BCHEM 365
Lecture 8
October 15, 2018

Pulsed field gel electrophoresis (PFGE)

- A special technique is used to determine the size of large DNA. There are two reasons for development of PFGE
- 1. The relationship between the log of a DNA's size and its electrophoretic mobility deviates strongly from linearity if the DNA is very large
- 2. The double-stranded DNA is a very rigid rod, very long and thin, making it fragile
- 3. The standard gel electrophoresis technique runs on a unidirectional constant electric field and works best up to 15 kb DNA beyond which DNA fragments migrate independent of size and produce a single large MW band at top of gel
- 4. The low concentration of agarose required to separate large MW DNA fragments is not strong and breaks easily
- Again beyond 15 kb, the large DNA fragments break very easily – swirling in a beaker or pipetting creates shearing forces sufficient to fracture DNA

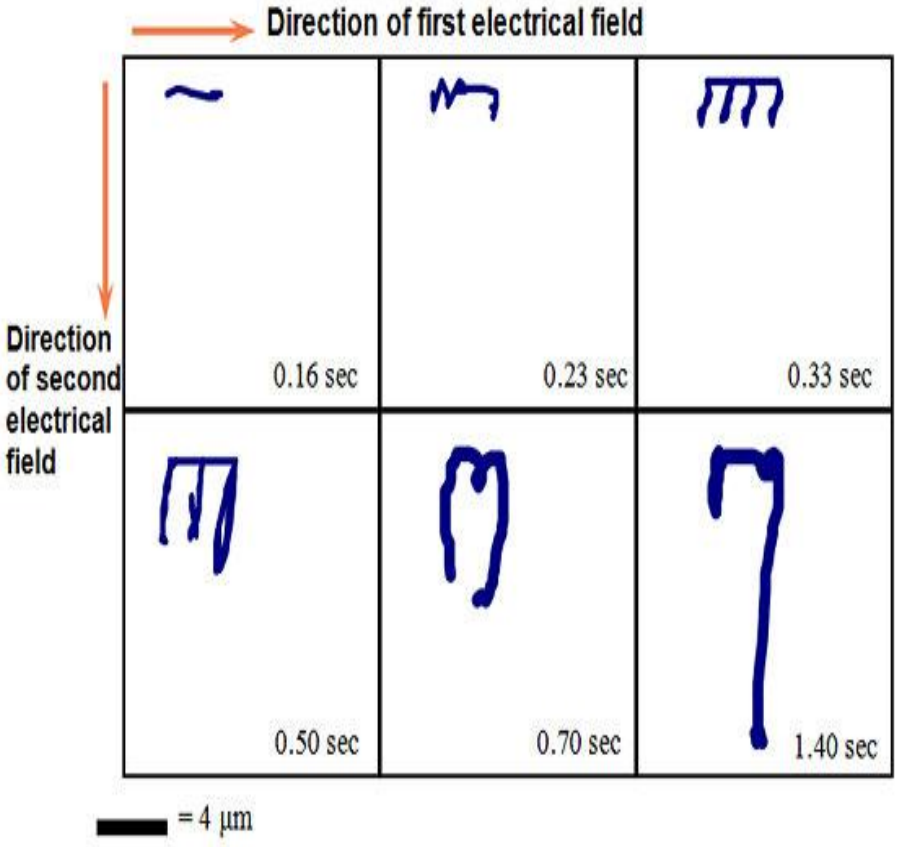
Pulsed field gel electrophoresis

- PFGE (developed by David Schwartz and Charles Cantor, 1984) is a variation of gel electrophoresis that can separate DNA molecules >15 kb to several million base pairs (Megabases, Mb) long and maintain a relatively linear relationship between the log of their sizes and their mobilities
- PFGE differs from normal gel electrophoresis. Normal gel electrophoresis uses constant voltage which runs down the gel in one direction. In PFGE two things keep changing at regular intervals: 1) The direction of current, 2) The holding time for each current direction may be same or different
- Direction of current: the current is periodically switched among three directions- one current runs through the central axis of the gel; the second current runs at 60^{o} from one side of gel; the third current runs at 60^{o} from other side of gel. Current may also be changed at 90^{o} to each other
- The pulse times are equal for each direction leading to a net forward migration of the DNA down the gel

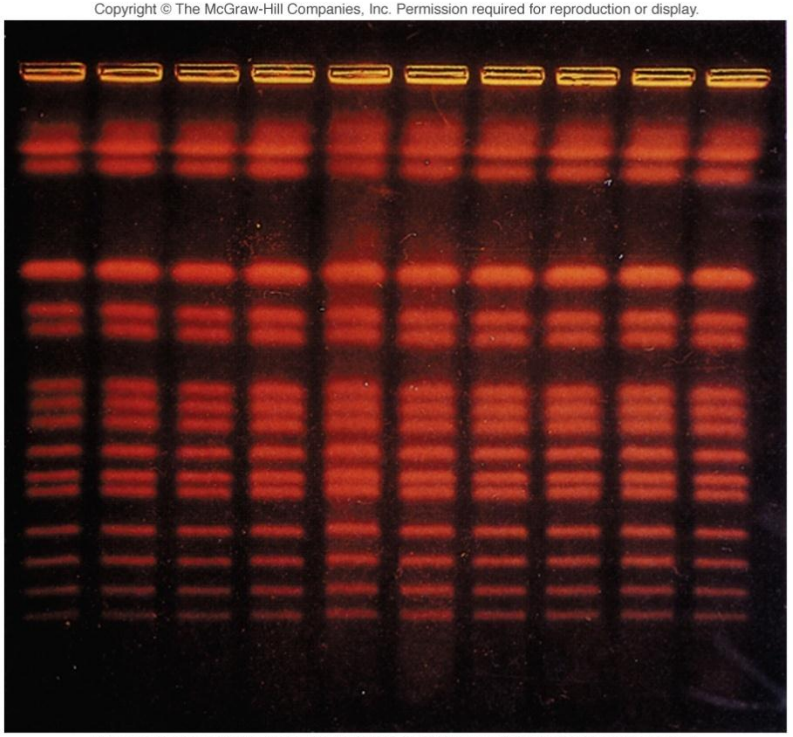
Pulsed field gel electrophoresis

The result is that:

- 1. Different DNA sizes respond to the changed current direction at differing rates
- 2. DNA molecules elongate upon application of an electric field and return to an unelongated (relaxed) state upon removal of the electric field
- The size-dependent relaxation rate introduces different rates of migration through the gel to effect separation
- Can be used to separate chromosomes



