Lab techniques are important for the MCAT, especially as the test examines your ability to interpret experimental data. These techniques are assessed in various parts of the med-pathway diagnostic question banks. This section will examine high yield experimental procedures in both the biological and physical sciences, including spectroscopy. The following topics are covered in this content review:

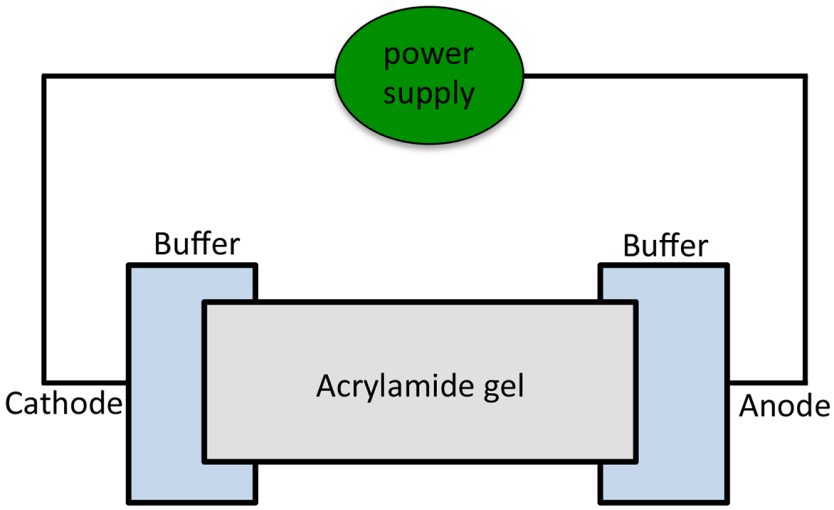
Electrophoresis with Reducing and Non-reducing SDS PAGE
Isoelectric Focusing **DNA sequencing** **Hybridization Techniques** **Generation of libraries** **Quantitative PCR and Analysis of Gene Expression** **Site directed Mutagenesis** **Western Blotting** **Polyclonal & Monoclonal Antibodies** **ELISA assay** **Chromatin Immunoprecipitation** **Flow Cytometry** **Chromatography and Protein Purification Tables** **Gel Filtration (Size Exclusion)** **Ion Exchange** **Affinity Chromatography** **Thin Layer Chromatography (TLC)** **Gas Chromatography** **NMR spectroscopy** **UV-Visible spectroscopy & Beer Lambert Law** **Infrared (IR) Spectroscopy** **Mass Spectrometry**

Electrophoresis

The basic analysis of protein structure is critical for understanding the molecular basis of human disease. Electrophoresis is one such analytical tool that utilizes an electric field to induce the migration of charged macromolecules (e.g. proteins and nucleic acids) through gels (acrylamide for proteins and acrylamide or agarose for DNA) for the purposes of separation and analysis. This is shown below.

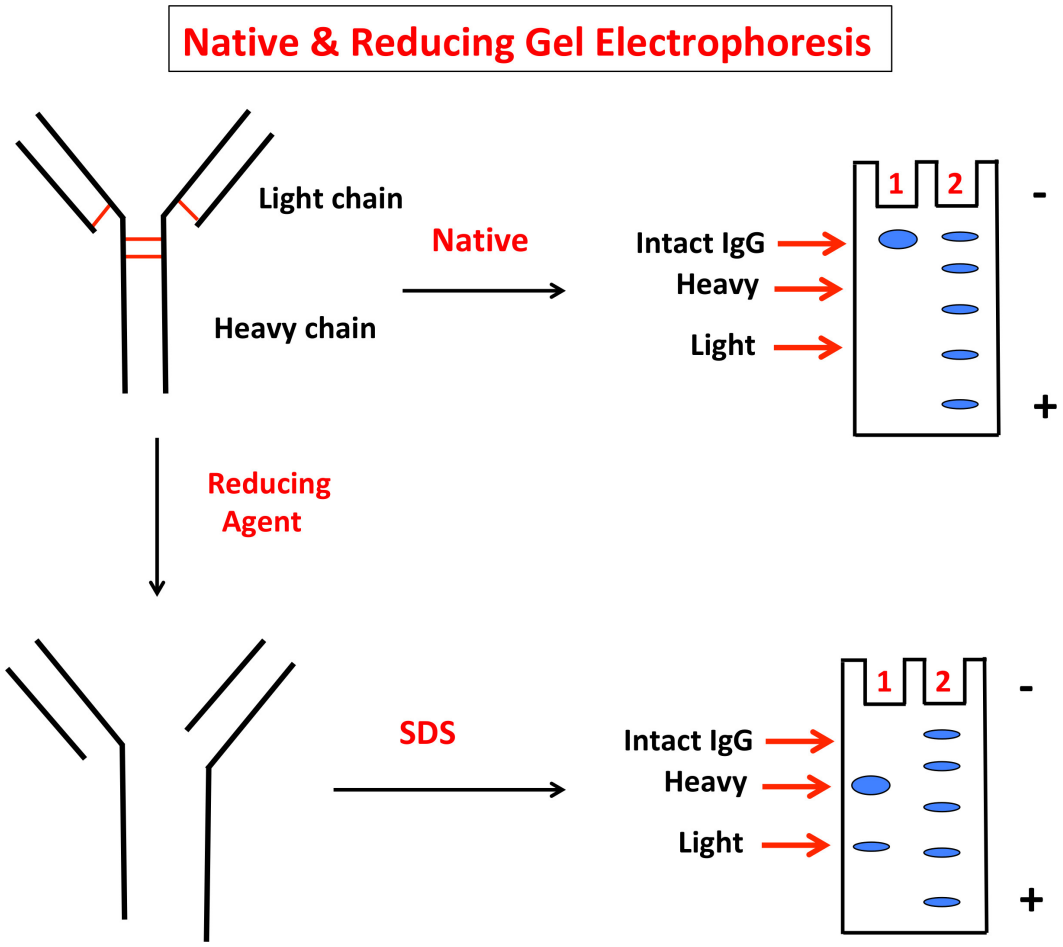
Once a voltage drop is established, proteins are accelerated due to electric force but are rapidly balanced by a growing frictional force (fv), resulting in a constant velocity. The resulting electrophoretic mobility (m) is defined as the velocity normalized by the applied electric field. This property is inversely dependent on the frictional coefficient (f),

which is the ratio of drag force to velocity and is a partially a function of the object's size and shape.

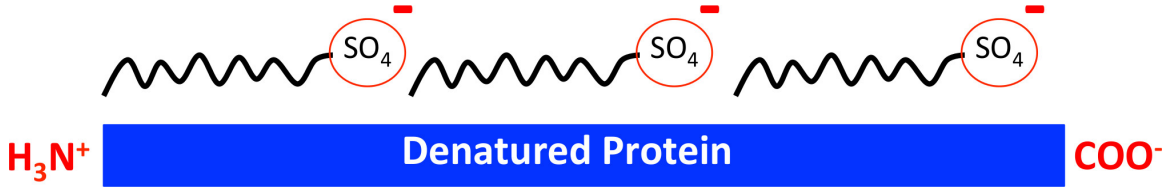


Electrophoresis is a common laboratory technique that you will encounter on the MCAT. Proteins are commonly separated in acrylamide gels. The cross-linked acrylamide forms a mesh where smaller proteins or peptide fragments migrate faster through the gel in an electrophoretic field. Both reducing and native gels are used and are shown below for the electrophoresis of a typical antibody (immunoglobulin).

As antibodies are heterotetramers composed of two heavy and two light chains covalently linked via disulfide bonds (red lines), they have distinctive electrophoresis patterns in reducing and native gels (Lane 1 on both gels: Lane 2 represents size markers).



Treatment of an antibody with a reducing agent in the presence of heat such as dithiothreitol (DTT) or β-mercaptoethanol will reduce the cystine bonds to free cysteine residues (–SH). This separates the heavy and light chains. When these chains are treated with sodium dodecyl sulfate (SDS; shown below) and subjected to polyacrylamide gel electrophoresis (SDS PAGE), the proteins are denatured (unfolded) and possess a uniform charge to mass ratio and will therefore migrate in the electrophoretic field as a function of their mass.



In the case of a native (non-reducing) gel electrophoresis, proteins migrate in their folded state as there is no SDS. In this application, proteins migrate in the electrophoretic field as a function of the charge to mass ratio as well as a function of their molecular shape. Note that in a native gel an intact immunoglobulin migrates as one species as the disulfide bonds are still intact. After electrophoresis of proteins in acrylamide gels, the proteins are often visualized by various staining techniques including silver and Coomassie Blue.

Isoelectric Focusing

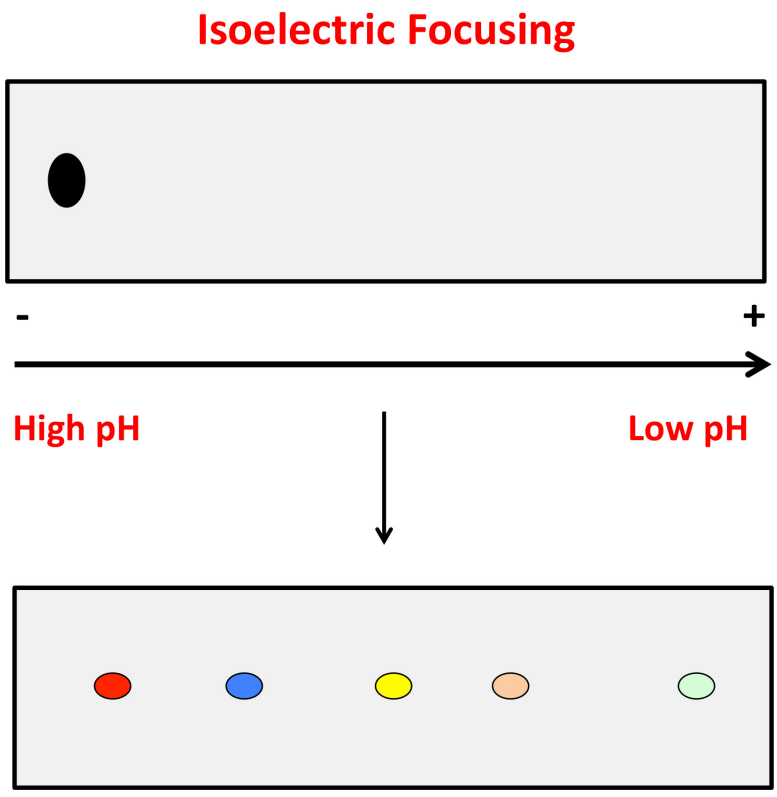
Isoelectric focusing is usually applied to separate proteins based upon their isoelectric points, the pH at which they exhibit an overall neutral charge. The charge of proteins considerably varies as a function of pH. At high pH values, proteins often exhibit an overall negative charge due to titration of carboxyl groups and the neutralization of basic groups:

If pH > pka then the protein is negatively charged

On the other hand, at low pH values proteins tend to have overall positive charges as the carboxyl groups have not been completely titrated and the amine side chains (lysine and arginine as well as histidine) are still in their positively charged form:

If pH < pKa then the protein is positively charged.

In an isoelectric focusing experiment a gel composed of ampholytes creates a stable pH gradient. The gels are created with large pores to eliminate separation effects based upon molecular weight. After a mixture of proteins (black circle) is added to the gel, an electric current is applied. The proteins will migrate to either the anode or cathode depending on their charge at the pH in which they were applied to the gel (usually around neutral pH). In the experiment shown, the red protein on the left has a higher isoelectric point than the green protein on the right, suggesting that the red protein has more basic residues than the green protein.

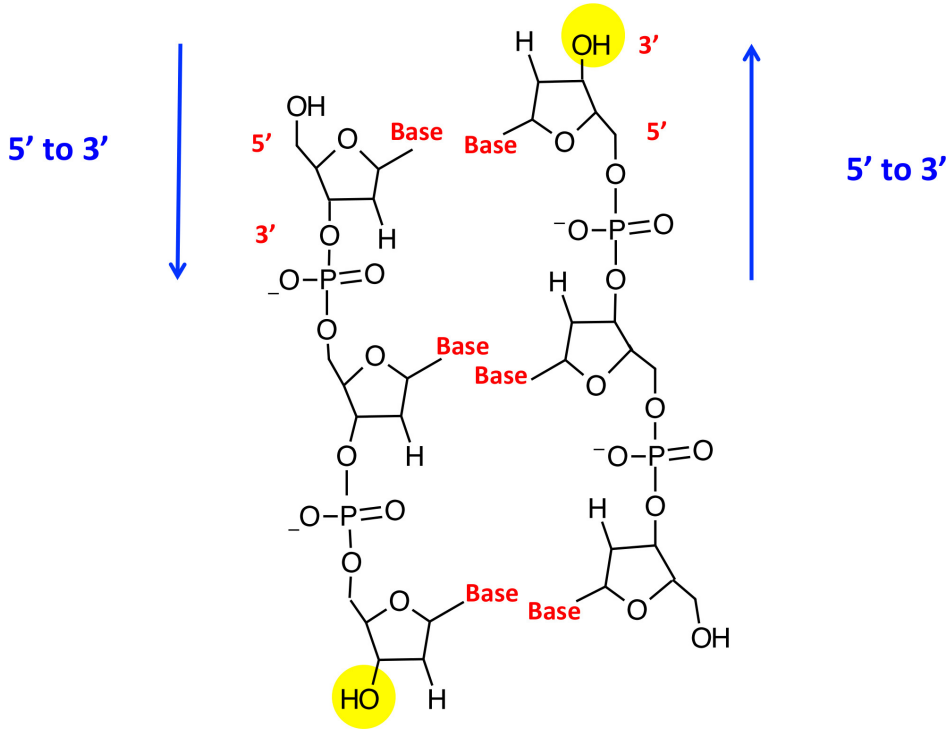


Nucleic Acid Techniques (Molecular Biology)

DNA sequencing by the Sanger dideoxy technique

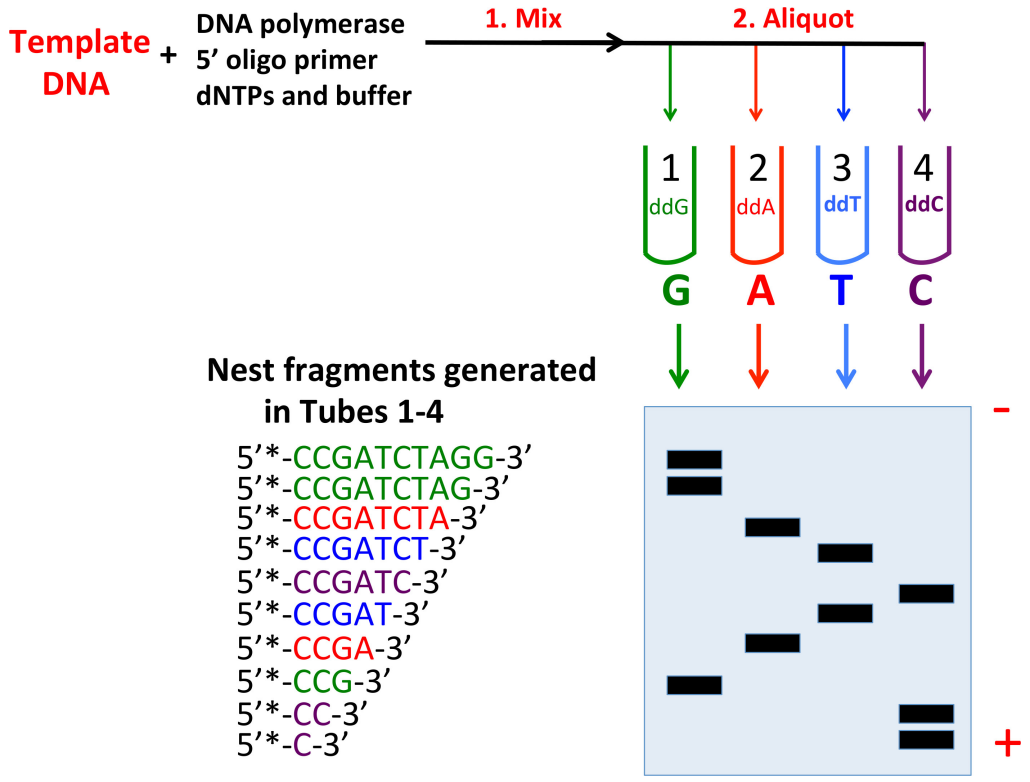
In the 1970s, Fred Sanger developed dideoxynucleotide triphosphates (ddNTPs or ddG, ddC, ddA, and ddT) for determining the sequence of DNA in a sample. These nucleotides have 3' H groups substituted for the 3'OH group normally used by template-driven DNA polymerase enzymes (hence, the term "dideoxy"). The 3'OH is an essential nucleophile for growing nucleic acid chains as shown below (yellow circles). Normally, the 3'OH group attacks the α-phosphate group in the dNTP to be incorporated into the growing chain. However, when a DNA polymerase reaction encounters a dideoxynucleotide (ddNTP), the nucleic acid chain is terminated upon incorporation of the ddNTP due to the absence of the free 3' OH group.

