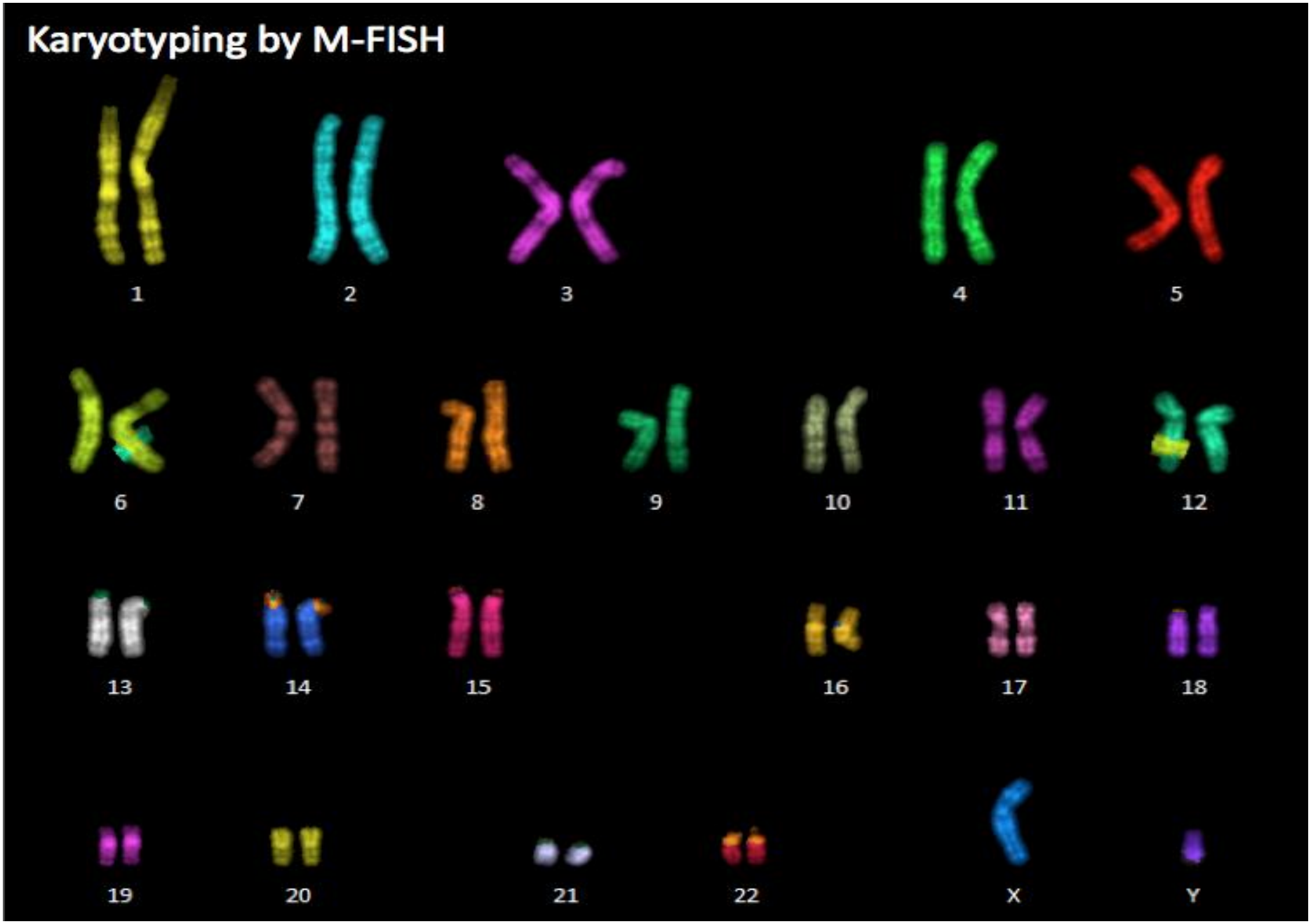


Denatured probe is applied to the slide/tissue

