I’m Eric Berg. I’m moderator for this reciprocation session, “Techniques in Gel Electrophoresis and Western Blotting.” Oh, what am I saying? Our speakers today are Amanda Weaver and Elisabeth Huff-Lonergan. Dr. Weaver is from South Dakota State University, and Dr. Lonergan is from Iowa State University. And to begin with, on behalf of AMSA, I’d like to present both of you with your speakers’ gifts, and you are welcome to use them in your presentation today. Other than that, as is the tradition with the reciprocation sessions, this is a free-flowing exchange of information. And our speakers would encourage you to put this in your reciprocation as well. So without any further ado, I’ll turn it over to our speakers. Thank you very much, Dr. Berg. I’ve been instructed I’m supposed to hold this microphone up to record things, but it’s not going to amplify my voice. So that’s what I’m going to do. So welcome, everyone, again. And on behalf of the host committee, thanks for coming out to SDSU for the RMC. So I’m going to start off talking about techniques in gel electrophoresis. And just to begin with a brief history and a review of SDS-PAGE, which most of you are probably familiar with. And then give a little bit of information on a technique that we’ve just recently been using to separate larger molecular weight proteins. I’ll then turn it over to Dr. Lonergan to discuss 2-D gels, visualization of gels, as well as immunoblotting.

Reports of electrophoretic separation of proteins date back to the 1800s. And in the early to mid-1930s they utilized sucrose to form their gels. And by the 1950s, they were workers were using starch gels. However, both of these methodologies provided for just modest separation of proteins. A breakthrough came in 1959, when Raymond and Whistler introduced acrylamide gels. Utilizing acrylamide gels, they were able to better control pore size as well as improve gel stability. Another advancement in this technique was, then, in the late ‘60s by Shapiro et al. in 1967 and Beber and Osborn in 1969, who reported improved separation using an anionic surfactant we know as SDS or lauryl sulfate. In a classic paper published in 1970 by Laemmli, utilized a 3% stacking gel to form a discontinuous gel. And this allowed for the concentration of proteins—proteins to be concentrated prior to entering the resolving gel. And Laemmli utilized this to discover unknown proteins in the bacteriophage T4. Just a little side note I thought was interesting, this happens to be the most commonly cited or the highest number of citations in the journal Nature. So I thought that was pretty interesting, that this technique is that widely used. And in fact, this methodology has been used across countless disciplines, from biochemistry to microbiology and also in the meat science arena.

As far as meat science applications, SDS-PAGE or gel electrophoresis has been utilized to identify different protein isoforms that we’re interested in studying. For myosin heavy-chain isoforms, which are... Any protein that may have different isoforms, we have the potential to separate those isoforms. In addition, this method has been used to identify post-translational modifications, such as phosphorylation or glycosylation events. And probably the most common application used in meat science would be detection of postmortem changes in muscle protein, or proteolytic degradation of susceptible proteins, likely by the calpain system. And just to illustrate this, this happens to be a western blot. So we had a polyacrylamide gel that was transferred to a membrane. Dr. Lonergan will explain this in more detail. But just to illustrate the fact, this has been labeled with monoclonal troponin-T. So we can see, across the top here, this is days postmortem storage from two to ten days. And we’re able to look at three different troponin-T isoforms, in this case, in bovine skeletal muscle. And also, we can see that over time we have a disappearance of the intact protein of these three main isoforms and the accumulation of breakdown products. And it’s just to simply illustrate that we can not only look at different isoforms but also look at this event of proteolysis using this system.

The most common form of one-dimensional gel electrophoresis is SDS-PAGE. And this capitalizes on the fact that charged molecules move in response to an electric field. And this movement is influenced both by net charge of the protein as well as resistance, ultimately allowing us to separate proteins based on molecular weight.
And we'll go through this in just a bit more detail.

[5] Sodium dodecyl or lauryl sulfate—SDS—is utilized to solubilize proteins to their primary structure. SDS also—and this is the SDS molecule here at the bottom—imparts a net negative charge approximately proportional to the mass of each protein. Therefore, negatively charged proteins, when run through the gel, will migrate toward the positive anode. And given these facts, mobility is a function of molecular weight in most cases. There are a few exceptions.