The Sanger DNA sequencing technique is described and illustrated below. Purified template DNA is enzymatically radiolabeled at the 5' end



with the addition of a phosphate group (represented by *). Template DNA is incubated in a cocktail containing multiple reaction components. This includes a suitable buffer containing Mg^{+2} and each of the four dNTPs.

After mixing in the enzyme (Step 1), the final reaction mixture is then aliquoted into four separate tubes (Step 2), each of which is spiked with a single ddNTP. In a single tube each reaction proceeds until the DNA polymerase adds a dideoxynucleotide into the nucleic acid chain. Because both normal deoxynucleotides and a specific dideoxy chain terminating nucleotide are present in a given reaction, a series or "nested set of fragments" is generated for each nucleotide. Each fragment ends with the incorporation of a chain terminating ddNTP. As each fragment varies in size, they can be separated by acrylamide/urea gel electrophoresis. In this procedure, small DNA fragments migrate faster than larger nucleic acid chains. After running the sequencing gel electrophoresis experiment, the following profile is obtained:



By reading the lanes of the gel from the bottom to top, the seqeunce can be determined as: 5'-CCGATCTAGG-3'. Therefore, the template sequence is the complement of this: "5'-CCTAGATCGG-3'.

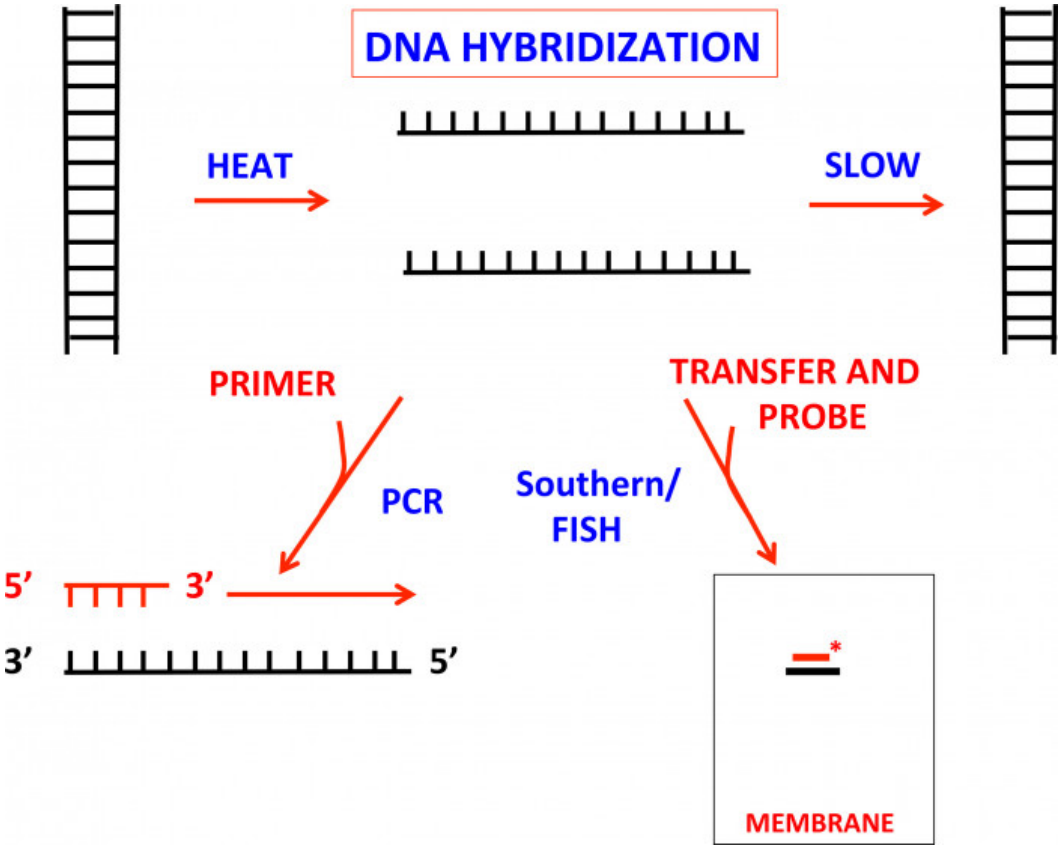
Nucleic acid hybridization

Nucleic acid hybridization is a fundamental aspect of biology and has been exploited in multiple, medically important techniques. This includes the polymerase chain reaction (PCR) and Southern blotting as well as fluorescent in situ hybridization (FISH). These techniques are outlined in the figure and discussed below.

Hybridization techniques are critical for analyzing gene expression. In a Northern blotting experiment, mRNA molecules are isolated and separated via gel electrophoresis in a manner analogous to Southern blotting. When single stranded, radiolabeled DNA probes are added, stable, complementary DNA-RNA hybrids can be detected. Therefore, the Southern blotting technique is for detecting DNA-DNA interactions and Northern blotting is used to detect DNA-RNA interactions.

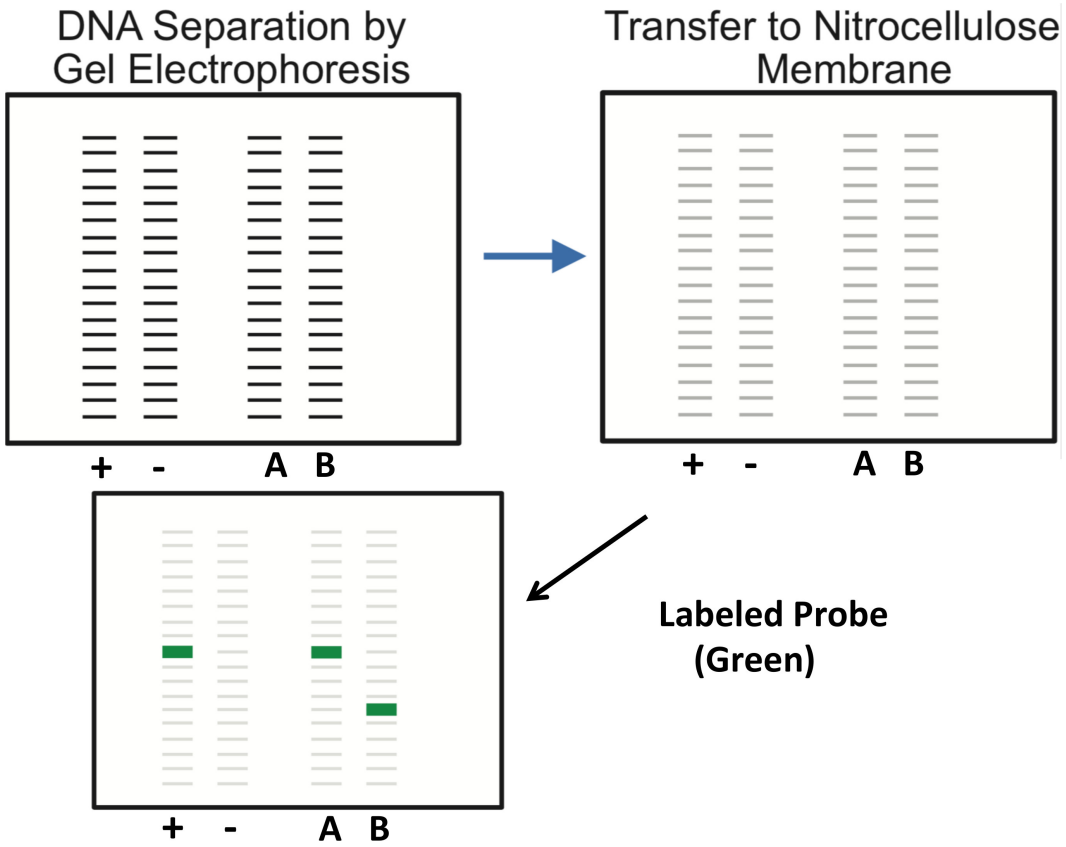
As the Watson-Crick model of DNA shows that the two antiparallel strands of DNA are held together through hydrogen bonding, DNA strands are easily separated with heat. The complementary single strands of DNA (ssDNA) re-associate after slow cooling. Alternatively, the single stranded DNA molecules can be used as templates to amplify DNA through the technique of PCR (Polymerase Chain Reaction). In this procedure (see below), primer sequences complementary to portions of the ssDNA are added. The mixture is cooled to allow annealing. In the presence of DNA polymerase enzyme and nucleotide triphosphates, the template DNA is amplified. Afterwards, the amplified DNA is heated, more primer anneals, and more DNA is amplified. After repeated cycles, a small sample of DNA can be exponentially amplified to significant levels used in various diagnostic techniques.

Alternatively, the ss DNA can be probed for the existence or alteration of various target sequences using techniques like Southern blotting or FISH. By fixing the single stranded DNA sequences in situ (i.e. membrane or glass slide), radiolabeled or fluorescent probes (i.e. Southern blot or FISH, respectively) can be used to query the presence of DNA sequences. Such techniques have wide applications ranging from forensic science to cancer diagnostics.



We will expand further on applying the technique of Southern blotting in the clinical context of disease. Imagine that two patients (A and B) are being screened for the presence of a disease that is caused by a deletion in a fragment of DNA. Below is a schematic of how a Southern blot would be set up in this case. The fragments of genomic DNA that are generated after restriction digestion are resolved on an agarose gel. This separates DNA fragments based upon their charge-to-mass ratio (i.e. size). Typically, a positive control (+) that uses DNA sequences

known to hybridize via complementarity is used as the probe. This could be from a normal individual. A negative control (-) will not hybridize to the target sequence and this DNA could be derived from a species that does not express the gene or region of DNA of interest. The resolved DNA is transferred to a nitrocellulose membrane and then probed with a radiolabeled probe (typically a smaller piece of DNA). The DNA is denatured on the membrane, allowing for the probe to bind to its complementary sequences. After washing, the filter is dried and exposed to film for autoradiography. Notice that the probe binds to the same fragment of DNA in the positive control and in Patient A. In contrast, the probe binds to a smaller fragment of DNA (faster mobility on the agarose gel) derived from Patient B, suggesting that this patient has a deletion.

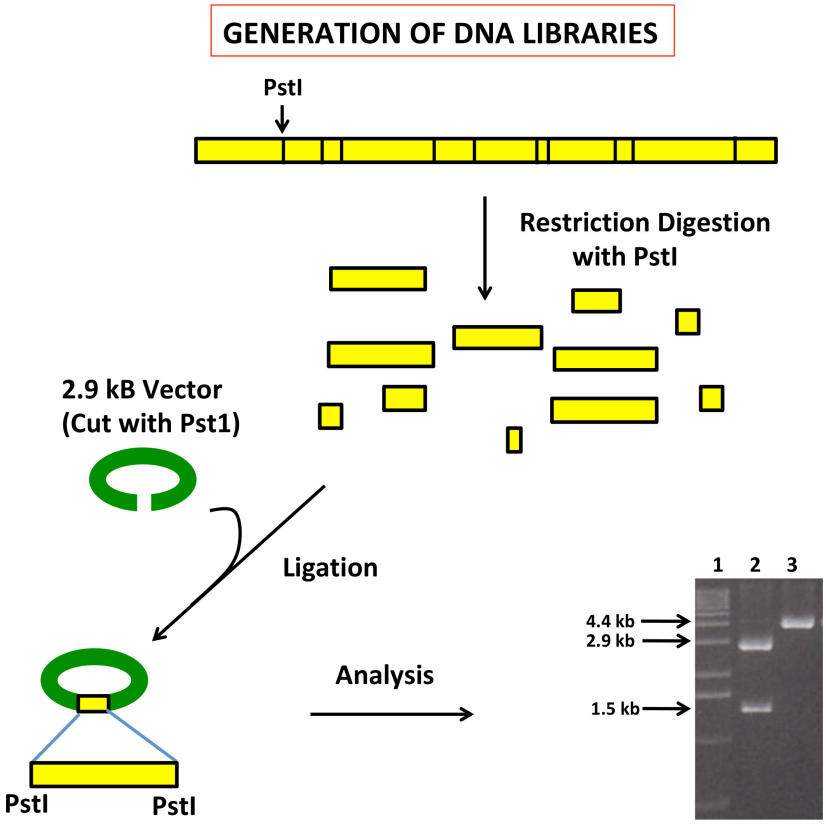


Generation of Libraries

The generation of libraries is specifically mentioned in the AAMC MCAT Content Outline that is available for free on our website. This classical technique was invaluable for identifying and cloning important genes for biochemical analysis.

Although the complete DNA sequencing of the genomes of numerous organisms has replaced the need for generating certain types of libraries, the practice is still useful. A simple schematic for generating a library is shown below.

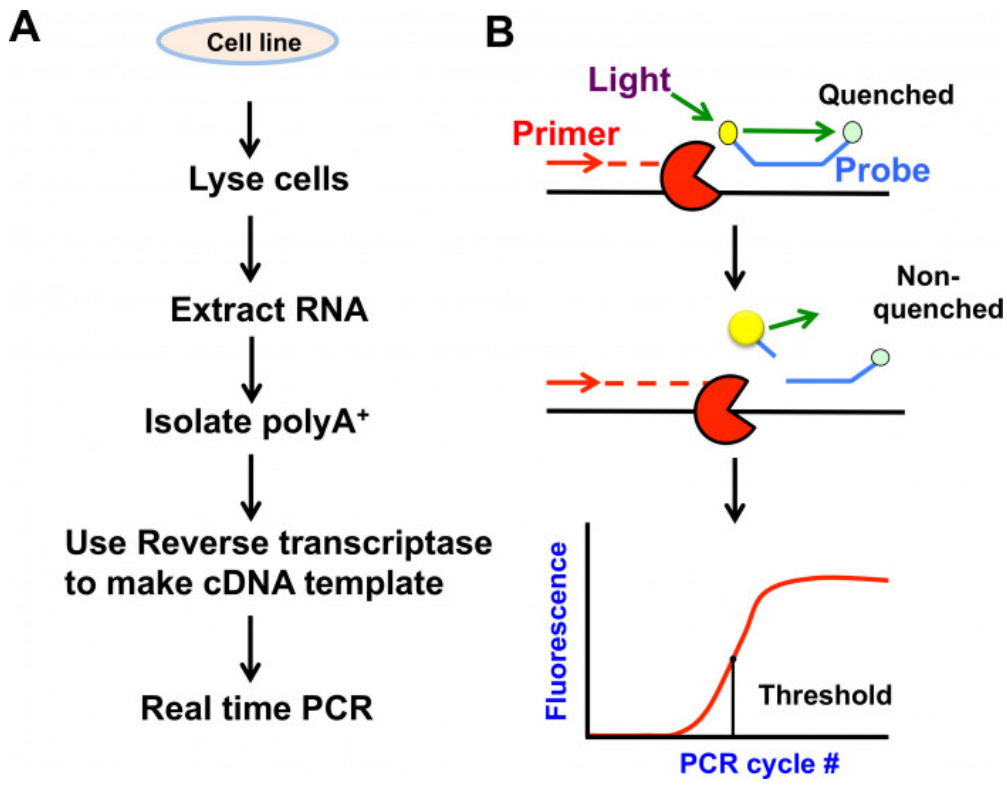
The genome of an organism (simplified with the yellow box) is treated with a restriction enzyme (PstI) that recognizes a 6 base pair palindrome (CTGCAG). After digestion of the DNA, multiple fragments of varying size are generated and ligated into a plasmid vector digested with PstI (shown in greens) that can replicate in bacteria. All of the PstI fragments should be represented in a library of plasmids (Only one is



shown in the picture). The ligation mixture is transformed into bacteria. Those bacteria that have taken up the plasmid are selected for via drug resistance (i.e. ampicillin). After isolation of plasmid DNA, restriction digestion with PstI is used to confirm the presence of a *PstI* insert derived from the genome of interest. For this, agarose gel electrophoresis is used. The DNA can now be studies for various purposes.

