

# Unit 4 Cram Sheet

## Introduction

In Unit 4, we are introduced to one of the most unfortunate of the Smith family's relatives: Diana Jones, the sister of Judy Smith. Diana is a Type II Diabetic who now has her diabetes under control because she has learned to regulate her sugar with an insulin pump. That was not always the case, and Diana's past choices will have a huge impact on her future.

## Lesson 4.1: Manufacturing Human Proteins

Because we are focusing on Diana Jones, a diabetic, our unit began with a study of the hormone insulin. Insulin is a protein that diabetics often use to regulate their blood sugar. In some forms of diabetes, the body does not make insulin, so sugar remains in the blood stream, causing damage to surrounding tissues. A diabetic who cannot make their own insulin must inject insulin from another source. Prior to the development of human insulin, people used to extract insulin from cow pancreases. Because this was not HUMAN insulin, it was less effective. Today, we have the technology to create human insulin, which means people have a safer source of insulin that is more effective.

As crazy as it sounds, this insulin is produced by bacteria. In Lesson 4.1, the big portion of the activity was learning how this happens. Though we created a glow-in-the-dark protein instead of insulin, the process is the same. It is outlined below.

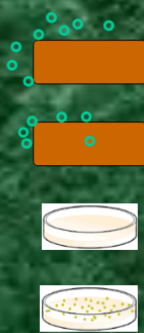
## **Bacterial Transformation**

Creating a human protein must begin with finding something to "grow" that protein. The easiest living organism to use for this purpose is bacteria. This involves using recombinant DNA technology to custom-design bacteria that can produce the proteins we want. This process has several steps, which will be discussed next.

Remember that a typical bacteria may contain plasmids: small, circular strands of DNA that replicate when the bacteria do and often contain antibiotic resistance (think rubber band made of genes). These plasmids can be modified by people through genetic engineering to contain other traits. To modify a plasmid, it is first sequenced so we know its composition. After its sequence is known, restriction enzymes are selected that will cut specific sequences of the plasmid DNA. After cutting with restriction enzymes, the plasmids are like a rubber band that has been cut with scissors. Either end of the cut contains "stick ends" - unpaired nucleotides that are able to bind to other, matching nucleotides. The gene to be added to the plasmids is then mixed with the cut plasmids. The sticky ends of the plasmid and the desired gene fragment bind, and are sealed together by DNA ligase. This creates a newly sealed plasmid that contains more DNA than the original. That extra DNA contains the gene for the protein we want the bacteria to produce. To make it simple, plasmids are changed to contain new genes, so they can produce human proteins. These modified plasmids will then be