[6] To facilitate the movement of these proteins, polyacrylamide serves as a chemically inert matrix. So we can form polyacrylamide gels that have various pore size. So the larger the pore, the larger the protein that's going to be able to move—or pass through that pore, and vice versa. Variations in pore size is going to alter the extent of migration—so how far proteins are able to move or migrate the gel—as well as the extent of protein separation. And finally, the molecular weight range that we're able to detect.

[7] There's two ways I want to point out that we're able to alter pore size. The first is by changing the acrylamide percentage. That can be altered anywhere from 3%—so a very low percentage acrylamide gel—up to a higher percentage, 15% 20% gels. And as most of you know, a lower percent acrylamide gel is going to have much larger pores, allow for very large proteins to pass through. And whereas we get to a higher percentage, we're going to allow for separation of smaller proteins or resolution of smaller molecular weight proteins. So changing the acrylamide percentage is going to shift the molecular weight range of the proteins we're capable of resolving. Another method to alter pore size would be to change the acrylamide-to-crosslinker ratio. We could use bisacrylamide or another crosslinker, such as DATT, DT or a 37.5:1, 100:1, or all the way up to a 200:1 ratio. And this'll give us a slight modification in resolving power. For instance, if we use a 200:1 ratio, again, allowing for separation of very large molecular weight proteins.

[8] Other alterations that have been reported with SDS-PAGE gels, Makowski and Ramsby, in 1993, reported on changing the pH of the resolving gel from the traditional 8.8 up to 9.2. And this improved resolution of low molecular weight proteins, those less than about 20 kilodalton. In addition, lowering the voltage over a—running with a lower voltage over a longer period of time also will improve the resolution of proteins.

[9] However, there are a few limitations. And there's one I want to point out to lead to what we've been working on in our lab. And that is the fact that SDS-PAGE gels tend to have a limited migration of higher molecular weight proteins. Workers have utilized 3% to 5% acrylamide gels—so, again, very low percentage acrylamide gels—to allow for separation of proteins such as titin, which would be the largest protein known, with a molecular weight of over 3,000 kilodalton. Also, utilization of 3% to 12% gradient gels has been utilized to also separate these proteins. However, these are challenging to pour. They're physically difficult to handle. And those of you that have handled them might want to pull your hair out, because they undergo physical distortion and/or tearing during staining or trying to transfer them and situations like that. Also, the transfer of large molecular weight proteins for immunoblotting is challenging as well using these low percent acrylamide gels.

[10] So to combat some of these issues, workers have added agarose to 2% acrylamide gels in two instances. And they've shown improvement in separation of intact titin—which is denoted as T1—and the breakdown product of titin, T2. However, this technique of adding agarose to 2% gels had a limited ability to separate different titin isoforms, specifically different cardiac titin isoforms, which they were interested in. Recently, a technique developed by Warren et al. in 2003, known as vertical agarose gel electrophoresis—or SDS-VAGE—showed improved separation of cardiac titin isoforms N2B and N2BA, which are slightly smaller molecular weight than the skeletal N2A isoforms. They also were able to improve separation of T1 and the T2 breakdown product of titin.

[11] So I want to briefly just point out some of the difference between SDS-PAGE and
SDS-VAGE. In the proceedings there will be more detailed methodology. However, I just want to point out some of the differences between these two systems. SDS-VAGE relies on the same basic principles as polyacrylamide gels. However, during sample preparation we use a gentle homogenization step to minimize any damage to these very large proteins. Samples are denatured in a typical urea/thiourea sample buffer at 60 degrees Celsius for ten minutes. To point out some differences in the sandwich assembly, which allows us to generate these agarose gels, we use a 1.57-millimeter T-spacer. A normal polyacrylamide gel, you may use a 0.75-millimeter T-spacer. So we're pretty much doubling the thickness of the gels, which gives them more stability. However, this thicker gel, a little bit heavier weight, has a tendency to want to slide out of the bottom of the gel sandwich during the electrophoresis. So in that case we've used about a one-centimeter-high, 12% to 15% polyacrylamide plug. And this just prevents the gel from sliding out of the bottom. It has, really, no other function. Another key point is that the gel sandwich and any other combs or pipettes that are going to come into contact with the agarose before it solidifies must be warmed to about 60 degrees Celsius just by putting in an oven. And that prevents the agarose from prematurely solidifying just by keeping that warm. And that's done after the polyacrylamide plug has polymerized.