Quantitative PCR & Analysis of Gene Expression

Quantitative or real time PCR (qPCR or RT-PCR) is widely used to determine gene expression levels. Unlike conventional PCR, RT-PCR is quantitative. The procedure is shown below. In Panel A, total mRNA is isolated from cell lines through the use of poly T affinity purification. As the vast majority of mRNA molecules are equipped with poly A tails, poly T affinity purification separates mRNA from other RNA species including ribosomal RNA which is abundant. Poly A+ RNA is composed of mRNA molecules devoid of introns; only the sequences encoded by the exons are isolated. The isolated mRNA molecules are used as templates in reactions catalyzed by reverse transcriptase. Originally discovered in retroviruses, reverse transcriptase uses mRNA as a substrate to generate complementary DNA (cDNA) molecules. This



reaction occurs through an RNA-DNA hybrid intermediate. The enzyme is also equipped with a RNA nuclease activity that degrades the RNA in the RNA-DNA hybrid, generating a single stranded DNA template that is used as a substrate for the DNA polymerase function of reverse transcriptase.

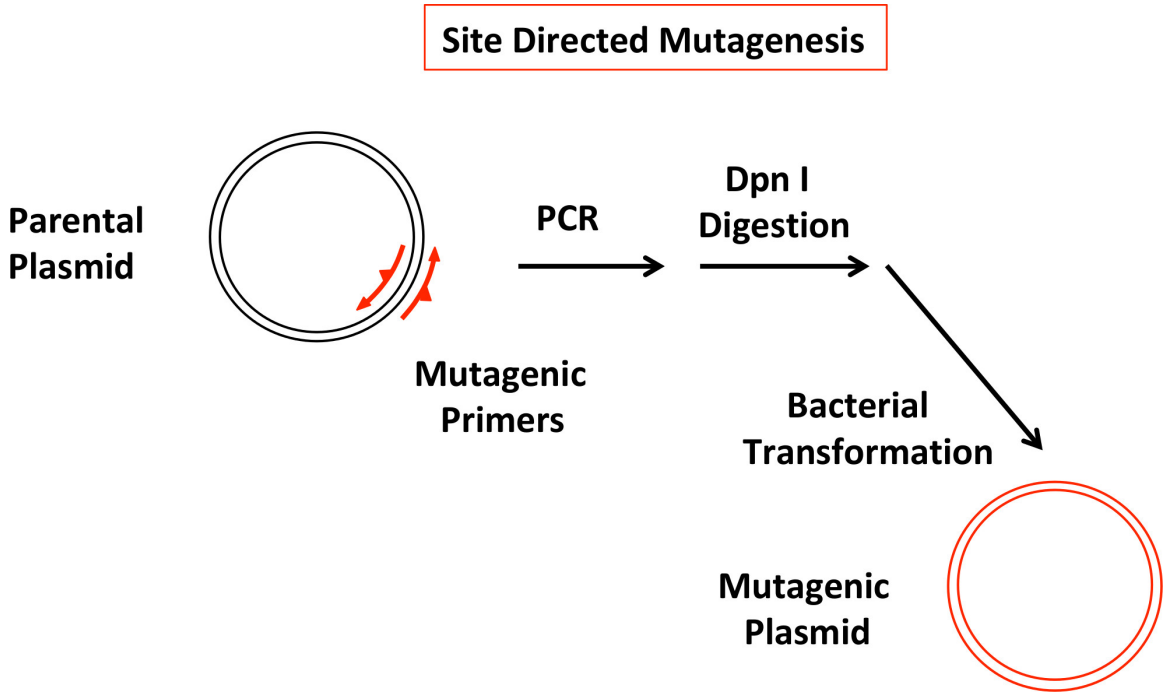
Panel B shows how the mRNA levels are quantitated. In one manifestation of the procedure, a fluorescent probe is hybridized to the internal region of the target gene whose expression levels are being measured. During the polymerization step of PCR, the polymerase will destroy the probe hybridized to the target DNA thereby liberating the fluorophore from the quenching agent. The amount of released fluorophore is related proportionally to the amount of input DNA and is measured in the qPCR technique

In qPCR, mRNA is isolated for the generation of complementary DNA (cDNA), which serves as a template for measuring the levels of various

genes. In one type of qPCR reaction format, a fluorescent probe hybridizes to the target sequence and emits a signal, when separated from its quenching agent, which is linearly related to the amount of DNA present. The probe is released from the quenching agent due to nucleotide degradation via the action of the elongating DNA polymerase. In each PCR cycle, DNA is exponentially amplified as a function of the PCR amplification efficiency, which can vary significantly for each gene due to a number of experimental factors.

Site Directed Mutagenesis

Site directed mutagenesis is a powerful technique for generating mutant enzymes and proteins for structure-function studies. There are multiple methods available for this. We will examine a version of the procedure that uses the *Dpn I* endonuclease, an enzyme that recognizes and cleaves methylated DNA sequences. The scheme is shown below.

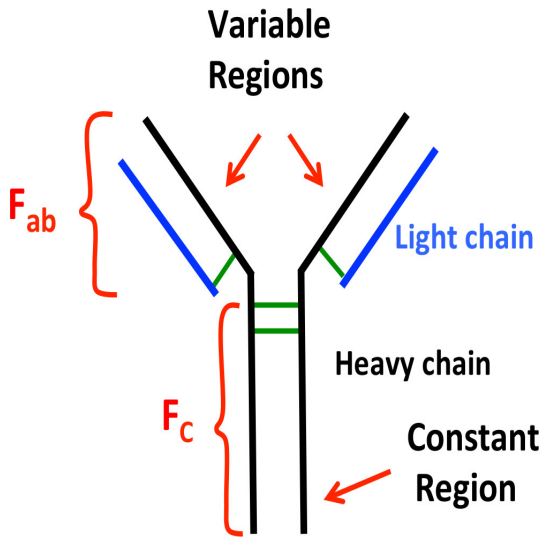


A parental plasmid containing the desired mutagenesis targeted gene of interest is isolated from a bacterial host that is capable of methylating DNA. That is, the parental plasmid DNA is methylated. After heating and cooling in the presence of mutagenic primers, PCR amplifaction is used in the presence of non-methylated nucleotides to generate complementary strands of DNA. The primers must be carefully designed to intoduce the desired nucleotide substitutions, yet still maintain the capacity to bind to the plasmid DNA. The PCR-amplified DNA is treated with DpnI, a procedure that will degrade the methylated, parental DNA that contains the non-mutated sequence. The remaining plasmid DNA should be non-methylated. This sample is transformed into a new bacterial host and transformants are screened for the presence of the mutation.

ANTIBODY TECHNIQUES

Antibodies are produced by B cells of lymphoid origin and are key reagents with a wide variety of clincal applications. In order to understand the various procedures that antibodies are used for, it is paramount to understand antibody structure and function. We saw above the heterodimeric nature of antibodies, but now we need to understand them further.

The “arms” in the classical Y structure of an antibody possess the antigen binding sites that are encoded in the variable regions. The variable region sequences recognize “epitopes” in antigens. Epitopes can be comprised of short sequences of amino acids or combinations of



sugars in glycosylated proteins. Therefore, each antibody has two antigen binding sites. This region is also called the Fab protion of an antibody. Such Fab fragments can be generated through proteolysis. Notice that the Fab protion of an antibody contains both heavy and light chain features.

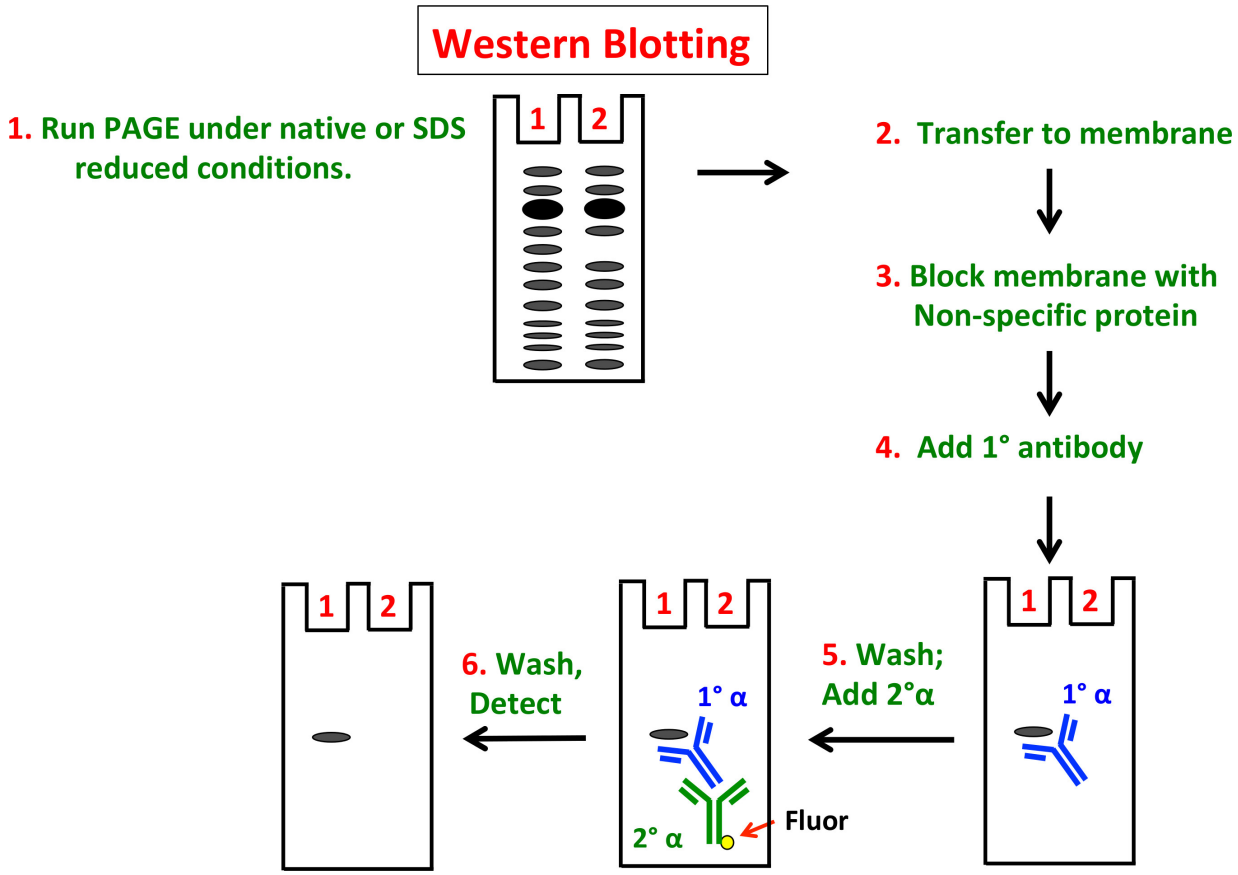
The Fc portion of the antibody contains the constant region and is composed solely of heavy chain amino acids. Historically, "Fc" means "Frgament crystalliazable" and is often thought of as the tail of the antibody. The constant region of the antibody defines the class of immunoglobulin. This is discussed further in the microbiology and immunology section. For now, we will examine the Fc and Fab portion of antibodies in the context of their utility in labatory techniques.

Western blotting

Western blotting is a technique used to detect specific proteins through antibodies that recognize specific epitopes. Epitopes can be unique to specific proteins or conserved in various proteins. The image below shows a schematic of a typical Western blot that indirectly detects an antigen of interest.

Step 1: A polyacrylamide gel is loaded with a protein sample of interest and subject to electrophoresis in order to separate the sample proteins. In the example, two lanes are loaded: 1) Normal, wild-type sample; 2) Mutant sample.

Step 2: After electrophoresis the proteins are transferred to a membrane for in situ analysis.



Step 3: The membrane is blocked with non-specific protein (ie. serum albumin) to reduce non-specific antibody binding to the membrane.

Step 4: A primary antibody (1° or α) that recognizes a specific protein is added. The antibody can be either polyclonal (a population of immunoglobuins that recognizes multiple epitopes within the same

antigen) or monoclonal (specifically created to recognize one epitope in an antigen).

Step 5: After incubation to allow for antibody binding, the filter is washed to get rid of excess and/or non-specfically bound 1° antibody. A secondary (2° or α) is added. This antibody specifcally binds to the constant region of the 1° antibody and is coupled to a fluor (or radiolabel) at its constant region.

Step 6: Excess secondary antibody is washed off and the filter is dried and prepared for detection of protein-immunoglobulin complexes. Note that there is a signal in Lane 1, but not Lane 2 as this sample is missing the protein due to mutation. In many cases, antibodies can recognize their epitopes under non-denaturing conditions. Therefore, SDS-PAGE is commonly used during elextrophoresis in Western blotting experiments. However, some antibodies will only recognize their epitopes under native conditions and the Western blot should be vonducted with a native gel.

Control experiments in Western Blotting

Although antibodies can be highly specifc for antigens, non-specific interactions are always a concern and must be controlled for. There are several methods for determining this:

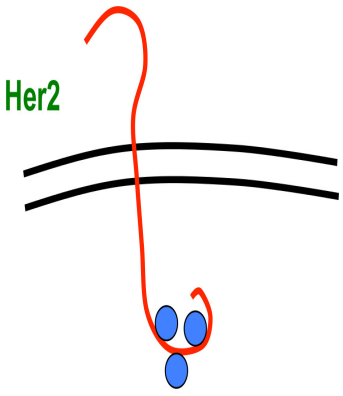
1) Use of pre-immune serum. Custom-made antibodies are dervied from injecting antigen into a host. Use of the serum prior to immunization should be give no signal, and is therefore considered a negarive control.

2) Use of secondary antibody alone. If the experiment is conducted with omission of the primary antibody, then there should be no signal detected. This is a negative control.

3) Use of recombinant protein. If a known antigen from a protein extract (i.e. cell line) is to be detected via Western blotting, then a small amount of recombinant protein derived from that antigen should be loaded in a separate lane of the gel and used as a positive control for the specificity and utility of the primary antibody.