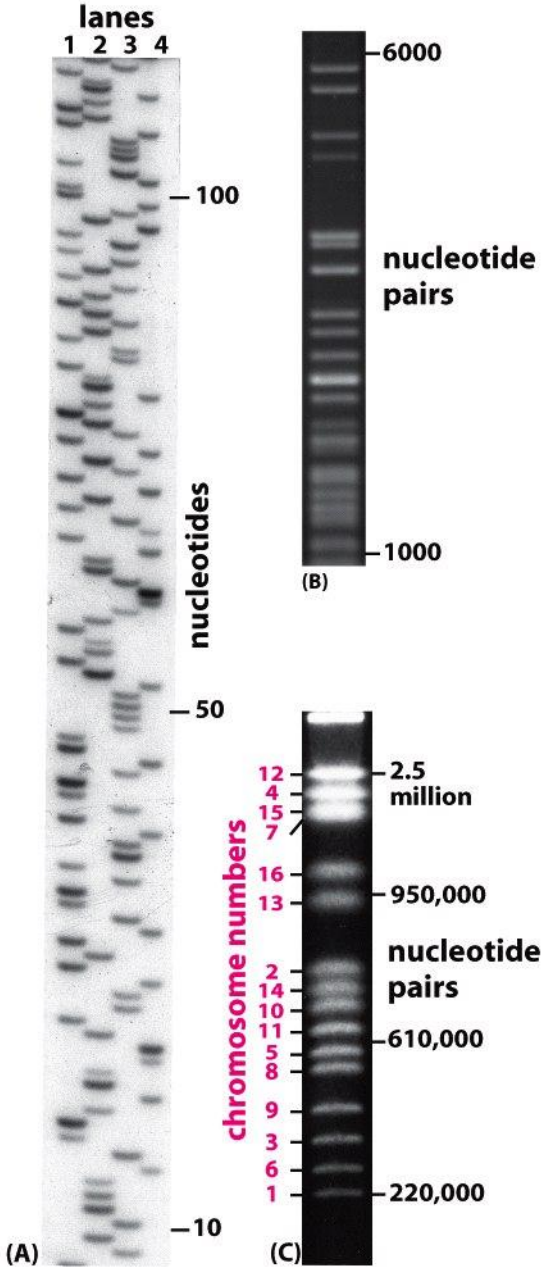
- PFGE of yeast chromosomes
- Identical samples of yeast chromosomes were electrophoresed in 10 parallel lanes and stained with ethidium bromide. The bands represent chromosomes having sizes ranging from 0.2 Mb (bottom) to 2.2 Mb (top)



(Source: Courtesy Bio-Rad Laboratories/CHEF-DR(R)II pulsed-field electrophoresis systems.)

Short summary

Three gel electrophoresis techniques for separating DNA molecules by size. Electrophoresis is from top to bottom. Largest and therefore slowest-moving DNA are near the top of the gel. Smallest and supercoiled DNA molecules are near the bottom



Applications of electrophoresis

- The phenomenon of nucleic acid hybridization is very important in molecular biology
- Nucleic acid hybridization reactions provide a sensitive way to detect specific nucleotide sequences
- Northern and southern blotting facilitate hybridization with electrophoretically separated nucleic acid molecules
- Southern blot is used to identify specific DNA fragments
- Northern blot is used to measure gene activiy as RNA transcripts
- Northern blot is similar to Southern blot but it contains electrophoretically separated RNAs instead of DNAs

Nucleic acid hybridization

- Heat an aqueous solution of DNA at 100^{o}C or expose to a high pH (pH≥13) and complementary base pairs that hold the two strands of the double helix together are disrupted, double helix rapidly dissociates into single strands
- This is DNA denaturation
- When complementary strands reform at a lower temperature renaturation or annealing occurs
- Similar hybridization can occur between any two complementary single stranded nucleic acid chains: DNA/DNA, RNA/RNA, RNA/DNA
- These specific hybridization reactions are widely used to detect and characterize specific nucleotide sequences in both RNA and DNA molecules
- The complementary strands which are used for detection of DNA or RNA on a blot are called **probes**