[12] The resolving gel in this case contains 1% agarose. And we use high-quality, SeaKem Gold Agarose. Also contains glycerol, Tris base, glycine, and 0.5% SDS. The solution is heated several times to a boil in a microwave and then poured into the pre-warmed sandwich assembly. And then we actually let this sit for just a few minutes, because there tends to be air bubbles that want to form. So letting that sit lets those rise so we don't have issues with that. Then 1.5-millimeter-thick combs are placed into the agarose, and it's just allowed to solidify for a few hours. For electrophoresis, we use this standard lower chamber buffer, containing 50 millimolar Tris base, 0.384 molar glycine, and 0.1% SDS. The sample is loaded through the upper buffer, which is the same as the lower chamber buffer. However, with 10 millimolar beta-mercaptoethanol is added as a reducing agent to keep these large molecular weight proteins in their reduced state through electrophoresis. Samples are run at about 15 milliamp constant current for anywhere from five to seven hours at 4 degrees C.

[13] So I want to point out some of the advantages of using this SDS-VAGE system. And this is from the original paper by Warren et al. And I'll orient you to this gel just briefly to point out that these last three samples are from cardiac muscle. The remaining samples are from different skeletal muscle tissues from different species. And as you can see, SDS-VAGE gels have improved mobility of titin. You can see that titin has migrated about 10.5 centimeters in this gel. Now, if you compare that with a normal polyacrylamide gel, where titin may migrate a few millimeters or a centimeter into the gel, this is a great improvement. And we've also found that you can run titin off of these gels. So do watch that. We also see Warren et al. reported improved resolution of titin isoforms. And just to draw your attention again, to the three cardiac samples, we have the N2BA and the N2B isoforms that have been separated in this case. And again, they're slightly smaller than the isoforms, the skeletal muscle isoforms. Also, improved separation of T1 and the T2 fragment. The T2 fragment is indicated by this arrow. And you can see that it's migrated about 11.5 centimeters into the gel, compared to the intact isoforms. These gels also provide improved gel stability. They're much easier to work with, to handle for staining or transfer—so much nicer from that standpoint—and can be useful, I believe, for characterizing other large molecular weight proteins, such as nebulin or obscurin. So I think there's a lot of room to use this technique for multiple applications.

[14] One last advantage is the fact that SDS-VAGE gels, when transferred, provide about 100% transfer efficiency. Again, from the original Warren paper, we see two gels that have been stained. Here, pre-transfer with Coomassie and post-transfer and the resultant western blot. And as you can see in the post-transfer gel, there's virtually no protein remaining in this gel. With a normal polyacrylamide gel—a low percentage polyacrylamide gel—most of that protein would remain in the gel. And some data from our lab—and I apologize. This was loaded a little heavy. But I think you can see, again, if we look across days postmortem storage from two to ten days,
we were looking at the intact titin isoforms as it disappears over time and the accumulation of the T2 degradation product.

But, as with everything, it's not perfect. There are just a few limitations I want to point out to this system I did mention that it's important to prevent air bubbles. And that can be accomplished just by allowing that resolving gel to sit at 60 degrees Celsius for a few minutes before it's allowed to solidify. Also, care must be taken to prevent premature solidification of agarose by keeping everything pre-warmed and moving fairly quickly when pouring the gel. Also, removal of comb from the solidified agarose can be a bit challenging, so it's important to be careful to prevent any breakage of the wells. And finally, these gels have a limited storage capability. They're good for about one to two days wrapped in plastic wrap at about four degrees Celsius. So with that, I guess we'll go to questions and I'll turn it over to Dr. Lonergan. Well, Amanda did a very nice job of covering one-dimensional gels. I'm going to take that a little bit further and talk very very briefly about 2-D gels and some of the newer technologies that are being used with two-dimensional gels. I'll talk, also, some about some detection methods, both old and some of the newer dyes and stains and fluoros that are being used. As well as western blotting, kind of a general overview of western blotting. And then, again, some of the more sensitive stains and some of the more exciting techniques, I think, that are out there and available to be used right now.