Polyclonal and Monoclonal antibodies

Polyclonal antibodies are comprised of multiple immunoglobulin molecules that recognize a single antigen at different sites (epitopes) of the molecule. Monoclonal antibodies (MAbs) are generated under laboratory circumstances from clonal cells (i.e. cells of the same origin) and have a monovalent affinity towards an antigen through the recognition of a single epitope. This is in contrast to polyclonal antibodies that recognize multiple epitopes on the same antigen. The generation of a homogeneous population of MAbs represents a major advance in medical research and was awarded the Nobel Prize in medicine. Multiple monoclonal antibodies have been approved by the FDA for clinical use. This includes Herceptin, an

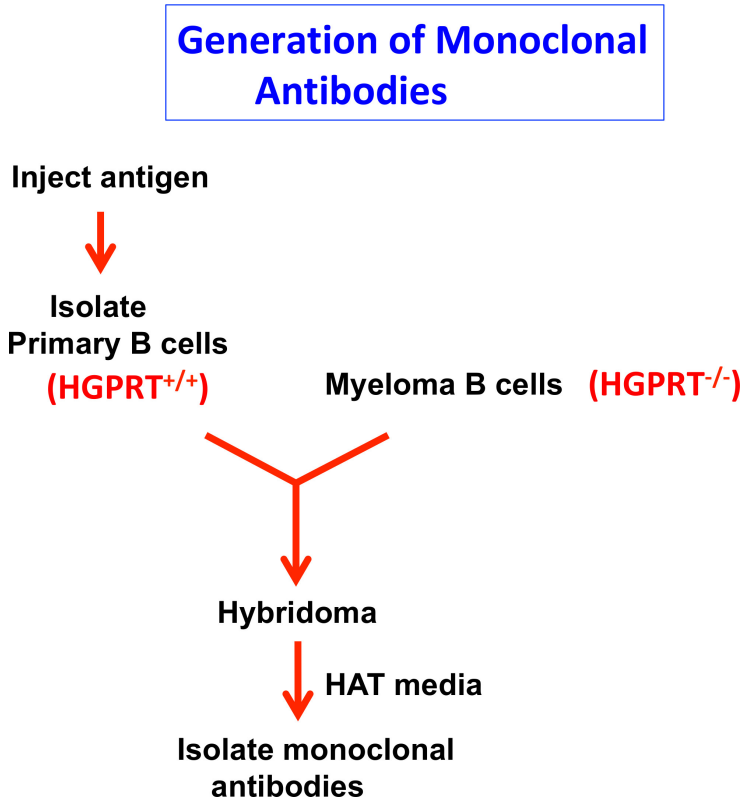


Structure of Her2 receptor.
Tyrosine phosphorylation occurs in cytoplasm and is shown in blue.

antibody that targets the Her2 tyrosine kinase and is used for the treatment of breast cancer (**Fig. 1**).

MAbs are generated from a cell line (hybridoma) derived from the fusion of two cell types. The first cell type, a primary B cell, cannot grow on its own, but can produce antibodies. The second cell type, a B cell derived from a myeloma, cannot produce antibodies, but can grow in its own. The strategy for generating MAbs is as follows.

When transformed, myeloma B cells (or plasmacytomas) deficient in antibody production due to lack of the enzyme hypoxanthine guanine phosphoribosyl transferase (HGPRT) are fused with freshly isolated, primary B cells derived from mice immunized with an antigen of interest, hybridoma cell lines are created (see Figure). Sequences containing clusters of charged and polar amino acids are often used as antigens for this procedure, as they are highly immunogenic. The primary B cells, which cannot replicate in vitro, produce antibodies and express HGPRT, an enzyme involved in the nucleotide salvage pathway, a cellular method to generate nucleotides de novo. Fused hybridoma cells are cultured in selective HAT media containing the nucleotide precursor hypoxanthine, the folate antagonist aminopterin, and

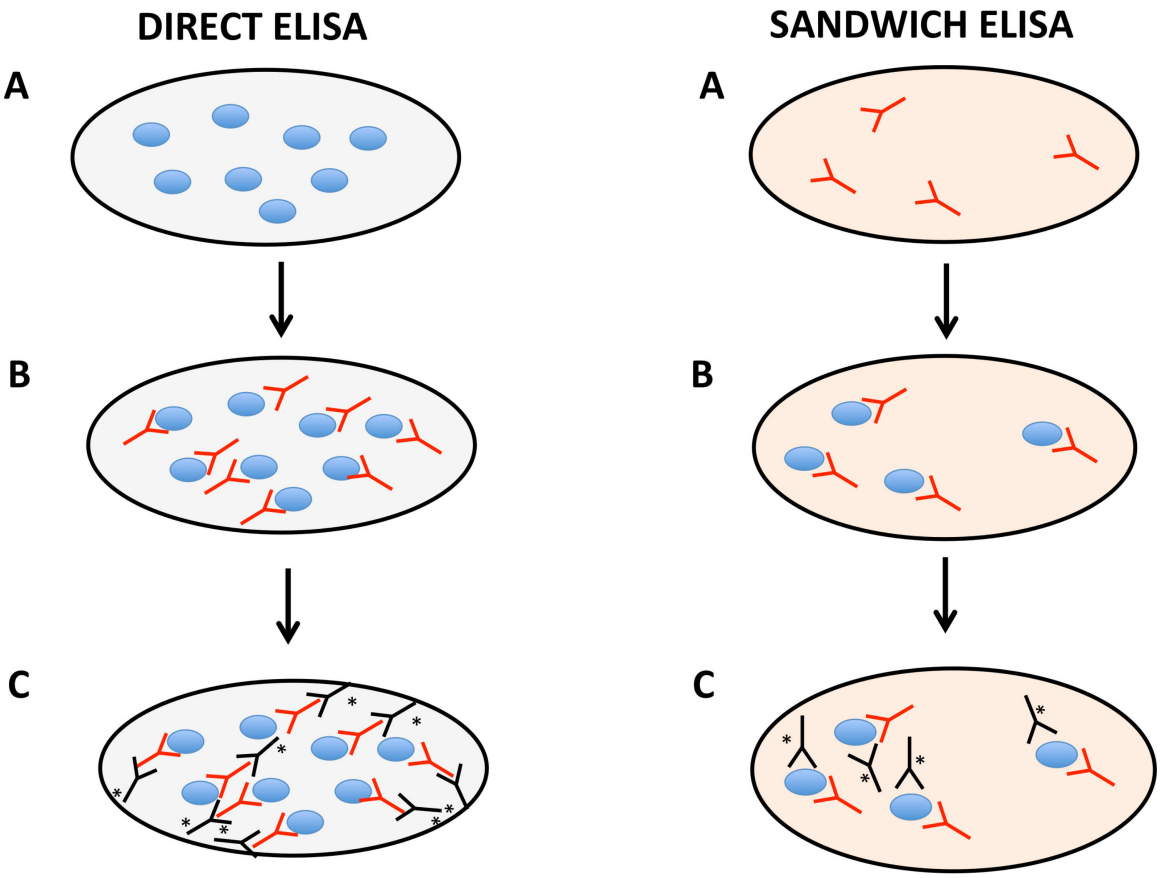


thymidine. Aminopterin inhibits dihydrofolate reductase (DHFR), an enzyme responsible for generating uracil from thymidine. This prevents de novo synthesis of DNA, the second pathway for generating nucleotides. The generation of the hybridoma generates a fused cell type that can synthesize DNA and has unlimited replicative capacity. Moreover, this cell line secretes only one antibody type, a monoclonal antibody that recognizes one epitope on an antigen.

ELISA

Enzyme-linked immunosorbent (ELISA or ELISA assay) is an important biochemical diagnostic test used in labs. They are used to determine the presence and amount of various proteins and metabolites in biological tissues. The direct and sandwich ELISA will be discussed here. A schematic is shown below.

In the direct ELISA, a solution containing the antigen to be measured is placed in a plastic coated well (solid state support) (**STEP A**). The antigen adheres to the surface through charged interactions with the well. Afterwards, the plate is coated with a protein (i.e. serum albumin) to saturate all binding sites. After washing to release all unbound material, a primary antibody is added that is specific for the antigen of interest (**STEP B**). After washing away the unbound antibody, a secondary antibody is added (**STEP C**). Importantly, the secondary antibody recognizes the constant region of the primary antibody (not



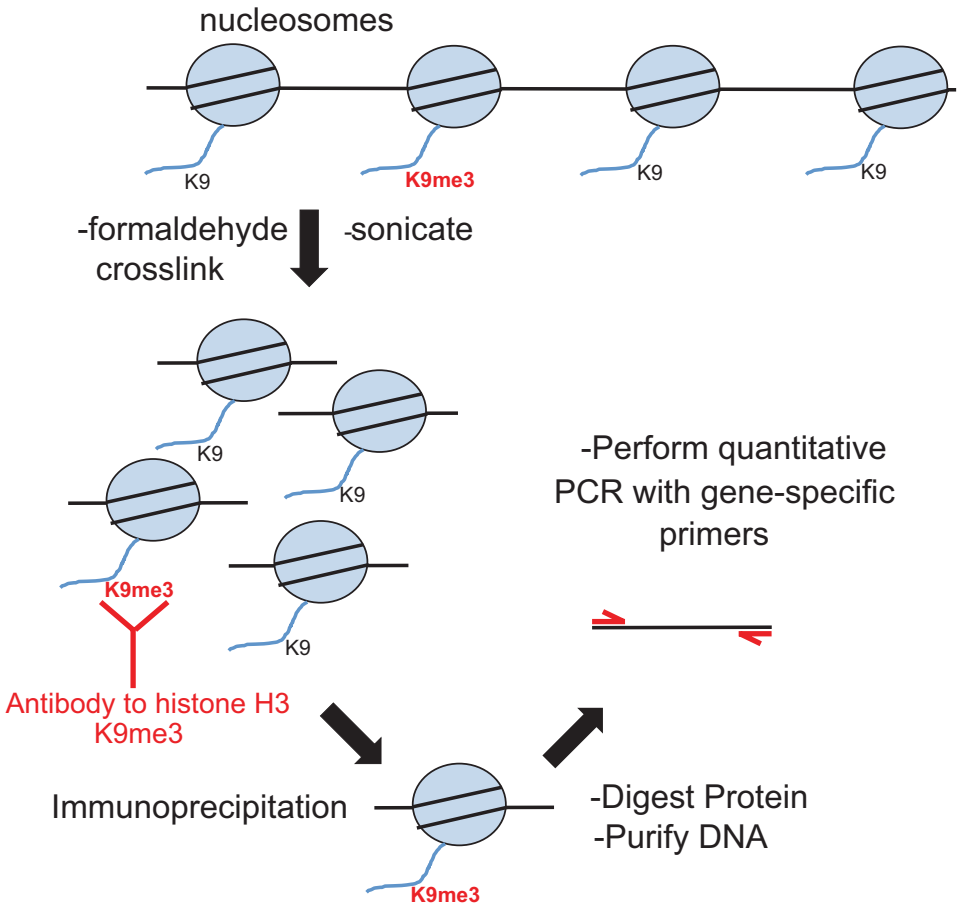
the variable region) and is labeled with a flour (*) that can be detected and quantitated. In some cases, the primary antibody can be labeled with a flour. In some cases, the secondary antibody is linked to an enzyme that performs a reaction that generates a product that can be detected by a spectrophotometer. Hence, the name "enzyme-linked" in ELISA. We will use the name "flour" as a general term.

The sandwich ELISA uses two antibodies that recognize different epitopes of the same antigen. In the sandwich ELISA, a "capture" antibody specific for an antigen of interest is added to the well (**STEP A**). After binding, serum albumin is added to block all potential binding sites on the surface. Afterwards, the well is washed and a mixture (i.e. cell extract or serum sample) containing the antigen of interest is added to the well. This allows for the formation of an antibody-antigen complex (**STEP B**). After washing, a second antibody specific for the antigen is added to the well. This antibody is labeled with a fluor for detection. The binding of both antibodies to the antigen forms the basis of the "sandwich" (**STEP C**).

Chromatin Immunoprecipitation (ChIP)

In addition to using antibodies as reagents in Western blotting experiments, researchers also use them for immunoprecipitation (IP) experiments.

Chromatin immunoprecipitation (ChIP) assays are used to determine the location of DNA binding proteins along the length of the chromatin fiber. They can also be used to determine the nature of histone modifications at a particular DNA locus. This includes examining the state of lysine methylation, including trimethylation at lysine residue 9 (K9me3). The experiment shown above used an antibody that specifically recognizes lysine 9 when it is tri-methylated. ChIP assays involve: 1) the treatment of cells with formaldehyde to allow protein-DNA crosslinking; 2) sonication of the cells to fragment the DNA; 3) immunoprecipitation of the protein-DNA fragments using antibodies, 4)

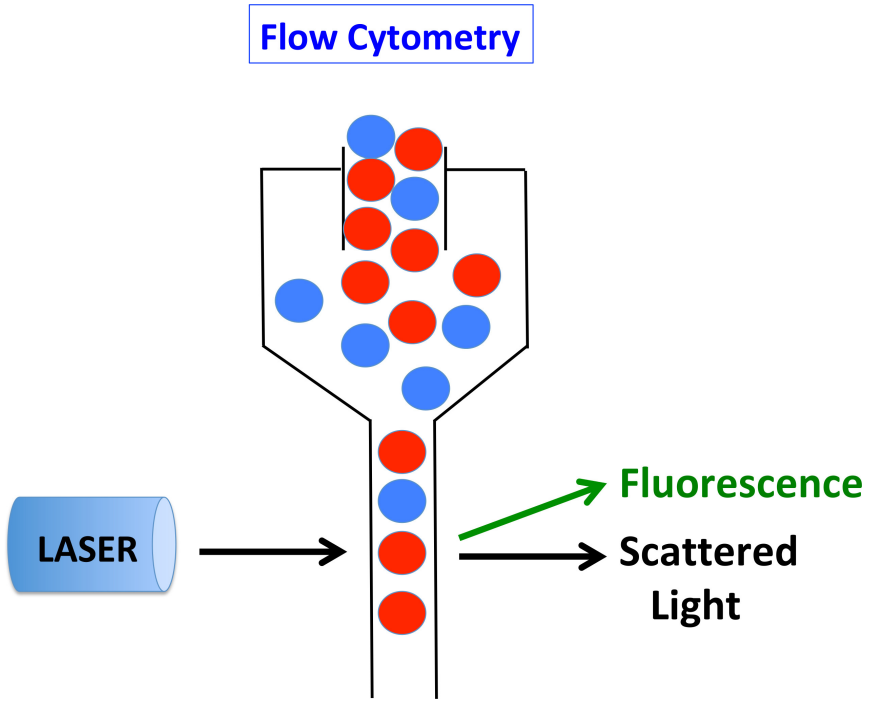


PCR using specific primers to quantify the associated DNA. The results of ChIP analysis of healthy versus FSHD patient cells are shown above. The presence of PCR-amplified DNA suggests that this DNA is associated with hostnes that contain the tri-methylation mark at lysine residue 9. Non-specific IgG antibodies, as well as the addition of no antibody, serve as negative controls.

