

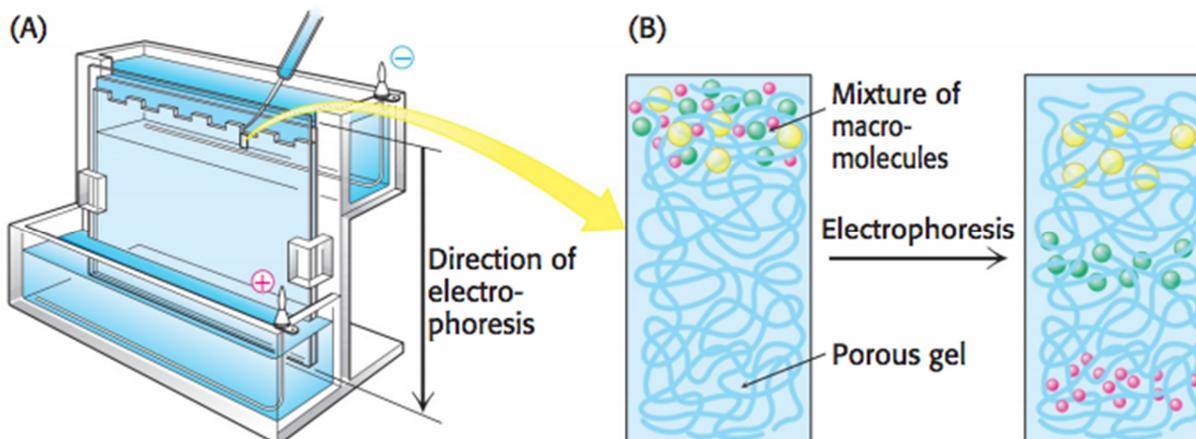
IWantAHighMCATScore's
Guide to MCAT Lab Techniques

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Gel Electrophoresis

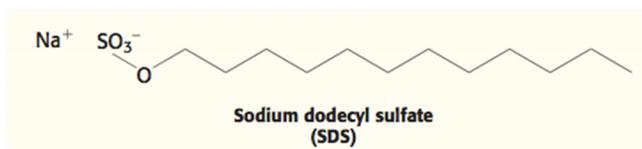
Purpose: Separation of proteins, DNA, or RNA based on size and/or charge



Macromolecules (proteins, DNA, or RNA) of interest are placed in the lanes of a gel. For proteins and small molecules of DNA and RNA, the gel will be polyacrylamide. For larger molecules of DNA (> 500 bp), the gel will be agarose. An electrical charge is placed across the gel. At the bottom is the **positively charged anode** and at the top is the **negatively charged cathode**. Keep in mind, since a voltage source is applied to gel electrophoresis, it follows the same principles as an electrolytic cell. Negatively charged molecules will travel toward the anode. Because of the resistance of the gel, larger molecules will have a harder time moving and thus, the molecules will be separated by size with the smallest molecules toward the bottom. The gel can then be stained for visualization, typically using Coomassie Blue dye. A lane will be loaded with a collection of molecules of a known size, called a ladder, which can be used to determine the size of the molecules being run. There are several different applications of gel electrophoresis:

Native-PAGE

Native-PAGE is a polyacrylamide gel electrophoresis method for proteins that occurs under non-denaturing conditions. This method will separate proteins by size while retaining their structure.



SDS-PAGE

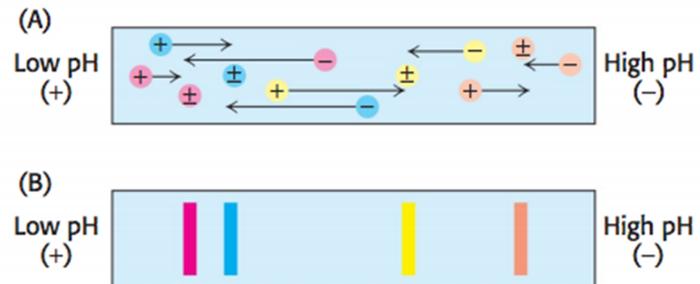
SDS-PAGE is a polyacrylamide gel electrophoresis method for proteins that occurs under denaturing conditions to separate proteins by mass. Negatively-charged sodium dodecyl sulfate (SDS) is added to the solution of proteins, denatures the proteins, and binds one SDS for every two amino acids, giving all proteins the same charge-to-mass ratio. Since all proteins have the same charge-to-mass ratio, they are separated solely on mass, with the smallest proteins found toward the bottom of the gel. SDS will only interrupt non-covalent bonds, so if disulfide bridges are present in the protein, they will not be broken. This is useful when analyzing proteins with multiple subunits.

Reducing SDS-PAGE

Reducing SDS-PAGE is exactly like SDS-PAGE, but with the addition of a reducing agent, like β -mercaptoethanol, which will reduce disulfide bridges and result in a completely denatured protein.