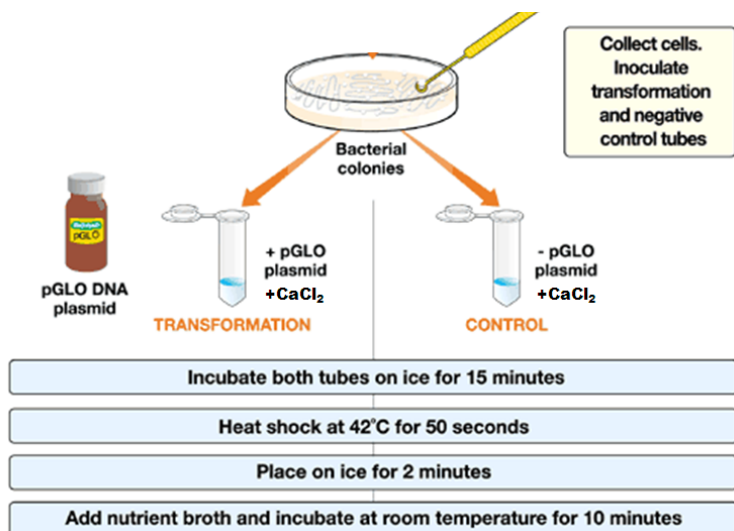
# Bacterial Transformation Lab

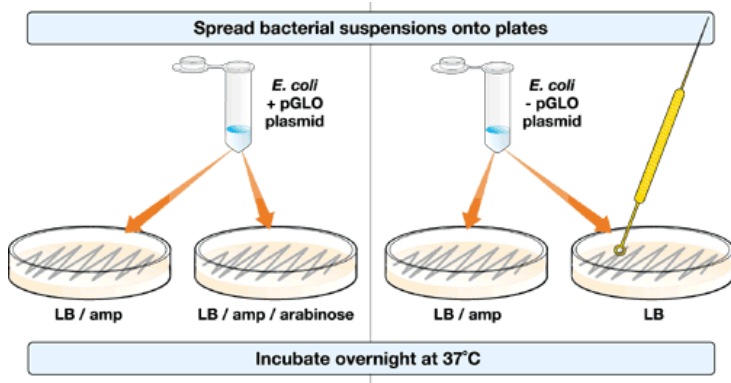
- Bacterial Cells and plasmid DNA are mixed
- Cells take up plasmid
- Cell/DNA mix is plated on nutrient agar with antibiotic
- Only cells which obtained plasmid DNA will grow... and glow



added to bacteria.

When choosing a bacterium for bacterial transformation, a simple, non-infectious bacteria is preferred - something like E. coli. The E. coli and the modified plasmid are mixed together in a vial and subjected to a form of shock. The bacteria are chilled in calcium chloride. This calcium chloride neutralizes the negative charge of DNA phosphate and phospholipids in the cell membrane, making it possible for plasmids to enter the bacterial cells. The cold slows cell membranes down and makes them more responsive to the calcium chloride. The bacteria then undergo heat-shock, which makes the cell membranes more permeable (more full of holes) that the plasmids can slip through. The two together - calcium chloride and heat shock - are critical in allowing the plasmids to slip into the bacteria, completing transformation. The bacteria are then allowed to recover in some nutrient broth, and the plasmids are sealed inside them. At this point, bacterial transformation is complete, and the bacterial cells are “plated” to allow the transformed cells to grow, reproduce, and make more of themselves. The plasmids used for transformation contain a gene for antibiotic resistance, and the bacteria are plated on agar containing a specific antibiotic. This prevents untransformed bacteria from growing, to ensure that bacteria used in later steps of the procedure actually contain the human protein desired.





## Protein Purification

We now have bacteria producing human proteins, but we are not done. In order to make those human proteins useful to people, we have to get them OUT of the bacteria. This is done by killing the bacteria and using a series of steps to extract the useful human proteins from the dead cell waste. The steps are described below.

The bacteria growing on a petri dish should contain the human protein of interest, since the gene holding the human protein coding also holds a code for protein resistance. A single, well-developed colony of these bacteria must first be transferred from a petri dish to a small vial. They are then grown overnight, then centrifuged to force the bacteria down into a pellet at the bottom of a microcentrifuge tube.

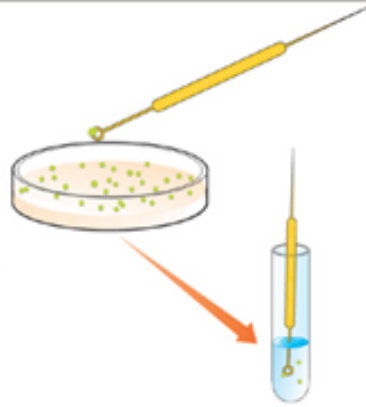
Once spun down, the pellet is resuspended in something called lysozyme, which ruptures the cell membranes. The cells are recentrifuged to help pellet the cell "waste" while the proteins remain in the fluid. Since we are trying to extract the proteins at this point, this fluid is saved.

To the protein-laden fluid, we add a binding buffer. This binds to the proteins in solution. This fluid is then run through a chromatography column for the first time. The first time the fluid is run through the tube with binding buffer, all the proteins will become bound up in the pellets within the chromatography column. We then added a slightly salty buffer, which forced unwanted (hydrophilic) proteins out of the chromatography column to be collected in a second test tube. This left just the desired proteins (which are hydrophobic) bound up in the pellets within the chromatography tube. It separated them from the remaining proteins the cell produced. At this point, all the human proteins created by the bacteria are trapped within the chromatography tube. A final solution, a low-salt buffer, is added to the chromatography column. As it trickles through, it separates the hydrophobic GFP proteins from the column, and they wash into a third test tube, where the desired proteins are finally captured. Please note: this discussion is about hydrophobic human proteins . . . the steps would be slightly different if we were talking about hydrophilic human proteins or some other form. Because GFP (the glowy protein) we extracted was hydrophobic, we have focused our discussion on that sort of protein.