We'll start off talking about two-dimensional gels. I'm going to assume that most of you have seen or heard about two-dimensional gels. But just, kind of, as a review, they are a very effective tool for separating complex mixtures. The concept of two-dimensional gels has been around for numerous years, since about the early 1970s, when it was first identified that one could improve separation of proteins by first separating proteins on the basis of isoelectric point and then completing the separation on the basis of molecular weight. That's the two dimensions that we have in a two-dimensional gel. These gels did not see a lot of popularity and use, however, due to the difficulty in effectively, reproducibly casting the first-dimension gel and getting very, very consistent results. In about the 1990s, early 1990s, there was a development of what are called IPG, or immobilized pH gradient, strips. These are essentially strips that have the pH gradient immobilized on them. And then they are cast onto a plastic backing and cut into strips, very thin strips, which does allow for these first-dimension gels to be very, very highly reproducible. And in the '90s—late '90s, early 2000—you began to see quite a few of the pharmaceutical companies starting to sell a lot of these strips and producing them on a mass-marketing basis. And that has greatly improved the separation that we see. It's also improved the reproducibility, because these are mass produced. One thing that is very exciting to me about two-dimensional gels is not only can you more effectively separate a complex mixture of proteins. But you can also, in many cases, more effectively study some of the post-translational modifications, specifically things like protein phosphorylation. They will migrate differently in the first dimension than they would in the second dimension. In a 1-D gel—which most of us are familiar with—a lot of times, those proteins that are post-translationally modified by phosphorylation or glycosylation might migrate to the same point. In this case we can separate those. So the concept of two-dimensional gels, first dimension is an isoelectric focusing gel, which is essentially a gel that contains an immobilized pH gradient, separates the proteins first on charge. Those gels are then laid on top of a traditional SDS-PAGE gel, and then further separation is based on size.

This is just an example of a 2-D gel that was done in our laboratory. Granted, this may be a little bit overloaded, but this is an example of a preparative gel, where we were trying to first run a very broad pH gradient to get an idea of where more of the samples—or most of the proteins in our sample were going to focus in the first dimension. And so we chose a seven-centimeter strip for the first-dimension gel—for the IPG strip—that had a pH of, at this end, from 10 to 3. And so the proteins that we see focusing in this region would be proteins that would be focusing more in the basic region. So these are more basic proteins. Proteins that typically look like they would have a charge of 7—or a pH of isoelectric point of 7 or greater. You can see that in this direction these are proteins that are separated on
the basis of isoelectric point. And in this direction the proteins are separated on
the basis of size. So if you look at these carefully, there are a lot of these
proteins that you see migrating as a chain. If you will, that we would have missed
in a one-dimensional gel, because in a one-dimensional gel they would have migrated
as a single band. Just an example, again, showing some of the differences in 2-D
gels and 1-D gel. This is a traditional 1-D gel, 15% polyacrylamide gel. A
two-dimensional gel, again, with a 3% to 10% at the top and then a 15% separating
gel, again, showing what you would miss. Now, there’s some things that I need to
point out about two-dimensional gels. They are very handy for a lot of applications.
But one thing you do have to note is typically you run one sample per gel. Okay? So
this is one gel, one sample. This, of course, in a traditional one-dimensional gel,
you can have multiple lanes. You can have as few as 5 lanes, depending on how
many—the type of comb you have—to as many as 15 lanes. So there are some things
that we do need to be considerate of when we start thinking about doing
two-dimensional gels. One of the first things, that I’ve already mentioned, is
sample number. Like I said, traditionally, one sample, one gel. So that can have
issues with reproducibility, meaning that you might have to run several gels on
the same sample to ensure that you are truly getting very, very good, high-quality
results. Another consideration is particularly from the first dimension. In the
first dimension we have to be very careful about how we prepare the proteins. For
instance, we cannot use SDS. It is an ionic compound that does denote a charge to
the protein. Well, since we’re separating proteins in this first dimension on the
basis of isoelectric point, that’s going to destroy what we’re doing. So we can’t
use SDS. That can have some limitations on how well we can solubilize a lot of
proteins. Okay? Particularly if you’re interested in membrane proteins, there are
some different tricks, etcetera, that you might have to call into play that you
wouldn’t have to with the one-dimensional gel. In most cases, when we’re loading
proteins, we don’t have the versatility that we do in the first dimension, we don’t have
what mentioned passive loading. And in this case the first-dimension gel is dried. We then take our sample.
We put it in the rehydration buffer. And then we apply that to that first-dimension
gel. And as that gel rehydrates, it, essentially, soaks up the rehydration buffer
and the protein. So that’s the typical way that you’ll see gels being loaded. That’s
the most common way. There are a couple other ways that we can do it. But if you
think about this, one challenge, then, is if you have some very, very high molecular
weight proteins, it can be very challenging to get them into the gel under most
any method that you use. So titin might not be a good candidate for this particular
study. Another thing, when you get into some very complex samples—as you’ll see in
some of the future slides—spot identification and protein identification can be a
challenge from the standpoint that you just have thousands of proteins. I’ve seen
gels where you can have as many as two thousand different spots located on
a gel. So identification of what you have on the gel can be a challenge and often
requires the use of a lot of sophisticated software to help identify some of those
proteins if you want to do some post-processing identification down the road.

