Experimental Biotechnology
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Lecture-30
Antibody-Antigen Interaction

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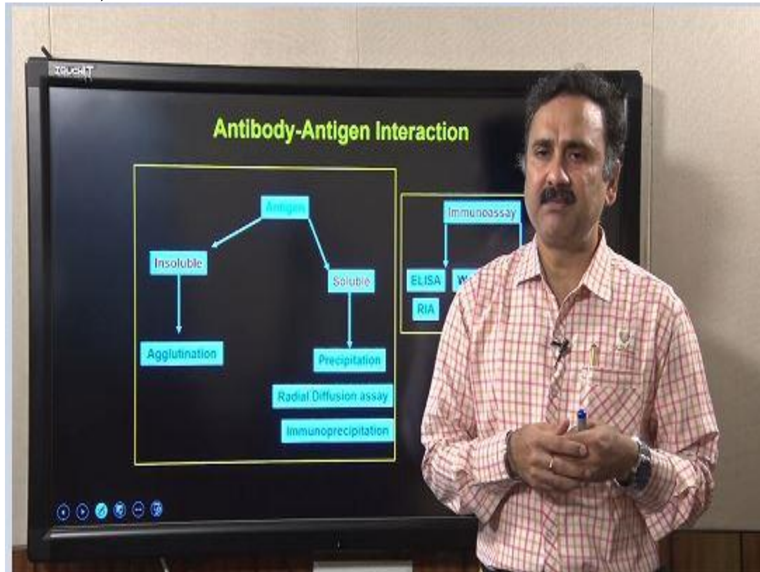

Hello everybody, this is Dr. Vishal Trivedi from Department of bio sciences and bio engineering IIT Guwahati and so, far what we have discussed we have discussed about the different types of antigen antibody interactions, where we have discussed about the explanation reactions we have discussed about the radio immunoassays we have discussed about the precipitation reactions and in the previous lecture, we have also discussed about the immunoassays. So, within the immunoassay we have discussed about the direct Elisa as well as the indirect Elisa. So, now, subsequent to that we are also going to discuss about the sandwich Elisa

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Sandwich ELISA

Sandwich ELISA- This setup is used to measure the level of antigen (such as insulin) in the serum. In the direct ELISA setup, a known amount of antibody specific antibody (capture antibody) to capture the antigen. The antigen is then recognized by the secondary antibody linked to the enzyme. A colorimetric substrate is used to measure the level of antigen.

Substrate

Capture antibody

Sandwich Elisa is actually a different types of setup this setup is used to measure the level of antigen such as the insulin in the serum and in the direct Elisa setup a known amount of antibody is specific antibody to capture the antigen. The antigen is then recognized by the secondary antibody linked to the enzyme. A colorimetric substrate is used to measure the level of antigen. So, that in the sandwich Elisa you use the 2 antibodies one is the capture antibody.

So, what you can do is first you take the well and then you coat this well with a capturing antibody and then you add your antigen of interest. For example, in this case it is insulin. So, you add your patient sample and then the insulin or the antigen is going to bind to this capture antibody, then you do a washing step and then you add the additional primary antibody. So, that actually is going to recognize the antigen again. And then you add the secondary antibody which is actually going to recognize this antibody instead of this antibody.

And then you are going to add the substrate and that actually is going to give you the product which is going to be colored in nature and that is how you can be able to measure the level of antigen present in the patient's sample or the biological fluid. So, the purpose of sandwich Elisa is to measure the antigen whereas, the purpose of indirect Elisa is to measure the level of antibody present in the blood.

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In this method, a capture antibody is used to collect the antigen from the sample. Afterwards, second antibody is used to detect antigen bound to the capture antibody. Second antibody is directed against the antigen using a unique distinct epitope. The antibody is linked to the biotin and that can be recognized by the avidin/streptavidin-HRP complex. In the last step, peroxidase substrate is used to get a readout.

So, in this method of capture antibody is used to collect the antigen from the sample. Afterwards a second antibody is used to detect the antigen bound to the capture antibody. The second antibody is directed against the antigen using a unique distinct epitope. The antibody is linked to the biotin and that can be recognized by the avidin or streptavidin-HRP complex. In the last step the prostate substrate is used to get a readout. So, when you use them, when you immobilise the capture antibodies, the antigen goes and bind to the capture antibody.

Then you use the detection antibody, which is actually going to use the different epitope present on to the same antigen. And then you have the option of either using the secondary antibody or you can use the biotin evident kind of recognition system and then you are going to add the substrate and that actually the substrate is going to be processed by the HRP. HRP means, horseradish peroxidase and that actually is going to give you the colored product and that colored product can be recognized by the calorimetric acids.

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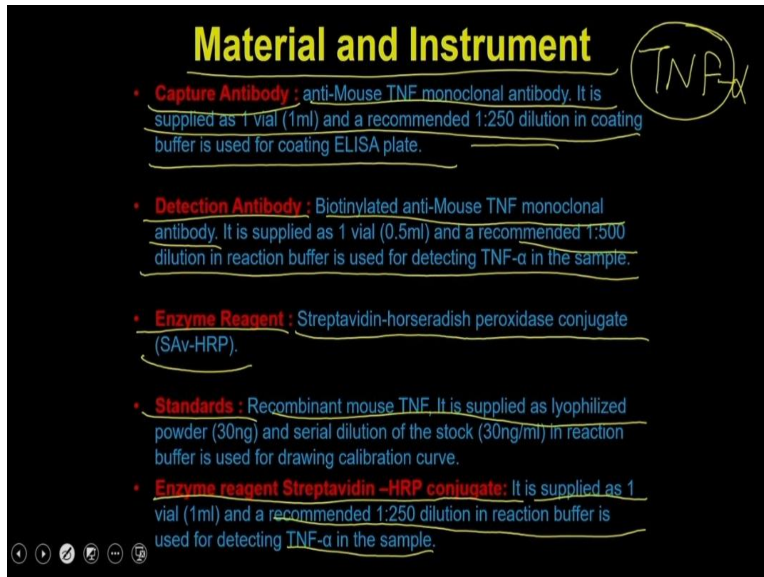

So, let us see how to do that and what are the materials are required. So, firstly if you require a capture antibody so, in this case, we are showing you the Elisa for the TNF alpha from the patient sample. So, you are using the anti mouse TNF alpha monoclonal antibody. It is 1is to 250 dilution is used for coating into the coating buffer using the Elisa plate. Then you require a detection antibody.