At this point, the third tube should hold the desired protein and nothing but the desired protein. Technically, creating and extracting the protein is complete. However, if you are making a human product, quality and purity MUST be checked before it can be sold.

Start with bacterial colonies transformed with pGLO plasmid DNA

Pick a single fluorescent green colony from the agar plate using a sterile inoculation loop



Inoculate into nutrient broth containing ampicillin and arabinose

Grow overnight at 32°C or 2 days at room temperature with shaking

## Day 1

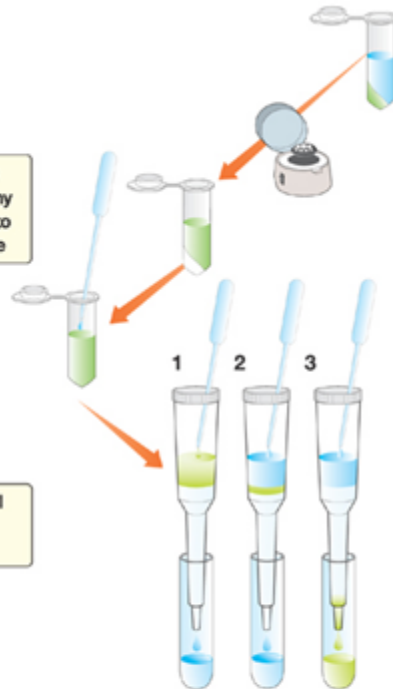
Transfer cell culture to micro test tube, then centrifuge and pellet cells



## Day 2

Add high-salt chromatography binding buffer to bacterial lysate

Load bacterial lysate onto columns



Resuspend cells, add lysozyme, and freeze to rupture cell membranes; then centrifuge bacterial lysate to pellet membranes and debris

1. GFP binds to chromatography matrix in high-salt buffer

2. Add medium-salt buffer to wash bacterial proteins from column

3. Add low-salt buffer to elute GFP

Collect three fractions

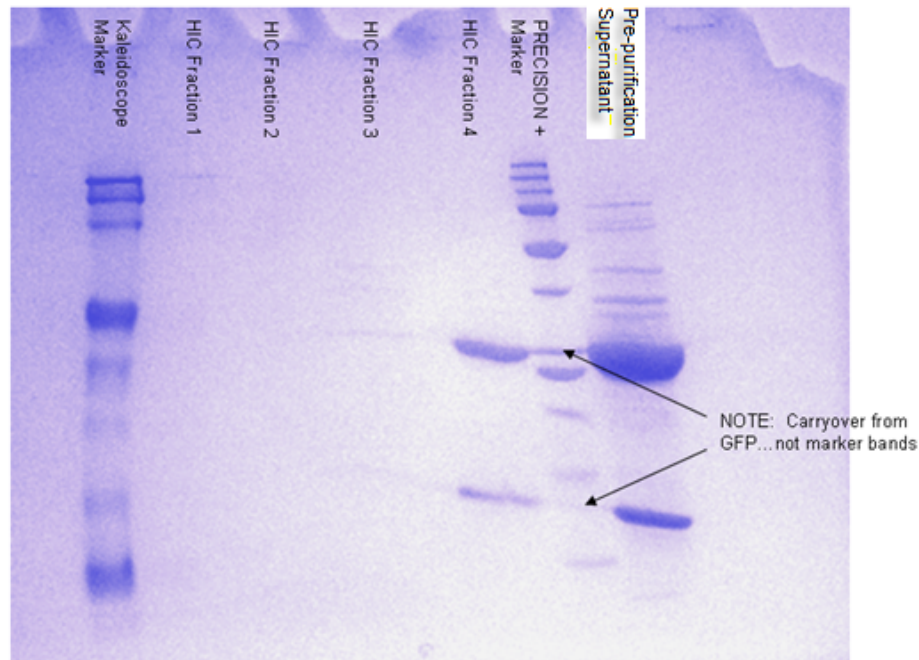
Separate GFP from bacterial proteins

**Extension:** Use protein gel electrophoresis to conduct quantitative and qualitative analysis of fractions

## Day 3

## Purity Confirmation with Gel Electrophoresis

The final step in creating human proteins is checking that purity using something called vertical electrophoresis, a form of gel electrophoresis. In this case, running buffer, fluid from each of the test tubes, and two forms of protein markers are run on a super-skinny acrylic gel. Separation occurs as it does for all forms of electrophoresis, with an electrical current performing the separation and larger fragments moving slower/traveling less far than smaller fragments. Below is a picture of the results. Note that in this case, the protein was NOT pure. If this were being created for humans, another attempt would need to be made to get to the proper level of purity.