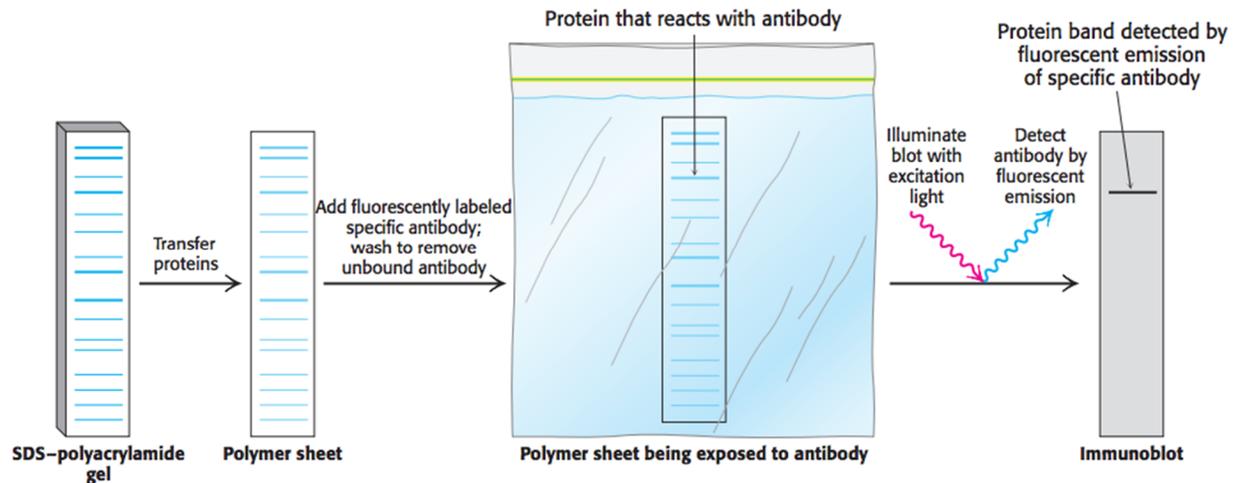
Isoelectric Focusing

Isoelectric focusing is a gel electrophoresis method that separates proteins on the basis of their relative contents of acidic and basic residues. A polyacrylamide gel with a pH gradient (low pH on one side, high pH on the other) is used. When proteins migrate through the pH gradient gel, they will travel toward the anode until the area of the gel with the pH that matches their isoelectric point (pI). When a protein is at its pI, it has a net charge of zero and will not be attracted to the positively charged anode so it will not move.



Western Blotting (Protein)

Purpose: Detection of a specific protein in a sample



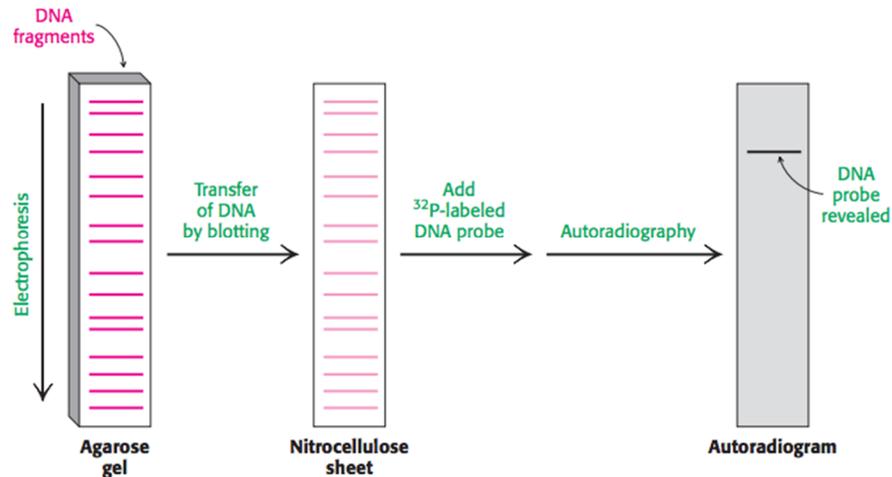
Step 1: Proteins from a sample are loaded into an SDS-PAGE gel and separated based on size

Step 2: Proteins from the gel are transferred to a polymer sheet and exposed to a radiolabeled antibody (sometimes using two antibodies; one specific to the protein of interest and another radiolabeled antibody that binds to the first antibody) that is specific to our protein of interest

Step 3: The polymer sheet is viewed used autoradiography. The protein of interest that is bound to the radiolabeled antibody will be visible.

Southern (DNA) and Northern (RNA) Blotting

Purpose: Detection of a specific DNA (Southern blot) or RNA (Northern blot) sequence in a sample



Step 1: The DNA strand of interest is exposed to restriction enzymes that cut the DNA strand into smaller fragments

Step 2: The newly cleaved strands of DNA are denatured using a solution of NaOH to create ssDNA strands

Step 3: The single stranded cleaved strands of DNA undergo gel electrophoresis, separating them by size. Smaller fragments will be found at the bottom of the gel. Polyacrylamide is used if the strands are less than 500 base pairs. Agarose is used if the strands are over 500 base pairs.

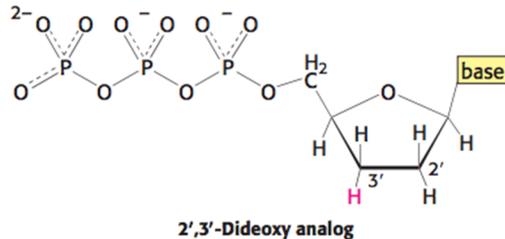
Step 4: The DNA from the gel is transferred to a sheet of nitrocellulose paper and then exposed to a ^{32}P radiolabeled DNA probe that is complementary to our DNA of interest.

Step 5: The nitrocellulose paper is then viewed using autoradiography to identify the strand of interest.

NOTE: These methods are nearly identical for Southern and Northern blotting. The steps listed above are for Southern blotting, however, the only difference is that Northern blotting uses RNA, so steps 1 and 2 are not done.

DNA Sequencing (Sanger Dideoxynucleotide Sequencing)

Purpose: Used to determine the sequence of nucleotides in a strand of DNA



Modified nucleotides, known as “dideoxynucleotides” (ddNTPs), are used in this method. ddNTPs are missing the OH group on the 3' carbon, thus they are unable to create a new 5'→3' phosphodiester bond. This allows us to control the termination of replication.