Flow cytometry

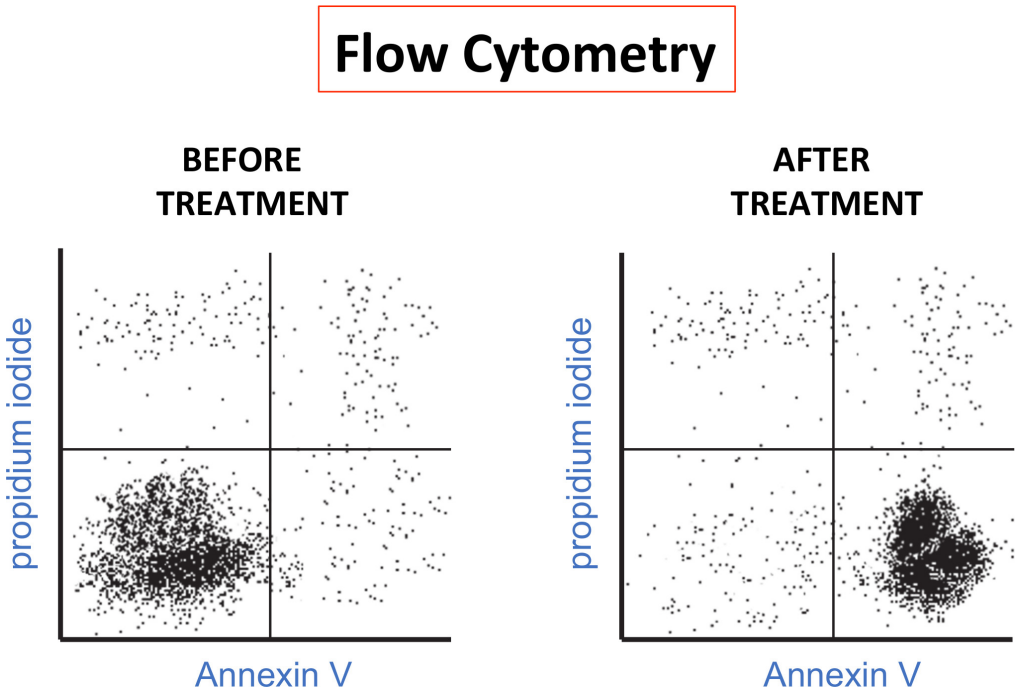
Flow cytometry is used to detect specific characteristics of cells in solution. It is often used to detect the presence of certain cell types in a biological sample. For example, imagine if a doctor was trying to diagnose a patient with mastocytosis, a condition where mast cells inappropriately accumulate in the bone marrow. As mast cells express CD117 on their surface, treatment of the sample from the bone marrow with an anti-CD117 antibody would label mast cells with the antibody. If

the antibody is fluorescently labeled, then the flow cytometer can specifically detect and quantitate the CD117+ cells in a population. Flow



cytometry is also used to measure cell size and purified cell populations.

In the procedure, a cell suspension is labeled with a fluorescent antibody and applied to the flow cytometer. The cells funnel down a chamber in single file and pass through a laser beam of light. As the light shines on the passing cells, those cells possessing the specific fluorescent label will emit light that can be used to quantitate the number of cells in the population. In addition, light is scattered in a forward and side fashion and this information is also used to generate information regarding cell size.



An example of a flow cytometry experiment is shown above. In this experiment, cells were treated with ionizing radiation (IR). As a control, a set of cells was set aside before treatment. After 72 hours, the cells were prepared for flow cytometry through treatment with propidium iodide (PI), a fluorescent dye that binds to and identifies DNA, and by addition of an antibody against Annexin V, a marker for apoptosis.

Each pixel in the flow cytometry experiment shows a cell that is stained with either PI and/or Annexin V. The experimental data show two things: 1) the amount of DNA as measured by the PI signal remains the same after treatment and; 2) the amount of Annexin V staining increases after treatment. Therefore, one can conclude that the treatment of the cells with IR induces apoptosis as witnessed by the increase in Annexin V staining. However, the total amount of DNA has remained the same during the 72 hours.

Chromatography

Chromatography is a commonly used technique for the purification of proteins and peptides. This technique has many applications but each one utilizes a stationary phase (i.e. beads on a column, silica plate) capable of interacting with a solution of molecules (cell extract). Depending on the nature of the stationary phase (charge, polarity, hydrophobicity), molecules will either interact and bind, or pass through with the mobile phase. This is the basis of separation: the intrinsic differences in physical properties. We will examine various forms of chromatography, starting with protein purification.

Protein Purification

Proteins and peptides are purified by various means that exploit differential physical properties, including size, charge, and polarity. Protein purification tables are often generated in order to evaluate the efficiency of various steps in the purification process. A hypothetical purification table for an enzyme is shown below.

1. Each major purification step is described and the total amount of protein is determined by a suitable assay (i.e. Bradford assay) is listed. The vast majority of purification schemes begin with a crude, soluble lysate derived from a cellular source. Crude extracts are commonly derived from the supernatant after a sample is centrifuged. In some cases, proteins may be associated with the membrane (i.e. peripheral or

integral). In these cases, the membrane fraction (pellet) is either washed or solubilized and then activity is measured.

2. Determination of target activity. In the table shown, enzyme activity, as determined via an enzyme assay, is listed in units that are typically identified by the amount of activity (i.e. cleavage of DNA for an endonuclease) per unit time. The temperature, pH, and buffer conditions are also typically included.

In some protein purification schemes, an enzyme is not the target of the purification. In these cases, the activity is often more difficult to determine. In the event that no quantitative assay is available, one way to determine activity is through either silver or Coomassie staining of the gels. After scanning, a quantitated value is determined for the protein band of interest (if it is known).

Purification Step	Protein (mg)	Enzyme (Units)	Specific Activity	% Yield	Fold Purified
1. Crude lysate	740	4800	6.48	100	1
2. Crude extract	620	4200	6.77	87.5	1.04
3. 45-55% Ammo. Sulfate cut	130	2541	19.5	52.9	3.0
4. DEAE pooled	65	2400	36.9	50.0	5.69
5. Gel filtration	4.2	1702	404	35.4	62.03

In the case of purification schemes for targets that have quantitative assays, specific activity is always included in the table. The specific

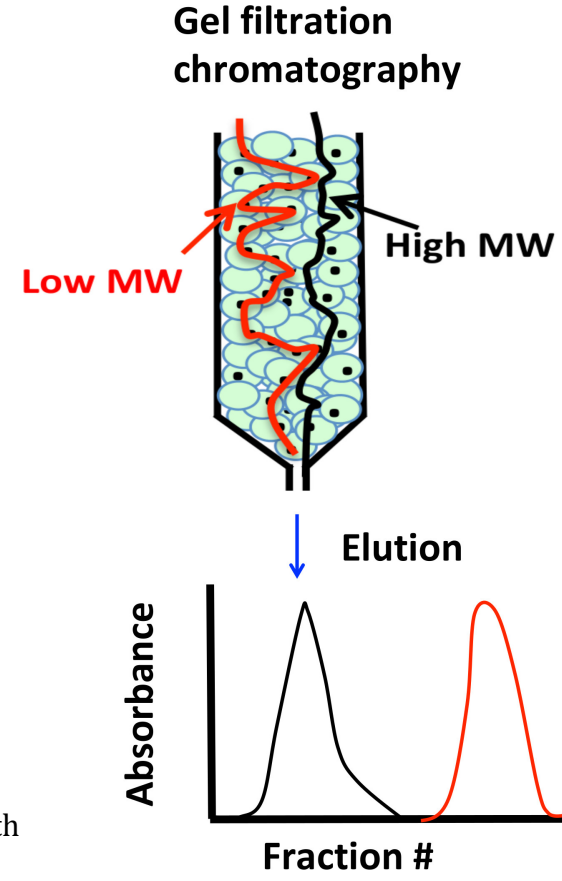
activity is derived from the ratio of the activity (enzyme units)/mg protein.

4. The percent yield for each step is calculated by dividing the yield for a given step by the yield in the first step (always given as 100%). The fold purification for each step is determined by dividing the specific activity of each step by the specific activity for the first step.

Three forms of chromatography exploiting these properties are described below. For the two examples of column chromatography, both high and low (i.e. gravity) pressure can be applied to move a sample through an absorption matrix.

Gel filtration (Size exclusion chromatography)

Agarose beads with defined pore sizes are used to separate molecules based upon differences in their molecular weights. This technique is most accurate with globular proteins. As the proteins flow through the column, lower molecular weight proteins are "trapped" within the pores of the agarose matrix. They are said to be "included". However, the



higher molecular weight forms cannot fit inside the pore. They are therefore considered “excluded”. In gel filtration, larger proteins elute first and the smaller molecular weight proteins elute later.

Ion exchange chromatography

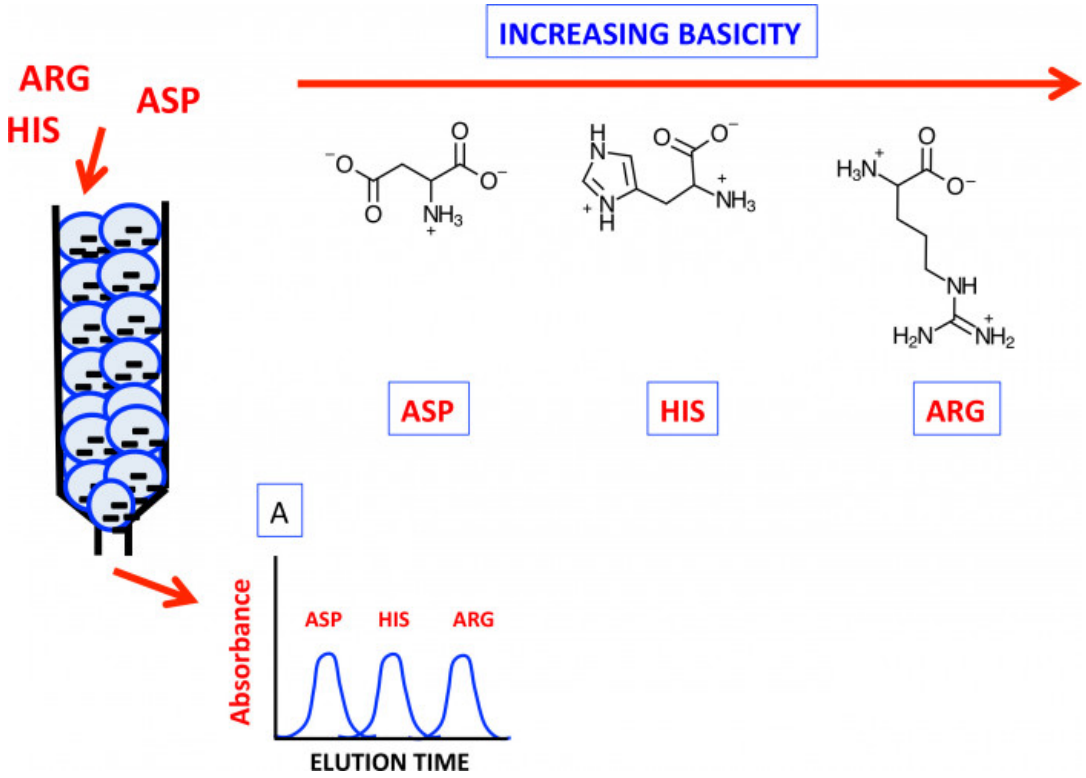
Ion exchange columns are composed of a solid matrix coated with a charged species. As amino acids, peptides and proteins have multiple charges (polyacids), they will exhibit an overall charge at any given pH. A positively charged matrix exchanges anions and a negatively charged matrix exchanges cations.

For this example, two species with differing, yet overall negative, charges are loaded onto a positively charged matrix. This anion exchange column will bind the negatively charged species. Any net positively charged species present in the sample would not be expected to bind. It would therefore be found in the flow through fractions. After binding, the column is exposed to increasing levels of salt (NaCl). This agent serves to disrupt the interaction between the negatively charged protein species and the column matrix. As the two column-bound species vary in the magnitude of their negative charge, the least negatively charged species would be expected to elute first from the column. This is independent of the size of the molecule.

Take the case of a solution of three amino acids (ASP, HIS, and LYS) at pH = 6.0 that was subjected to a cation exchange column (negatively charged matrix). After binding of the amino acids, a pH gradient was use for elution? What is the order of elution of each amino acid?

Appreciate that a cation exchange column is composed of a matrix consisting of negatively charged species (see image below). This means that those molecules with the least positive charge at pH = 6.0 (the pH of the buffer) will elute first. In other words, the least basic species (or most acidic) will elute first. At pH = 6.0, aspartate has a net charge of -1 and since the pKa of the histidine side chain is approximately 6.0, then 1/2 of the histidine molecules will be charged and the remaining 1/2 will be uncharged (determined from using the Henderson Hasselbach equation). Therefore, the net charge of histidine at pH = 6.0 is ~ +0.5. Both nitrogen groups in arginine would be positively charged as the pKa for each group is approximately 12. The net charge on arginine is therefore +1 (-1 + 1 + 1 = +1). Therefore, the amino acids in order of increasing positive charge (basicity) are ASP, HIS, ARG.

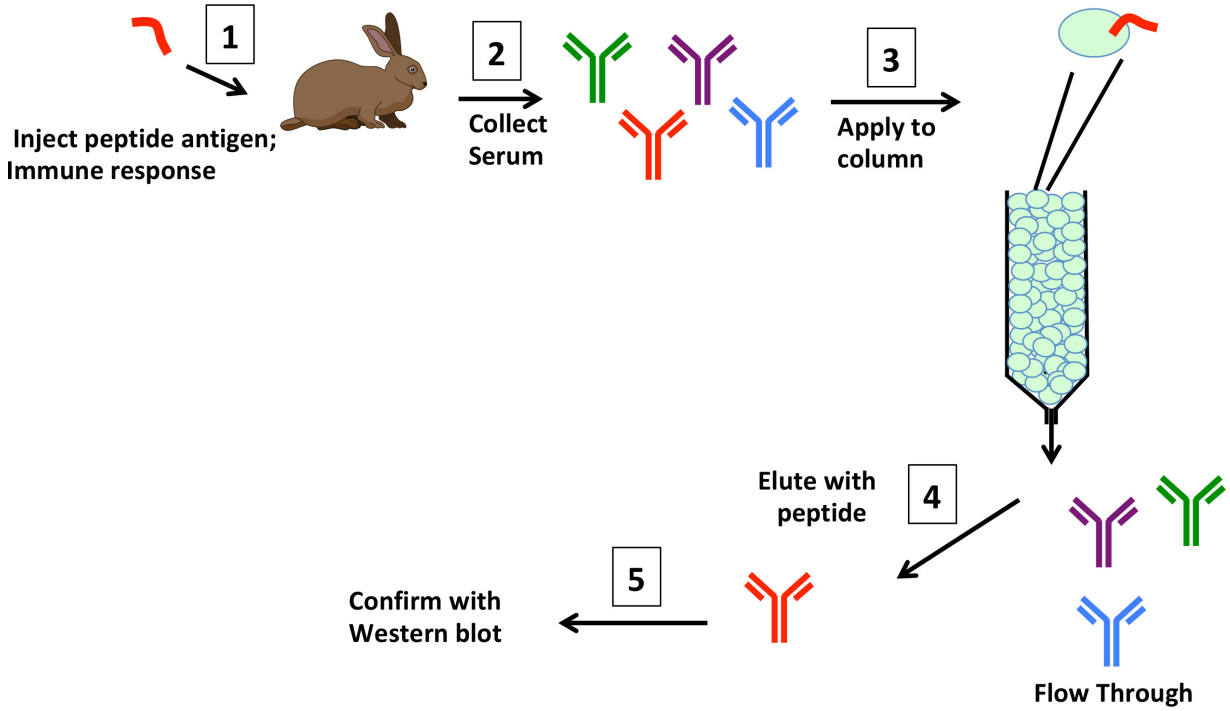
The binding step occurs at pH = 6.00. At this pH the negatively charged ASP would bind to the column with the least strength as it has only one positively charged nitrogen and two negatively charged carboxyl



groups, ASP elutes first followed by HIS and then ARG.