## Lesson 4.2: Organ Failure

So, we now know how to make human proteins. The procedure just discussed is how many human proteins are made today, including the human protein insulin. Insulin, you may recall, is commonly administered to people with diabetes in order to regulate their sugar levels. People who use insulin are called diabetics. Unregulated diabetes can cause many problems, starting with diabetic symptoms: frequent urination, constant thirst, rapid weight loss, etc. Complications can then begin to build up. These complications include diabetic retinopathy (vision loss), diabetic neuropathy (nerve damage), fatigue, etc. Finally, kidney function can be affected. Unregulated diabetes can lead to something called kidney failure, where the kidneys shut down and are unable to work properly. This means that blood does not get filtered properly, toxins build up in the blood, and the body becomes poisoned. It will lead to death if not treated properly.

In Lesson 4.2, we met Diana Jones, the sister of Judy Smith. Diana was a long-term diabetic who, in the past, did not manage her diabetes well. Because of this, permanent damage to her kidneys occurred, and we learned that Diana

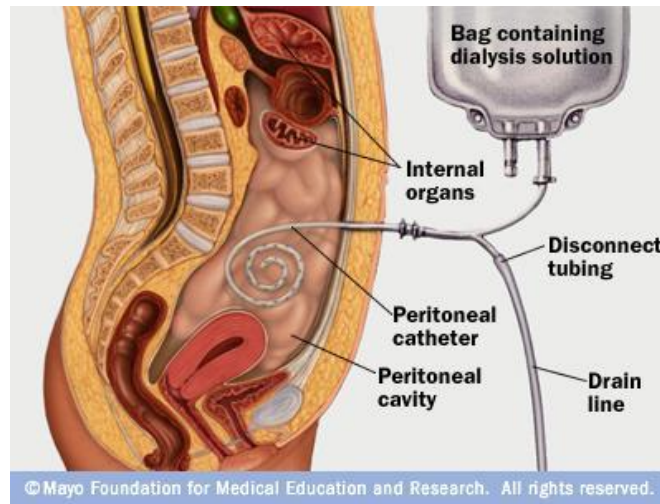
was facing a diagnosis of ESRD, or end-stage renal disease. Her kidneys had failed. This disease is diagnosed using a few different tests, whose results are shown below for Diana:

| Test:                            | Result(s):                                                                                         |
|----------------------------------|----------------------------------------------------------------------------------------------------|
| Blood Urea Nitrogen Levels       | 60 mg/dL                                                                                           |
| Blood Creatine Levels            | 2.8 mg/dL                                                                                          |
| Blood Potassium Levels           | 7.1 mEq/L                                                                                          |
| Red Blood Cell Count             | 3.6 million cells/mcL                                                                              |
| Glomerular Filtration Rate (GFR) | 13 mL/min                                                                                          |
| Urinalysis                       | Presence of red blood cells<br>Presence of white blood cells<br>High levels of albumin (300 mg/dL) |
| Blood Pressure                   | 140/90                                                                                             |
| EKG                              | Normal                                                                                             |

All these different tests offer clues to the diagnosis of ESRD, which is confirmed with the kidney-based tests: GFR and Urinalysis. If the kidneys fail, what options are available.

Though a kidney transplant is the best option for people with ESRD, it is not always possible. While waiting for a transplant (or when a transplant is not an option) people can undergo dialysis. Dialysis is an artificial process that removes waste products and excess water from the blood when the kidneys can no longer function.

