A. Other Causes of Hyperbilirubinemia – Hypersplenism

1. Know the following definitions:
   a. Hypertrophy – enlargement of organ or part due to an increase in size of its constituent cells
   b. Hyperplasia – the abnormal multiplication or increase in number of normal cells in a normal arrangement in a tissue
   c. Cytopenia - deficiency of cellular components in the blood
   d. Splenomegaly – enlargement of the spleen

2. Know the following facts about hypersplenism:
   a. Overactive spleen caused by hypertrophy of macrophages or caused by congestion of the spleen (slowing transit time of blood through the spleen)
   b. The condition leads to destruction of up to 90% of platelets and 45% of red blood cells
   c. Criteria for diagnosis (all must be present):
      • Cytopenia
      • Splenomegaly
      • Compensatory marrow hyperplasia
   d. Causes of hypersplenism:
      • Infection
      • Inflammation
      • Hemolytic Disease
      • Splenic Vein Hypertension
B. Basics of the Antibody Immune Response – Antibody production

1. Diagram of process

![Diagram of process]

2. Facts on process diagrammed above:
   a. There are two types of lymphocytes: B lymphocytes and T lymphocytes
   b. Primary lymphoid tissues
      • This is location where mature B cells and T cells are formed from stem cells and then released into the blood and the lymph
      • This process happens without antigen (antigen independent)
      • The lymphoid tissues are:
        ➢ Bone marrow partially and other unknown location for B cells
        ➢ Thymus for T cells
   c. Secondary Lymphoid Tissues
      • Place where the antigen is concentrated and the specific immune response is generated
      • Location
        ➢ Spleen
        ➢ Lymph Nodes
d. The Clonal Selection Theory
Antigen stimulated proliferation of monoclonal subset of mature lymphocytes (see point 2 in diagram)

- There is a specific B cell for each antibody produced of given specificity. Thus there are countless B cell subtypes each dedicated to the production of plasma cells producing one clone of antibodies with one epitope specificity. Antigen binds to the one subtype of B cell that has antibodies on the surface that specifically bind it causing a proliferation of this particular subset of B cells. The antigen also interacts with T cells (although how, is not been defined, as there is not a specific receptor on the cell surface for antigen) that cause the proliferation of T cells that either promote the further proliferation of the B cell subset (T helper cells) or suppress it (T suppressor cells)
- The antibody receptors on the B cells are either monomers of IgM or IgD. These antibodies have identical specificity to IgG and IgM antibodies produced by their progeny
- This occurs in the Secondary Lymphoid Tissue
e. Plasma cells are the cells which produce the antibodies. These cells do not divide.

3. Primary response to antigen
   a. This is response when body first exposed to antigen
   b. The response is primarily production of IgM (small amount of IgG) by the plasma cells
   c. Memory clonal B cells specific for that antigen are produced which prime the body for next exposure to the antigen

4. Secondary response to antigen
   a. Subsequent exposure of the body to antigen activates the clonal memory cells specific for that antigen to produce plasma cells
   b. The antibody response gives a higher concentration (titer) of antibody than the primary response with the production of IgG primarily (some IgM)
   c. This is why vaccines are effective. The initial immunization shots consist of injecting proteins of the infectious agent (or inactivated infectious agent) which lead to the production of memory cells. These memory cells can then be activated quickly and with a high antibody titer response if the infectious agent enters the body, which allows the body to destroy the infectious agent before it can replicate in significant amounts

5. Other facts about antibody production
   a. In order to elicit an immune response the substance must have a large molecular weight (or be attached to a large molecular weight species) and be foreign to the body
   b. An epitope is the part of the antigen that binds specifically to the antibody. A antigen can have many different epitopes which select different B cells to produce antibodies with specificity for the particular epitope
   c. The reason for the body not making antibodies to self components is thought result from the action of T suppressor cells
C. Other Causes of Hyperbilirubinemia—Disorders in which antibodies bind to RBCs

1. Know that there are hemolytic disorders in which antibodies bind to red blood cells which are taken up by macrophages in the spleen. They are stopped by these macrophages because the macrophages have receptors for the Fc portion of IgG (thus since all IgG have a Fc region, the macrophages will bind any RBC that has an antibody bound to it and thus the RBC will be hemolyzed as it stays in the harsh macrophage environment. Also, some antibody–coated RBCs are broken down by complement or complement intermediate (C3b or C3bi) are bound to the RBCs which bind specifically to macrophages which have receptors for these complement intermediates.