[18]Okay. So that’s 2-D gels, kind of, in a nutshell. We’ll come back to them in a
minute. I did want to spend a couple minutes, though, talking about different
staining techniques that we use in the lab and what some of the advantages of some
of those are. And I’ve got some examples on some future slides. The most one
that you will see is Coomassie Blue R-250 stain, which is a very cheap, very
inexpensive, very simple stain to use and works quite well for most applications.
Staining time for this particular method is about 30 minutes to 6 hours, depending
upon the thickness of the gel, how much protein is on the gel, etcetera. And the
sensitivity is somewhere in the 30 to 50 nanogram range. And it’s a very good
process that you’ll see. And this is what you see most often. If you want to get
more sensitive, there are stains such as silver stain, which depends upon having
silver deposited, actually, onto the proteins. It’s a much more sensitive method, as
you can see. Can detect anywhere from 0.6 to 1.2 nanograms. Staining time is much
more—is much improved, but it is very hands-on and very, very labor-intensive. Where
as with the Coomassie stain, you can pretty much take your gel out of the running
apparatus, throw it in stain, walk away. With silver stain, there are several
post-processing steps where you’re changing solutions throughout the staining
process. A couple of others that are useful, copper and zinc stains. Both of these
are referred to as negative stains. And that means that they stain the gel, not the
protein. They also do not fix the protein in the gel, as these two methods do. So if you anticipate wanting to do something with a protein down the road—say, use it in a western blot or excise it out of the gel and use it in, say, future experiments—copper and zinc stains might be the way to go, because you’re not altering the protein in that case.

[19] Couple of quick slides just to show some of the different staining techniques. This is actually compliments of Bio-Rad. They—these are all the same samples on all these gels that I’m going to show you. The only difference is in each lane is a decreasing amount of protein. And this is common that you would see for Coomassie.

[20] This would be silver stain. And again, we’re starting to see more and more lanes start to show up with the silver stain than what we saw with the Coomassie.

[21] The zinc stain. And in this case what we have done—what they’ve done is they’ve laid this gel onto a black background. Okay? So it looks like these proteins are stained. They’re not. They’re actually—the black background is showing through. And this is really what it looks like; then, The gel itself is kind of a fuzzy, milky white color. Okay? And sensitivity of zinc stain actually approaches many of the silver stains that are available.

[22] Okay. Some of the newer stains that have come out that are quite nice would include some of the fluorescent stains. One is referred to as Sypro Orange. It does have a sensitivity greater than Coomassie, although it’s slightly less than silver staining. It’s a very rapid stain, very easy stain to use, much like Coomassie. You, actually, just put the gel in the stain and let it sit until it is stained. This particular stain does not fix the proteins in the gel. So if you want to do any further processing with this gel, you can go ahead and use it for transfers, since you’re not actually fixing the protein in the gel. One thing with both of these stains, you have to have a camera and a light source, or, actually, a source that can excite the fluorescent dyes and the appropriate filters in order to allow these dyes to actually be visualized. Another stain that you’ll see commonly used—probably more with 2-D gels— is the Sypro Ruby. And in this particular stain it does fix. Sypro Ruby is pretty much on par with silver stain. It has some advantages, in that it does identify and stain glycoproteins, lipoproteins, and metalloproteins. And these are often very difficult to stain, so very useful from that standpoint. It’s used a lot in 2-D gels, because the Sypro Ruby stain does not interfere with further processing steps—such as mass spectrometry—to further identify proteins. Another very useful technique, another new class of stains that has been looked at are the phosphoprotein stains. And in this case this is an example of a gel that was stained for total proteins. So you can see all the spots on this 2-D gel that were generated. That same gel was then stained with the phosphoprotein stain. And in this case it was Pro-Q Diamond from Molecular Probes. And these phosphoprotein stains will stain only those proteins—or theoretically, only those proteins that are phosphorylated. So we can actually look at these two images of the same gel and identify which proteins are potentially phosphorylated just from which ones come up with this stain. One thing I forgot to mention, that is an advantage if you’re using these fluorescent stains, is you can image the same gel. You just change the wavelength that’s being passed through that gel and change the filter on the camera. And you can actually image the same gel. And then you can electronically overlay the images to see what spots line up. And in this case this is an overlay of these two images, and you can see those that come up black, actually, are identified—the proteins in your total protein stain gel that are the phosphoprotein stains. And if you were going to go ahead and identify these proteins, you could actually use this to pick the samples from that gel and go ahead and do further identification or further work. It’s a very useful technique.