Two options of dialysis are available for people with kidney failure: hemodialysis and peritoneal dialysis. Hemodialysis involves using a machine to filter the blood outside the body. A person is hooked up to an intravenous line that removes their blood a little bit at a time, filters it, and pushes it back into the body. Review this procedure at <http://www.mayoclinic.com/health/hemodialysis/MY00281> (this website has multiple pages that are all useful - don't just use the one called "Definition." The second option, peritoneal dialysis, is a bit different. Here, the body's own peritoneum (the fatty membrane covering the intestines, is used to filter the blood and collect wastes. Review this procedure at <http://www.mayoclinic.com/health/peritoneal-dialysis/MY00282>. (Note that, like the other page, there's lots of information not on this first page. Use the menu on the left side of the screen for additional information.)



So, Diana has ESRD, and is temporarily using dialysis to extend her life. Without this treatment, her own blood would poison her. Diana knows this needs to be a temporary treatment, and thankfully she has lots of family willing to donate a kidney for her.

### **Lesson 4.3: Transplant**

The reality is, there are far more people in need of organs than there are willing donors. Some facts to keep in mind: A name is added to the national transplant waiting list every 13 minutes. It is estimated that more than 70 people's lives are saved by organ transplantation each day. Unfortunately, because there are more people in need of an organ transplant than there are organ donations, it is estimated that almost 20 people die each day waiting for a donated organ. Organ donation and allocation are strictly regulated by federal guidelines which ensure that organs are distributed in a fair and equitable manner. The first step in allocating donated organs is to match the recipient with the donor. The donor and recipient not only need to have compatible blood types but also need to have compatible tissue types. Currently, the average national wait time for a donated kidney is longer than three years. There are more than 78,000 people in the United States presently waiting for a kidney transplant. When possible, family remains the best option for kidney transplants. They are more likely to be matches, and getting a donation from a relative allows people in need of kidneys to bypass the long wait time and get kidneys sooner.

We began by discussing the rules and regulations surrounding organ donation. It became clear that deciding who receives donated organs is not always a clear-cut issue and involves many difficult decisions guided by federal policies. The final decision as to who receives an organ involves multiple policies outlined by NOTA (the National Organ Transplant Act) and OPTN (the Organ Procurement and Transplantation Network).

#### **The National Organ Transplant Act**

- Outlaws the sale of human organs.
- Specifies that the OPTN establish medical criteria for organ allocation including compatibility of the donor and the recipient and medical urgency (medical urgency only considered for heart, liver, and intestine transplantations).

- Excludes social criteria such as celebrity status, wealth, or prison status, from medical criteria which are not permitted in consideration of organ allocation.
- Specifies that a potential candidate for organ transplantation whose test for HIV is positive, but who is in an asymptomatic state should not necessarily be excluded from candidacy for organ transplantation, but should be advised that he or she may be at increased risk of morbidity and mortality because of immunosuppressive therapy.

The OPTN Allocates Organs based on the following criteria:

- Compatibility of the donor and recipient.
- Geographical proximity between donor and recipient.
- Time on a waiting list.
- Age of recipient (preference given to children).

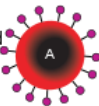
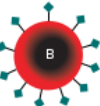
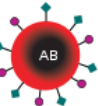
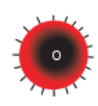
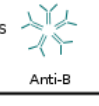
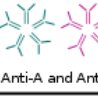
These rules and criteria seek to reduce the subjective reasons people might select one recipient over another. This needs to be an emotionless, fair process based on need. Again, that isn't always easy, and may not seem fair.

After being placed on the waiting list, people waiting for kidneys have certain tests performed so that a likely match can be found either from the donor banks or from a family member. Donors receive many of the same tests. These will be discussed next.

One of the first – and simplest – tests when finding an organ match is blood typing, which works to find out the blood type of both potential donors and recipients. There are four common blood types: A, B, AB, and O. Blood is also classified as positive or negative based on Rh factors. Blood type and Rh factor simply discuss antigens on the outside of cells. Blood cells labeled “A+” contain both A antigens and Rh antigens. Someone who is “A-” contains A antigens, but not Rh factor. The following charts summarize blood types:

| Blood Type | Genotype             |          | Can Receive Blood From: |
|------------|----------------------|----------|-------------------------|
| A          | $i^A i$<br>$i^A i^A$ | AA<br>AO | A or O                  |
| B          | $i^B i$<br>$i^B i^B$ | BB<br>BO | B or O                  |
| AB         | $i^A i^B$            | AB       | A, B, AB, O             |
| O          | $ii$                 | OO       | O                       |

|                     | Group A                                                                                       | Group B                                                                                        | Group AB                                                                              | Group O                                                                                                    |
|---------------------|-----------------------------------------------------------------------------------------------|------------------------------------------------------------------------------------------------|---------------------------------------------------------------------------------------|------------------------------------------------------------------------------------------------------------|
| Red blood cell type |            |            |  |                       |
| Antibodies present  | <br>Anti-B | <br>Anti-A | None                                                                                  | <br>Anti-A and Anti-B |
| Antigens present    | A antigen                                                                                     | B antigen                                                                                      | A and B antigens                                                                      | None                                                                                                       |