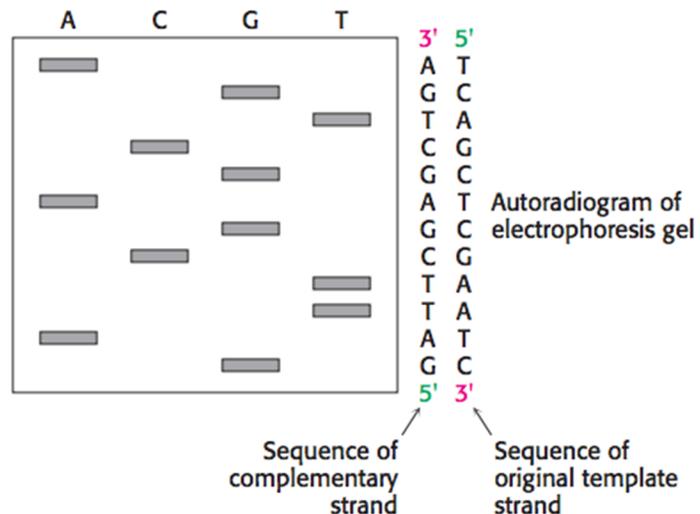
Step 1: The DNA strand of interest is denatured using an NaOH solution to create a ssDNA strand that we can use for replication

Step 2: The ssDNA strand of interest is added to a solution containing:

1. A radiolabeled DNA primer that is complementary to the gene of interest
2. DNA polymerase
3. All four dNTPs (dATP, dTTP, dCTP, dGTP)
4. A very small quantity of a single ddNTP (e.g., ddATP)

This step is done once for each of the four nucleotides in separate solutions.

Step 3: Each solution containing a specific dNTP and ddNTP are placed in their own lane of a gel and ran under gel electrophoresis. The gel is transferred to a polymer sheet and autoradiography is used to identify the strands in the gel.



For each respective nucleotide, the insertion of a ddNTP will terminate replication and create various strands of different length that correspond to that specific nucleotide. Therefore, the gel can be read from bottom-to-top to determine the nucleotide sequence. The smaller the fragment, the further it travels in the gel.

Chromatography

Purpose: The separation of two or more molecules from a mixture

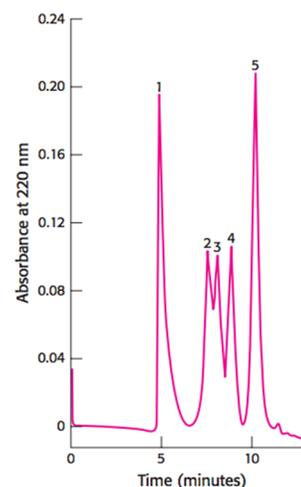
There are several different types of chromatography that can be used for separating or analyzing a mixture of two or more molecules based on their properties. Traditionally, there are two components to chromatography; a **stationary phase**, which is typically polar, and a **mobile phase**, which is typically non-polar. Polar molecules are separated from a mixture by staying with the stationary phase, while non-polar molecules stay with the mobile phase. However, if **reverse-phase** is specified, then the properties of the two phases are switched. This also changes based on the type of chromatography used, as some methods use ligands or gel beads.

Liquid Chromatography

In liquid chromatography, silica is traditionally used as the stationary phase while toluene or another non-polar liquid is used as the mobile phase.

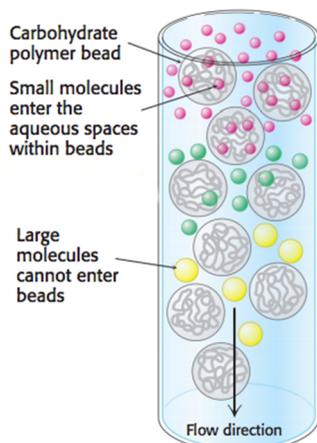
High-Performance Liquid Chromatography (HPLC)

HPLC is a type of liquid chromatography that utilizes high pressures to pass the solvent phase through a more finely-ground stationary phase, which increases the interactions between the molecules and the stationary phase, giving HPLC a higher resolving power. Molecules can then be determined based on their absorbance and elution time as seen on the right. In **normal phase HPLC**, stationary phase is polar, mobile phase is nonpolar; in **reverse phase HPLC**, stationary is nonpolar, mobile is polar.



Gas Chromatography

Gas chromatography (also known as gas-liquid chromatography) is used to separate and analyze molecules that can be vaporized. The mobile phase is an inert or unreactive gas, such as helium or nitrogen, while the stationary phase is a thin layer of liquid or polymer that surrounds the walls of a tube. The stationary phase allows more polar molecules to elute slower, giving them a higher retention time.

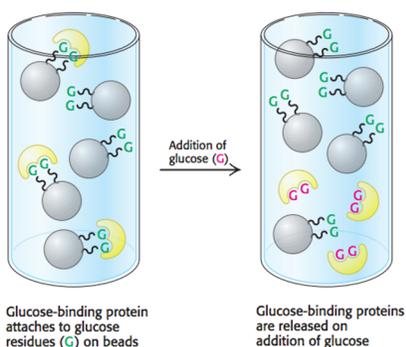
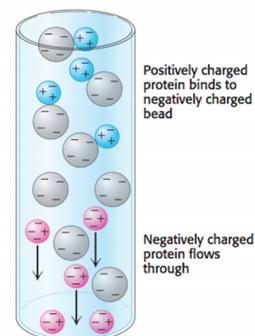


Gel-Filtration (Size Exclusion) Chromatography

Gel-filtration chromatography (also known as size-exclusion chromatography) is used to separate molecules by size rather than polarity. Smaller molecules can enter the porous gel beads, allowing them to elute later, while larger molecules that do not fit will elute faster. The gel beads can be viewed as the stationary phase, while the solution in the column can be viewed as the mobile phase.

Ion-Exchange Chromatography

Ion-exchange chromatography will separate proteins by their net charge. The column is filled with charged beads, either positive or negative. In cation-exchange, negatively-charged beads are used which attract positively charged proteins and negatively-charged proteins will elute first. In anion-exchange, positively-charged beads are used which attract negatively-charged proteins and positively-charged proteins will elute first.