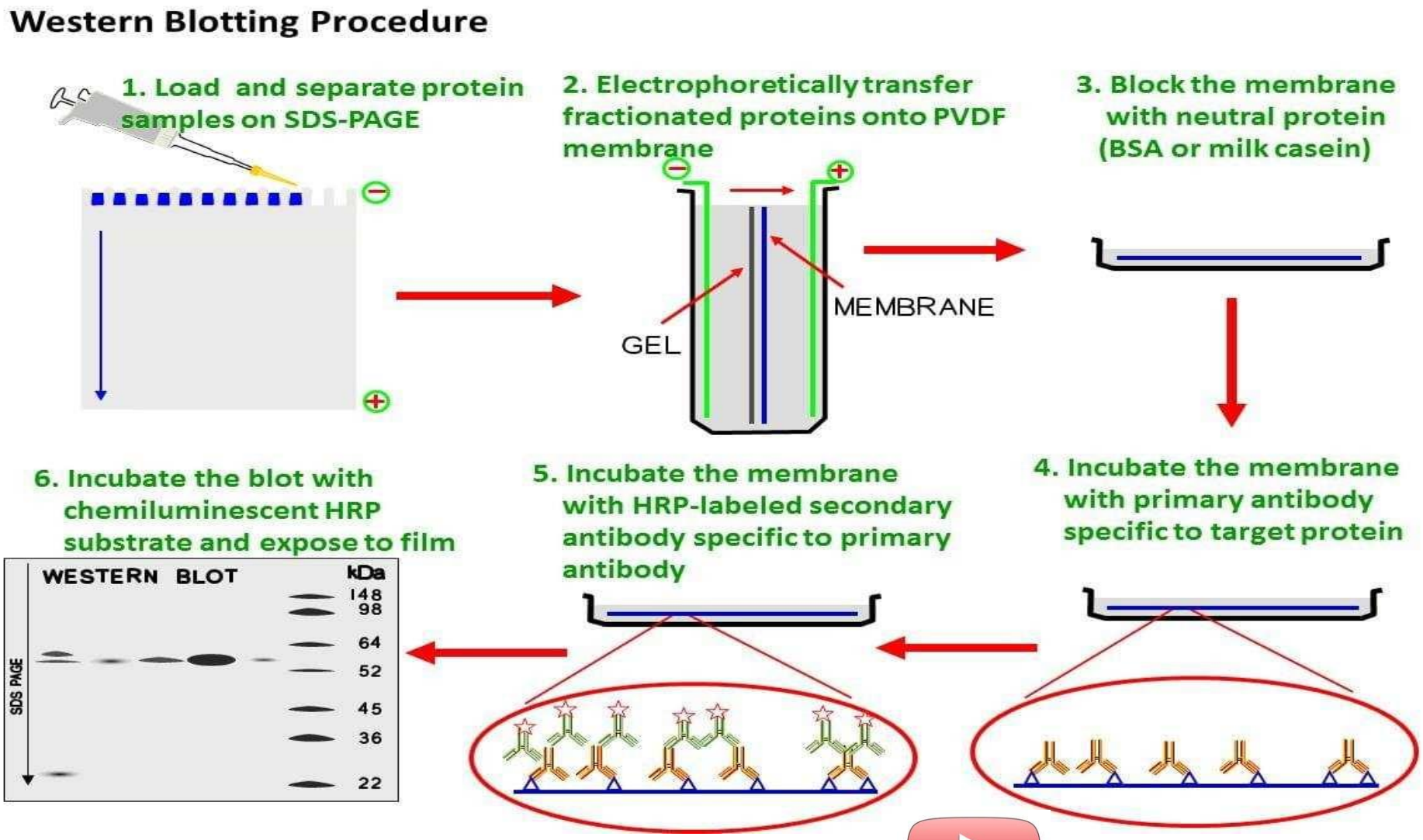
Slide is incubated to allow hybridization