Blood tests are a quick, easy, cheap way to determine people who are not good donors. If donor and potential recipient do not have the same blood type, the recipient cannot get a kidney from that donor. People who are blood type matches then have further testing done. Keep in mind that the test works by checking for agglutination – clumping of blood. A person’s blood is mixed with “Anti-serum” for different blood types. Type “A” blood mixed with “Anti-A” serum will agglutinate, meaning that the person has A antigens. The same is true for Type B blood – it will agglutinate with Anti-A and Anti-B serum, meaning both A and B antigens are present. This test takes advantage of blood’s habit of agglutinating. This means nothing bad – it just is a way of finding out what types of antigens are present on blood cells. Agglutination with a certain “anti-serum” means that whatever the serum was testing for is present. In the picture below, agglutination (spotty sections) show which traits different blood types have. Note that Anti-D is the same as Anti-RH, and determines whether blood is + or -.

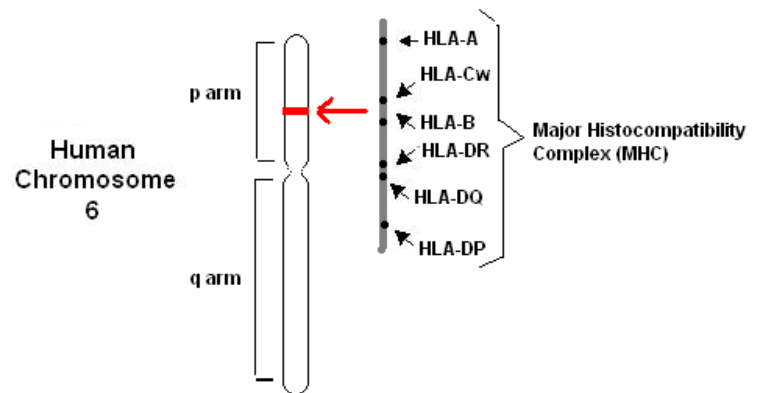
| Anti-A                                                                              | Anti-B                                                                              | Anti-D                                                                              | Control                                                                             | Blood Type |
|-------------------------------------------------------------------------------------|-------------------------------------------------------------------------------------|-------------------------------------------------------------------------------------|-------------------------------------------------------------------------------------|------------|
|    |    |    |    | O-pos      |
|   |   |   |   | O-neg      |
|  |  |  |  | A-pos      |
|  |  |  |  | A-neg      |
|  |  |  |  | B-pos      |
|  |  |  |  | B-neg      |
|  |  |  |  | AB-pos     |
|  |  |  |  | AB-neg     |
|  |  |  |  | Not valid  |

Blood typing allows elimination of people who are NOT good donors for people in need of transplant. If blood types don’t match, it is highly unlikely that other, more important cell markers won’t match, either. People who do match then undergo further testing.

After blood typing testing is completed, the next test completed is HLA typing. First, a bit of background: Everyone has several antigens located on the surface of his/her leukocytes (white blood cells). One of the most important groups of antigens is called the HLA (Human Leukocyte Antigen) group. The HLA antigens are responsible for stimulating the immune response to recognize tissue as “self” or “non-self”. It tells white blood cells which tissues belong to the body and should be left alone, and which should be attacked as foreign materials. These antigens are controlled by a set of genes on chromosome 6 called the Major Histocompatibility Complex (MHC).

HLA typing involves testing for the presence of different versions of this gene. There are multiple different

versions of this gene broken down into two classes: Class I and Class II. Each class holds several types: HLA-A (there are multiple versions), HLA-B (lots of types) and HLA-Cw (lots of types). Class II holds HLA-DR, HLA-DQ, and HLA-DP – and again, multiple different types. For a transplant to be successful, as many of these need to match as possible. The more that match, the more likely that the recipient's body will not reject the transplanted organ. For this reason, tissue typing



is an essential step performed prior to transplantation.