2. Know the types of immune hemolytic disorders and the details given below:
   a. **Alloimmune hemolytic anemia disorders**—meaning antibodies are made to foreign components which bind to RBCs. Examples are:
      1’. Imperfectly matched transfusions
      2’. Antibodies made to intestinal bacteria that also bind to RBCs
      3’. Hemolytic Disease of the Newborn
   b. **Autoimmune hemolytic anemia disorders**—meaning antibodies are made to self components
      1’. Warm antibody autoimmune hemolytic anemia disorders (IgG antibodies).
         a’. Mechanism
         In this disease IgG binds to RBC at warm temperatures, causing splenic sequestration by binding of antibody-coated RBCs to Fc receptors on the splenic macrophages
         b’. Types
         - Idiopathic (meaning it is the primary disorder)
         - Secondary to the following; lymphomas, leukemias, other cancers, lupus (collagen vascular disease)
      2’. Cold agglutinin autoimmune hemolytic anemia (IgM antibodies).
         a’. Mechanism
         In this disease, binding of IgM occurs at colder temperatures (hemolysis worse in the winter). Although there is complement activation it is stopped (and thus no lysis) because there is cleavage of the complement factor at the C3b stage. Hepatic macrophages take up these RBCs because it has receptors to C3b and C3bi which are coated on the RBCs
         b’. Types
         - Idiopathic
         - Secondary to lymphoma (chronic) or certain infections (acute)
      3’. Paroxysmal Cold Hemoglobinuria (IgG antibodies)
         a’. Mechanism
In this disease IgG antibodies and complement bind to RBCs at lower temperatures. With increase in temperature lysis of RBCs occurs because of activation of complement proceeding to completion. This is an intravascular hemolysis.

b’. Types
- Most cases are secondary to infection
- Some idiopathic cases

c. Drug-induced immune hemolytic anemia disorders – certain drugs elicit the hemolysis by different mechanisms:

1’. Drugs that bind to RBCs, with subsequent antibodies made to the drug (remember that antibodies are not made against small molecules unless it is attached to bigger component). These antibodies thus bind to the RBCs via the bound drug, which bind to splenic macrophages through their Fc receptors. Penicillin is an example.

2’. Innocent bystander, drug binds to protein, antibodies bind to the protein-bound drug, complement is activated which breaks up both the protein and anything around it (including RBCs). An example is sulfonamide.

3’. Methyl dopa. A small percentage of patients taking this drug develop hemolysis. The drug interferes with the suppression of production of autoantibodies (IgG). These IgG antibodies bind to self components on the RBCs causing sequestration by the splenic macrophages.

3. Know the following details of Hemolytic Disease of the Newborn

a. Overview
This occurs for Rh negative woman (Rh is a particular antigen system on red blood cells) which have there second or more Rh positive pregnancy. The first Rh positive fetus elicits a primary antibody response (since Rh antigens are foreign to the mother), as the delivery exposes large quantities of fetal blood to the maternal blood (or an abortion or ectopic pregnancy). In the next Rh positive pregnancy a small number of fetal RBC cells gain access to the maternal blood system (this is a normal process although the fetal and maternal compartments are considered to be separate) which elicits a big antibody secondary response. These antibodies pass readily through the placenta barrier from the maternal blood to the fetal blood. These maternal antibodies to the Rh antigens bind to the fetal RBCs and the ultimate hemolysis of RBCs.

b. Clinical presentation without intervention
1’. Anemia eventually lead to congestive heart failure (CHF) (the heart overworks as it cannot deliver oxygen to tissues) and edema [the edema arises because of the CHF and also because of a destroyed liver due to extramedullary hematopoeisis (fetus pulls out all stops to make more RBCs) which leads to a decrease in albumin concentration in blood leading to edema].
2’. Intrauterine demise will usually occur. If baby survives to birth lungs cannot expand and liver cannot conjugate bilirubin (kernicterus).
3’. Kernicterus is not a concern of the developing fetus (although it is with the neonate) because mother performs the function of removal of bilirubin. The mother also performs the function of respiration.

