Experimental Biotechnology
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Lecture-29
Immunoassay - Part 1

Hello everybody, this is Dr. Vishal Trivedi from department of bioscience and engineering IIT Guwahati and what we were discussing, we were discussing about the different types of immunological tools, what you can use. So, in our previous lectures, we have discussed about how you can be able to generate the polyclonal as well as the monoclonal antibodies and then subsequent to that, we were discussing about how the antibodies are interacting with the antigen.

And how that interaction can be exploited to design different types of immunological tools to answer the different types of biological problems. So, following that discussions, today, we are going to discuss few more analytic few more immunological tools, where the antibody and antigen and are interacting with each others.

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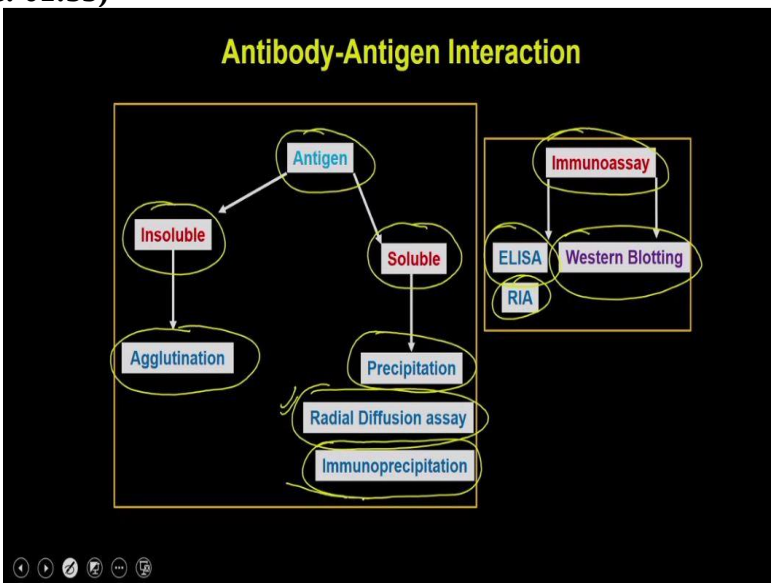

So, this is what we were discussing so, far that the antigen if it is insoluble nature, you can actually be able to perform the agglutinations or if it is soluble in nature, you can be able to perform the precipitation as well as the radial immunoassay as well as the immunoprecipitations. So, in the today's lecture, we and subsequent to that, we have also planning to discuss about the different types of immunoassay which are also been based on the antibody antigen interactions

such as ELISA RIA and a western blotting. So, subsequent to the radial immunoassays, we are now going to discuss about de immunoprecipitations.

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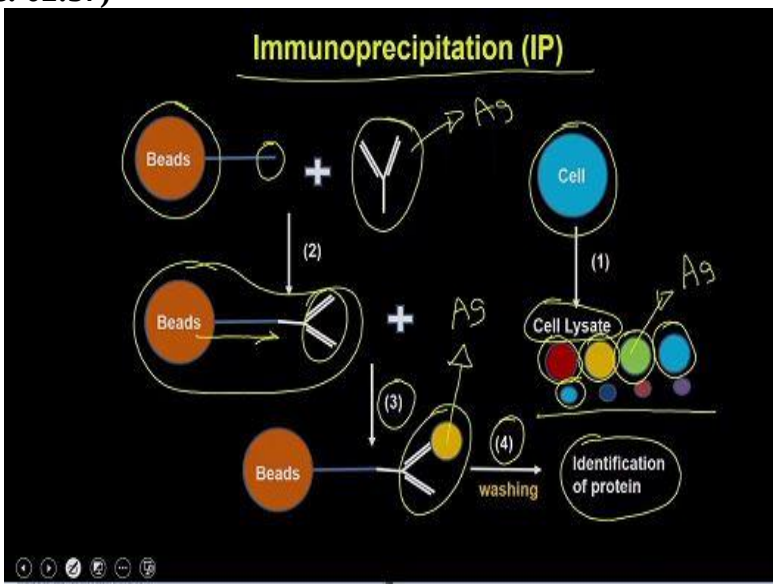

So, immunoprecipitation the basic principle is very simple, the principle is that where you are going to take the empty beads and these empty beads are actually going to have the functional groups. So, with the help of these functional group, what you are going to do is you are going to attach the particular antibody, these antibodies are actually going to be a specific for a particular antigen. And then what so this beads is going to be prepared.