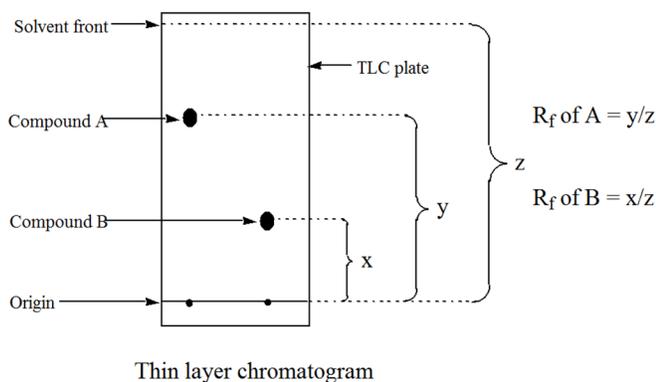


Affinity Chromatography

Affinity chromatography will separate proteins based on their affinity for a specific ligand. Beads that are bound to a specific ligand will be used and proteins with a high affinity for that ligand will bind to the beads, allowing proteins with a low affinity to elute first. The high affinity proteins are then eluted by increasing the concentration of the free ligand in the column, which competes for the active site of the bound proteins.

Thin-Layer Chromatography

Thin-layer chromatography consists of a small sheet of medium that is coated in an adsorbent material, such as silica gel. The polar silica is the stationary phase. The molecules of interest are added to the bottom of the sheet and the sheet is placed in a non-polar liquid, such as heptane, until it reaches the origin. The mobile phase then travels up the plate using capillary action, allowing the molecules to move with it if they are relatively non-polar. The spots are then visualized using UV light.



The relative distances traveled between the molecules is represented by the R_f value, which is measured as the ratio of the distance the molecule traveled from the origin to the distance the solvent front traveled from the origin.

Distillation

Purpose: Used to separate two or more molecules from a solution

Simple Distillation

Simple distillation is used to separate two molecules from a solution when their boiling points differ by 25° C or greater.

Fractional Distillation

Fractional distillation is used to separate two molecules from a solution when their boiling points differ by less than 25° C.

Vacuum Distillation

Vacuum distillation is used to separate two molecules from a solution when their boiling points are high and risk changing chemically.

Polymerase Chain Reaction

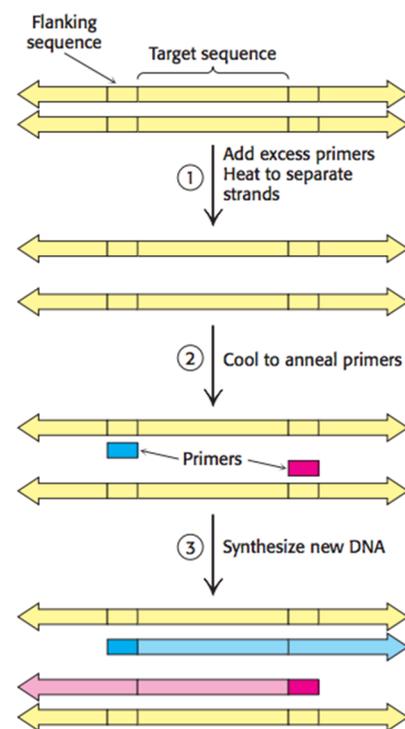
Purpose: Used to amplify a small quantity of DNA by several orders of magnitude

Step 1: DNA strands and complementary DNA primers are heated to 95° C for 15 seconds to separate the strands.

Step 2: The solution is abruptly cooled to 54° C to allow the primers to anneal to each ssDNA.

Step 3: The solution is heated to 72° C and new complementary strands are synthesized using *Taq* DNA polymerase.

Step 4: The cycle is repeated until the desired quantity of DNA is synthesized.



Spectroscopy

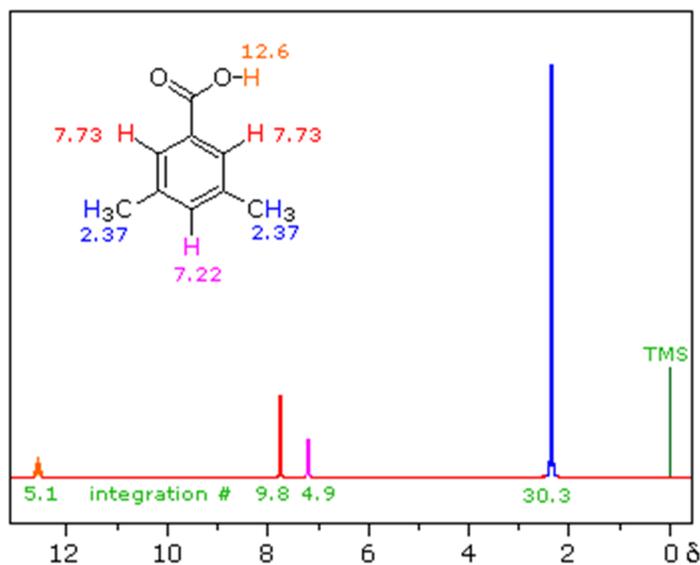
Purpose: Used for structural determination of molecules

¹H-NMR Spectroscopy

The application of nuclear magnetic resonance regarding the ¹H isotope within the molecules of a substance.

Chemical shift: The chemical shift on the x-axis (δ ppm) represents the amount of deshielding of electrons that is caused by an adjacent heteroatom or pi bond.

- 0 - 5 ppm → Alkane region
- 3 - 5 ppm → Alkane with a heteroatom region
- 5 - 7 ppm → Alkene region
- 6 - 8 ppm → Aromatic region
- 9 - 10 ppm → Aldehyde region
- 10 - 13 ppm → Carboxylic acid region



Integration: The integration of the peak determines the number of equivalent hydrogens a signal represents.