[23] One—the last technique that I’m going to talk about on identification is relatively new, and it’s the 2-D DIGE format. That stands for two-dimensional fluorescence difference gel electrophoresis. And this particular technique is very, very useful if you’re wanting to compare more than one sample. And this particular technique does allow you to load multiple samples on a single gel. Remember, I said that was a limitation of traditional 2-D gels. This is a tool that can help us
overcome some of these differences. Now, you’re never going to get up—or, at least, with current technologies, we’re not going to get up to 10, 15 samples per gel. But we can do two, potentially three, samples within a single gel.

[24] And this is an example of a 2-D DI GE gel. We’ve got samples from one animal that are labeled with the green dye, samples from another animal labeled with the red dye. And here, if we have proteins or spots that are in common when we overlay the two images, we get this yellow spot. Okay? Going to briefly walk you through how this is done in order to do the 2-D DI GE. Obviously, you have two samples. You label them with two separate dyes. And the most commonly used dyes are referred to as the Cy3 dyes and the—the Cy dyes. And Cy3 and Cy5 are the two most commonly used. Cy2 is another commonly used dye that you’ll see. These dyes come in a couple of different formats. This particular format that’s most commonly used are dyes that are going to label the amine-end of proteins. So you take your sample, you label it with the respective dyes, and then you actually mix the samples together. Okay? But they’re separately labeled. You then go through the 2-D electrophoresis process, and you generate this single gel. You take it to your imaging system, whether that’s a Typhoon laser imager or a particular camera that’s equipped with the appropriate excitation and emission wavelength filters. You image that gel twice. So you image it first to excite the Cy3 dye. You image it, then, to allow the Cy5—the proteins labeled the Cy5 dyes to be imaged. And then you can overlay those two images. And when you get the overlaid image, you’ll see, actually, the color change from the red and green dyes to indicate which proteins are in common in those two samples. So this is a very, very effective way to do multiple samples, in this case two samples. Though I didn’t put it into this particular slide, this is also a very effective way that you can use to incorporate a standard. And if you’re doing an experiment, what you might do is you might, in addition to having your two samples on each gel, you might then also take a set of samples to be each sample in your experiment. So say you had ten animals in your experiment. Mix them label them with a third dye, and that would give you an internal standard for your experiment that would be run on every gel. And then the concept is the same. You would just have three images that you would overlay. So very, very powerful technique, very, very useful, and one that I think you’re going to see a lot more of in the future.

[25] Okay. On to my last topic—so hopefully, we have time for questions and discussion—and that is the concept of immunoblotting. Immunoblotting is going to be a very useful technique using—it’s essentially a powerful extension of either SDS-PAGE or the 2-D PAGE technologies. It’s going to allow us to very effectively identify some specific proteins that we see within our gels.

[26] I’ll just walk you through, kind of, the basic steps. I’m sure many of you are familiar with this. I know some of you are very familiar with this technique. But first of all, we start out with separation of proteins with SDS-PAGE or with 2-D PAGE. It can be used with that system. Proteins are then transferred to a membrane such as nitrocellulose. Or PVDF is another very commonly used membrane, has a little bit more strength than does the nitrocellulose and maybe a little bit lower background. We then apply an antibody that’s specific for a particular protein. So if you’re interested, say, in troponin-T, you would be applying a protein that specifically—or an antibody that specifically recognizes troponin-T. We then apply an antibody that’s specific for the first antibody, or for the primary antibody that we used. And that’s a second—that’s referred to as a secondary antibody. And what’s special about this particular antibody is that it is tagged with a reporter molecule that will allow us, then, to ultimately visualize whatever this primary antibody recognized.