Wash off non-hybridized probe

Detection of fluorescent hybridization signal by fluorescence microscopy


Karyotyping by M-FISH
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
X
Y

Protein Expression and Localization

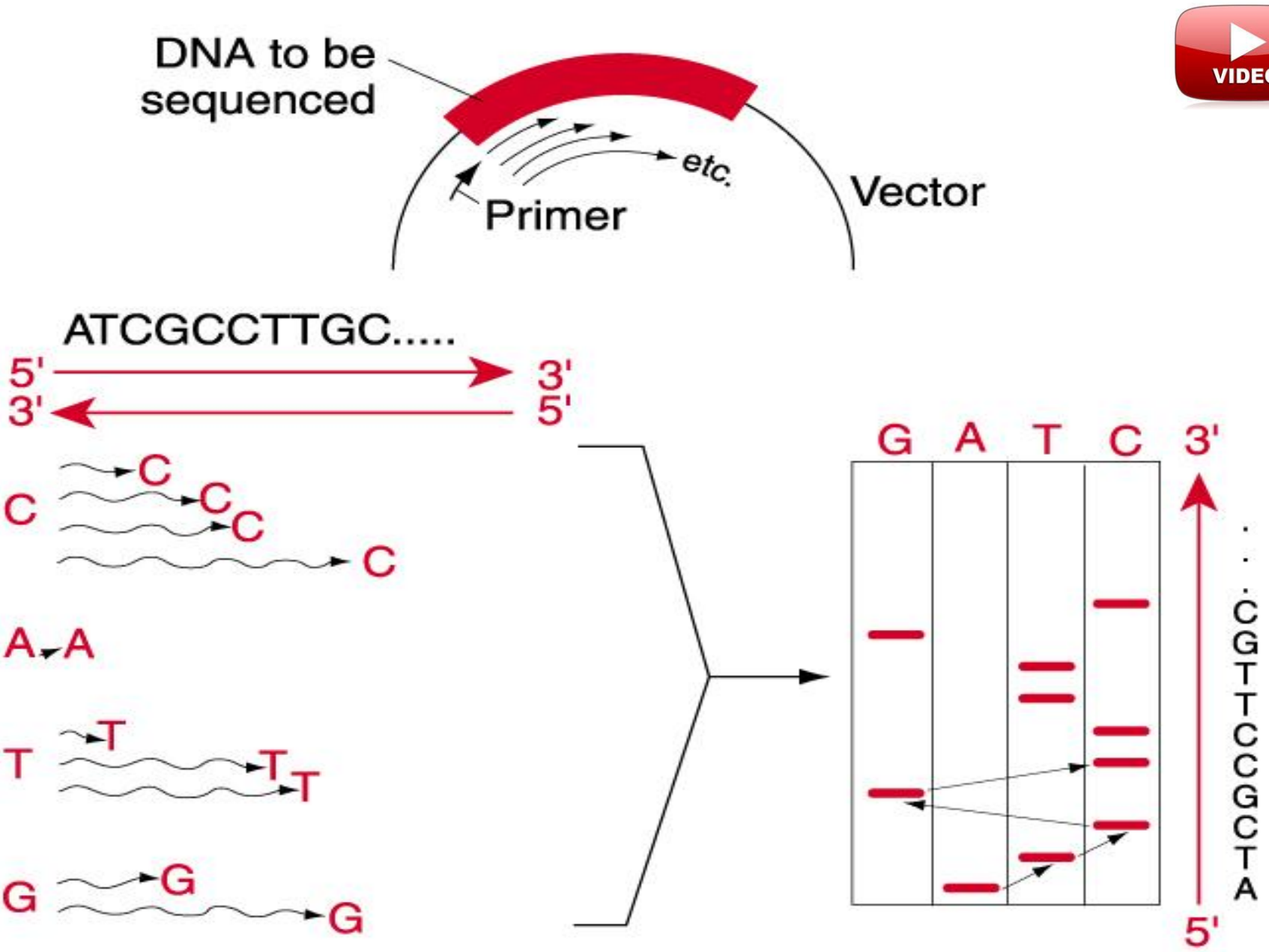


VIDEO

DNA Sequencing

- DNA sequencing is the process of determining the sequence of nucleotide bases (As, Ts, Cs, and Gs) in a piece of DNA.
- DNA sequencing is used to
  - ✓ Identify genes,
  - ✓ Determine the sequence of promoters and other regulatory DNA elements that control expression,
  - ✓ Reveal the fine structure of genes and other DNA sequences, and
  - ✓ Confirm the DNA sequence of cDNA and other DNA synthesized *in vitro* .