So biotinylated anti mouse HR TNF alpha monoclonal antibody is used and the recommended 1 is to 500 dilution is required in the reaction buffer for detecting TNF alpha in the sample then you require the enzyme substrate. So, streptavidin-horseradish peroxidase conjugate is being used as a detection system and then you require the standards. So, in the case of this you actually need the mouse TNF alpha standards.

So, that; you can be able to perform the standard reaction. So, that you will be able to measure the level of TNF alpha into the patient samples and the enzyme treatment HRP conjugate. So, this is the substrate what you require to use and recommended dilution is 1 is to 250 in a to detect the TNF alpha in the sample.

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Material and Instrument

- Coating Buffer - 0.2 M Sodium Phosphate, pH 6.5 : Weight 12.49 g Na2HPO4, 15.47 g NaH2PO4 and make up the volume to 1.0 L. Adjust the pH to 6.5. Prepare freshly and use within 7 days of preparation, stored at 2-8°C.
- Assay Diluent-PBS with 10% FBS, pH 7.0. Freshly prepare and use within 3 days of preparation, store at 2-8°C.
- Wash Buffer – PBS with 0.05% Tween-20. Freshly prepare and use within 3 days of preparation, stored at 2-8°C.
- Substrate Solution - Tetramethylbenzidine (TMB) and Hydrogen Peroxide.
- Stop Solution -1 M H3PO4 or 2 N H2SO4
- 96-well ELISA flat bottom plates are recommended
- Microplate reader capable of measuring absorbance at 450 nm
- micropipettes
- Tubes to prepare standard dilutions
- Plate sealers or parafilm

Apart from that you require a coating buffer. So, this is the composition of the coating buffer. The coating buffer has to be prepared fresh and you have to use that in the span of 7 days. Then you require the Assay diluent. So assay diluent is a PBS which actually can be used to prepare the different dilutions of samples. Then you require the wash buffer. So, wash buffer contains the PBS plus tween-20 and then you require the substrate solution which is the TMB and the hydrogen peroxide.

And then, once the reaction is over, then you require the stop solution which contains the H3PO4 or the sulfuric acid. Then required the 96 well plate for doing the Elisa and then you require the microplate reader which actually can be able to capture the absorbance at 450 nanometer and then you require the micropipette tubes and the plate or the parafilm.

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Procedure

Step 1: Specimen Collection and Handling: Specimens should be clear, non-hemolyzed and non-lipemic. In the case of cell-culture, remove any particulate material by centrifugation and assay immediately or store samples at ≤ -20°C. Avoid repeated freeze-thaw cycles. Where as in the case of patient blood, use a serum separator tube and allow samples to clot for 30 minutes, then centrifuge for 10 minutes at 1000 x g.

Remove serum and assay immediately or store samples at ≤ -20° C. Avoid repeated freeze-thaw cycles.

Step one in the step one you have to collect the specimen or the sample from the patient or the biological fluid. So, in the step one you have to do a specimen collection and the handling. The specimen should be clear, non hemolyzed and non lipemic. In the case of cell culture, remove any particulate matter by centrifugation and assay immediately or stored the sample at -20 degrees Celsius. Avoid repeated freeze-thaw cycles.

So, even if you preserve the sample at -20, you should not repeat the freeze thaw cycle because as many times you will do the freeze thaw cycle is actually going to damage the antigens, what is present inside the sample and that actually is going to reduce its cross reactivity. Whereas, in the case of patient blood, use a serum separating tube and allow the sample to clot for 30 minutes and then send a fuse for 10 minutes.

So, in the case, if you are processing with the patient blood, you do not need the blood component you only require the serum component or the fluid component. So, what you do is you just allow the blood to clot and then you take the clear supernatant for detecting the antigen either it is TNF alpha or insulin or any other antigens. You remove the serum and assay immediately or you store them at -20 degree. You avoid the repeated freeze thaw cycles. So, that is very important that you should not do the repeated freeze-thaw cycle because that actually is going to reduce the reactivity of the antigen against the antibody.

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Step 2: Preparation of TNF-α Standard dilution: **Dissolve the vial content into 1ml** deionized water to yield a stock standard 30ng/ml. Allow the standard to equilibrate for at least 15 minutes before making dilutions. For preparing different concentration of TNF-α solution, initially prepare a 1000 pg/mL standard from the stock standard. Vortex to mix. Dilute this stock into different dilutions as per the calculation given in the Table.

Table: Preparation of the TNF-α dilutions

TNF-α concentration (pg/ml)	TNF-α (µl)	Assay Dilution Buffer (ml)	Total volume
1000	300	300	600
500	300	300	600
250	300	300	600
125	300	300	600
62.5	300	300	600
31.25	300	300	600
15.62	300	300	600

Step 2 you have to prepare the TNF alpha standard dilutions. So what you have to do is you start with the 1000 Pico gram per ml and then you do the serial dilutions like as we discussed for the indirect Elisa as well, and ultimately you are going to get the 15.56 Pico gram per ml dilutions. So this is exactly what you have to do, you have to start with the stock solutions and then first to prepare the 1000 pico gram per ml. You take the 300 microlitre you diluted and that is how you actually are diluting every time you are diluting half into the individual eppendorf.