In us on a parallel, what you are going to do is you are going to break the cell and you are going to prepare a cell lysates. And in this cell lysate, you are going to have the different types of proteins, which I am showing you with the different types of beads or different types of color beads. So these are all the potential antigens, which actually can interact with this antibody, and actually can give you the which actually can be isolated from these particular cell line cell lysate.

So in the third step, what you are going to do is you are going to incubate these bead bound antibody with the cell lysate, which actually contains different types of antibody, different types of antigens, and then when you do the incubations does one some of these proteinaceous factors of antigens are going to bind the antibody and then you are going to do a initial washing step and then you are going to do the identification of this particular antigen with the help of several

techniques such as you can run it on the SDS page or you can do it like the silver staining or you can do the western blotting with the additional antibodies how to perform this assay.

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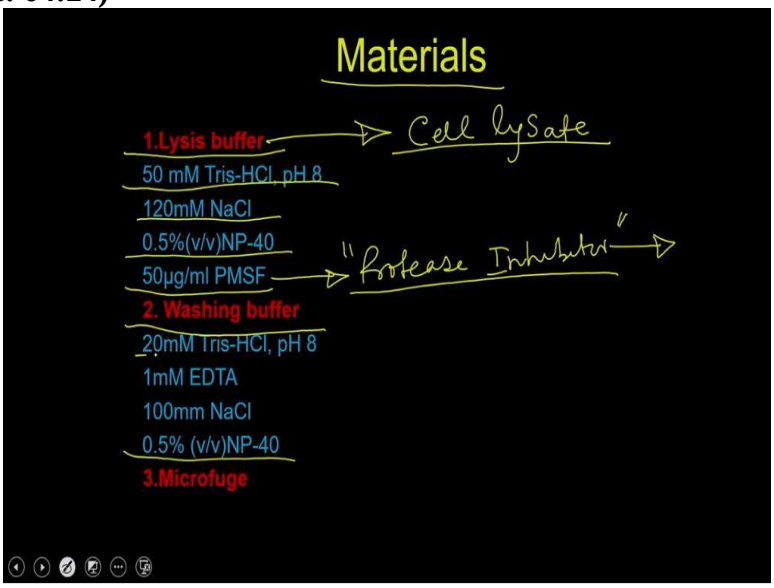

So, to perform this assay, these are the following material what you require first you require a lysis buffer this lysis buffer is required for preparing a cell lysate. So it has all the components like the buffer, then NaCl and then it has the detergent and then it also has the protease inhibitor. So this is a protease inhibitor which you have to add just before reconstituting the lysis buffer. So protease inhibitor is actually going to protect the cell lysate from the degradation by the proteases which is going to be present in the cell lysate.

Apart from that, you also require a washing buffer. So, washing buffer is exactly this competition like the buffer then you require EDTA NaCl and NP 40. And then you also require a small centrifuge so that you can be able to do the piloting and all that.

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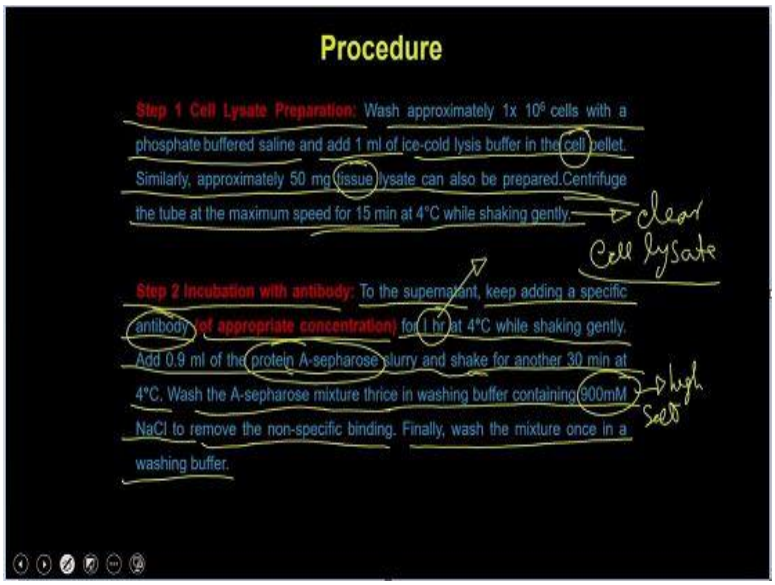

In the first step, you are going to prepare the cell lysate. So, you wash the 10 to power 6 cells with a phosphate buffer saline and then you add 1 ml of ice cold lysis buffer in the cell palate. Similarly, you can approximately 50 mg tissue lysate can also be prepared. So, either you are going to have the individual cells or you are going to have the tissue either irrespective of the case, you have to break open the tissue to release the individual cells and then you can actually be able to add the lysis buffer or if you had the individual cells.