Nucleic acid hybridization

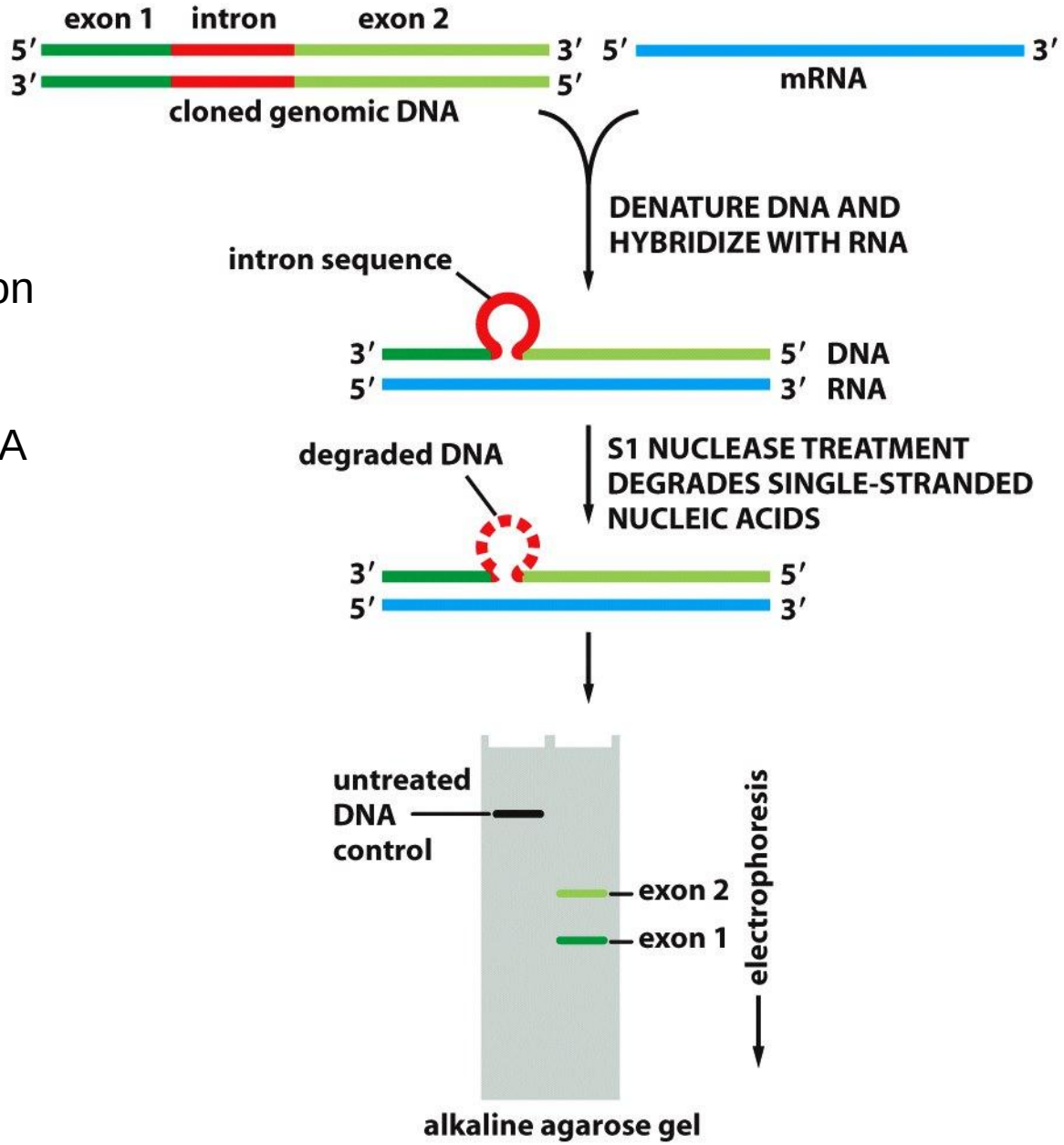
DNA probes

- These are single-stranded DNA molecules used to detect complementary sequences. They are usually labeled with a radioactive or chemical marker for detection. Can range from 15 to 1,000 nucleotides long
- DNA probes are very sensitive. Can detect complementary sequences present at concentrations as low as one molecule per cell therefore it can determine the number of copies of a DNA sequence present in a cell
- Can be used to find related but nonidentical genes
- Can be used to find a gene of interest in an organism whose genome has not yet been sequenced
- Can be used in hybridization reactions with RNA and allows one to determine if a particular gene is being transcribed

Nucleic acid hybridization

- In this case, a DNA probe that contains part of a gene's sequence is hybridized with RNA purified from the cell to see whether the RNA includes nucleotide sequences matching the probe DNA, and in what quantities
- After hybridization is complete, the DNA is treated with specific nucleases to determine the exact regions of the DNA that have paired with the RNA molecules.
- Through this, one can determine the start and stop sites for RNA transcription, as well as the precise boundaries of intron and exon sequences in a gene

Use of nucleic acid hybridization to determine the region of a cloned DNA fragment that is present in an mRNA molecule



RFLP

- Restriction-fragment length polymorphism
  - Cut genomic DNA from two individuals with restriction enzyme
  - Run Southern blot
  - Probe with different pieces of DNA
  - Sequence difference creates different band pattern

Recognition site for enzyme: GTATCC

Southern blot

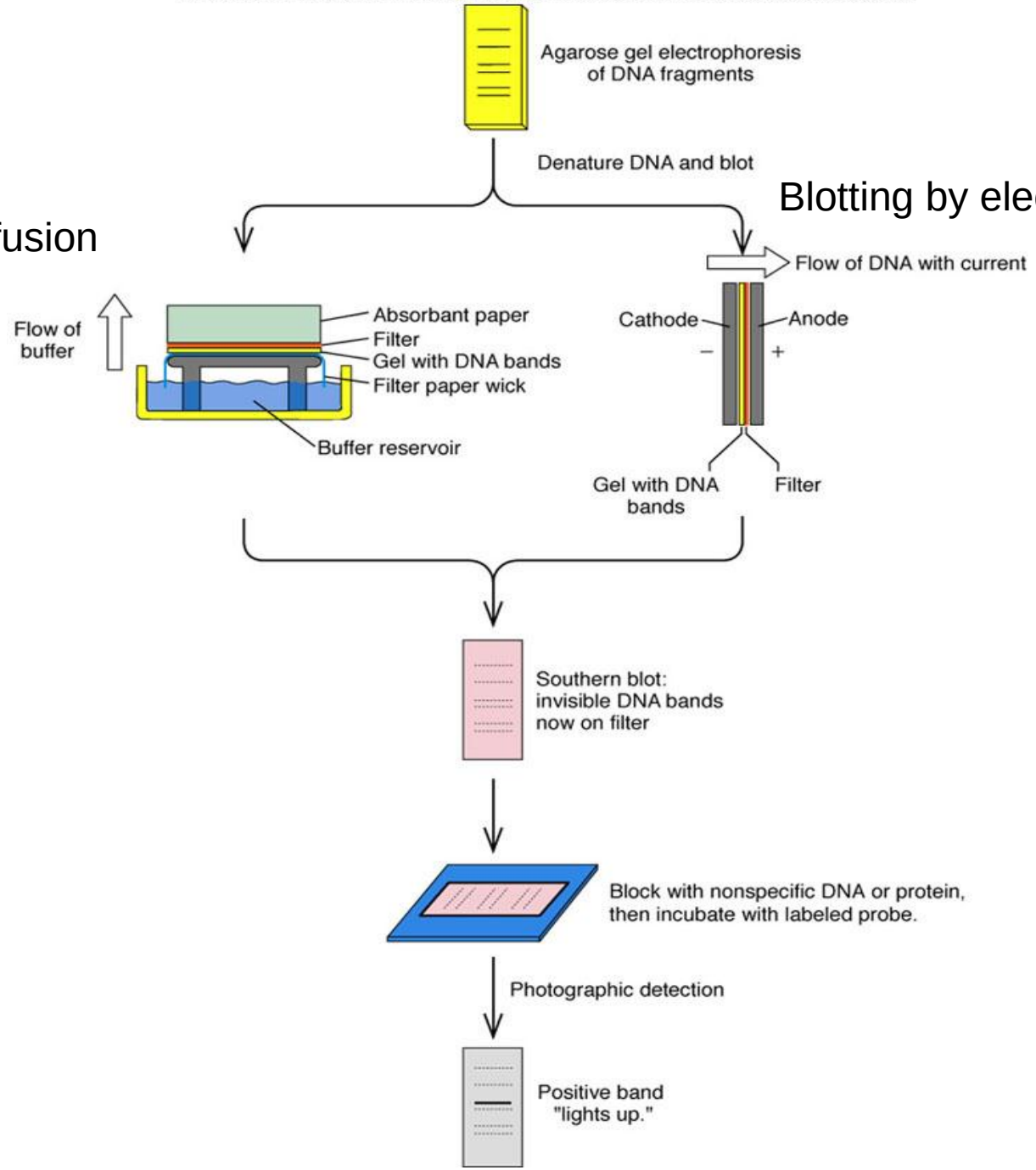
- Named after its inventor, Edward Southern
- In this method, isolated DNA is first cut into readily separable fragments with restriction endonucleases
- Double-stranded fragments are then separated on the basis of size by agarose gel electrophoresis
- Denature the DNA with a base to obtain single-stranded DNA fragments.
- Transfer the DNA single strands from the gel to a nitrocellulose material
  - By diffusion, in which the buffer passes through the gel, carrying the DNA with it
  - By electrophoresis
- Hybridize the blot to a labeled probe and detect labeled bands by autoradiography or phophorimaging
- This probe will form base pairs with its complementary DNA sequence and bind to form a double-stranded DNA molecule.
- The degree of hybridization of a complementary probe is a measure of the amount of the specific target sequence in the sample.
- The probe is labeled before hybridization either radioactively or enzymatically (e.g. alkaline phosphatase or horseradish peroxidase).
- Those fragments complementary to a DNA probe are identified by blotting and hybridization