And that is how you are going to prepare the complete dilutions. So, you take whatever the content comes along with the kit into a deionized water to get stock of 30 nanograms per ml. And then you can make the dilutions like this. And you vortex the mixture for that it is going to be a homogeneous dilutions and then only you are actually going to prepare the serial dilutions. Because once you add the things, you have to mix this very thoroughly so that it is actually going to be homogeneous and then only you take out an additional 300 microlitre diluted to make the additional dilutions.

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- **Step 3 ELISA plate coating:** Add 100 μL diluted Capture Aantibody to each well. Incubate overnight at 4°C. Aspirate and wash 3 times with wash buffer.
- **Step 4 Blocking :** **Add** 200 μL Assay Diluent to each well. Incubate 1 hr RT. Aspirate and wash 3 times with wash buffer.
- **Add** 100 μL standard TNF-α or sample to each well. Incubate it for 2 hr at RT.
- **Step 5:** Aspirate the sample and wash the plate 5 times with wash buffer.
- **Step 6 Detection:** Add 100 μL Working Detector (Detection Ab + SAv-HRP) to each well. Incubate for 1hr at RT. Aspirate the detector solution and wash 7 times with wsh buffer. Add 100 μL Substrate Solution to each well and incubate 30 min RT in dark. Stop the reaction by adding 50 μL Stop Solution to each well. Read the ELISA plate at 450 nm and the at 570 nm (it is required to substract background absorbance).

The step 3 you have to do the coating. So add the 100 microliters diluted capturing antibody to each well. And then you incubate overnight at 4 degree and Aspirate and wash 3 times with the wash buffer. So now you have coated your coating antibody. Then you do the blocking. So, exactly the same way you have to do the blocking, so that there will be no nonspecific interaction of the antigen to the plastic of that particular well. And then you add the 100 microliters of standard TNF alpha or sample and incubate it for 2 hours at room temperature.

Then in the step 5, you aspirate the sample and wash the plate 5 times with the washing buffer, and then step 5, you have to do detection. So, add the 100 microliters of detectors to each well. So, detector is a streptavidin-HRP conjugate. Incubate for one hour at room temperature aspirate the detector solution wash it and then you incubate it with the substrate solutions and that actually is going to give you the reactions after the sometime and that actually it can be read out with the help of the spectrophotometer.

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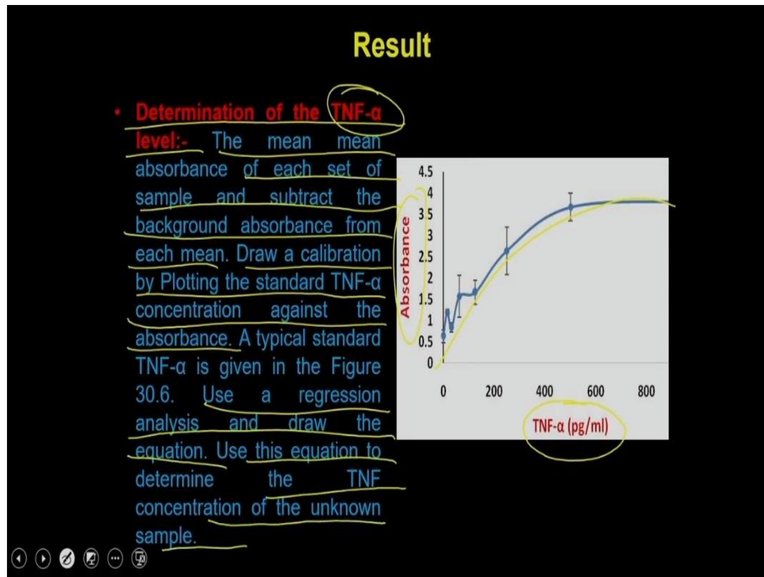

Now, determination of the TNF alpha level, so, we are just giving you an example of TNF alpha, but that can be done with any antigens, the mean absorbance of the each set of the sample and subtract the background absorbance from the each mean. Draw a calibration by plotting the standard TNF alpha consultation against the absorbance. So, you actually absorb you can plot the absorbance on the y axis and you plot the TNF alpha concentration on the x axis.

And that actually is going to give you curve and use a regression analysis and you can be able to draw the equation of this particular curve and then you use this equation to determine the TNF alpha concentration into the unknown samples. So, this is all about the sandwich Elisa to determine the level of antigen into the patient sample or the biological fluid.

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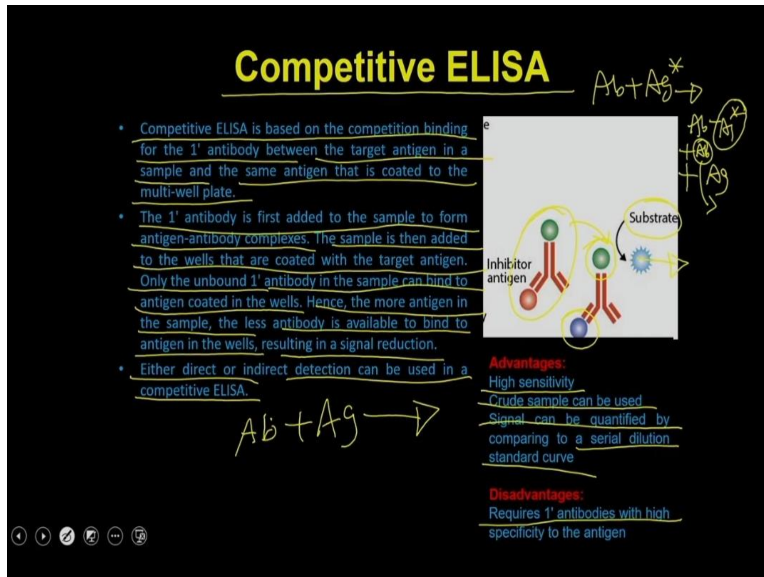

Now, this is the competitive Elisa. So, competitive Elisa is actually based on the competition binding for the primary antibody between the target antigen in a sample and the same antigen that is coated onto the multi well plate. In this case, what you are doing the primary antibody is first added to the sample to form the antigen antibody complexes. The sample is then added to the; well that are coated with the target antigen, only the unbound primary antibody in the sample can bind to the antigen coated in the well.