[27] So just a, kind of, brief schematic. We have our SDS-PAGE or our 2-D gel here.

[28] We transfer these proteins to our membrane and then go through our detection system.

[29] Now, really briefly, I’m going to describe how we actually transfer these proteins. Whether or not you are using 1-D PAGE or 2-D electrophoresis, by the time your gel is run, your proteins are going to be negatively stained. Because even with
a 2-D system, we do switch the proteins over to being solubilized with SDS. Okay? So our proteins are going to be negatively charged. We're going to use electrophoresis, essentially, to get these proteins to move out of the gel and onto the membrane. So in this case we would create a sandwich that includes the membrane, the gel, and then, essentially, blotter paper and sponge to, kind of, make sure we have a very tight connection between the gel and the membrane. We then insert it into a tank that contains a buffer that is very similar to a traditional electrophoresis buffer, may or may not contain SDS. And we make sure that this particular sandwich is oriented so that the membrane is always towards the positive electrode. Okay? Because remember, our proteins are negatively charged. And we want them to move— as the electric current passes through, we want them to move from the negative to the positive, so they're stuck onto that membrane. If you flip them around, trust me, they'll go right out into your transfer buffer. It's happened. Okay?

[30] So just, kind of, a general overview. In this case this is our membrane. These are our little proteins that are stuck to our membrane after we've done our transfer.

[31] Commonly, virtually almost every step includes a step that is a blocking step. And that's where we add a nonspecific or a nonrelevant protein to that membrane. So we would take something like nonfat dry milk or BSA or some other irrelevant protein and use that to block any of the protein binding sites on that membrane. Okay? These membranes have an extremely high affinity for proteins. And remember, antibodies are proteins.

[32] So we want to prevent our antibodies from seeing this membrane at all. We just want them to see those proteins that are stuck on the membrane. So we'll block them with an irrelevant protein, a protein that we're pretty sure our primary and our secondary aren't going to recognize.

[33] We then apply our primary antibody.

[34] We wash off whatever primary antibody didn't bind.

[35] We then apply our secondary antibody.


[37] And we activate that tag.

[38] And we're actually able to see exactly where on that membrane that protein is. Now, this is going to be very powerful. It will allow us to see, say, if you're looking at degradation of proteins, you can actually—if your primary antibody, hopefully, recognizes your degradation product, you can see that. Even if it doesn't, you can often see decreases in the intact protein using this particular method.

[39] Why is this so powerful? Well, you can take a very complex situation, as you would see with a typical SDS-PAGE gel here. We've got quite a few proteins in each individual lane. Identifying troponin-T, for instance, and its degradation products would be quite difficult to do very easily. But if we use immunoblotting or western blotting, you can see that we clear that picture up very nicely, and we're able to see exactly where those particular proteins are.

[40] So some things to think about if you're going to try to optimize immunoblotting. Some things to optimize signal intensity, obviously, the more protein you load, often the more intense the signal will be, because there's more protein there to be detected. You kind of have to be careful about this, though, because you can, actually, create some false-some background issues if you have too much protein. Transfer, of course, obviously, the more efficient the transfer of the protein from the gel, the better your signal is going to be. Certain membranes can help. PVDF generally has lower background than nitrocellulose, although some of the new nitrocellulose membranes are also very good at giving low background. Transfer
buffer composition, if you had a very high molecular weight protein, such as titin, using SDS in your transfer buffer can, actually, facilitate movement. One thing you remember, though, if you do use SDS in your transfer buffer, you can have some heating issues. So you had better make sure you've got some really good cooling system for your transfer unit. Transfer time and temperature, obviously, increase time, increase temperature, you're going to increase transfer efficiency. But you've also got to worry—especially with temperature—about denaturing your protein. So you do want to, kind of, minimize any increases in temperature. Some other issues—which we'll talk about in just a minute, which is going to be very critical to some of the sensitivity that you have. Some other things to think about: background, your choice of blocking agent. Again, that's going to be dependent upon your sample. You may have to try a couple of different blocking agents. Obviously, your choice of antibody and the antibody concentration are all going to influence background. So if you use a higher antibody concentration of either the primary or the secondary, you're going to stand a very good chance of getting higher backgrounds. So these are all things that might have to be considered if you're going to try to optimize a particular immunoblotting procedure.