Southern blot

- Wherever the probe encounters a complementary DNA sequence, it hybridizes, forming a labeled band corresponding to the fragment of DNA containing the gene of interest

Blotting by diffusion

Blotting by electrophoresis



DNA fingerprinting

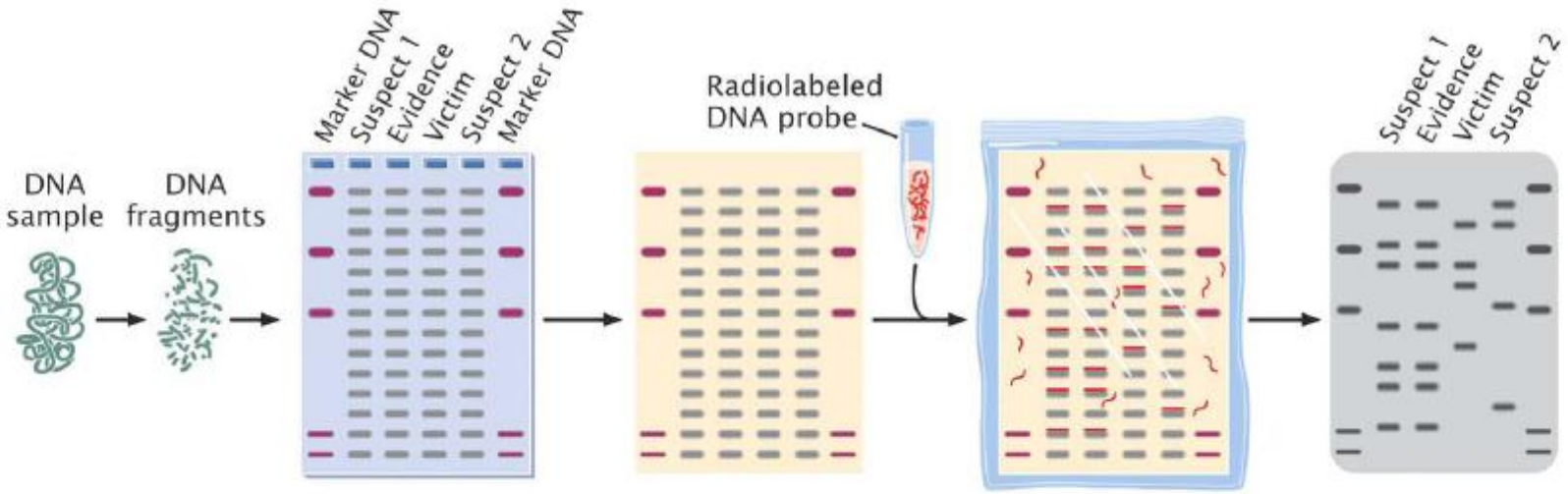
- It is a Southern blot technique
- Used in forensic labs to identify perpetrators, that is, suspects who have left blood or any DNA-containing tissue on a crime scene
- Makes use of sequences of bases repeated several times: minisatellites
- Individuals differ in the pattern of repeats of the basic sequence
- Two individuals have an extremely rare chance of having exactly the same pattern. Such patterns are like fingerprints hence DNA fingerprinting
- To do it, first cut the DNA with restriction enzyme such as *HaeIII* (GGCC nucleotide sequence)which cuts on either side of the repeat regions. This produces fragments bearing the repeated regions
- Perform electrophoresis of the fragments, denature and blot
- Probe with a labeled minisatellite DNA and detect bands with X-ray film

DNA fingerprinting—use of DNA sequences to identify a person

Used in paternity/forensics

Chance to 2 people having same set of bands is low unless monozygotic twins

If examine many markers—probability that 2 people have same sets of patterns—extremely small



DNA fingerprinting used in other criminal investigations:

- Identify human remains
- Identify source of other organisms—anthrax in mail

Northern blot

- A technique used in molecular biology research to study gene expression by detection of RNA (or isolated mRNA) in a sample.
- Northern blotting involves the use of electrophoresis to separate RNA samples by size, and detection with a hybridization probe complementary to part of or the entire target sequence
- Say you have cloned a cDNA and you want to know how the corresponding gene is expressed in a number of tissues
- Begin by collecting RNA from the tissues, then perform agarose gel electrophoresis of the RNA and blot them to a nitrocellulose or nylon support
- Hybridize the northern blot to a labeled cDNA probe.
- Wherever an mRNA complementary to the probe exists on the blot, hybridization will occur, resulting in a labeled band that can be detected by X-ray film. If you run the electrophoresis along with an RNA marker, you can tell the size of the RNA bands

Electrophoresis in the detection of sickle-cell anaemia

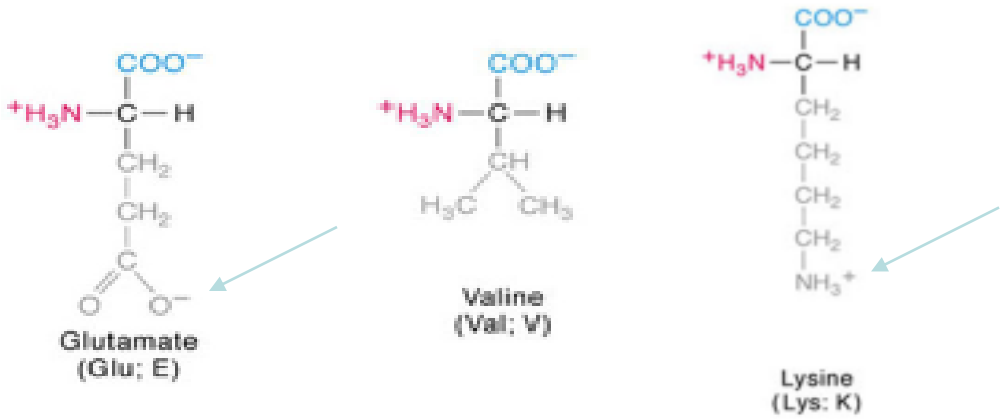
- Hemoglobin (Hb) is the iron-containing oxygen-transport metalloprotein in red blood cells
- In most humans, the hemoglobin molecule is an assembly of four globular protein subunits (tetramer), each composed of a polypeptide chain tightly associated with a non-protein heme group.

- There are variants of hemoglobin
- Normal hemoglobin, called hemoglobin A (HbA) consists of two alpha chains, and two beta chains as subunits non-covalently bound, each made of 141 and 146 amino acid residues, respectively. This is denoted as $\alpha_2\beta_2$.