Affinity chromatography

Besides gel filtration and ion exchange, affinity chromatography is a major method for protein purification. One common use of this technique is to affinity purify antibodies. Let's take the case where a researcher generated an antibody against a peptide sequence in a target protein of choice. In the first step, the chemically synthesized peptide is injected into a host. This will elicit an immune response and the generation of antibodies against the peptide. The antibody (red) is collected from the serum, but there are numerous additional antibodies in the serum represented by green, blue, and purple structures. Affinity chromatography is used to separate the antibody of interest (red) from the other antibodies. For this, the peptide antigen is covalently linked to agarose beads by any of several techniques not discussed here. After generation of this affinity matrix, the serum is applied to the column. After binding, the column is washed to dislodge any non-specific interactions. Only those species with affinity towards the peptide will bind to the column; the other antibodies will flow through. The antibodies specific for the peptide of interest are eluted by addition of excess competitor peptide to the column. This is the same peptide that was used to generate the antibodies. The antibodies are collected in the eluate and assayed by Western blotting.

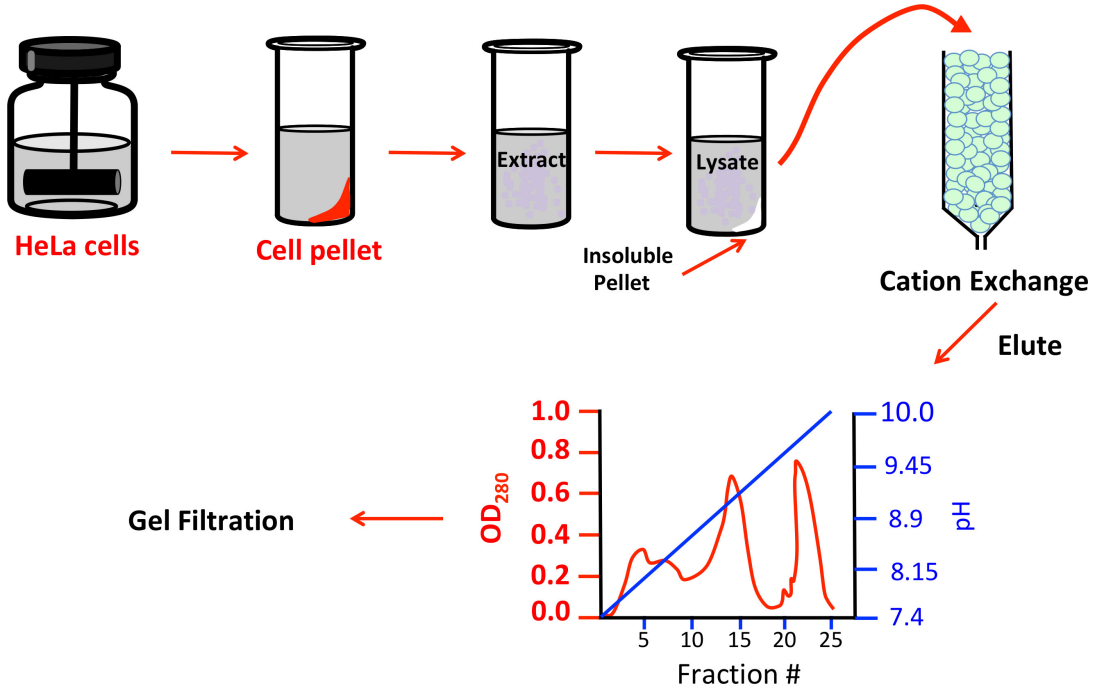


Living cells are first treated with formaldehyde to crosslink proteins to DNA. This "freezes" whatever proteins are associated with DNA at the time of chemical addition. The cells are then lysed and subject to sonication with high-energy sound waves that shear the DNA. An important consideration of this step is to generate a relatively uniform distribution of protein/DNA fragments so that regions of DNA can be isolated (thus, **choice A is incorrect**) that is of equal relative concentration between the two states that will be compared (making **choice B incorrect**). A specific antibody is then added to the DNA/protein fragments (**choice C is incorrect**) and is then used for immunoprecipitation (IP). Following the IP step, proteins are digested before DNA purification. The DNA is then subjected to PCR using gene-specific primers. The length of the two primers used in the PCR does not have to be identical, only that they are specific (**choice D is correct**).

PROTEIN PURIFICATION SAMPLE PASSAGE

A lysosomal enzyme, tentatively identified as X123, was overexpressed in a mammalian cell line in preparation for purification. The enzyme was believed to be stable and to hydrolyze proteins through recognition of a KFERQ sequence motif. The experimental purification scheme was described as follows.

As shown in the schematic, 2.0 L of HeLa cells were grown in spinner flasks, harvested, and pelleted by centrifugation. The cells were resuspended in phosphate buffered saline (PBS; 0.150 M NaCl, 3.0 mM KCl, 1mM Na_2HPO_4, 2.0 mM KH_2PO_4, pH = 7.4) and sonicated, a procedure that disrupts cell membranes and membrane-bound organelles. The crude extract was obtained and centrifuged at 12,000g for 10 minutes at 4°C. The supernatant was applied to a cation exchange column. After binding, the column was eluted with Tris-based buffer gradient ranging from pH 7.4-10.0. 0.3 mL eluates were assayed for protein content by measuring optical density at 280 nm. Fractions 10-15 were pooled, dialyzed in hydrolase buffer, and applied to a gel filtration column. A single peak was observed from this column (not shown). Enzyme activity was only detected for fractions 10-15. The data were plotted in a purification table.



Purification STEP	Protein (mg)	Enzyme Units	Specific Activity
Crude Extract	680	2850	4.19 U/mg
Lysate	647	2700	4.17 U/mg
Cation Exchange	132	1650	12.15 U/mg
Gel Filtration	24	1221	50.8 U/mg

A second purification scheme was designed to improve the yield of the enzyme. In this experiment, a new step prior to the anion exchange column was introduced. For this, the salt concentration of the cell lysate was adjusted to 1.0 M with KCl and this new sample was directly applied to a hydrophobic column. After elution of bound protein, fractions were dialyzed in enzyme buffer. Active fractions were pooled and both the

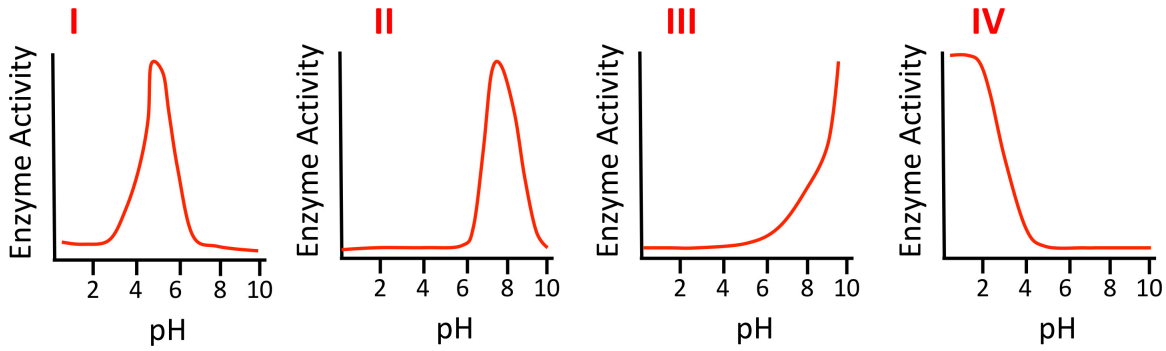
cation exchange and gel filtration steps were performed as described above. The purification table is shown below.

Purification #2

Purification STEP	Protein (mg)	Enzyme Units	Specific Activity
Crude Extract	720	2920	4.55 U/mg
Lysate	701	2806	4.00 U/mg
Hydrophobic Column	203	4240	20.88 U/mg
Cation Exchange	160	5890	36.81 U/mg
Gel Filtration	46	7132	155 U/mg

PASSAGE QUESTIONS

1. Which of the following enzyme activity vs. pH profiles best describes the purified target enzyme?



A. Profile I
B. Profile II
C. Profile III
D. Profile IV

2. During the second purification procedure, a hydrophobic column was used. Bound protein is most effectively released through which of the following procedures?

A. Decreasing salt gradient from 1.0 M to 0.1 M NaCl
B. Increasing pH gradient from 4-10.
C. Increasing salt gradient from 0.1 M to 1.0 M NaCl
D. Decreasing pH gradient from 10-4.

3. The incorporation of the hydrophobic column improved the enzyme X123 purification scheme. Which of the following statements best describes the reasons for this?

A. Decreased binding of lysosomal enzymes to column.
B. Removal of inhibitor of lysosomal enzyme X123.
C. Emulates hydrophobic environment of lysosome.
D. Binds hydrophobic residues to stimulate enzyme activity.

4. The fold purification after the cation exchange column in the first purification scheme is approximately:

A. 2.0
B. 3.0
C. 6.0
D. 12.0

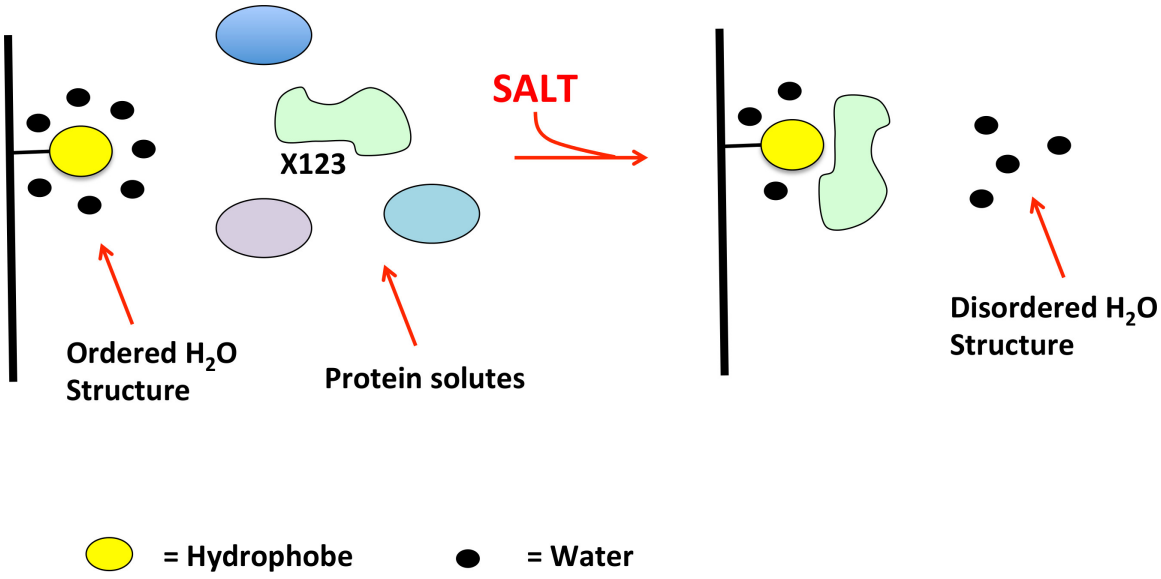
ANNOTATIONS To Protein Purification Passage

1. **Choice A is correct** (Profile I). Although the passage never explicitly states the precise enzyme assay conditions, appreciate that lysosomal enzymes have optimal pH activity around 4-5. Only profile I conforms to this. Choice B is wrong because the pH optimum is ~ 8.0, well above the optimal pH for the acidic lysosome. Do not confuse the fact that the enzyme eluted from the ion exchange column around pH 9.0 (fractions 10-15). This reasoning can also be used to eliminate choice C, a profile that has the most enzyme activity at the pH ~ 9.0. Choice D is wrong because no enzyme activity is detected between pH 4-5.

2. **Choice A is correct**. Hydrophobic chromatography exploits the hydrophobic effect. We will describe this below, but you could also infer what the answer choice was without any extended knowledge of this phenomenon. First, note from the passage that the salt concentration of the lysate was increased to 1.0 M with KCl. This suggests that high salt concentrations favor the binding of proteins to hydrophobic columns. Therefore, by implication, reducing the salt concentration will promote elution of protein from the column.

The hydrophobic effect describes the orientation of water molecules around a hydrophobe. In the case of hydrophobic chromatography, proteins bind to the matrix in a reversible fashion as a function of their hydrophobicity. The column contains solid state, hydrophobic structures such as phenyl sepharose (yellow hydrophobe). In a low salt environment, water molecules orient themselves around the hydrophobe in an orderly fashion. (Water also orients itself around the protein solutes too, but this is now shown). However, in the presence of salt from the buffer, the solvation of the hydrophobe is reduced as the water can now interact with the salt ions. As the solvation of the hydrophobe decreases, hydrophobic regions of the protein become exposed, causing hydrophobic interactions between those proteins with exposed hydrophobic sequences and the stationary hydrophobe that is

part of the column matrix. As a result, there is more disorganization of water structure, demonstrating that the hydrophobic effect is driven by thermodynamics (i.e. entropy).



3. **Choice B is the credited answer**. As the purification of a lysosomal enzyme was conducted from cell extracts, as opposed to purified lysosomes, one might expect the presence of factors that could bind and to X123 and interfere with its activity. In principle, the hydrophobic column could purify such factors away from X123. Further, the purification of X123 from whole cell extracts could also explain the apparently low specific activities observed in the purification table. Choice A is incorrect because the column would be expected to increase the binding of lysosomal enzymes. Choice C is incorrect because the lysosome has an acidic environment, not a hydrophobic one. Choice D is incorrect because the enzyme assay (and therefore its detected activity) is performed in solution, not on the column.

4. **Choice B is correct**. The fold purification for each step is determined by dividing the specific activity of each step by the specific activity for

the first step. As calculated from the table, the fold purification is: 12.15/4.19 = 2.89.

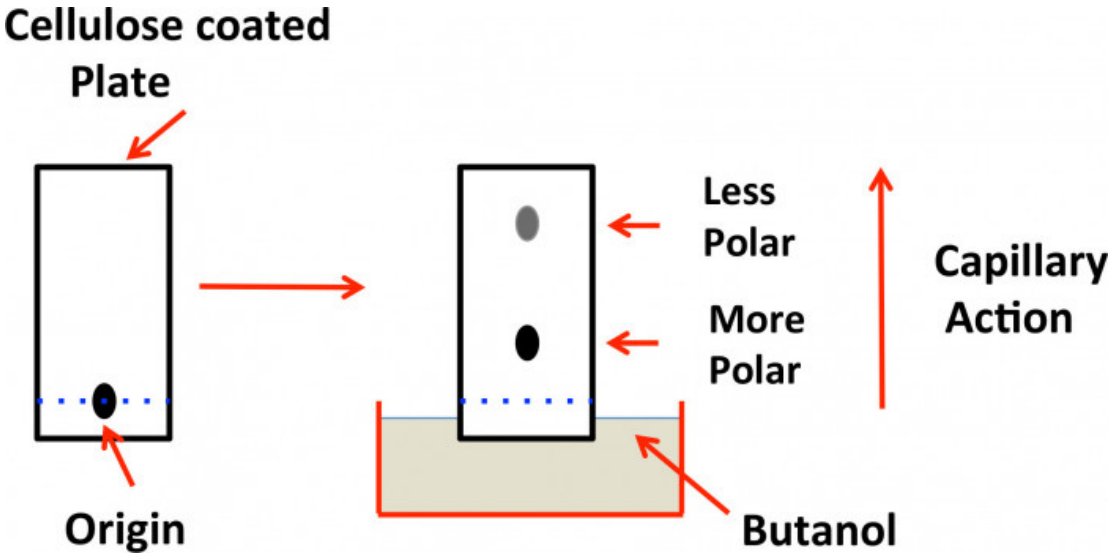
Purification STEP	Protein (mg)	Enzyme Units	Specific Activity
Crude Extract	680	2850	4.19 U/mg
Lysate	647	2700	4.17 U/mg
Cation Exchange	132	1650	12.15 U/mg
Gel Filtration	24	1221	50.8 U/mg

Thin Layer Chromatography)

TLC is useful for separating amino acids and small peptides based upon differences in polarity. Detection is determined through radiolabeling or staining techniques such as ninhydrin for amino acids. In the example shown, a sample containing two molecules that vary in polarity are spotted into a cellulose coated TLC plate. The origin is marked as shown. Afterwards, the samples are immersed into a trough of butanol (a more nonpolar medium). Through capillary action, the molecules move up the plate. However, as polar molecules interact more favorably with the polar, cellulose surface, their mobility is hindered relative to more nonpolar molecules that unfavorably interact with the matrix. This is how separation is achieved. By determining the distance traveled from the origin as a function of time, the ratio to front (RF) value can be determined. A relatively lower R_F value indicates a more polar molecule.