You can actually treat it with the lysis buffer and that actually is going to give you the cell lysate then you centrifuge this mixture at for a full speed like the 15,000 G and that actually is going to give you the clear cell lysate which means it is actually going to remove all the degrees from the cell lysate. Then the step 2, you are going to do the incubation with the antibody to the supernatant keep adding a specific antibody.

So, this specific antibody means that antibody which is against a particular antigen at appropriate consultation, so, that depends on the amount of antigen present as you recall in a previous lecture itself, where we discussed that the antigen antibody precipitation reaction is this documentary governed which means, if you require proper precipitations the amount of antigen as well as the amount of antibody has to be in equimolar ratios.

So, if you add a little antibodies or very high amount of antibodies, the precipitation is not going to be adequate, you add that for 1 hour at 4 degree which shaking this 1 hour can be overnight

also in some cases when the antigen is very difficult to bind or you require the more number of recovery then you add the 0.9 ml of a protein A-sepharose. So protein A-sepharose is the bead which actually is going to bind the antibodies and in shake for another 30 minutes at 4 degree.

You watch the A-sepharose mixture tries in washing buffer containing 900 milli molar NaCl. So, this is the high site consultation which you are going to use, so, that you will remove the nonspecifically bound protein to the antibodies as well as to the beads to remove the nonspecific binding. Finally, wash the mixture once in a washing buffer. So, now, once you are done with the washing with the license buffer as well as washing with the washing buffer, your sample is now ready. And now what you can do is you can elute the antigens from the antibodies and then you can be able to analyze them.

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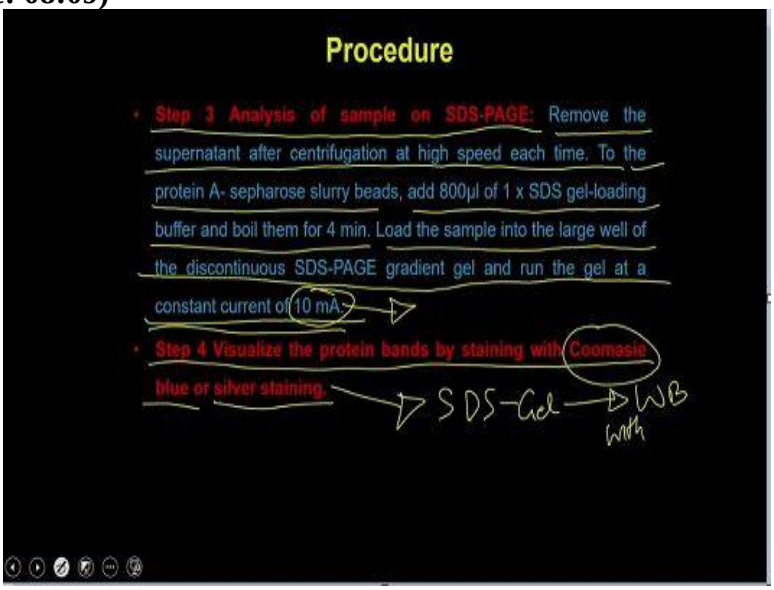

So in the subsequent step in the step 3, you are going to do the analysis or sample on the SDS page. So, you remove the supernatant after the centrifugation at high speed to the protein A-sepharose slurry beads at 800 microliters of 1 x SDS gel loading buffer and boil it for 4 minutes load the sample into the large well of the discontinuous SDS page gradient gel and run the gel at a constant temperature of 10 milliamps.

So, once you have the beads, which actually have the antibodies and antibodies are binding to the particular antigen from the cell lysate you take those beads, wash it remove the supernatant and then you add the 1 x SDS loading buffer and then you heat it and boil it. So in that process all the

intergenic determinant because the antibody is going to be denatured when you do so, and the antibody is going to release the antigen into the supernatant. And that supernatant you can load it onto the SDS page.