- Hemoglobin S ($\alpha_2\beta^S_2$) –is found in sickle cell anemia. a variation in the β-chain gene, causing a change in the properties of hemoglobin which results in sickling of the red blood cells.

Electrophoresis in the detection of sickle-cell anaemia

- HbS arises from a point mutation of the HBB gene on the short arm of chromosome 11. This gene encodes the beta globin chain. Mutation in the β-globin which causes codon = GAA and GAG of the gene (the amino acid glutamic acid at the sixth position) to be replaced with codon GUA and GUG (the hydrophobic amino acid valine)



- HbS therefore assumes a less negative charge. In HbC, glutamic acid is replaced with lysine making it slightly more positive (or better still, much less negative than HbA and even HbS

Electrophoresis in the detection of sickle-cell anaemia

- The hydrophobic valine causes Hb to be less soluble at low oxygen concentration (i.e., deoxygenated Hb), enhances hydrophobic interaction among the β- chains, and leads to aggregation and distortion in the shape of the red blood cells

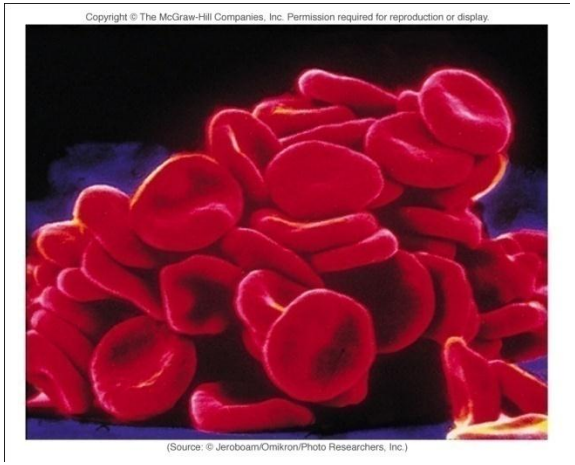
Electrophoresis in the detection of sickle-cell anemia

- Hemoglobin C ($\alpha_2\beta^C_2$) –variation in the β-chain gene. This variant causes a mild chronic hemolytic anemia.
- Heterozygotes have one normal and one sickle-cell gene and their red blood cells contain one HbS and one HbA and are said to have the sickle-cell trait. Others have one HbS and one HbC; Homozygotes have two HbS genes and have sickle cell anemia

Electrophoresis in the detection of sickle-cell anemia

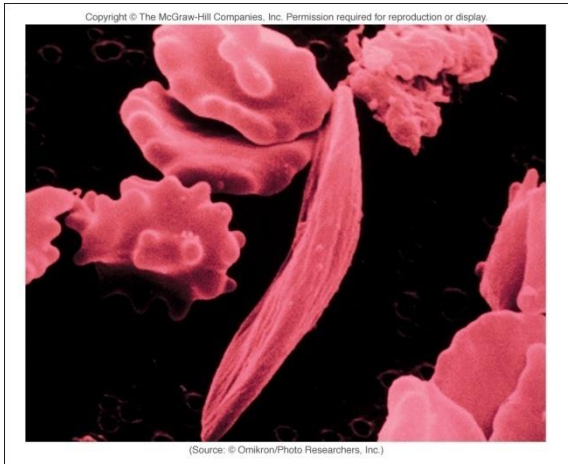
- Sickle-cell anemia, is a disease caused by a defective gene which produces a life-long blood disorder characterized by red blood cells that assume an abnormal, rigid, sickle shape (biconcave in normal cells).
- Sickle cell anemia affects about 1 in 500 infants and is common among blacks

A



(Source: © Jeroboam/Omikron/Photo Researchers, Inc.)

B



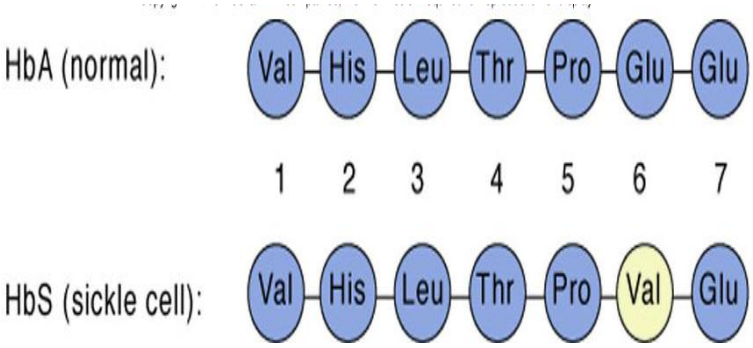
(Source: © Omikron/Photo Researchers, Inc.)

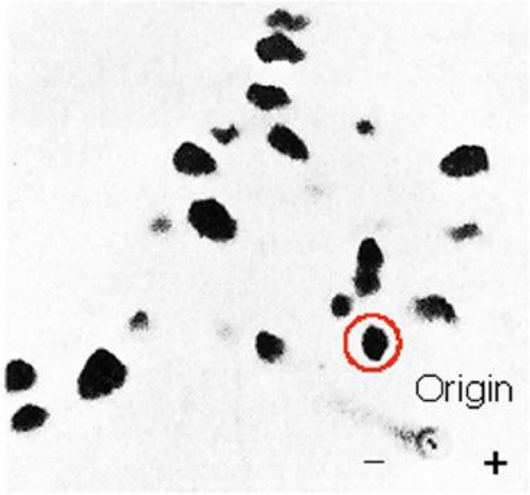
A) Normal red blood cells are disc shaped, biconcave, flexible, move freely through blood vessels. B) Sickle cells (like crescent), stiff, elongated, do not move freely, clog and stick to blood vessels, causing pain in limbs

Electrophoresis in the detection of sickle-cell anemia

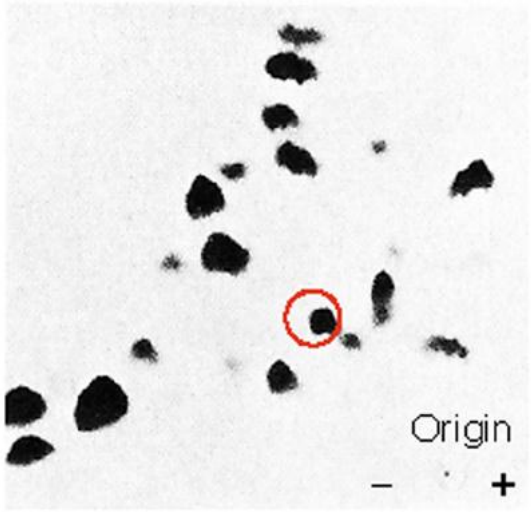
- During electrophoresis at alkaline pH, HbS with its less negative charge will migrate more slowly toward the anode than HbA does. HbC will migrate even more slower than HbS because it is much less negative
- Vernon Ingram in 1957 used a 2-dimensional fingerprinting method to find the difference between HbA and HbS. He digested the two globin subunits to obtain peptides and separated the peptides first by paper electrophoresis. Then the paper was turned 90^{o} and the peptides separated further by paper chromatography in the second dimension
- The peptides appeared as spots on the paper; different peptides with different amino acid composition gave different pattern of spots. The patterns were named fingerprints.
- Comparing the fingerprints of HbA peptides and HbS peptides, he observed that all spots matched except for one, which had a much slower mobility in the HbS fingerprint than in the normal HbA fingerprint indicating that it had an altered charge, arising from a different amino acid composition
- He found that they differed in only one amino acid: the glutamic acid in the sixth position in HbA becomes valine in HbS

Fingerprint of hemoglobin A and hemoglobin S. All spots are identical except for one peptide which moves up and to the left in HbS





Hemoglobin A



Hemoglobin S

(Source: Dr. Corrado Baglioni.)