Neighbors: The number of peaks determines the number of neighboring hydrogens that are ≤ 3 bonds away. The number of peaks equals the number of neighbors + 1.

- Singlet → No neighboring hydrogens
- Doublet → One neighboring hydrogen
- Triplet → Two neighboring hydrogens
- Quartet → Three neighboring hydrogens
- Quintet → Four neighboring hydrogens
- Sextet → Five neighboring hydrogens
- Septet → Six neighboring hydrogens
- Multiplet → Seven or more neighboring hydrogens

^{13}C -NMR spectroscopy

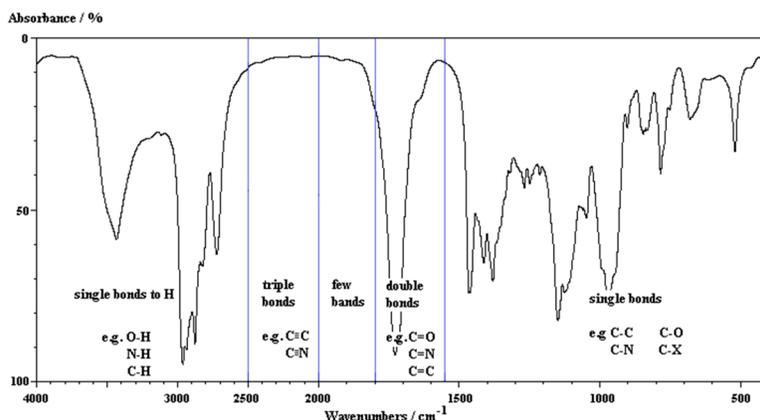
The application of nuclear magnetic resonance regarding the ^{13}C isotope within the molecules of a substance

Chemical shift: The chemical shift on the x-axis (δppm) represents the amount of deshielding of electrons that is caused by an adjacent heteroatom or pi bond.

- 0 - 70 ppm \rightarrow Alkane region
- 90 - 120 ppm \rightarrow Alkene region
- 110 - 160 ppm \rightarrow Aromatic region
- 160 - 200 ppm \rightarrow Carbonyl region

IR Spectroscopy

IR spectroscopy is a method that is used to identify certain functional groups within a molecule. Only molecules that have a dipole moment will show absorbance. The x-axis is reported in wavenumbers (reciprocal centimeters) and the y-axis is reported in percent absorbance.

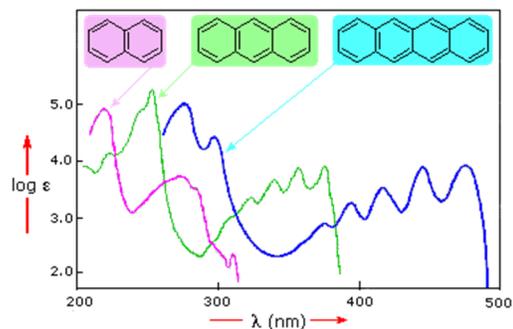


Important regions:

- 1700 - 1750 \rightarrow Carbonyls (sharp peak)
 - 1720 - 1740 \rightarrow Aldehydes
 - 1700 - 1725 \rightarrow Ketones
 - 1735 - 1750 \rightarrow Esters
 - 1700 - 1725 \rightarrow Carboxylic acids
- 3200 - 3600 \rightarrow OH groups (broad peak)
- 3300 - 3400 \rightarrow Amines
 - The number of peaks are relative to the number of hydrogens on the amine (e.g., 1° amines will have two peaks, 2° amines will have one peak)

UV-Vis Spectroscopy

As the number of conjugated pi bonds increase, the energy gap between the highest occupied molecular orbital (HOMO) and lowest unoccupied molecular orbital (LUMO) decreases, which means that light of a lower energy is absorbed. This results in light with a longer wavelength to be emitted (as seen on the right). If a molecule absorbs green light, we see it as red.



Autoradiography

Purpose: To visualize the location of a radioactive substance in a molecule or structure

In the scope of the MCAT, autoradiography is used primarily as an imaging technique to identify radiolabeled atoms that are in a molecule, often as part of a Southern, Northern, or Western blot. The radiolabeled substance is placed in contact with a photographic emulsion containing silver halide crystals. The radiation of the radiolabeled substance converts the silver halide crystals into metallic silver, producing an image.

X-Ray Crystallography

Purpose: To visualize the structure of a molecule, used often with proteins

In x-ray crystallography, a crystalline molecule causes a beam of incident x-rays to diffract into many specific directions. A three-dimensional image can be constructed by measuring the different angles and intensities of the diffracted rays.

Immunoprecipitation

Purpose: Used to purify proteins from a solution

In immunoprecipitation, a protein of interest can be precipitated from a solution by adding bead-conjugated antibody that is specific to the protein of interest. The antibody is bound to some sort of solid bead, which can be used for either extraction with a magnetic or by centrifuge.