In this case, we are using the gradient SDS page so that the resolution is going to be even better. And then you run it for at constant current for 10 milliamps. Because we want to, we do not want to create the heat into the system so that there will be a denaturation of the antigen. That is why you have to run it at a 10 milliamps current at a very, very slow rate. So that it is actually going to resolve the samples, but it will not going to heat the gels.

Then you are going to 4 the step 4 you can visualize the proteins with the help of either the coomasie blue (())(09:45)blue staining or the most sensitive silver staining at this is stage what you can also do is you can take the SDS gel, and you can actually can do the western blotting with a specific antibody. For example, if I am expecting actin to be presented with a bound to the antibodies, what I can do is I can just take this supernatant load it onto the SDS page.

I can do a staining with the coomasie or silver staining to check that I am getting a band very close to these to the level of the actin, but alternatively what I can do is I can just simply transfer that gel onto a nitrocellulose membrane and then I can do a western blotting with the NT actin antibodies, and that actually is going to give me the specific information about the protein what is present into the SDS page whether it is a actin protein or some other proteins.

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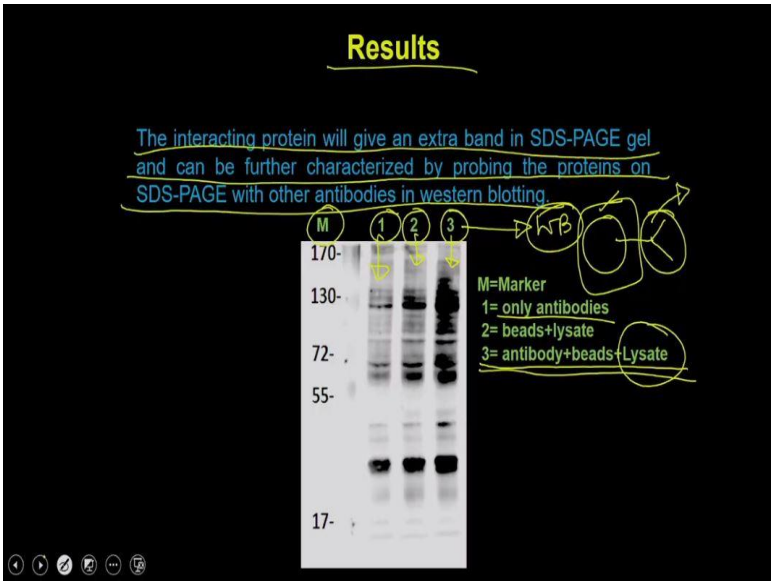

The results the interacting protein will give an extra band in the SDS gel and can be further characterized by probing the protein on the SDS page with another antibody in a western blot. So, this is a typical result what you see. So, in the lane 1 we have loaded the molecular markers lane 1 is actually only antibodies, lane 2 is actually the beads plus lysate and the lane 3 is actually the complete reaction.

So, what you can see is the lane 1 is showing some band because some of the protein will bind to the antibodies, the lane 2 is showing more number of bands and those are the nonspecific band which are actually binding to the antibody as well as binding to the beads. So, these 2 are actually going to be it these 2 the protein which are binding to the beads as well as the protein which are binding to only antibodies or has to be subtracted.

And then you can actually be analyzed the sample which is actually in the number 3 where the antibody as well as the bead plus lysate is present. And in this what you see is there are specific bands which are appearing into this and now what you can do is you can also do a western blotting of this sample with the specific anti antibodies or anti antigen antibodies whichever the antigen you are expecting that should present in this particular immunoprecipitations.

Or in some cases, if you are not sure, then you can go through with the molecular weight and then you can probably can make a you know intelligent guessing and then actually be able to

probe the SDS page with the particular antibody and to recognize or to identify this particular protein in a generic approach, if you do not know any of these things or you do not know even the pathway, then what you are supposed to do is you have to take out that particular protein from the gel you have to do (())(12:36).

So, that it should produce the peptides and then you can send this peptide for the mass spectrometry and then you can do the proteomics studies and then actually that also will allow you to identify this particular protein. Now, apart from the immunoprecipitation, where you are actually using the antibody as a probe, so, that it will bind to the beads and then you can use that bind bead bind antibodies for getting the proteins from the cell lysate or getting the antigen from the cell lysate.