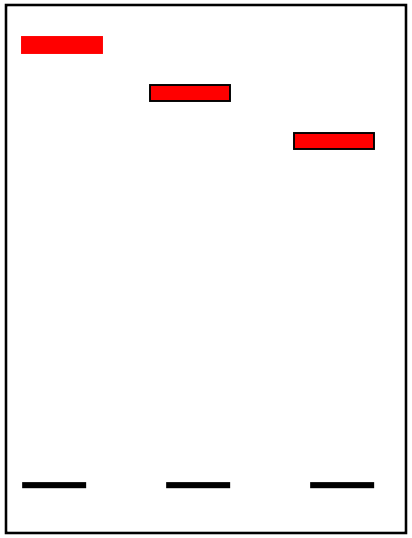
Electrophoresis in the detection of sickle-cell anemia

- Normal hemoglobin remains soluble under physiological conditions
- Sickle cell hemoglobin precipitates when blood oxygen level falls (e.g., during exercise), forming long, fibrous aggregates. Such cells are impeded in their path through the tiny capillaries, so they clog and rupture them, starving parts of the body of blood and causing internal bleeding and pain
- The hemoglobin electrophoresis test precisely identifies the hemoglobins in the blood by separating them. The separation of the different hemoglobins is possible because of the unique electrical charges they each have on their protein surfaces, causing them each to move characteristically in an electrical field.

Hemoglobin electrophoresis test

- Electrophoresis is carried out on cellulose acetate paper using an alkaline buffer at pH 8.6 to 9.2 and run for 30 min at 250 to 300 Volts. At the end of the process, the paper is stained and dried at 60 to 100^{o}C. The pattern of the bands is a function of the differences in the mobilities of the variants.

Paper electrophoresis of haemoglobin A, S, and C



+ anode

- Cathode as origin

HbA HbS HbC

Serum protein electrophoresis (SPEP)

- This is a technique used to investigate the levels of specific proteins in the blood called albumin and globulins. It is used clinically to diagnose and monitor a variety of diseases, especially disorders of the immune system, liver dysfunction, kidney diseases, impaired nutrition, some genetic disorders, and cancer
- Blood serum contains two major protein groups: albumin and globulin. Both albumin and globulin carry substances through the bloodstream
- In this method, serum samples are placed on electrophoresis gels and exposed to an electric current to separate the two major serum protein components into five smaller group which differ in shape, size, and electrical charge. The intensity of the fractions are measured with a densitometer

Serum protein electrophoresis (SPEP)

- Albumin
  - More than half of the protein in blood serum is albumin. Albumin proteins keep the blood from leaking out of blood vessels. Albumin also helps carry some medicines and other substances through the blood and is important for tissue growth and healing

- Globulins
  - Alpha-1 globulin. Made up of α1-antitrypsin and α1-acid glycoprotein. High-density lipoprotein (HDL), the "good" type of cholesterol, is included in this fraction. α1-antitrypsin protects tissues from enzymes of inflammatory cells, especially elastase. In its absence, elastase is free to break down elastin, which contributes to the elasticity of the lungs, resulting in respiratory complications such as emphysema, or COPD (chronic obstructive pulmonary disease) in adults and cirrhosis in adults or children.

Serum protein electrophoresis (SPEP)

- Alpha-2 globulin. A protein called haptoglobin, that binds with haemoglobin, is included in this fraction.
- Also present are α2-macroglobin, a large plasma protein found in the blood and produced by the liver. It functions as an inhibitor of coagulation by inhibiting thrombin and an inhibitor of fibrinolysis by inhibiting plasmin.
- Also in this fraction is α2-antiplasmin which is responsible for inactivating plasmin, an important enzyme that participates in fibrinolysis and degradation of various other proteins.
- Ceruloplasmin, officially known as ferroxidase or iron(II):oxygen oxidoreductase, is the major copper-carrying protein in the blood, and in addition plays a role in iron metabolsm is an alpha-2 globulin

Serum protein electrophoresis (SPEP)

Beta globulin. This fraction consists of transferrin, LDL and the complement system. The **complement system** is a biochemical cascade (a group of plasma proteins) that helps clear pathogens from an organism