c. Treatment
1’. Administer anti (Rh-Ig) antibody after delivery of first Rh positive baby. This minimizes the development of the primary antibody response because it removes the antigen (fetal RBCs) from circulation before it can stimulate the mother’s immune system.
2’. Intrauterine transfusion and earliest possible delivery

d. Testing
1’. Test RBCs of mother and father for Rh antigens. Necessary to this condition developing is Rh negative mother and Rh positive father.
2’. Mother is screened for erythrocyte antibodies.
2’. Bilirubin is monitored in the amniotic fluid after 22 weeks (once every 1 to 3 weeks) to access the severity of hemolysis. When severe hemolysis is indicated intrauterine transfusion is started. After this point bilirubin testing stops because blood is inevitably spilled causing positive error in the test.

D. Other Causes of Hyperbilirubinemia—Pre hepatic causes
- Very common cause of elevated bilirubin is decreased hepatic circulation, which would mean that bilirubin will not be as efficiently processed. This will lead to an unconjugated hyperbilirubinemia
  - Right-sided congestive heart failure
  - Portosystemic shunt

E. Other Causes of Hyperbilirubinemia—Hepatic and biliary causes
1. Drugs can competitively interfere with the uptake and storage of bilirubin, and inhibit the conjugation step leading to unconjugated hyperbilirubinemia
2. Name for condition of impaired storage of bilirubin is Rotor’s Syndrome leading to unconjugated hyperbilirubinemia (serum bilirubin 3-8 mg/dL)
3. Congenital disorders of decreased glucuronyl transferase
   a. Gilbert’s Syndrome
      - 50-90% decrease in glucuronyl transferase activity
      - Benign, asymptomatic
      - Higher prevalence in males (3-7% of population)
      - Serum bilirubin 1-7 mg/dL
   b. Crigler Najar Syndrome Type I
      - Complete absence of glucuronyl transferase activity
      - Severe jaundice and kernicterus leading to death in infancy or early childhood
      - Serum bilirubin >20 mg/dL
   c. Crigler-Najar Syndrome Type II
      - Glucuronyl transferase activity is less than 10%
• Jaundice in late childhood, seldom kernicterus, normal life
• Serum bilirubin is 10-19 mg/dL
4. Dubin-Johnson Syndrome – congenital defect in bilirubin carrier in bile canaliculi which transports conjugated bilirubin from the hepatocyte to the bile. This leads to conjugated hyperbilirubinemia (serum bilirubin around 10 mg/dL)
5. Hepatocellular Disease
   a. Leads to conjugated hyperbilirubinemia due to diminished secretion of the bilirubin into the bile (remember this is an energy expending process and thus is most susceptible to injury)
   b. Most common hepatocellular diseases are hepatitis and cirrhosis
6. Cholestasis – conditions that lead to diminished or stopped flow of bile
   a. Classifications
      • Intrahepatic – various conditions that injure the biliary vessels in the liver (DO NOT NEED TO KNOW THE VARIOUS CONDITIONS FOR THE TEST)
      • Extrahepatic – conditions that lead to blockage of bile ducts outside the liver include:
         ➢ Gallstones
         ➢ Strictures
         ➢ Cancerous obstructions
   b. This leads to a conjugated hyperbilirubinemia because the conjugated bilirubin is not removed from the hepatocyte (since conjugated bilirubin is building up in the bile because the bile is not flowing). The excess conjugated bilirubin in the hepatocyte thus leaks into the blood.
7. Know the facts (and the reasons for the differences) in the following table which shows how to distinguish between hepatocellular disease and cholestasis