Radioimmunoassay

Purpose: Used to determine the concentration of a protein of interest in a given sample

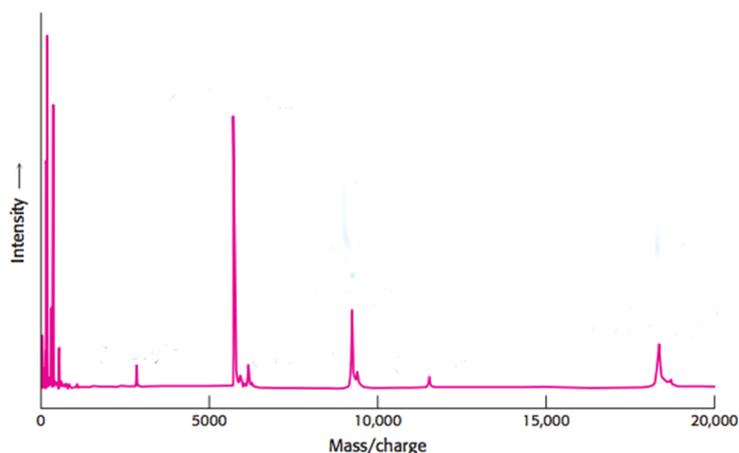
Similar to ELISA, the wells of a plate are coated with the primary antibody that is specific to our protein of interest. Next, the radiolabeled protein, typically containing a tyrosine residue labeled with ^{125}I , is added to the wells and binds to the primary antibody. The concentration of that radiolabeled protein is determined by counting the gamma emission. Next, a sample of the protein of interest in an unknown concentration is added to the wells, where it competes for the active site on the primary antibody, displacing an amount of the radiolabeled protein. The concentration of the radiolabeled protein is determined again by counting the gamma emission. The difference in concentrations provides the concentration of the protein of interest.

Mass Spectrometry

Purpose: Used to determine the molecular weight of a compound and aid in determining the molecular structure

In mass spectrometry, the sample is vaporized and subjected to ionizing conditions. The charged molecule collides with an electron, resulting in the ejection of an electron from the molecule, making it a radical. The charged radical can undergo fragmentation or being detected.

The x-axis represents the mass/charge ratio (m/z), which essentially just means the molecules mass using only the lowest isotopes of the atoms involved (e.g., ^{12}C , ^1H , ^{35}Cl). The y-axis represents the intensity, or relative abundance of the molecule, usually given as a percentage.



Base peak: The tallest peak. This does not always represent the actual intact molecule, as it may sometimes be a fragment of the molecule that is found in higher abundance.

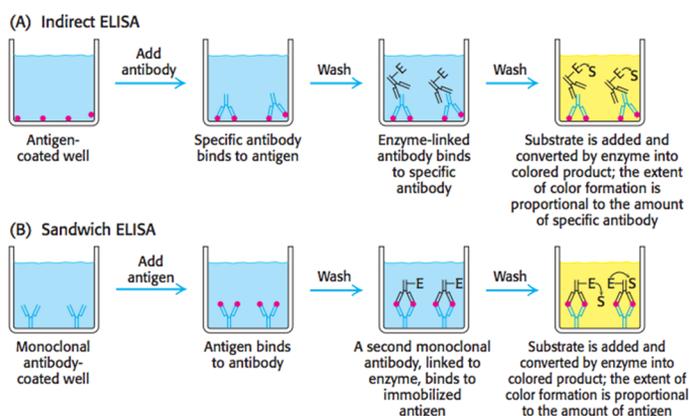
Molecular ion peak (M): The peak that represents the molecule. The m/z value of this peak represents the molecular weight of the molecule.

M+1 peak: The relative abundance of ^{13}C in the molecule. Found in a relative abundance of 1.1%. So if there is a M+1 with a m/z value of 4.4, it means that there are 4 carbons present ($4.4/1.1 = 4$).

M+2 peak: The relative abundance of either ^{37}Cl or ^{81}Br in the molecule. ^{37}Cl will be found in a 3:1 ratio relative to the M peak (e.g., if the M peak has a relative abundance of 90%, if the M+2 peak is at 30%, it means there is chlorine present in the molecule). ^{81}Br will be found in a 1:1 ratio relative to the M peak (e.g., if the M peak has a relative abundance of 90%, if the M+2 peak is also at 90%, it means there is bromine present in the molecule).

Fragments: There are three types of fragmentation that can occur, which can help identify the structure of the molecule. 1) Alkane fragmentation, 2) alcohol dehydration, and 3) alpha cleavage. These are most likely beyond the scope of the MCAT.

Enzyme-Linked Immunosorbent Assay (ELISA)



Purpose: Used to identify the concentration of a molecule of interest in a given sample

ELISA is an assay that uses primary antibodies that are specific to a molecule of interest and secondary antibodies that are specific to primary antibodies and are conjugated with a fluorophore, so their presence can be measured via spectrophotometry. There are two different methods of ELISAs that are used.

Indirect ELISA

The molecule of interest (in this case, considered an antigen) is coated to the wells of a plate. A primary antibody that is specific for that antigen is added and washed to remove any unbound antibodies. Next, the secondary antibody is added. The secondary antibody is linked to an enzyme, often horseradish peroxidase (HRP), that will be converted into a fluorophore when reacted with an oxidizing agent, such as hydrogen peroxide. This will cause a change in color, which can be detected using photospectrometry. The absorbance of the well is compared against a serial-diluted standard of known concentration, and based on a determined curve using the standards, the concentration of the molecule in the well can be determined.

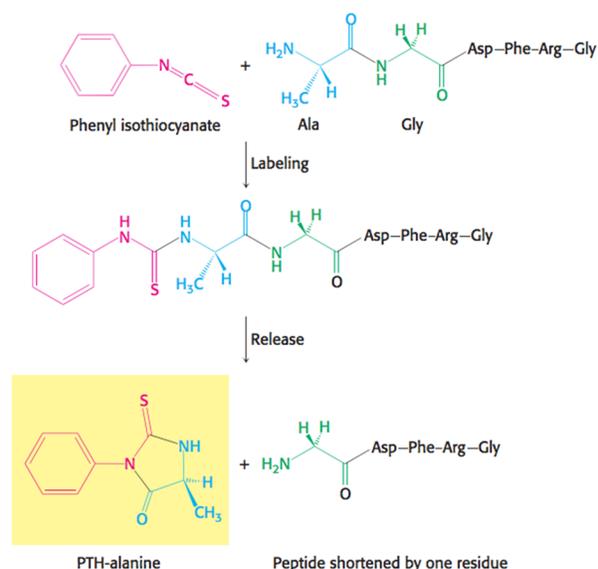
Sandwich ELISA

The primary antibody that is specific to our molecule of interest is coated to the wells of a plate. Next, the molecule of interest is added to the wells and then washed to remove any unbound molecules. Next, the secondary antibody is added. The secondary antibody is linked to an enzyme, often horseradish peroxidase (HRP), that will be converted into a fluorophore when reacted with an oxidizing agent, such as hydrogen peroxide. This will cause a change in color, which can be detected using photospectrometry. The absorbance of the well is compared against a serial-diluted standard of known concentration, and based on a determined curve using the standards, the concentration of the molecule in the well can be determined.