Hence, the more antigen in the sample, the less antibody is available to bind the antigen in the well resulting in a signal reduction which means, what is what this means is that you first you make the antigen antibody antigen complexes into the sample. So, what you do is you take the sample you add the antibody, so, what will happen is the antibody will go and bind to the antigen. So, as I said you know antigen antibodies are always making a complex in the equimolar ratio. So, suppose; you added the 10 microgram of antibody.

So, what will happen? The 10 microgram of antibody is actually going to form the complex with the 10 microgram of antigen. So, now, imagine that you have the more amount of antibody. So, suppose, I added the 100 microgram of antigen antibody and the level of antigen presenting your sample is only the 20 micrograms. So, what will happen is the rest 80 microgram antibodies are still available, so, they will go and bind to the antigen what is available onto the plate and that is how they are actually going to give you the readouts.

But if you have more amount of antigen for example, if you have 80 microgram of antigen, then you only have the 20 antibody molecules available, which means, as the amount of antigen in the biological fluid is available. It is actually going to keep reducing the available antibody for making an interaction with the antigen which is present onto the plate. This means, there will be a competition of antigen between the antigen which is present onto the plate and the antigen what is present on to the sample and that is why this is called as the competitive Elisa.

Either direct or the indirect detection can be used in the competitive Elisa which means, you have an antigen which is actually been coated onto the plate. And you have an antibody which is bound to the antigen which is in the sample and then when you add them to this, so, antibody plus antigen is so, antigen star is making a complex like antibody antigen star complex. So, when you have the antigen which is coated onto the plate, this antigen is actually less.

So you have the excess antibody and that excess antibody is going to react with this antigen and that so, this antibody has an enzyme so, that actually is going to process the substrate and will give you the color. The advantage of this essay is that it is highly sensitive it is can be used with the crude sample and you can be able to use the mute the signal whatever you get can be quantified either by with the serial dilution standard curve.

So, you can actually use the different amount of antigen into the solution and that actually will allow you to measure the level of antigen. The disadvantage is that you require a primary antibody which is very very specific for at that particular antigen. It should not have the cross reactive.

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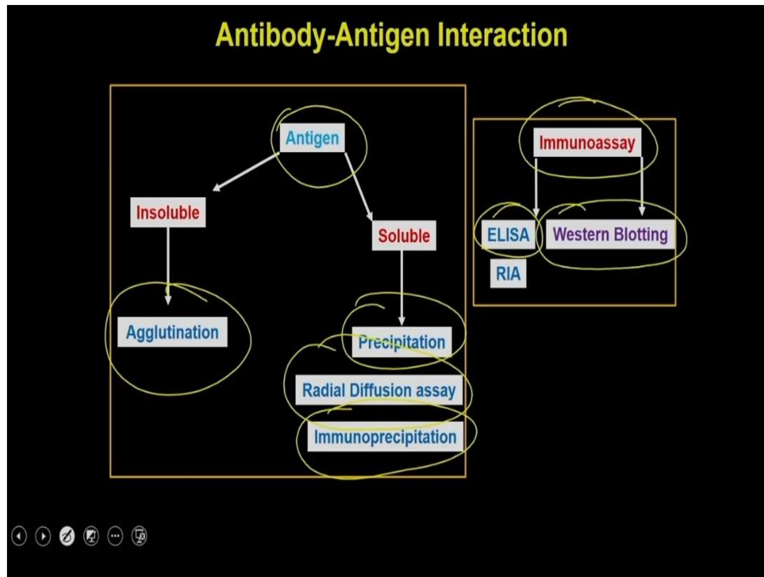

So, this is all about the antigen antibody interactions. We discuss about the agglutination we discuss about the precipitations we discuss about radio amino acids and we discuss about the immunoprecipitations. Within the immuno assay also we discuss about the Elisa. And now, we are going to discuss about the western blotting.

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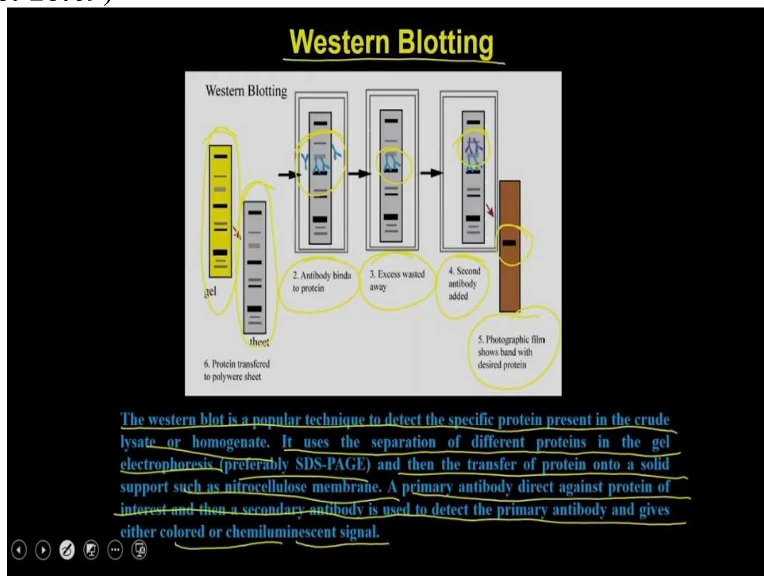

So, western blotting is a technique to detect the proteins what is being bloated onto the nitrocellulose membrane. So, the western blotting is a popular technique to detect the specific protein present in the crude lysate or the homogenate. It uses the separation of different proteins in the gel electrophoresis preferably SDS-PAGE and then on the transfer of the proteins on to a solid support such as the nitrocellulose membrane.