- The first methods for sequencing DNA were developed in the middle 1970s by Fred Sanger,
- Early DNA sequencing was technically challenging and slow.
- Resources were expensive, and the reactions required complex conditions to work.
- It therefore took several years to sequence just one or two genes.
- We can now sequence an entire human genome, all 3.2 billion letters, in a matter of hours and for much less money.


DNA to be sequenced
Primer
etc.
Vector
ATCGCCTTGC.....
5'
3'
3'
5'
C
C
C
C
A
A
T
T
T
T
G
G
G
G A T C 3'
. . . CGTTCCGCTA
5'

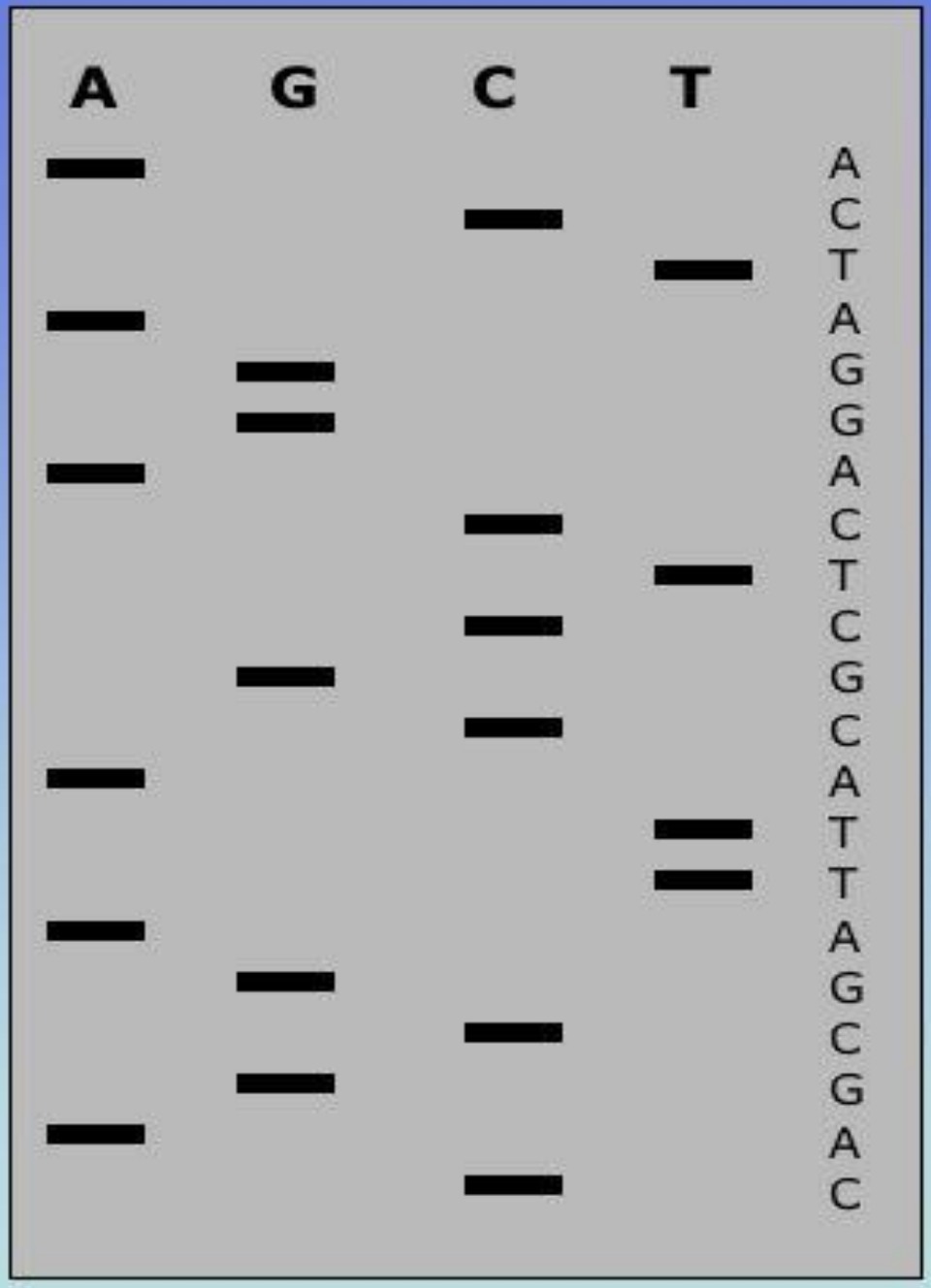


Figure 1: Manual Sequencing Using Radiolabeled ddNTPs

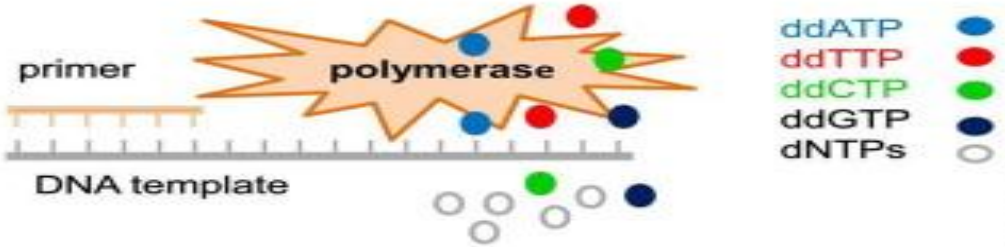
Manual sequencing using the Sanger method originally used radioactively labeled ddNTPs. Because there was no method to detect the difference between the A,G,C, and T bases, the reaction for each bases was done separately and loaded into separate lanes on a polyacrylamide gel as shown.

Sanger Sequencing

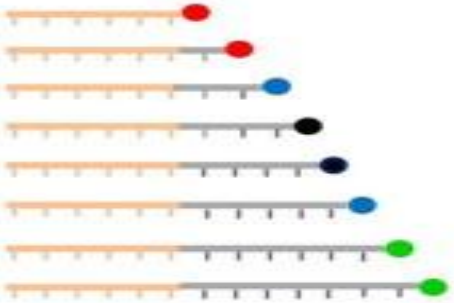
- Sanger sequencing, also known as the chain termination method.
- Classical Sanger sequencing requires
  - ✓ A single-stranded DNA template,
  - ✓ A DNA polymerase,