HLA typing is performed today using molecular techniques. The DNA is isolated, amplified with PCR, and sequenced to figure out which alleles are present. Again, potential donors and the recipients are tested, with the best match possible being found.

Once a match is found in this manner, there is yet another test done on the person receiving an organ: Antibody screening. This test is also known as a Panel Reactive Antibody (PRA) screening. Here, a small amount of the organ recipient's serum is mixed with cells from 60 different individuals in separate vials. This determines how many different HLA antibodies a person has in his or her blood, and how likely a person is to reject a transplanted organ. The more antibodies present in the blood, the more the body will fight a foreign organ. Ideally, PRA testing should result in the person's serum reacting less than 50% of the time with the blood of the 60 individuals. When patient serum is mixed with the random samples, agglutination is interpreted as a positive result. Agglutination means the body's serum reacted, and this is not a good sign if it occurs in more than 50% of samples. It means the body will likely reject a transplanted organ fairly quickly.

Finally, there is one more test performed before a transplant can occur: the crossmatch test. Here, the donor's white blood cells are mixed with the serum of the recipient. Hopefully, agglutination will NOT occur. This final check is to determine if the recipient's body will attack the donated organ. Agglutination means a transplant will not be successful, and is considered a + crossmatch test. If this occurs, a new donor must be found. If, however, there is no agglutination, the crossmatch test is considered negative, and the transplant will happen. The crossmatch test is usually done early, and again right before surgery. It is absolutely critical that no reaction occur, as it means the body will reject the donated kidney very quickly.

So, testing is completed, and now it's time for transplant surgery. This is really a two-step procedure: kidney must be removed from the donor, and surgically implanted in the recipient.

Kidney removal from the donor is done laparoscopically, a surgery that is much less invasive (causes less damage)

than traditional methods. *Laparoscopy* is a minimally invasive surgical technique that uses small cylindrical tubes, called *trocars*, to enter the abdominal cavity. The trocars allow entry of a fiber-optic video camera, called a *laparoscope*, to view the inside of the abdominal cavity. Smaller trocars are used to allow entry of the instruments necessary to perform the surgery. Laparoscopic nephrectomies, compared to traditional open nephrectomies, require less pain medication, shorter hospital stays, and a shorter time before the patient can return to work. Physicians hope that the change in the surgery required to donate a kidney will encourage more people to become donors.

Immediately after the kidney is removed from the donor, it is transported to an operating room where the recipient is waiting, already unconscious and prepped for surgery. The recipient is more completely opened, and the kidney implanted without the old kidneys being removed. Here, the new kidney is attached to the iliac or femoral artery in your lower abdomen, and its one ureter is directly attached to the bladder. The body is stitched up, and sent to recovery. If all goes well, at the end of surgery the recipient has a functional, working kidney and can lead a more normal life, free of the demands of dialysis. Visit <http://www.mayoclinic.com/health/kidney-transplant/MY00792> for additional information.

We ended this lesson by going over the different careers involved in transplant: anesthesiologist, transplant surgeon, surgical (perioperative) nurse, and pharmacist. Be sure to review these careers. They will not be discussed here.

Something to consider: a heart transplant is a different procedure than a kidney transplant. How would it differ?

#### **Lesson 4.4: Building a Better Body**

We ended unit four with a study of how the body can be built better. We discussed tissue engineering, xenotransplantation, and bionics. Xenotransplantation, the transplantation of living tissues or organs from one species to another; and tissue engineering, the replacement of damaged organs or tissues with engineered organs or tissues created in the laboratory, are two of the technologies likely to become used in the future to create/engineer/procure organs for human use. Currently, these technologies have limited use – we use valves from cows and pigs for transplant, and grow human skin and bladders through tissue engineering. However, the future holds a great deal of promise for these two technologies.

Bionics is like a fusion of human and machine, replacing parts with machines that have similar or different functions. When we discussed prosthetics and the myoelectric arm, we were looking at one form of bionics. It is also being used to give some vision back to the blind through the use of electrodes, cameras, and sensors. Again, right now not much is done with this technology, but more will be done in the future.