A primary antibody directed against the protein of the interest and then secondary antibody is used to detect the primary antibody and give either the color or the chemiluminescent substrate. So, what you have is you have the proteins present on the SDS that you transfer on to the nitrocellulose membrane, then you add the primary antibodies, the primary antibody will go and bind to the their target antigens, then you remove the step with the washing.

So, the washing will remove the nonspecifically bound antibodies, then you are going to add the secondary antibodies and that actually is going to bind to the primary antibody and then ultimately you can be able to add the colorimetric substrate or the chemiluminescent substrate. And that actually is going to give you the signal next to the protein where the anti primary antibody is bound. So, let us see how to perform the western blotting.

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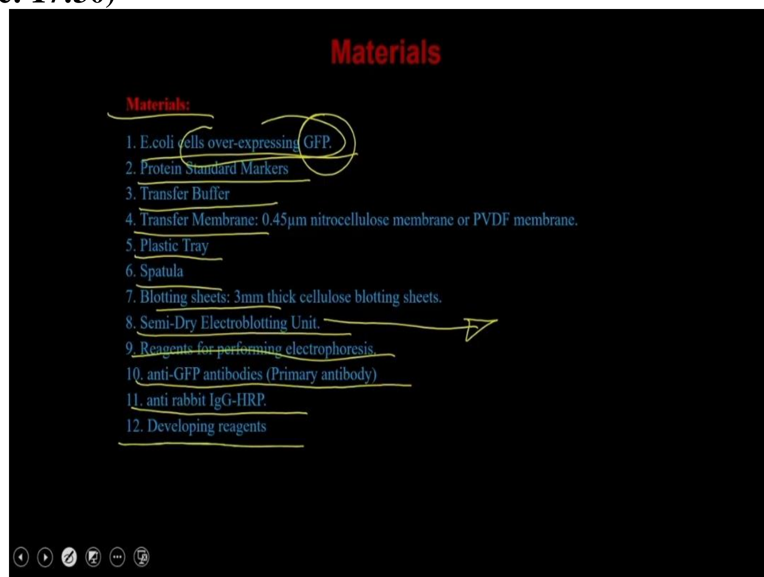

So, the material what you requires the material what you require. If the cells. In this case, the E.coli cells which is overexpressing the GFP, you require the protein standards transfer buffers, transfer membrane like the nitrocellulose membranes, the plastic tray, spatula, blotting sheets, semi dry electro clotting units, that is required to electro blot the proteins from the gel to the nitrocellulose membrane then you require the reagent for performing the electrophoresis you require the primary antibodies to detect the GFP into the proteins they require the secondary antibodies which is coupled with HRP and then you require the developing reagents.

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Procedure

STEP 1: Preparation of Sample: **Preparation of sample depends on the sample type.**

For Tissue: For solid tissue such as tumor or whole liver, brain, it is first mechanically broken down into individual cells using a blender, homogenizer or by sonication. Once individual cells are obtained it will be processed as described.

For Cells: Individual cells are incubated with the lysis buffer containing detergent along with protease and phosphatase inhibitor cocktail to protect the sample from degradation.

STEP 2: Electrophoresis of Sample: Sample was resolved on SDS-PAGE as described previously.

STEP 3: Transfer of protein gel on blotting membrane:

A. Preparation of Transfer membrane: Cut the membrane of the same size as gel.

A1: For nitrocellulose membrane: Place the membrane in the transfer buffer and observe that the liquid has wicked the membrane. Areas appeared as white spot needs special consideration.

A2: For PVDF membrane: Immerse the membrane into the 100% methanol for 15-30mins. Decant the methanol and submerge the membrane into the transfer buffer for additional 10-30mins.

In the step 1, you have to prepare the samples so, the preparation of the sample depends on the cell type for a tissue First, the follows tissue such as the tumour or the whole liver or the brain, it is first mechanically broken down into the individual cells using a blender and then homogenise it for the sonications. Once the individual cells are obtained, it will be processed as described below. For the cell, individual cells are incubated with the lisis buffer containing detergent along with the protease as well as the phosphate inhibitor cocktail to protect the sample from the degradation.

So the phosphate inhibitor cocktail only you will use if you are interested to probe the antibodies or group the antigen which is phosphorylated. So because you do not want a phosphatase to remove the phosphate from the phosphorylated proteins. Step 2 you have to electrophoresis the sample. So, your sample is well resolved on the SDS page, then the step 3 you transfer the protein gel onto the blotting membrane.

And then the before you do the transferring of the blotting membrane, you have the multiple steps like you have to prepare the transfer membrane. So you have to cut the membrane into the same size as the gel like if you have a gel of this size you have to cut the membrane of this size. And then you have to process the membrane in different way depending on which membrane you are using. So for example, if you are not using the nitrocellulose membrane, place the membrane in the transfer paper.

And observed that the liquid has wicked the membrane at yours appeared as a white spot needs a special consideration which means if you add the transfer buffer, this membrane is going to be completely wet. There will be no white spot present on this membrane which means this membrane will not be visible. It is actually going to submerge into the transfer and it will take up the same color as your transfer buffer that will indicate that the membrane is that has taken up the transfer buffer.

If you are using the PVDF membrane, which is actually a hydrophobic membrane, then what you have to do is you immerse the membrane into 100% ethanol methanol, because then you have to do a charging step because the PVDF membrane has to be because it is a hydrophobic membrane. So, it will not bind the protein very nicely. So, it has to be charged with a polar solvent. So, what you have to do is you first incubate the PVDF membrane into 100 percent methanol for some time and then you add the transfer.

And then you add the transfer the transfer effort should take up the PVDF the buffer and it should be also does not can show any white color. Decant the methanol and submerge the membrane into a transfer buffer and additional 10 to 30 minutes. So, with this the transfer membrane is going to be ready and then you have to set up the electroplating.