Edman Degradation

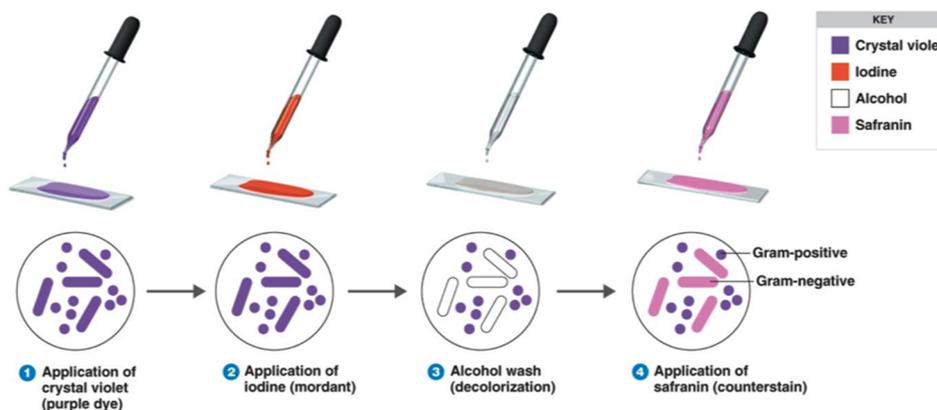
Purpose: Used to sequence the amino acid residues in a protein

Edman Degradation is conducted by removing one amino acid residue at a time from the N-terminus of the peptide. Phenyl isothiocyanate is added to the N-terminus of a polypeptide, which then cyclizes and breaks off, leaving an intact polypeptide that is shortened by one residue. The PTH-amino acid hybrid can then be analyzed using chromatographic techniques to identify the amino acid. This can be repeated until every amino acid residue in the polypeptide is identified. This method is limited in the fact that it can only accurately identify polypeptides less than 50 residues.



Gram Staining

Purpose: Used to differentiate bacteria into two groups, gram-positive or gram-negative, based on the content of their cell wall



The sample of bacteria is heat-fixed to a slide and crystal violet dye is added. Iodine is added to the bacteria, which binds to the crystal violet dye and traps it in the bacterial cell. The bacteria is then washed with alcohol to remove any dye that was not taken up by the bacteria. Safranin is added as a counterstain, so that the bacteria that did not stain with crystal violet can be visualized.

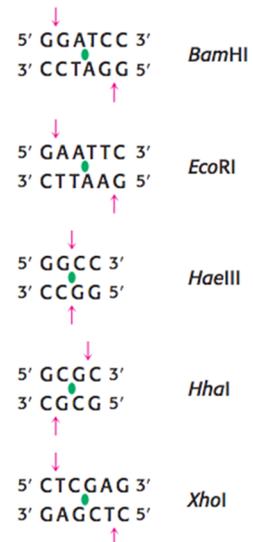
Gram-positive bacteria: Appears purple on the slide. Contains a thick peptidoglycan layer that takes up the stain.

Gram-negative bacteria: Appears pink on the slide. Contains a thin peptidoglycan layer sandwiched between two lipid bilayers that take up the safranin stain.

Restriction Fragment Length Polymorphism (RFLP)

Purpose: Used to identify differences in homologous DNA sequences based on the length of fragments caused by restriction enzymes

RFLP allows us to determine differences in two or more DNA sequences of the same gene. Most often, this will be comparing a wild type (WT) gene with a mutated version of the gene. The DNA strand is exposed to a restriction enzyme, also called a restriction endonuclease. Restriction enzymes recognize a specific palindromic sequence (the sequence is the same in the 5' → 3' direction in both strands) in the DNA and cleave both strands. Once the DNA sequence is cleaved with the restriction enzymes, it can be ran through gel electrophoresis. If a mutation has occurred, there may be a difference in the number and length of strands between the WT and mutant genes, as the restriction points may differ.

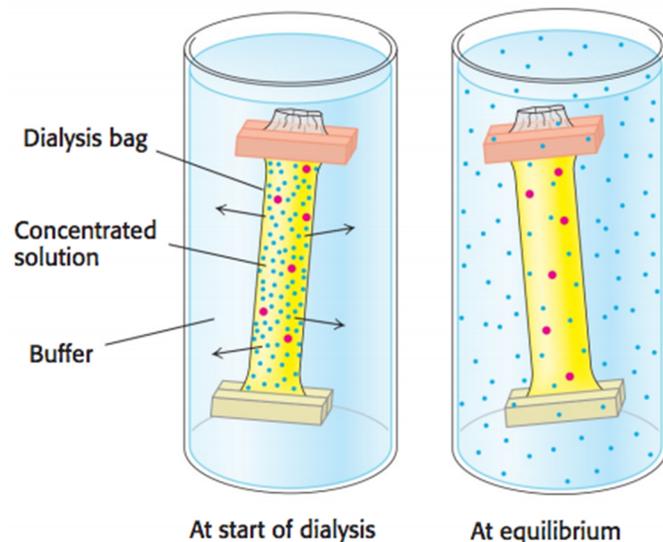


Salting Out and Dialysis

Purpose: Purification of proteins in a solution

Adding salt to a solution containing proteins can allow you to selectively precipitate out proteins. Salting out is due to the competition between the salt ions and the protein for water to keep the protein in solution. The salt concentration at which a protein will precipitate varies for each protein.