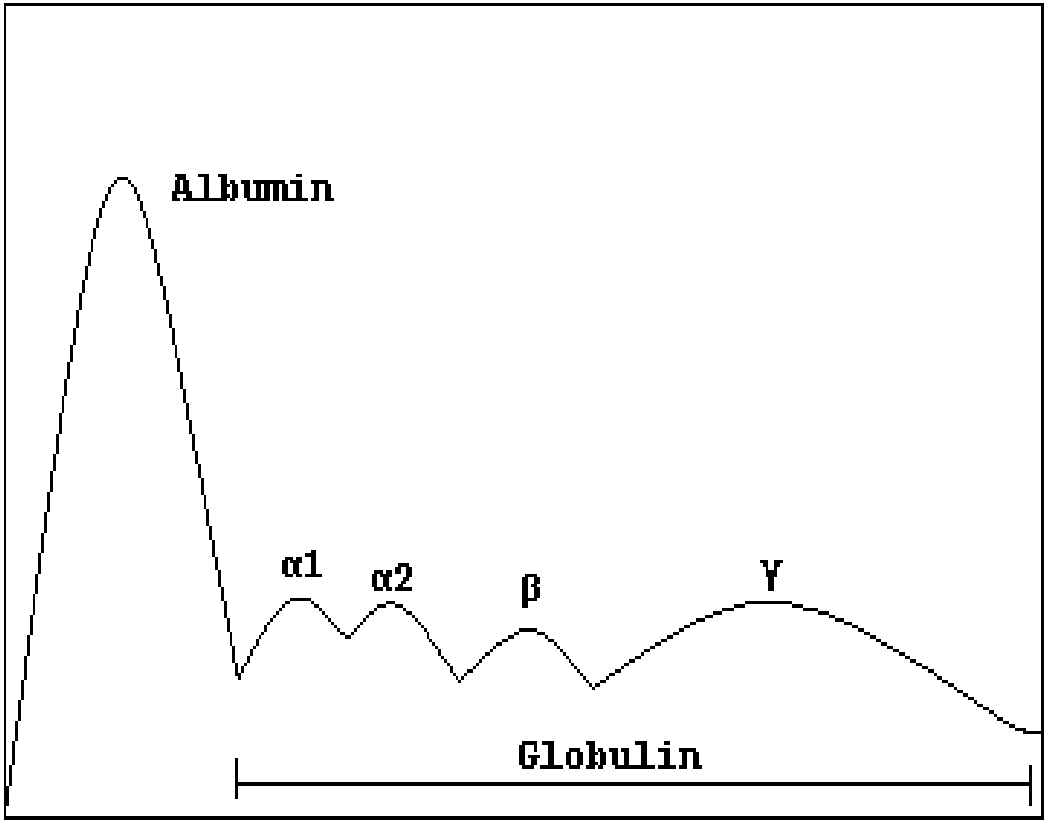
Transferrin is for iron transport

LDL is a type of lipoprotein that transports cholesterol and triglycerides from the liver to peripheral tissues. Also known as bad cholesterol.

Serum protein electrophoresis (SPEP)

- Gamma globulins
  - This fraction is made up of immunoglobulin (IgA, IgD, IgE, IgG and IgM) and paraproteins
  - The immunoglobulins are also called antibodies. They help prevent and fight infection. Gamma globulins bind to foreign substances, such as bacteria or viruses, causing them to be destroyed by the immune system
  - Paraproteins are a group of proteins most often associated with benign MGUS (monoclonal gammopathy of undetermined significance), and multiple myeloma

Each of these five protein groups moves at a different rate in an electrical field and together form a specific pattern. The topography of the pattern helps identify some diseases.



Classification of serum proteins: (1) Albumin (2) Alpha-1 globulin (3) Alpha-2 globulin (4) Beta globulin (5) Gamma globulin

Serum protein levels

- Protein tests may be measured in grams per deciliter (g/dL) or milligrams per deciliter (mg/dL) depending on the laboratory performing the test.

Total serum protein: Normal levels are 5.5 to 9.0 g/dL

- Albumin: Normal levels are 3.5 to 5.5 g/dL
- Globulins: Normal levels are 2.0 to 3.5 g/dL
- A/G ratio (albumin to globulin ratio): Normal levels are greater than one

Normal levels of a serum protein electrophoresis

- Alpha-1 globulin: Normal levels are 0.1 to .4 g/dL
- Alpha-2 globulin: Normal levels are 0.5 to 1 g/dL
- Beta globulin: Normal levels are 0.7 to 1.2 g/dL
- Gamma globulin: Normal levels are 0.5 to 1.6 g/dL

Abnormal results

- **Albumin levels**

  Abnormally high may indicate severe dehydration.

  A decreased level of albumin is common in many diseases and is especially important in liver disease Abnormally low may signal malnutrition, liver or kidney disease, lupus, rheumatoid arthritis, Hodgkin disease, uncontrolled diabetes, hyperthyroidism or heart failure.

  Globulin levels

  Alpha-1 globulin elevated in inflammatory diseases. Absence indicates a genetic disorder called juvenile pulmonary emphysema

  A decrease in alpha-1-globulin is seen in nephrotic syndrome and absence could indicate possible alpha-1-antitrypsin deficiency which eventually leads to emphysema

Abnormal results

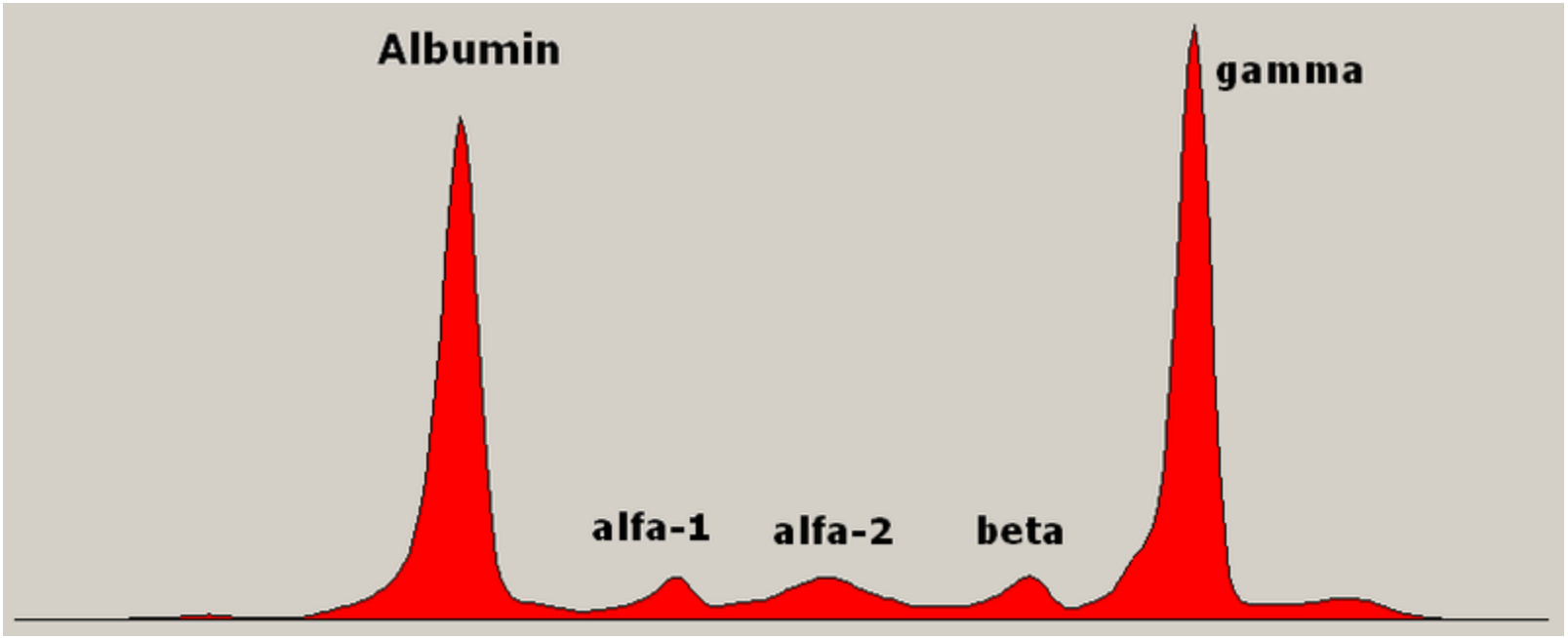
Alpha-2 globulin elevated in nephrotic syndrome of the kidney (from the macroglobin fraction). Decreased in hyperthyroidism or severe liver dysfunction and in blood clotting problems

A normal alpha-2 and a raised alpha-1 is typical in hepatic metastases and cirrhosis.