As TLC separates things based upon their polarity and the most polar molecules will be closest to the origin. This is because “polar migrates lower and slower”. The silica surface of the TLC plate is polar and the

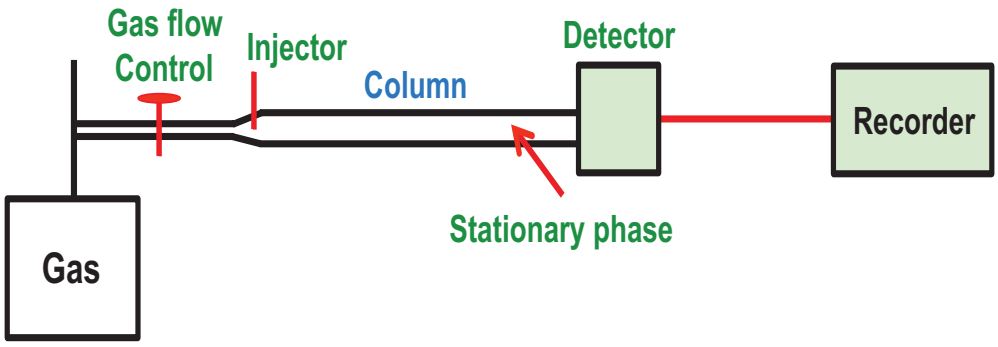
polar molecules will interact more with the surface compared to a relatively less polar molecule, retarding their migration up the plate.



Gas chromatography

Recall that like many forms of chromatography, there are two phases: a mobile phase and a solid, stationary phase. In gas-liquid chromatography, the mobile phase is usually an inert gas and the stationary phase is a liquid-gel phase (a hydrophobic resin in this case) that coats the walls of the column.

In a gas chromatography experiment, a sample is injected to the column where it is immediately vaporized with heat. As gas is introduced into the column, the vaporized molecules are carried into the column where they can interact with either the gaseous or liquid phase. The amount of time a molecule interacts with the stationary phase (liquid gel) depends on its volatility. The retention time reflects this; higher retention times signify a stronger interaction with the liquid phase. This would translate into having a higher boiling point (less volatile). In this separation technique, the most volatile component will be detected first.

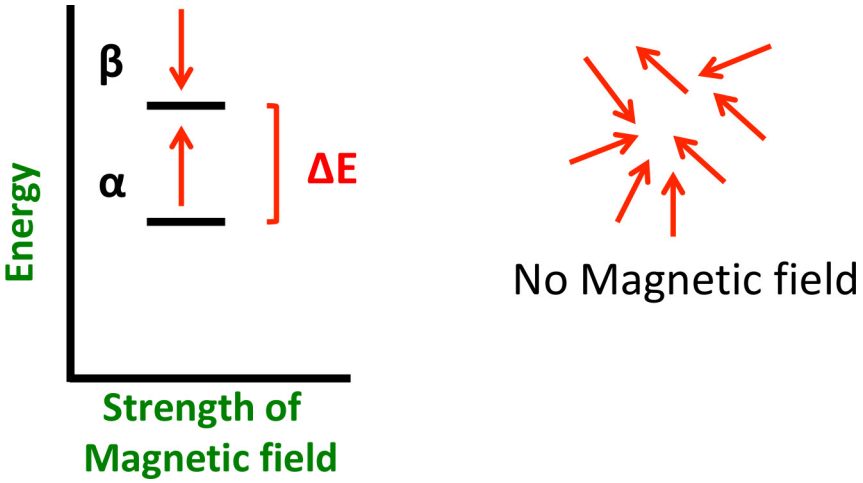


Spectroscopy

Spectroscopy is a powerful method for determining the structure of molecules. Most molecules exist in the grounded state, their lowest energetic form. Addition of energy to the molecule, in the form of light (i.e. photon) and/or energy, often causes some of the bonds (i.e. functional groups) within the molecule to absorb energy and adopt an excited state. At some point, the molecule will return to the ground state and release light or energy. This section examines multiple forms of spectroscopy that will appear on the MCAT. This includes nuclear magnetic resonance (NMR), infrared (IR) spectroscopy, and ultraviolet (UV) spectroscopy.

NMR

NMR was developed by scientists in order to study atomic nuclei. One major application of NMR is to determine the structure of molecules. Nuclei spin and generate magnetic fields. In the absence of an applied magnetic field, the nuclei are randomly oriented. However, in the presence of a magnetic field, the nuclei align either with or against the field (spin up or α and spin down or β). The α and β spin states are separated by an amount of energy that is proportional to the strength of the applied magnetic field (B_0).



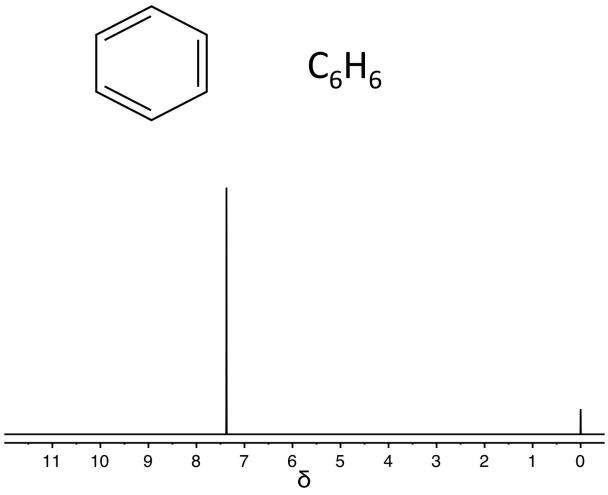
NMR spectra can be generated for various types of atomic nuclei, but the MCAT will examine four major properties associated with ^{1}H NMR:

1) Chemical Equivalency of Protons
2) The Chemical Shift
3) Splitting
4) Integration

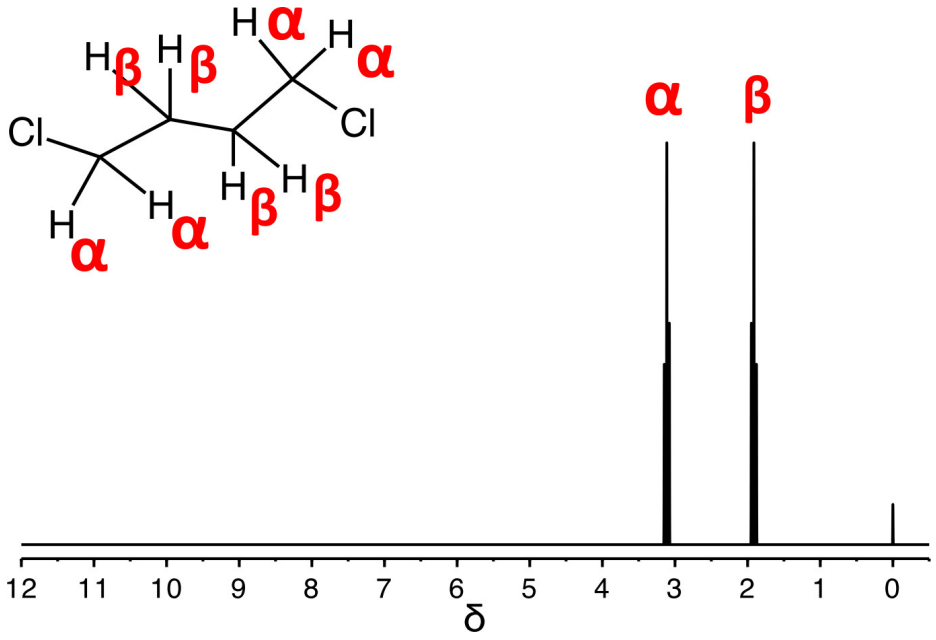
1) NMR Spectrum Signals and Chemical Equivalency of Protons

Protons (or hydrogen atoms in the vernacular of NMR) that exist in the same molecular environment are referred to as chemically equivalent protons. Benzene (C_6H_6) is one such example and its spectra is shown below. Because all of the hydrogen atoms of benzene are in equivalent environments due to the symmetry of the molecule, they each generate signals of the same frequency. Therefore, there is only one peak in its NMR spectra.

Chemically Equivalent Protons

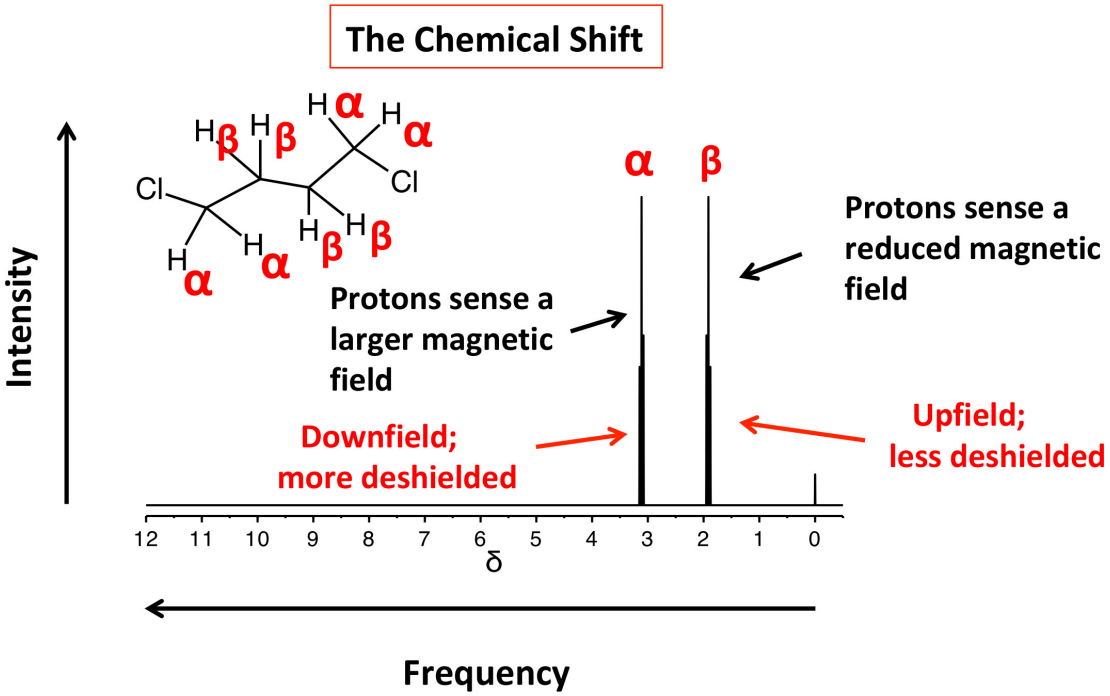


In contrast to benzene, the hydrogen atoms in 1, 4 dichlorobutane exist in two distinct environments as shown. Because of this, there are two distinct NMR peaks for 1, 4 dichloroethane as shown below.



2) The Chemical Shift

The position of the two resonance peaks for 1, 4 dichlorobutane is called its chemical shift value. The phenomenon of "shielding" underlies the principle of the chemical shift. Recall that the underlying concept behind NMR is the magnetic behavior of the nucleus of the atom (hydrogen). However, as the nucleus of an atom lies behind a cloud of swirling electrons the applied magnetic force is reduced from this phenomenon. Those atoms that contain more electron density in the environment of the proton (hydrogen atom) being examined via NMR spectroscopy are more shielded from the applied magnetic force. Those protons that experience a smaller applied magnetic field due to shielding require a lower resonance frequency. They are said to be "upfield" in the spectrum. This explains why the α protons in 1, 4 dichlorobutane are more "deshielded" and have a more downfield shift than the β protons; the electronegative chlorine groups pull the electron density away from the protons and deshield it. All chemical shifts are measured relative to the standard trimethylsaline (TMS).



Additional factors influence the chemical shift. For example, the more acidic a hydrogen atom is, the more deshielded it is. This is particularly true when the hydrogen is bonded to an electronegative atom such as O or N. The electronegativity of these atoms pulls the electrons away from the hydrogen atom, creating a deshielding effect that lowers the resonance frequency. Further, as hybridization influences acidity, the hydrogen atoms in ethylene (CH_2=CH_2) are more deshielded (relatively downfield) relative those in ethane (CH_3CH_3).

A table of relative chemical shift values for some important functional groups are shown. The values are approximations and expressed in parts per million (ppm). Note that the more acidic a hydrogen atom is, the more downfield its resonance frequency is. This includes the acidic hydrogen atoms that are stabilized by resonance (aldehydes and carboxylic acids).

Relative Chemical Shift Values

Chemical Group	Resonance Frequency (ppm)
Alkyl	0-2.0
Allylic, Alkyne	2.0
Benzylic, alkyl halide	3.0
Vinyl	5.5
Aromatic	7.0-8.0
Aldehyde	9-10
Carboxylic Acid	10-12

3) Splitting

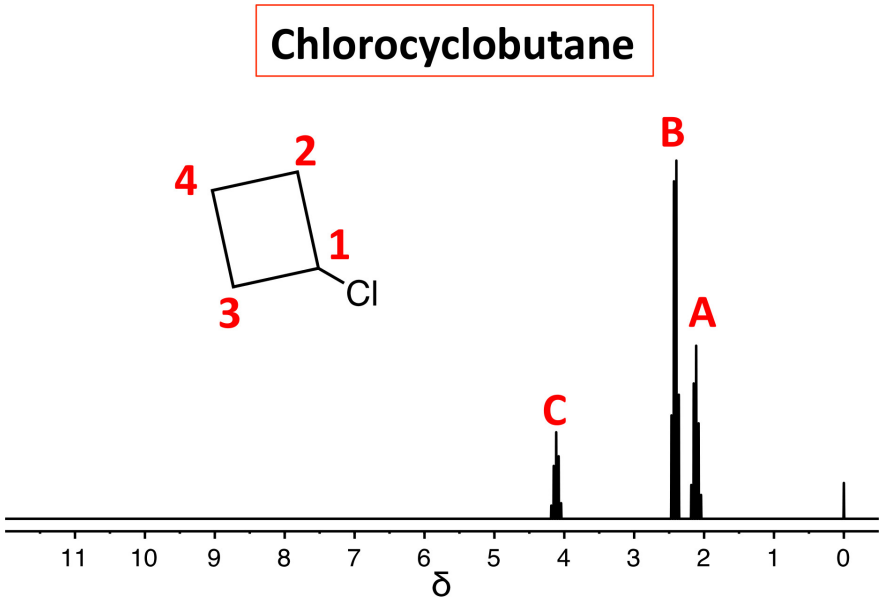
Spin-spin splitting patterns are observed when hydrogen atoms in different electronic environments interact with each other. This is most prominent with hydrogen atoms on adjacent atoms. The rule to remember is the n + 1 splitting rule: the level of splitting is a function of the number of nonequivalent, neighboring hydrogen atoms, or n. Note from the structure of 1,4 dichloroethane that both the alpha and beta hydrogen atoms are each adjacent to 2 nonequivalent hydrogen atoms (n = 1), meaning that each peak will split into a 2 + 1 = 3, or triplet. A closer examination of the spectra presented above for this molecule shows that it is indeed a triplet.

4) Integration

Integration is the measurement of the area under the peak and is proportional to the number of protons that gave rise to the peak.

NMR Problem

Below is the NMR spectrum of chlorocyclobutane. Assign the hydrogen atoms at positions 1-4 with peaks A, B, and C.