As far as detection methods, some of the more commonly used detection methods that we've seen would include just the color development system. And the most common of these you'll see is the 4CN system, or the 4-chloro-1-naphthol—which, essentially, is done directly on the membrane. It causes a colored precipitate, actually, to form right on the membrane. And you actually see the bands appear on the membranes themselves. This is typically referred to as a colorimetric detection system.

Another detection system which you see used a lot more in recent years, are some of the chemiluminescent detection systems. And this is an example of one. And these particular systems are dependent upon the oxidation of luminal, which is in the solution that you apply to the membrane, by the HRP—or the horseradish peroxidase tag—that's on the secondary. Okay? So in this particular case we activate the tag by adding hydrogen peroxide and luminal. And we actually get the emission of light from these samples. And if you've done this in the darkroom and you had a really intense signal, you can actually see that light on your blots. And it's kind of cool. But ten years ago, we were doing this, and we were actually—actually 12, 15 years ago—we were using autoradiography film or phosphoimaging devices. What I didn't put up here, there also are CCD cameras that are very, very good at picking up these very low-intensity light signals. And that's currently what most of us are using now, are these CCD cameras.

Just an example, chemiluminescence systems. These are actually the same samples. Chemiluminescence, actually, tends to be a lot more specific and a lot more sensitive than the traditional colorimetric systems.

One last one that I wanted to bring up is fairly new, probably within the last three, four years. And this particular system is marketed by GE, and it's referred to as their ECL-Plex. GE bought Amersham which was the company that first developed a lot of the chemiluminescent techniques. And their chemiluminescent brand is ECL. They've modified this to ECL-Plex. This is a really cool system because if you think about what I just told you about western blotting, typically, we're only going to be identifying one protein on a gel or on a western blot. Typically, if you're going to do a western, you're thinking, "I'm only going to identify troponin T," for example, "on that gel." The reason for that is you've got your primary. You've got your secondary on there. If you want to detect something else, you need to strip those away and put new ones on. Well, in this case this ECL-Plex utilizes secondary antibodies that are fluorescently tagged. Okay? So they're going to allow us to have different signals for different proteins. Now with the chemiluminescence that I showed you, this is your only signal that you get. You get black—or actually, light signal. This is reversed. And so even if we had multiple primary antibodies in here, we might have multiple bands. But we don't know which one that signal is coming from. So we're not any better off than we were with just staining. In this case,
though, we used two different antibodies. And in this particular instance there's two different proteins, beta-tubulin and ERK1/2. So they used a primary antibody against beta-tubulin, a primary antibody against the ERK1/2, and then two different secondaries. This particular secondary was labeled with the Cy5 fluorescent dye. And then this one was labeled with the Cy3 fluorescent dye. You put both secondaries on at the same time. They find their respective primary antibody. You take it to an imaging system that has the proper filters for this, and you receive an image like this. And you can simultaneously see both proteins at the same time. Now, tremendous tool to be able to use if you wanted to see simultaneous changes in the same sample of a particular protein, great tool. One thing you have to remember, though, is you have to make sure that your secondary antibodies aren't going to cross-react with your primary antibodies. So that's the one caveat to that. And typically, the way we get around that is we will use what's called a monoclonal antibody. It's produced in mouse. It's specific for one epitope. And we would use, then, a secondary antibody that only detects mouse proteins. So it would only detect the mouse primary. For this other protein, we might choose an antibody that was produced in rabbit. Okay? Then our secondary antibody would be an antibody that only recognizes rabbit proteins. So we do have to be careful there, because we'd be right back to the same thing if we used the antibodies—both antibodies that identified mouse, for example. So a new technique, a very powerful technique, and one that I think you might see a little bit more of use in the future.

So we talked a lot about technologies. What's the take-home message? All of these technologies have the capacity to provide a lot of new information. The one caveat, and hopefully the take-home message that you have from all of this, is the key is to ask the correct question and then match the appropriate technology and the use of that technology to the right question. So half your work is done if you state your problem well, and then identify the technologies that you want to use to identify your answer.