  - ✓ A DNA primer,
  - ✓ Normal deoxynucleosidetriphosphates (dNTPs), and
  - ✓ Modified nucleotides (ddNTPs) that terminate DNA strand elongation.

- These ddNTPs lack a 3′-OH group that is required for the formation of a phosphodiester bond between two nucleotides, causing the extension of the DNA strand to stop when a ddNTP is added.
- The sequence will continue to extend with dNTPs until a ddNTP attaches.
- As the dNTPs and ddNTPs have an equal chance of attaching to the sequence, each sequence will terminate at varying lengths.
- Each ddNTP (ddATP, ddGTP, ddCTP, ddTTP) also includes a fluorescent marker.
- By convention, **A** is indicated by green fluorescence, **T** by red, **G** by black, and **C** by blue.
- A laser within the automated machine used to read the sequence detects a fluorescent intensity that is translated into a "peak."

reaction mixture:
- primer
- DNA template
- DNA polymerase
- dNTPs
- fluorescently labeled ddNTPs

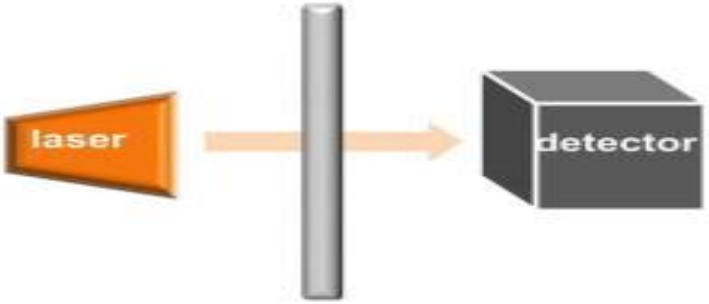


chain extension
& chain termination

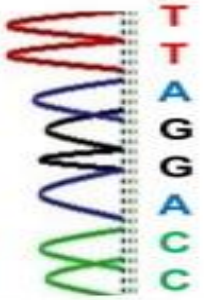


VIDEO

gel electrophoresis
& fluorescence detection



computational sequence analysis



Thanks for your attention