Beta globulins elevated in hypercholesterolemia and iron deficiency anemia. Abnormally low levels can indicate malnutrition

Gamma globulins elevated in chronic inflammatory diseases, in autoimmune diseases such as lupus or rheumatoid arthritis, cancer, kidney or liver disease, infection, multiple myeloma (a bone cancer), or Hodgkin disease (cancer of the lymphatic system).

Abnormally low levels can indicate , gastrointestinal diseases such as Crohn's disease, liver or kidney disease, blood clotting problems, emphysema, leukemia, hemolytic anemia, or problems with the immune system.



Serum protein electrophoresis showing a paraprotein (peak in the gamma zone) in a patient with multiple myeloma

Cardiac markers in myocardial infarction

- Myocardial infarction is the destruction of heart tissue resulting from obstruction of blood supply to the heart muscle
- It is the medical term for heart attack. This is most commonly caused by occlusion (blockage) of a coronary artery following rupture of a vulnerable atherosclerotic plaque, which is an unstable collection of lipids (like cholesterol) and white blood cells (especially macrophages) in the wall of an artery.
- The resulting ischemia (restriction in blood supply) and oxygen shortage, if left untreated for a sufficient period of time, can cause damage or death (infarction) of heart muscle tissue (myocardium)

Cardiac markers in myocardial infarction (MI)

- Heart attack is a leading cause of death for both men and women all over the world.
- Important risk factors :

  -previous cardiovascular disease (such as angina, a previous heart attack or stroke)

  -old age (especially men over 40 and women over 50)

  - tobacco smoking

  - high blood levels of certain lipids (triglycerides, LDL or "bad cholesterol")

  - low levels of high density lipoprotein (HDL, "good cholesterol")

  -diabetes, high blood pressure, obesity, chronic kidney disease

  -excessive alcohol consumption, the abuse of certain drugs such as cocaine

  - chronic high stress levels

Cardiac markers in myocardial infarction (MI)

- Patient will receive a number of diagnostic tests, such as electrocardiogram (ECG), a chest X-ray and blood tests to detect elevations in cardiac markers (abnormal protein levels signifying heart muscle damage).
- Commonly used markers are the creatine kinase-Myocardial B fraction (CK-MB) fraction and the troponin I (TnI) or troponin T (TnT) levels.
- These proteins normally reside in the heart cells but are released into the blood stream after a heart attack, although elevated levels may also signify problems with other muscles in the body so these tests are not specific for myocardial infarction

Cardiac markers in myocardial infarction (MI)

- Until the 1980s, the enzymes, serum glutamic oxaloacetic transaminase (SGOT) and lactate dehydrogenase (LDH) were used to assess cardiac injury

  -Cardiac troponins T and I which are released within 4–6 hours of an attack of MI and remain elevated for up to 2 weeks, have nearly complete tissue specificity and are now the preferred markers for assessing myocardial damage.

  -Elevated troponins in the setting of chest pain may accurately predict a high likelihood of a myocardial infarction in the near future. New markers such as glycogen phophorylase isozyme BB are under investigation

Cardiac markers in myocardial infarction (MI)

- Cytosolic creatine kinase (CK) enzymes consist of two subunits: *B* (brain type) or *M* (muscle) type. Three different isozymes therefore exist: CK-MM, CK-BB and CK-MB. The genes for these subunits are located on different chromosomes, *B* on 14*q*32 and *M* on 19*q*13. Isozyme determination has been used extensively as an indication for myocardial damage in heart attacks.
- Isozymes are enzymes that catalyze the same reaction but differ in their physical properties as a result of differences in their amino acid sequence which is genetically determined.
- Isozyme patterns differ in tissues. Skeletal muscle expresses CK-MM (98%) and low levels of CK-MB (1%). The heart muscle, in contrast, expresses CK-MM at 70% and CK-MB at 25–30%. CK-BB is expressed in all tissues at low levels and has little clinical relevance.
- Increase in myocardial-specific enzyme CK-MB is considered an evidence of acute myocardial infarction

Cardiac markers in myocardial infarction (MI)

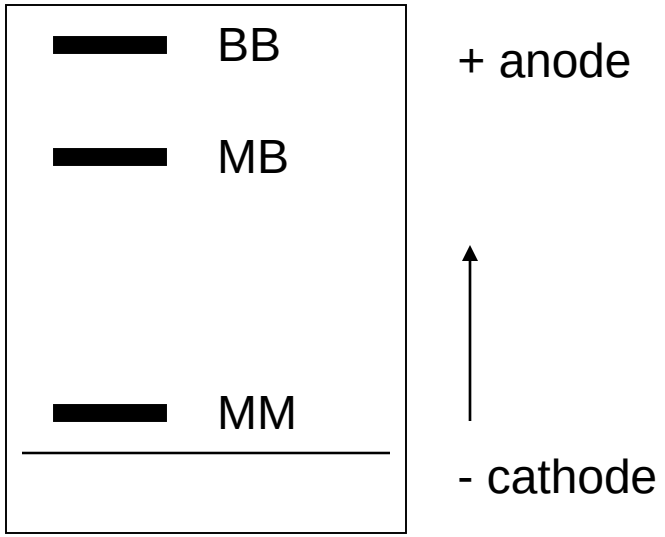
- Troponins are measured in the blood to differentiate between unstable angina and MI. Cardiac troponin T and I are measured by immunoassay methods

Cardiac markers in myocardial infarction (MI)

- CK-MB is known to exist in two forms: CK-MB_2, the gene product, and CK-MB_1, which is modified upon release into the bloodstream. Carboxypeptidase cleavage of the C-terminal lysine residue of the M subunit transforms CK-MB_2 into CK-MB_1.
- In healthy individuals, CK-MB_2 is in equilibrium with the modified CK-MB_1 subform at a ratio of approximately 1:1. In the early hours of myocardial infarction, the abrupt release of CK-MB_2 from myocardium produces an upward shift in the serum CK-MB_2/CK-MB_1 ratio, usually before total CK-MB ($CKMB_2$ + $CKMB_1$) exceeds normal levels.
- Determinations of the serum CK-MB_2/CK-MB_1 ratio are also proving useful in diagnosis of myocardial infarction

Cardiac markers in myocardial infarction (MI)

- Electrophoretic separation of the isozymes using agarose, cellulose acetate or polyacrylamide as support media achieves distinct bands that can be quantified by fluorescence densitometry.
- Separation is possible because the M and B subunits have different charges on them. The mobility of these isozymes at pH 8.6 toward the anode is BB> MB> MM with the MM remaining close to the point of application.
  The method is sensitive from 2 - 5 U/L



Electrophoretic separation of
CK-MM, CK-MB, and CK-BB