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B. Assembly of transfer cassette:

1. Remove the stacking gel from the PAGE and equiliberate the gl in transfer buffer for 10-30mins.
2. Place a pair of blotting sheet (already saturated with transfer buffer) on the anode plate (usually this plate is black colored).
3. Place the transfer membrane on the top of blotting sheets and remove trapped air bubble by rolling test tube or glass rod.
4. Place the PAGE gel on the top of membrane and gently remove trapped air bubble by rolling test tube or glass rod.
5. Place another blotting sheet (already saturated with transfer buffer) on the top and remove trapped air bubble by rolling test tube or glass rod.
6. Finally keep cathode plate (usually red colored) and tight the transfer cassette by four screws.

So, assembly of the transfer cassette. So, what you have to do is remove the stacking gel from the page and incubate the gel in a transformer for 10 to 30 minutes. Place a pair of blotting sheets onto the anode plates. So, transfer operators is having a an anode on one side and the cathode on one side and then you have to set up the things like this, you have to first put the filter papers then you have to keep your nitrocellulose membrane or then you keep your gel.

And then you keep your transfer membrane or you can do reverse that you put first put the filter paper then you put the gel then you put the membrane and then you keep the next layer of the filter papers. You have to ensure that there is no bubble present between the gel and nitrocellulose membrane because it does there will be an air bubble present that area is not going to be will not going to allow the transfer of protein from the gel to the nitrocellulose membrane.

So place the tancet membrane onto the top of blotting sheets and remove the trapped air by rolling the test tube or the glass rod. Then you place the PAGE gel on top of membrane and gently remove the trapped air bubble by rolling the test tube you place the another blotting sheets onto the top. And then remove the trapped air bubble by rolling the test tube or the glass rod and finally keep the cathode plate usually the red colored cathode plate and tide the transfer captured by the 4 screws.

So, once you have done that, you have the 4 screw on all the sides and that actually you are going to put to screw the this particular gel cassette it and then you connect it to the electrode to the power card and that actually will allow the transferring of the protein from the gel to the to the nitrocellulose membrane.

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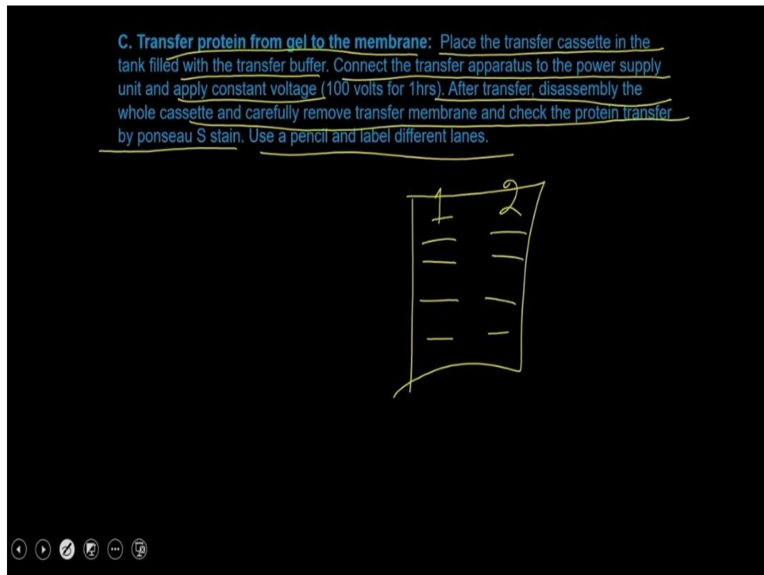

Then you transfer the protein from the gel to the membrane plus that place the transfer Cassette in the tank filled with the transfer buffer connect the transfer operators to the power supply and apply the constant voltage after the transfer disassemble the whole cassette and carefully remove the transfer membrane and check the transfer by the punch shoe stain use a pencil and label the differently which means if you got these lanes and these membrane you have to write the lane number.

So that that lane number can be used subsequently when you are doing the chemical plant development or you are using the autoradiogramy or any other method, this number will actually allow you to identify the particular lane.

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STEP 4: Blocking: Wash the membrane with distilled water to remove any remaining ponseau S stain. Put the membrane in blocking buffer containing 5% skimmed milk (for detection of cytosolic protein) or 5% Fat free BSA (for detection of phosphor protein) for 30 mins.

STEP 5: Probing: In western blotting, probing can be done in two different ways.

A. Two Step Probing: In two step probing scheme, membrane is first probed with the primary antibody to recongnize the protein of interest. (Generation of monoclonal or polyclonal antibody is discussed in the later lectures). Membrane is probed with the primary antibody with an appropriate dilution for 1hrs at room temp (RT). Membrane is washed with buffer containing non-anionic detergent Triton X-100 and reprobed with another antibody directed against the primary antibody. The secondary antibody is coupled with an enzyme (either HRP or alkaline phosphatase) or a fluorescent dye. Washed membrane is incubated with the secondary antibody with an appropriate dilution for 1hrs at room temp (RT). Membrane is washed with buffer containing non-anionic detergent Triton X-100 and developed. Use of two different antibodies increases sensitivity as well as giving flexibility to plan multiple probing.

B. One Step Probing: In one step probing, primary antibody contains enzyme or fluorescent label for detection. One step probing is not very common.

The step 4 you have to do the blocking the blocking exactly the same and then step 5, you have to do the probing. So, in the western blot the protein can be done in 2 ways. In the 2 step probing you have to first add the primary antibody followed by the secondary antibodies. So, you have to take the appropriate dilution of the primary antibody you have to add the primary antibody followed by the washing and then you had have to add the secondary antibodies or in a 1 step probing.

What you have to do in one step itself you add the primary antibody which is containing the enzyme which means the primary antibody which is labelled with the enzyme or the fluorescent probe, and that can be used. One step probing is very uncommon, because the one step probing does not allow you to do because the sensitivity level is very low with the one step probing.