Many people are also using or also performing a slightly derived version of this, which is called as the pull-down assay. So, in the pull-down assays, you are either using the antibodies or you are also using the some other protein which is bind to the beads. So, let us discuss about the pull-down essay which is actually a modification of the immunoprecipitation assays.

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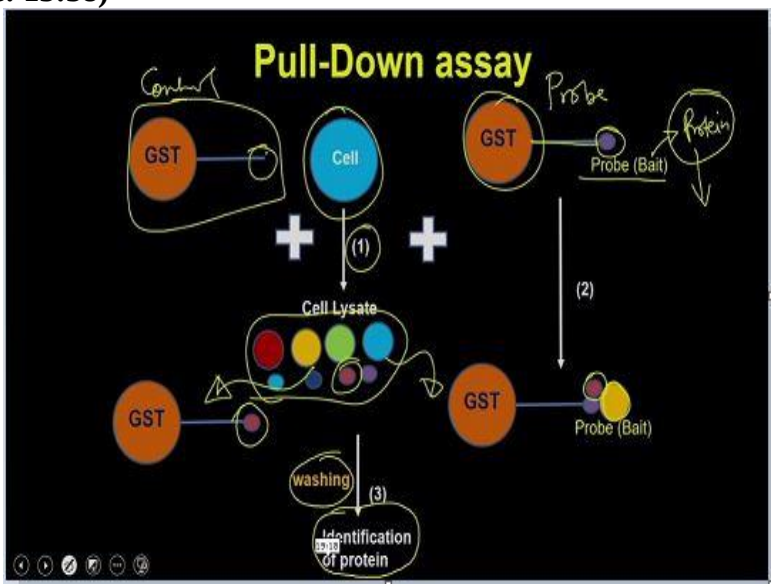

So, in a pull-down essay, what people are doing is they are taking a GST beads and GST baits has the functional group. So, that GST group functional group is present and that functional group. So, they are preparing the 2 baits one is the control bead where you have the GST baits with a functional group the other one is the probe bead or probe bead. So, where you are actually

having the GST beads and on the functional group you have the probe this probe is also called as the bait protein.

So, that bait protein is a specific protein on which to against which you are interested to see how many proteins are interacting and then what you do is you take the cell you bracket open with the lysis buffer to generate the cell lysate and the cell lysate actually contains the different types of antigens and then what you do is divide the cell lysate into 2 reactions and then you incubate these with the these 2 preparation of the beads and then these 2 beads are actually going to bind the nonspecific protein.

For example, in this case it has bound this particular protein so this and whereas in this case it has bound the yellow color protein as well as the this pink color protein. So now what you do is you do a washing and then you do the identification of the protein with the help of the different types of analytical dyes available or different types of western blotting via experiments. So, how to perform the pull-down assay.

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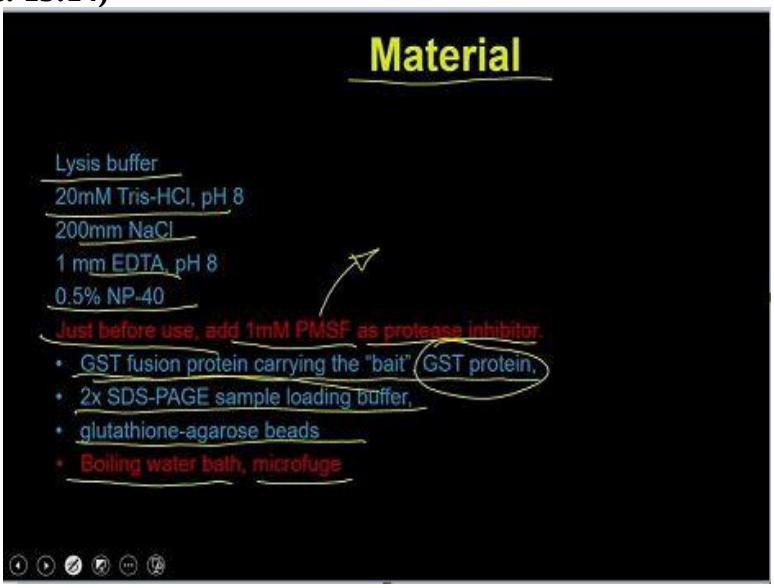

So, in the materials what you require is the lysis buffer, so, lysis buffer has the buffer NaCl EDTA and then you have the NP 40 and then just before the use you can add the 1 milli molar PMSF of to PMSF service also a protease inhibitor which actually going to protect the sample from the degradation by the proteases then you require the GST fusion protein carrying the bait or the GST protein. So, these are the control protein these are the probe proteins and then you

require the SDS page sample loading buffer or then you require the glutathione-agarose beads and then you require the boiling water bath as well as the microfuge diffuse.