<table>
<thead>
<tr>
<th>Laboratory Test</th>
<th>Cholestasis</th>
<th>Hepatocellular Disease</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum Bile Acids</td>
<td>Result: Very high</td>
<td>Result: moderately elevated</td>
</tr>
<tr>
<td></td>
<td>Reason: Bile acids are normally secreted into bile, with decreased bile flow the bile acids back up in the hepatocyte and the blood</td>
<td>Reason: With hepatocyte damage the bile acids are not excreted in the bile efficiently backing up in the hepatocyte and blood, however not as much as if bile is not flowing</td>
</tr>
<tr>
<td>Steatorrhea (fat in feces)</td>
<td>Result: Present</td>
<td>Result: Not present</td>
</tr>
<tr>
<td></td>
<td>Reason: With no bile flow fats are not absorbed from the intestines and thus is excreted in the feces</td>
<td>Reason: Bile flow still occurs at a reasonable rate in hepatocellular disease so fats are absorbed from the intestines</td>
</tr>
<tr>
<td>Serum Alkaline Phosphatase</td>
<td>Result: &gt; 3x normal</td>
<td>Result: &lt; 3x normal</td>
</tr>
<tr>
<td>(ALP)</td>
<td>Reason: backing up bile causes increased synthesis of ALP which leaks into the blood</td>
<td>Reason: Damage to hepatocytes causes release of ALP from the cell into the blood</td>
</tr>
<tr>
<td>Serum Cholesterol</td>
<td>Result: Increased</td>
<td>Result: Decreased</td>
</tr>
<tr>
<td></td>
<td>Reason: With decreased bile flow the reaction of cholesterol to bile acids is shifted towards the reactant (cholesterol) because the product bile acid is not removed into the bile. Therefore there is excess cholesterol present in the hepatocyte which backs up into the blood</td>
<td>Reason: Damage to hepatocyte compromises its ability to synthesize cholesterol</td>
</tr>
<tr>
<td>Serum Transaminases (AST, ALT)</td>
<td>Result: Mildly elevated</td>
<td>Result: Elevated</td>
</tr>
<tr>
<td></td>
<td>Reason: The cause of increased clotting time for cholestatic disorders is the lack of vitamin K needed for synthesis of coagulation proteins. Vitamin K is a fat soluble vitamin that needs bile in the intestines to be absorbed. With decreased bile flow diminished levels of vitamin K are available. With IV administration vitamin K is made available and thus coagulation proteins can be synthesized</td>
<td>Reason: In hepatocellular disease there is more damage to hepatocyte than in cholestatic disease leading to greater release of hepatocytes contents into the blood. AST and ALT are two enzymes present in hepatocytes that are released when the cell is damaged</td>
</tr>
<tr>
<td>Prothrombin time response (clotting time decreases back to normal) to IV administration of vitamin K</td>
<td>Result: Yes</td>
<td>Result: No</td>
</tr>
<tr>
<td></td>
<td>Reason: The cause for increased clotting in hepatocellular disease is that there is diminished function of the liver, including its ability to synthesize coagulation proteins. Vitamin K availability is not the problem.</td>
<td>Reason: The cause for increased clotting in hepatocellular disease is that there is diminished function of the liver, including its ability to synthesize coagulation proteins. Vitamin K availability is not the problem.</td>
</tr>
</tbody>
</table>
8. The usually concentration of serum bilirubin in chronic hemolytic anemias is 3-4 mg/dL.

F. Hepatitis
1. Hepatitis is inflammation of liver caused by viral or toxic agent.
2. Know the components of the virus
   - Nucleic acids
   - Protein coat
     - Protects nucleic acids
     - Some proteins involved in binding to receptors on host cells
   - Lipid envelope (only some viruses)
   - Replications enzymes (some viruses have this)
3. DO NOT NEED TO KNOW MECHANISM OF VIRAL REPLICATION OR MECHANISM OF VIRAL INJURY TO HOST
4. Know that enzymes that do NOT have lipid envelope are active in intestinal tract, those that have a lipid envelope are inactivated in the intestinal tract because of bile acids disrupting the envelope.
5. Know the phases of acute hepatitis which ultimately leads to recovery
   a. Incubation
      - Period of viral reproduction without symptoms
      - Lasts 1 to 5 months depending on virus type
      - Patient infectious
   b. Preicteric
      - Period when symptoms (flu-like) appear
      - Prior to appearance of hyperbilirubinemia
      - Enzymes AST and ALT in serum rise
      - Patient has highest virus concentration (most infective) at end of the incubation period and early days of acute symptoms
   c. Icteric
      - Appearance of jaundice
      - Symptoms begin clearing
   d. Convalescence
      - Recovery phase

The time period for the combined preicteric and icteric phase is a few weeks to several months.