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STEP 6: Blot Development : There are multiple ways to develop the blot and detect the protein present on the membrane.

Table: Different Reagents Available to develop western blot.

System	Reagent	Reactions
Chromogenic		
HRP Based	4-chloro-napthol	Oxidized Product to form purple precipitate
	$DAB/NiCl_2$	Forms dark brown precipitate
	TMB	Forms dark purple stain
Alkaline phosphatase	BCIP/NBT	BCIP hydrolysis produces indigo precipitate after oxidation with NBT.
Luminescent		
HRP Based	Luminol/H2O2	Oxidized luminal gives off blue light.
Alkaline Phosphastase	Substituted 1,2 dioxetane phosphate	Dephosphorylated susbtrate gives off light

A. Colorometric Detection: Wash the membrane with TBS to remove detergent. Place the membrane into the colorimetric reagent and protein bands appeared in 10-30mins. Stop the reaction by washing in distilled water. Air dry the membrane and photograph for permanent record.

B. Chemiluminescent Detection: The different chemiluminescent reagents are given in the Table 16.1. Transfer the membrane into the chemiluminescent reagent. Soak the membrane for 30sec to 5mins. Drain off the reagent and wrap the membrane into the plastic wrap. Place it in film cassette and expose the membrane to film for a few seconds to several hours.

C. Fluorescent detection: Secondary antibodies labeled with fluorescent dye and captured in the scanner.

Then you have to do the block development. There are multiple ways to develop the block which is present onto the membrane. You have the HRP based chromogenic substrates like 4-chloro-napthol DAB or TMB or you can have the alkaline phosphatase enzyme also. So; you for that you can use the BCIP/NBT system or you have the luminescent substrates like for HRP, you have the luminol and H2O2 or for alkaline phosphatase you have the substituted 1 2 dioxetane phosphate.

So, all these substrates are allowing you to detect the proteins what is being bloated onto the nitrocellulose membrane either you have the colorometric methods or you have the chemiluminescent methods. So, both the methods are actually allowing you to detect the proteins colorimetric detection has the lower sensitivity compared to the chemiluminescent detections.

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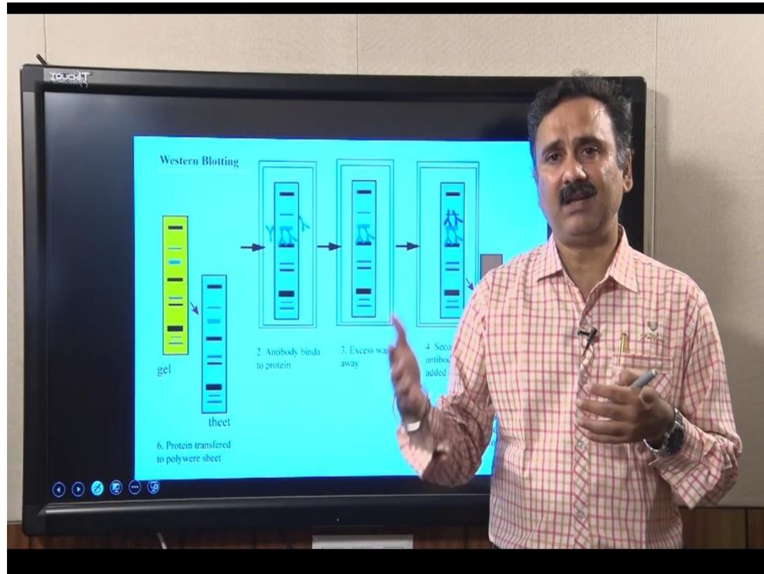

So, this is about the western blotting. So, we have discussed all these steps and then we have also discussed about how to perform these steps. And now, to show you how to do these experiment, I will like to take you to my lab and where the students are going to perform these experiments, where they are actually going to discuss each and every steps how to perform and what are the different steps you should take, how to remove the trapped air from the air bubbles or trapped air between the gel as well as the sandwich.

How to process the membrane? So, that it will actually going to be useful for transferring of the proteins from the SDS page to the nitrocellulose membrane or the PVDF membrane. And with these steps, you will be able to understand the how to perform the western blotting in your laboratories.

(Video Starts: 26:40)

In this video, we will demonstrate you how to do a Western blot and how to analyse the result using ACL electro-chemiluminescence substrate. So, here what we will do we have to run gel first, then we will transfer the transfer method how to do the transfer will show in this video. In previous video we have already shown that how to run how to prepare SDS pages and how to run protein samples. So, in this video, particularly we are interested in factors associated with the western blotting.

For doing Western blot we need membrane and transfer buffer and the Transfer Medium this one is we use to transfer this run gel to membrane. So, here membrane can be 2 kinds. One is

nitrocellulose which has low protein binding efficiency and the hydrophobic nature another membrane is PVDF this is hydrophobic membrane and higher protein binding capacity. So, the processing for Western blot is different from different for nitrocellulose and PVDF.

If you are using PVDF membrane, we have to take we have to cut the part whether if you have readymade pre cut pre cut blots, then normally if you have if you are taking from a binding, you have to cut precisely how many wells you want. So, after that, you have to label front on the black where the front side can be used for transferring the protein and that can be used in previous step further steps also like antibody incubation. So here for if you want to use PVDF membrane you have to charge with the methanol.

So, since the PVDF is a hydrophobic membrane, you cannot directly transfer the transfer in the first you have to keep in methanol for at least 20 minutes. So after this can be called as charging. So after this, we will use that for transfer. So this is pre soaked in methanol and equilibrated in Transfer buffer. So, here by doing transfer we need to consider a few things the buffer always should be in chilled condition.

Otherwise during this transfer at high voltage it will generate high temperatures so that may degrade your protein or decrease the efficiency of the transfer. That is why we need to keep the buffer always in chilled condition and let start the presentation. So we need a gel so we are already finished the gel, in addition to that, we also need sponges, which will give cushion to the gel, so that the gel may not destroyed during the transfer.