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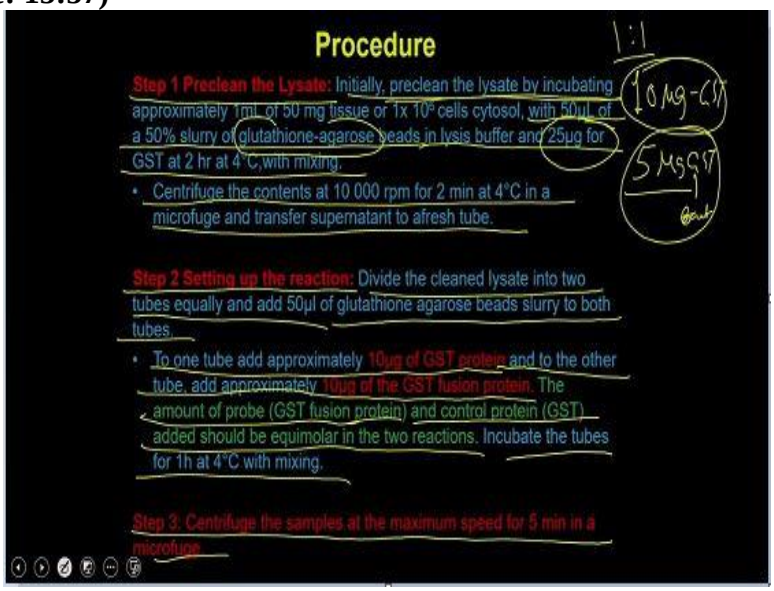

The step 1 you are going to do the preclean the lysate. So, initially you will prepare the lysate by while baking open the cell with the help of the lysis buffer and then you are going to do a preclean in the preclean what you are going to do is you are going to take the lysate and then you are going to add the 50 microliters of glutathione-agarose bead in a lysis buffer and 25 microgram of GST at 2 hours. So, that actually is going to remove the proteins which are interacting with the GST beads or with the GST alone.

And that actually is going to be a preclean step which means it is actually going to reduce the background of the proteins what is present in your reactions, then what you do is you centrifuge the content for 10 minutes and then you transfer the supernatant to a fresh tube, then you are going to set up the reactions. So, divide the cleaned, the lysate into 2 tubes equally and add 50 microliters of glutathione-agarose beads to the 2 tubes to one tube you add approximately 10 microgram of GST protein.

And to the other tube, you add approximately 10 microgram of GST fusion protein, the amount of flow protein and the amount of the GST protein has to be equal, which means equal means in terms of the equi molar ratios. So, if you have the GST of one molecular weight and bait is of one molecular weight this means, if you if I am adding the 10 microgram of GST so then and if

the GST and the bait is presenting the 1 is to 1 ratio then why what I have to do is I have to add the 5 microgram of GST bait protein.

Because that actually is going to make these 2 beads in a equimolar ratio. So, you have to add the control beads which actually contains the GST bound to it as well as the probe beat in equi molar ratio which means you have to calculate the molecular weight of the bait protein and add it into the GST and then only you can be able to calculate the molar ratios of both the GST as well as the GST bait complexes.

So, the amount of probe which means the GST fusion protein and the control protein added should be an equi molar ratio incubate the tube for 1 hour at 4 degree with mixing, then the step 3, you centrifuge the sample at the maximum speed and in a microfuge, which means like 15,000 G.