6. Do NOT need to know the histiological changes that occur for hepatitis

7. Know the following about Hepatitis A
   a. No long term consequences – most all go to full recovery
      - No carriers
      - No development of a chronic condition
   b. Mode of transmission - oral
      Since the virus does not have a lipid envelope it is present in the intestinal tract. Thus the mode of transmission is through introduction of fecal
material into the body, including ingestion of water or food contaminated with fecal material.
c. High risk in developing questions that have poor sewage disposal, military camps, homosexuals
d. The virus is an RNA virus without lipid envelope
e. Markers
   • IgM-HAV is a marker for recent exposure
   • IgG HAV provides immunity
8. Know the significance of the following markers for Hepatitis B
   a. HBsAg
      • Earliest marker to rise
      • Indicator of development of carrier or chronic state if still present after 6 months
   b. HBeAg
      • Indicates infectivity
      • Development of chronic state if elevated for > 20 weeks
   c. Anti-HBe
      • Indicates loss of infectivity
   d. Anti-HBs
      • Indicates recovery from acute disease
      • Individual has immunity to subsequent exposure (8-10 years)
      • Can result from either past exposure or immunization
      • If present, not chronic disease or carrier
   e. Anti HBc(IgM)
      • Indicates recent infectious exposure
      • Useful in window period
   f. Anti-HBc (IgG)
      • Definitive marker for past exposure (not present with immunization by vaccine)
9. Know the values of pertinent markers (i.e. whether they are present or not) during the following stages of Hepatitis B (as given below):
   a. Infectious
      • HBeAg (positive)
      • HBsAg (positive)
      • Anti-HBe (negative)
      • Anti-HBs (negative)
   b. When the patient has passed from an infectious period to not being infectious
      • HBeAg (negative)
      • Anti-HBe (positive)
   c. Window period
      • HBeAg (negative)
      • HBsAg (negative)
      • Anti-HBe (positive)
      • Anti-HBs (negative)
      • Anti-HBc (IgM) (positive)
d. Recovery is indicated
   - HBeAg (negative)
   - HBsAg (negative)
   - Anti-HBs (positive)
   - Anti-HBc (IgM) (positive)

e. Past exposure (not recent)
   - HBeAg (negative)
   - HBsAg (negative)
   - Anti-HBe (negative)
   - Anti-HBc (IgM) (negative)
   - Anti-HBc (IgG) (positive)
   - Anti-HBs (IgG) (positive)

f. Immunization by vaccine but no past exposure
   - HBeAg (negative)
   - HBsAg (negative)
   - Anti-HBe (negative)
   - Anti-HBc (IgM) (negative)
   - Anti-HBc (IgG) (negative)
   - Anti-HBs (IgG) (positive)

g. Long term infectious carrier
   - HBeAg (positive)
   - HBsAg (positive)
   - Anti-HBe (negative)
   - Anti-HBs (IgG) (negative)

10. Know the various outcomes of a hepatitis B exposure
    - No manifestations (60-65%)
    - Acute Hepatitis (20-25%)
      - Recovery (99% of acute cases)
      - Fulminant condition (1% of acute cases)
    - Chronic Hepatitis (5-10%)
      - Chronic Persistent (33% of chronic cases)
        - Smoldering disease that eventually resolves in several years with no lasting consequences
      - Chronic Active (67% of chronic cases)
        - Progression to cirrhosis and eventual death either from hepatic failure or development of liver cancer
    - Carrier (5-10%)
      - No signs of disease but is infective (although there is a carrier classification that has elevated HBsAg but is not infective)
      - Some carries develop liver cancer