So this is the Cassette we will use for the transfer. So, this is negative side of Cassette and this is the positive side. So, we are going to keep gel on negative side and the positive side the blot membrane. So, when we apply voltage from this side to this side, the negative protein it will be transferred, it will be move to positive side and it will be a captured in the membrane. So first for doing these sponges we need to keep and also this maybe give some nonspecific binding to membrane.

So, what we will do, we will put blotting sheets on top of this. So, after this you have to remove air bubbles if any present. So, once you inserted the blotted sheet, then you have to keep your gel. So, here when we have to remember that gel after finishing the SDS is running you have to keep in Transfer buffer. So, that it will give identical condition or equilibration kind of thing during transfer. So, that protein transfer may be easy. So, this is the gel and keeping on the negative side.

So, after that we have to overlay with the membrane. Next we have to remove any air bubbles. We have to overlay another blotting sheet and remove the air bubbles. Each and every time when you introduce something you have to remove a air bubbles. So, this is the final sheet. So, this is the positive side of the Cassette. Just how to like this, these are the screws we have to tighten it up then only the contact between the gel and the membrane will be sufficient to get transferred.

First you do not tight initially you just keep and after that press the positive side of that cassette then tight the screw. So, all these things should be done in the transfer buffer only unless specified. So this is the chilled transfer buffer. Now we are going to do transfer. Pour sufficient buffer. And keep this ice pack also if the chilling is not sufficient, then there may be heat generation. So, in order to prevent that, we will use this ice pack. So, this will keep the buffer cool till the transfer end of the transfer.

So, once that is over, you directly take out the cassette and keep. If there is a buffer insufficiency, you can add on top of that. Make sure that the cassette completely submerged, so that the transfer will be proper and there are no air bubbles. So, once the setup is over, now, you can transfer. Transfer is going well. So how much voltage we need to give, it depends on transfer to transfer it varies. Generally in our lab we will give at least 2 hours of transfer it 120 volts which is sufficient to transfer when low molecular weight proteins also.

But, from instrument to instrument also it varies. We need you needed to optimise before doing transfer. After 2 hours, we have to remove the blot and incubate with the blocking buffer. So, I am going to stop here remove the cassette keep that in a tray, remove the screws properly gently

remove the Sponges. Take out the blot and keep it in blocking buffer. In this condition, we have to keep if you are keeping it room temperature, it is for 2 hours at least.

If you are keeping in 4 degrees Celsius, you can keep overnight the blocking buffer contents skim milk or BSA along with tween-20. The western blot transfer it is all depends on the efficiency, how precisely you are doing the transfer. For example, you should not use your bare hands while handling the blot or gel. So, whatever the proteins present on your fingers, it will transferred into gel or membrane which will give high background during development of the blot.

So, always use gloves. Apart from that, while handling the instrument, make sure that there may be possibility of electricity the shock may happen sometime. So, we have to that time also we need to use gloves and after finishing the transfer you have to clean all the apparatus properly and try it for the next time use. After the blocking of the membrane we have to remove the membrane and incubated with the primary antibody without washing. The main purpose of the blocking is that it will occupy non specific sides other than the respective protein.

So that when antibody comes it will bind to that specific protein and gives no noise. So, after this, we will incubate with the primary antibody for overnight at 4 degrees Celsius then wash 3 times at least 15 minutes each with the TBST buffer or PBST buffer. And again treat with incubate with the secondary antibody suitable secondary activity for 5 hours at 4 degrees Celsius or 2 or 3 hours at room temperature. After that, we need to wash properly at least 3 times then we will develop the blot with the electro chemiluminiscent substrate.

In earlier Western blot; how to do western blot video we showed how to transfer the proteins to membrane. So, we are we incubated with the primary antibody, following secondary antibody and wash it thoroughly. Now, here we show how to develop a blot. For developing the blot, we need chemiluminiscent substrate. In most of the commercially available kits, luminal is the one of the substrate we used for this purpose. So, luminal in presence of hydrogen peroxide.

And peroxide gel which present in the secondary antibody, (()))(40:08) this horseradish peroxidase converts luminal to excited state luminal by deprotonating and oxidizing. So, this excited state product gradually leaves the energy by releasing luminescent protons that light will be detected using this instrument. So, these are the commercially available Chemiluminiscent substrate solutions. So, it is available from a wide range of companies we have to mix one ratio so, we have to take out the blot.

Then the buffer whatever present properly so, keep blot in between a plastic paper files that we will take a chemiluminiscent substrate. After that slowly press and remove air bubbles. This is the tray we use it for developing blot. So, we have to open the system. Properly align the tray and shift blot to that tray. Once it is over, you have to just close here we have to select application. We want blots that is chemiluminescent and what exposure you have 2 options manual, auto. Auto 2 options are there optimal auto exposure, rapid auto exposure.

We will choose optimal auto exposure. So, you can enlarge the blot, once it is over, just say. So, this is the develop blot. So, as we can see the bands pattern. So, this is how we develop Western blot through electro-chemiluminescent substance. So, in this video we will demonstrate how to transfer proteins to blot and what are the precautions to be taken during the western blot? And also, how to develop the blot and what is the main principle behind the development. So, I hope this will help you to understand the basic outline mechanism.

(Video Ends: 44:07)

So, with this we have discussed the demo of the western blotting and in the western blotting demo the student have discussed many aspects related to the western blotting. So with these we have discussed about the antigen antibody interactions we discuss about the agglutination reaction we discuss about the precipitation reactions. And then lastly, we have also discussed about the different types of immunoassays like Elisa as well as the western blotting.

So, with this I would like to conclude our lecture here in the subsequent lecture we are going to discuss few more immunoassays related to this particular immunology and we are also going to see how those tools are helpful in answering the different types of biological problems. So with this I would like to conclude my lecture here. Thank you.