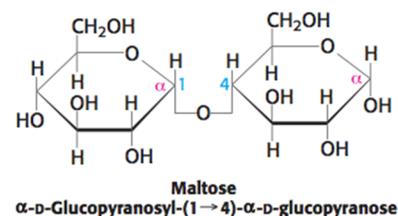
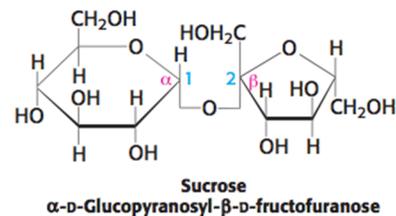
After a protein has been precipitated, the salt can be removed via dialysis. The solution is placed in a dialysis bag and then added to a hypotonic solution. The ions will pass through the semipermeable membrane of the dialysis bag, while the protein of interest is left inside.



Reducing Sugar Tests

Purpose: Used to identify the presence of a reducing sugar in a solution

A reducing sugar is any sugar that is capable of acting as a reducing agent due to the presence of a free aldehyde or ketone functional group. All monosaccharides are reducing sugars, since they are capable of mutarotation, and therefore, will be found in the open-chain form to some degree. However, not all disaccharides, oligosaccharides, or polysaccharides are reducing sugars, since some glycosidic bonds are between two anomeric carbons (classified as a 1→2 bond), preventing mutarotation. Sucrose is an example of a non-reducing disaccharide while maltose is an example of a reducing disaccharide (as seen in the figure on the right). Certain reagents can test for the presence of a free aldehyde or ketone group in order to identify the presence of a reducing sugar.



Tollen's Test

Tollen's reagent tests for the presence of an aldehyde and can distinguish between aldoses and ketoses. Ketones do not react unless they are α -hydroxy-ketones. A positive Tollen's test is characterized by the precipitation of elemental silver.

Tollen's reagent consists of $[\text{Ag}(\text{NH}_3)_2]\text{NO}_3$.

Benedict's Test

Benedict's reagent tests for the presence of an aldehyde. Ketones do not react unless they are α -hydroxy-ketones. A positive Benedict's test is characterized by a change in color from clear blue to brick-red with the formation of a precipitate.

Benedict's reagent consists of a mixture of sodium carbonate, sodium citrate, and copper (II) sulfate pentahydrate.

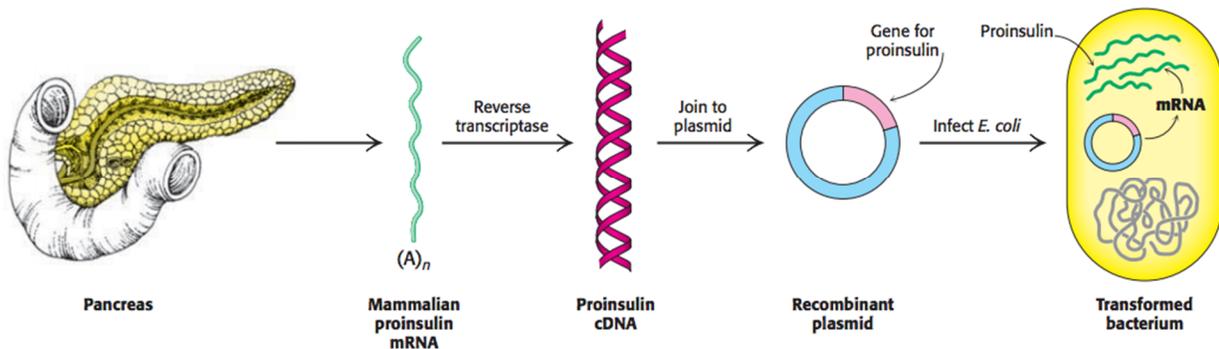
Fehling's Test

Fehling's solution tests for the presence of an aldehyde. Ketones do not react unless they are α -hydroxy-ketones. A positive Fehling's test is characterized by a change in color from clear blue to brick-red with the formation of a precipitate.

Fehling's solution consists of two parts; Fehling's A and Fehling's B. Fehling's A consists of aqueous copper (II) sulfate. Fehling's B consists of potassium sodium tartrate and sodium hydroxide.

cDNA Libraries

Purpose: Used to create and house complimentary DNA (cDNA) strands that can be expressed in



bacterial vectors

In order to synthesize proteins for things like medication, such as insulin, we must clone the DNA sequence for that protein in a bacterial vector. However, eukaryotes have the ability to undergo post-transcriptional modification of hnRNA to create mRNA while prokaryotes do not. Therefore, we can not insert eukaryotic DNA directly into a bacterial vector since it still contains introns. Instead, we must synthesize a strand of cDNA from the protein of interest's respective mRNA.

The mRNA transcript of the protein of interest is isolated and a complementary DNA primer containing many thymine repeats and a free 3'-OH group is added, which anneals to the poly-A tail of the mRNA. Next, the mRNA is exposed to reverse transcriptase and an excess of the four dNTPs so a complementary cDNA strand can be synthesized, creating a cDNA-mRNA hybrid. The hybrid strand is hydrolyzed using an alkaline solution. A primer is added to the new single-stranded cDNA and the complementary strand is synthesized by DNA polymerase. The new double-stranded cDNA can be inserted into a bacterial plasmid vector by using restriction enzymes and the bacteria will synthesize the protein of interest.