11. Know that chronic hepatitis is indicated if there is hepatic inflammatory disease beyond 6 months. Know that the only way that Chronic Persistent and Chronic Active Hepatitis can be distinguished is through a biopsy in which Chronic Active Hepatitis shows the distinguishing features of piecemeal necrosis and bridging necrosis
12. Know other facts about Hepatitis B
   a. The virus is a DNA virus with a lipid envelope
   b. That the mode of transmission is through parental incidents [direct contact of
      infected blood to person’s blood system via needle stick, syringe needle
      (drug user), infected blood transfusion, cut or injured skin], sexual contact, or
      perinatal
   c. Besides blood virus is present in saliva, breast milk, vaginal secretions,
      semen, ascetic fluid
13. Know the following for Hepatitis D infection
   a. There needs to be the co-presence of HBsAg along with the Hepatitis D
      virus (RNA virus). This can occur either through coinfection of HBV and
      HBV or a superinfection in which HDV infects a HBV carrier
   b. Markers:
      • Anti-HD (IgM) – marker for recent acute infection
      • HDAg – if persists for long time indicates chronic state
      • Anti-HD (gG) – confers immunity
   c. The superinfection route has a high incidence of chronic disease (10-40%) or
      fulminant (10%)
14. Know the following about Hepatitis C
   a. That it is an RNA virus
   b. Outcome from acute infection
      • Recovery (20%)
      • Chronic Hepatitis (80%)
        ➢ 20% of these cases develop into cirrhosis which can then
        progress to liver cancer
      • Some cases do go to fulminant condition leading to death
15. Know about the testing for Hepatitis C virus
   a. The testing strategy is:
      • Use enzyme immunoassay (EIA) test for screening test
      • For positives use RNA immunoblot assay (RIBA) for confirmations
   b. Each of the above tests for antibodies to HCV antigens, which can be
      detected 80-90 days after infection
   c. The EIA test tests for several different antibodies to HCV antigens at the
      same time. A positive is indicated if one or more of the antibodies is present,
      however it cannot distinguish which antibodies are present. The test is
      sensitive but has many false positives thus requiring a second confirmation
      test
   d. The RIBA test has four HCV antigens immobilized on a strip, each antigen at
      a different location of the strip. Antibodies to HCV antigens from a patient
      sample will bind to the particular immobilized Ag which can be detected by
      further chemical steps. This test will be able to identify which particular
      antibodies are present since each Ag is present in a particular location on the
      strip. Results are as follows:
      • 2 or more HCV antibodies identified is confirmation of HCV
      • no HCV antibodies identified means test is negative for HCV
      • 1 HCV antibody present is indeterminant
e. The newer generation EIA and RIBA tests included more antigens immobilized to the well for EIA (see point 16 below) or the strip for RIBA which made the test more sensitive in detecting the disease (shortening the time from the incidence of infection to detection of HCV virus in blood)
f. An RNA test has also been developed. It has the following features:
   - Earliest detection of any test 1-3 weeks after infection
   - Used for monitoring progression of disease and to determine viral load, which is used to predict effectiveness of and for monitoring of interferon treatment
   - Problematic because RNA mutates extensively compared to DNA so the tests have to be continually updated

g. Detecting HCV in blood as early as possible after infection is imperative for blood banks

16. Know how an EIA test is done
   a. Several HCV antigens are added to plate well which stick to the plate well (which is made up of a hydrophobic polymer and thus the antigens stick through hydrophobic forces). Note that after this step a protein such as BSA is added to the plate well to cover any active sites on the plate well.
   b. Sample is added. If there is any antibodies to HCV present then these antibodies will bind tightly to antigens immobilized on the plate well.
   c. A wash step is performed to wash off any component in the sample not bound to the plate well.
   d. A reagent antibody is added. This antibody binds to IgG. The reagent antibody is labeled with an enzyme that can be used for quantitation. This labeled antibody will bind to HCV antibodies bound to antigen immobilized on the well.
   e. Another washing step is performed to wash off any unbound labeled antibody.
   f. Substrate to the enzyme is added and the reaction is allowed to occur for some time to generate a product, which is usually colored (substrate is colorless). This solution is then measured spectrophotometrically to determine absorbance.
   g. The absorbance is proportional to the concentration of product which is proportional to the amount of labeled antibody present, which is proportional to the amount of HCV antibodies present.