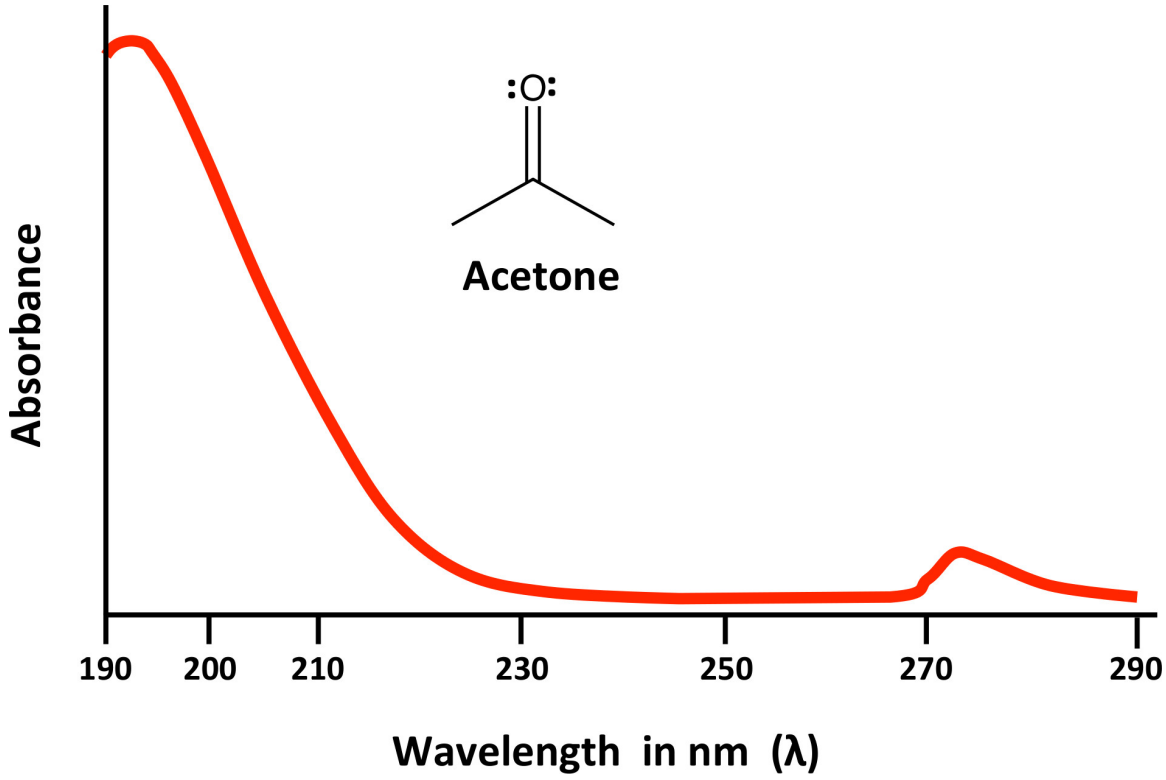


Immediately appreciate that the hydrogen atoms at positions 2 and 3 are in identical chemical environments due to symmetry. There are a total of four hydrogen atoms that contribute to this peak. This is contrast to the hydrogen atoms at positions 1 and 4 that give rise to peaks composed of two hydrogen atoms. Therefore, through the principle of integration peak B is composed of the four protons bonded to carbon atoms 2 and 3. Next, observe that the hydrogen atoms at position 1 and 4 differ through the presence of the C-Cl bond at position 1. Because of the strong polar bond, those hydrogen atoms bonded to carbon 1 will be deshielded and have a downfield shift and will represent peak C. The hydrogen atoms bonded to carbon 4 will be the most shielded and lies furthest upfield in peak A. Note that each peak, though the principle of splitting, appears in the form of a multiplet.

Ultraviolet (UV) and Visible spectroscopy

UV (180-400 nm) and visible light possess the right amount of energy to promote an electron from one molecular orbital to another. UV spectroscopy reveals structural information about molecules, particularly transition state metals and those compounds with π bonds. The technique can detect bonding and nonbonding electrons.

Molecules that absorb light with the energy necessary to excite an electron to a higher molecular orbital enter the "excited state". For transition metals ions in solution, the d orbitals absorb visible light and change colors. The absorbance spectrum for acetone is shown below. Acetone contains a carbonyl chromophore: the region of the molecule that absorbs UV/visible light. As acetone contains both π electrons and lone pair electrons, there are two absorbance peaks: A major one near 190 nm and a smaller one representing the lone pair electrons near 270 nm. The major peak is referred to as the "lambda max" or λ_{max}.

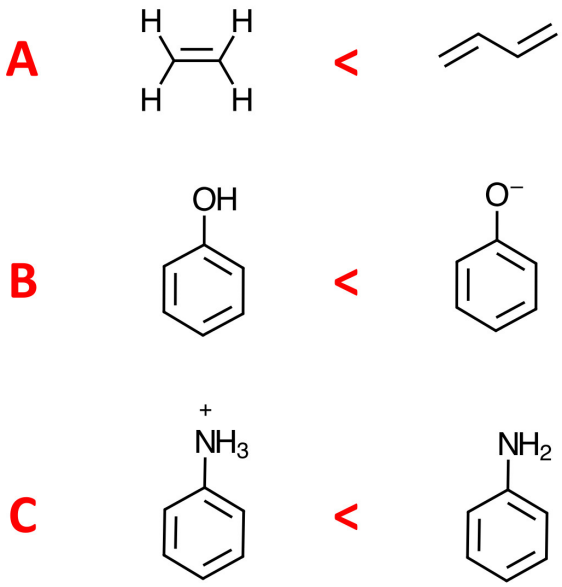


Trends in UV/Visible Spectroscopy: Effects on λ_{max}

The absorption spectra of molecules change as a function of the introduction of more conjugated double bonds and the availability of lone pairs of electrons to interact with π bonds. The effect of this is to reduce the energy required to achieve the transition from the ground state to the excited state. As energy is inversely related to the wavelength, the λ_{max} increases.

The image below shows a few trends that you should be familiar with. In Row A, the introduction of conjugated double bonds from ethylene to 1, 3 butadiene increases the λ_{max}. In Row B, the ionization of phenol to the phenolate ion generates a lone pair that interacts with the conjugated π electrons in the parental benzene ring. In Row C, the loss of a proton from aniline at high pH generates an amine with a lone pair of electrons that can interact with the conjugated π electrons in the ring.

Trends in Absorption: Changes of λ_{max}



Application of UV/Visible Spectroscopy

As the absorption spectra of a molecule can change through the loss of protons, this property can be exploited in enzyme assays such as that for lactate dehydrogenase.

Pyruvate + NADH + H^+ ➔ Lactate + NAD^+

The λ_{max} of NADH is 340 nm. Therefore, through monitoring the decrease in absorption at 340 nm, the reaction catalyzed by lactate dehydrogenase can be monitored.

The Beer Lambert Law

The amount of electromagnetic radiation absorbed and emitted by compounds can be used to analyze the structural nature of molecules.

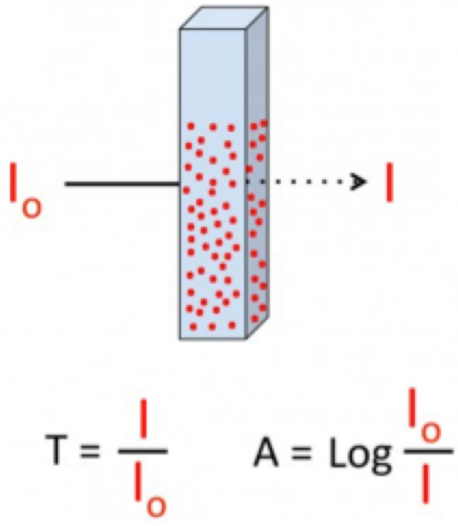
This is because the absorbance of a compound depends on the concentration of absorbing species (chromophores) in solution.

When monochromatic radiation of initial intensity (I_0, photons s^{-1}) is directed at a solution (protein or nucleic acid) containing solute molecules, light is absorbed and emitted with an intensity of I (See below). The % of light in the beam that reaches the detector is called the % transmittance (% T). However, most measurements use absorbance: (A) = -log I_0/I. The absorbance of molecules is dependent on multiple factors and is described by the Beer-Lambert Law:

$$\mathbf{A = \varepsilon cl}$$

ε = the molar extinction coefficient (liter mol^{-1} cm^{-1}); c = concentration (mol/litre); l = path length (cm).

Aborbance



Infrared (IR) Spectroscopy

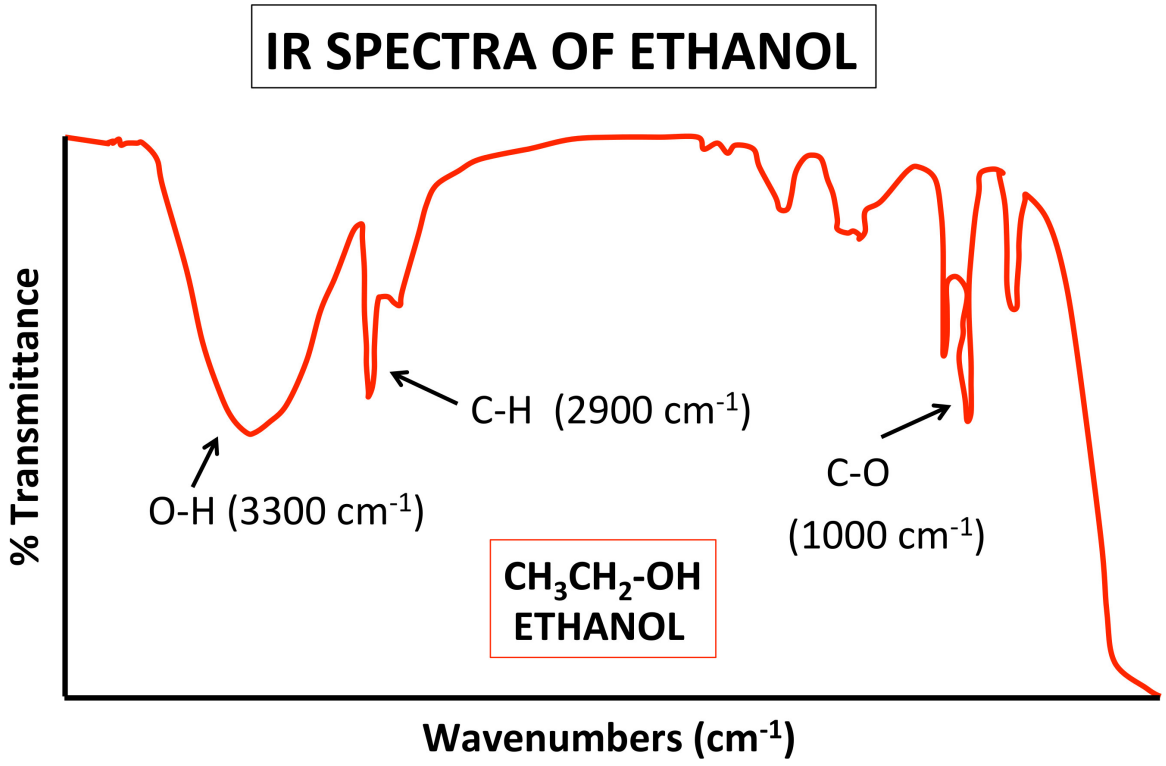
Infrared (IR) radiation is used to examine molecules through the identification of their functional groups. IR radiation has the appropriate amount of energy that corresponds to the stretching and bending frequencies that are characteristic of various covalent bonds within molecules. The frequency of electromagnetic radiation used in IR experiments is expressed in the wavenumber (ν), which represents the number of waves per cm. The unit of the wavenumber is cm^{-1}. The infrared spectrum ranges from 4000-6000 cm^{-1}.

Be familiar with the common functional groups and their corresponding stretching frequencies. The table below describes this further.

COMMON IR ABSORPTION VALUES

FUNCTIONAL GROUP	ABSORPTION WAVELENGTH (cm^{-1})	COMMENTS
Alkyl C-H	3300-2700	Ubiquitious
Alkyl C=C	2250-2100	Medium/Weak
Alcohol R-OH	3600-3200	Strong, broad peaks
Carboxylic Acid -OH	3300-2500	Strong, very broad
Amine N-H	3500-3300	Medium, broad
Carbonyl C=O	1780-1650	Strong peaks

The IR spectra for ethanol is shown below; note the presence of the broad alcohol peak.



Notable Trends in IR Spectra

There are some notable trends in IR spectra that are worth mentioning:

1) As the dipole moment of the bond increases, the intensity of the absorption increases.

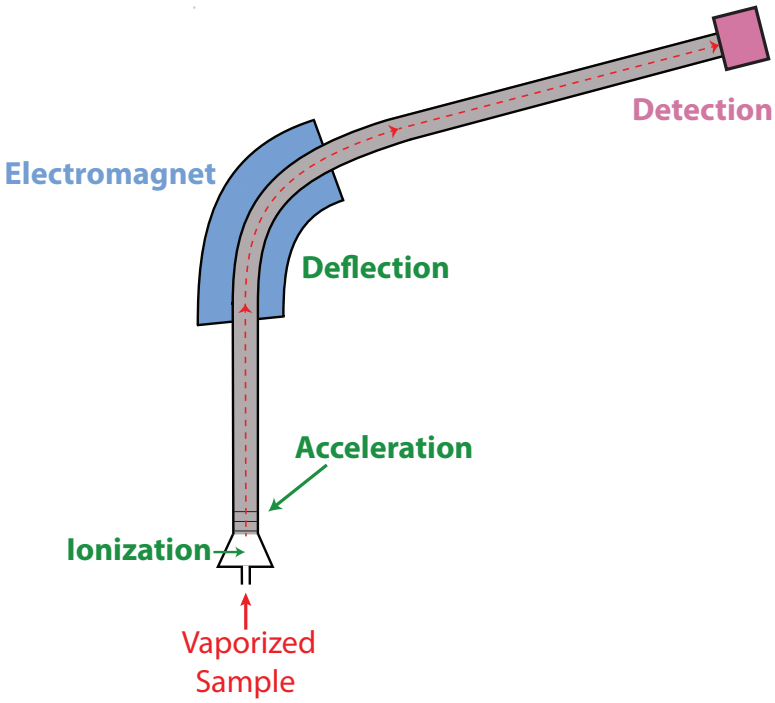
2) Lighter atoms show absorption bands at larger wavenumbers.

3) The stronger the bond, the more energy is required to stretch it. This is because heavier atoms vibrate at lower frequencies:
C=C (1650 cm^{-1}) > C-C (1200 cm^{-1})

Mass Spectrometry

The MCAT Content Outline specifically lists mass spectrometry or "mass spec". This powerful technique is used in research and clinical labs to identify the presence of compounds such as peptides.

Initially, a sample such as a mixture of proteins is digested by proteases, creating a complex mixture of peptides that are separated by liquid chromatography (LC). These samples are then vaporized and injected into the MS device a shown below. In this first step, or ionization, removal of one or more electrons from atoms generates a positive ionic charge within the atoms that make up the peptide fragments. The ionization step establishes the overall mass to charge ratio (m/z) of the peptides. The positively charged peptide fragments are then accelerated so that they have the same kinetic energy. The next step involves the use of an electromagnet and results in deflection of the peptides as a function of their mass and their overall positive charge. An important feature of this step is the degree of deflection depends on the m/z ratio but the electromagnet itself does not determine the m/z ratio. The



deflected peptides are deflected and detected, allowing the mass to be calculated based upon the amount of current produced. A mass spectrum is shown that plots the relative abundance of the detected peptide vs. the charge to mass ratio. Mass spec is a highly sensitive technique and can distinguish between isotopes of the same atom.