17. Know that Hepatitis E is the other virus that is transmitted orally through ingestion of fecal material or water or food contaminated with fecal material. The virus is an RNA virus that does not have a lipid envelope.
G. **Statistics**
1. Be able to do all the problems in the problem set or that were example problems in class.
2. Know (memorize) the following formulas and how to calculate the from the formulas if you are given a set of data:
   - Mean
   - Median
   - Standard deviation
   - % coefficient of variation
3. Know the definition of accuracy and precision
4. Know that for a probability distribution function:
   - That it is a continuous function of frequency (or number) of data points having a particular value versus the value measured
   - That the plot is normalized such that the area under the curve is 1
   - The probability of having a data point within a certain range is given by the area under the curve in that range
5. Know the following characteristics of the Gaussian Distribution
   a. It is a probability distribution characterized by $\mu$ and $\sigma$
   b. The distribution is symmetric bell shaped curved centered on $\mu$
   c. That the area under the curve is:
      - $\mu \pm \sigma$ is 0.683 (68.3% of the area)
      - $\mu \pm 2\sigma$ is 0.955 (95.5% of the area)
      - $\mu \pm 3\sigma$ is 0.997 (99.7% of the area)
      - $\mu \pm z\sigma$ is $2z$ (z value given in the table handed out in class)
6. Know (memorize) the formula for Z value and be able to state in words what it means.
7. Know that the conditions and conclusions of the central limit theorem:
   a. One of the following conditions must apply:
      - The original population is Gaussian, $n$ can be any value ($n$ is sample size)
      - $n \geq 30$
   b. Conclusions
      - The sample means have a gaussian distribution
      - The mean of the sample means equals $\mu$ (the true mean of the population)
      - The standard deviation of the sample means [called standard error of the mean (SEM)] is $\sigma/(n^{1/2})$
8. Know the following general formulas giving the range in which the true mean of population is in relation to an experimentally determined sample mean
   a. When the true standard deviation of the population (σ) is known or can be accurately determined (if sample size n ≥ 30)
      \[ X \pm (Z)^* \left[ \frac{\sigma}{(n^{1/2})} \right] \]
      (Estimator of μ) (Reliability Coefficient)[Std Dev of the Estimator]
   b. When the true standard deviation of the population is not known or cannot be accurately determined (n < 30)
      \[ X \pm (t)^* \left[ \frac{s}{(n^{1/2})} \right] \]
      (Estimator of μ) (Reliability Coefficient)[Std Dev of the Estimator]
      where s is the sample standard deviation

9. Know that in point 8 above that one chooses the confidence limits by choosing the appropriate reliability coefficient value.
   - Thus if z values can be used (σ is known), then one determines the level of desired confidence and finds this value as a fraction on the z table (remember to double the value given on the table handed out in class) and reads off the z value having this value. Thus if one wants to define the range in which there is 88.12% confidence given by the equation in point 8a, one would choose a z value of equal to .4406, which is z = 1.56.
   - If t values should be used (equation in point 8b) then one looks at the t table for the given confidence (note that the t table handed out in class gives fraction values of 0.5, 0.6, 0.7, 0.8, 0.9, 0.95, 0.98, 0.99, which give confidences of 50%, 60%, 70%, 80%, 90%, 95%, 98%, and 99% respectively). One then reads off the t value directly. Thus if one wants to define the range in which there is 80% confidence, for a sample size of n=10, then the t value is 1.383 is used for the equation in point 8b (reading off the value for υ=9).

10. Know that a t distribution:
   - Is centered around 0
   - Is symmetrically shaped
   - Is less peaked in the center and has higher tails than the Gaussian distribution, to take into account greater uncertainty when σ is not known
   - Has a different plot depending on the value of υ (n-1)
   - The t distribution becomes more like the Gaussian distribution the higher the value of υ and at very high sample sizes is Gaussian (because σ can be accurately estimated at values of n ≥ 30)
   - Assumes that the population itself is Gaussian

11. Be able to know how to use the table of t values. Particularly:
   - How to calculate the number of degrees of freedom (υ = n-1)
   - When to use t table (when σ is not known or can accurately be determined)
12. Know (memorize) the formulas and the assumptions for calculating if two means from two populations are different.
   a. For the case in which the $\sigma$ is known for each population and assuming each population is Gaussian distributed (or $n \geq 30$ for each population) then use the following equation for determining the range of the true difference of the population means ($\mu_1 - \mu_2$):

   $$(X_1 - X_2) \pm (Z)^* \left[ \left( \frac{\sigma_1^2}{n_1} + \frac{\sigma_2^2}{n_2} \right)^{1/2} \right]$$

   Estimator of ($\mu_1 - \mu_2$) (Reliability Coefficient)[Std Dev of the Estimator]

   If zero is within the range defined by the above equation then there is no significant difference between the true means $\mu_1$ and $\mu_2$ as determined by the sample means

   b. For the case in which $\sigma$ is NOT known for either population (assumes that the $\sigma$ is the same for each population) then use the following equation for determining the range of the true difference of the population means ($\mu_1 - \mu_2$):

   $$(X_1 - X_2) \pm (t)s_p^* \left[ \left( \frac{1}{n_1} + \frac{1}{n_2} \right)^{1/2} \right]$$

   Estimator of ($\mu_1 - \mu_2$) (Reliability Coefficient)[Std Dev of the Estimator]

where $s_p$ is the pooled standard deviation from the sample standard deviation determined from each of the populations (i.e. $s_1$ and $s_2$) as given below

$$s_p^2 = \frac{[(n_1- 1)s_1^2 + (n_2 - 1)s_2^2]}{(n_1+n_2-2)}$$

and note that the value for the number of degrees of freedom to use for determining the appropriate t value is:

$$\nu = n_1 + n_2 - 2$$

If zero is within the range defined by the above equation then there is no significant difference between the true means $\mu_1$ and $\mu_2$ as determined by the sample means

13. Know the definition of quality control:
   Quality control is procedures that assess the performance of a methodology with respect to precision and accuracy.

14. Know the following about errors:
   a. Random errors ($2s$) increase the variability of the measured values (precision)
   b. Systematic error ($|\mu - X|$) is a shift of the measured value from the true value (accuracy)
   c. Total error = random error + systematic error = $2s + |\mu - X|$

15. Know how controls are used to assess quality control
   a. The control is a sample in similar matrix to that of patient samples is rigorously measured over a period of time to determine $X$ and $s$
b. The controls are run with patient samples to verify that the technique is giving results that have sufficient accuracy and precision. This is done by comparing the results for the controls with the range $X \pm$ limits (usually $\pm 2s$) established as given in 15a. If the results for the control is within this range then the run is acceptable (called “in-control”) if it is outside this range the run is not acceptable (called “out-of-control”).

c. Two controls at two different concentration are usually run. One of the concentrations is in the normal range (healthy individual) and the other is at a level indicative of disease.

d. Frequency that the control is run depends on the stability of the methodology. It can be run each day, each shift or each run. In rare cases it may be run once a week.

e. A Levy-Jenny plot is a plot with the $X \pm$ limits verses days follows the performance of each control (one plot for each level control for each analyte) over a number of days (one control value per day is plotted). It can identify (know what each looks like):

- Trend (steady slow uni-directional change in control value measured indicating a problem such as a reagent slowly degrading)
- Shift (sudden change in the control value measured indicating a problem such as a change in technologist doing the test or different lot of reagent used)
- Random Error (wide fluctuation of control value measured indicating a problem such as loss of temperature control)

16. Power function assesses the effectiveness of a particular quality control procedure in detecting a systematic or random error.

- It is a plot of probability that a control is beyond the limit (or a rule violated) verses the amount of error
- The power function detecting systematic error is a plot of probability of detecting a systematic error (i.e., that the measured control value will be beyond the limit) verses the magnitude of the systematic error (given in number of standard deviations)
  - The probability of false rejection is given by the intersection of the plot with y axis
- The power function detecting random error is a plot of probability of detecting a random error (i.e., that the measured control value will be beyond the limit) verses the magnitude of the random error (given as the multiple increase of the standard deviation “s”)
  - The probability of false rejection is given as the value at x=1 (meaning there is no increase in my random error)

17. Know how to calculate the value of a power function for given limits (such as 1s, 2s, or 3s) for:

- A certain systematic error (for example 1.5*s)
- A certain increase in random error (for example a factor of 2.5)

18. When the number of controls per run are increased (with the run being unacceptable if any of the controls are out) this has the following effects:
a. Advantage – increases the sensitivity in detecting the error (i.e. curve shifts upward)
b. Disadvantage – probability of false rejection increases

18. Comparison of using 2s limits to 3s
   • 2s limits has much higher probability of detection of error but also has higher probability of false rejection