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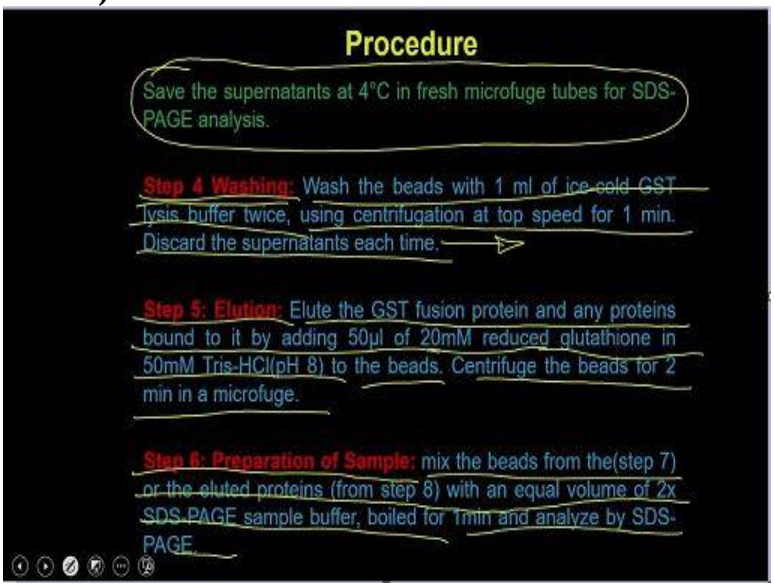

Then you go to the step 4 and step 4 is a washing step. And remember that whatever you are doing, whether you are doing the immunoprecipitation or do the pull-down assays, you always have to keep the supernatants you have to keep saving the supernatant. So, that if there will be no protein present after the final step, you can be able to cross verify at what step you have done something wrong so that the protein complexes are bond to the beads, but they have been detached so that you can actually vary or you can be able to optimize the buffer condition.

So that deep, it actually allows the protein complexes to come and bind to the beads, because that is very important to monitor. So that is why at every stage when you are doing the spinning and throwing the supernatant instead of throwing the supernatant you preserve that supernatant into additional eppendorf tubes so that you can be able to cross verify if there anything goes wrong with the experiments. So in the step 4, you are going to do a washing so wash the beads with 1 ml of ice cold GST lysis buffered twice, using a centrifuge at the top speed for 1 minute.

And then you discard the supernatant although I am writing discard the supernatant but as I said, you know you have to preserve the supernatant for cross verification if anything goes wrong. Then the step 5 you are going to do elution to elute the GST fusion protein and any protein bound to it by adding the 50 microliters of 20 milli molar reduced glutathione in 50 millimeter HCl to the beads, centrifuge the beads for 2 minutes in a micro centrifuge and then you prepare the sample. So, mix the bead or the eluted protein with an equal amount of 2 x SDS buffer and boil it for 1 minute and analyze on to the SDS page.

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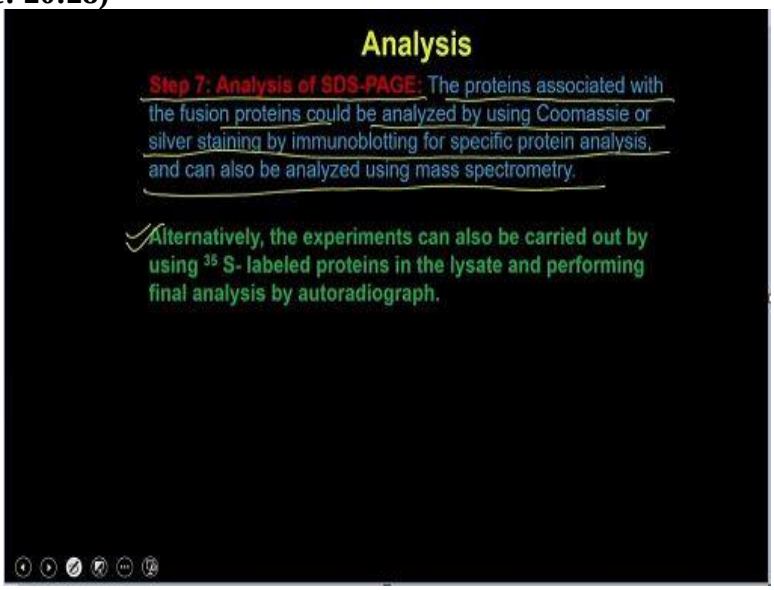

The step 7 you are going to do the analysis on the SDS page the protein associated with the fusion protein could be analyzed by using the coomassie or the silver staining by immunoblotting for a specific protein analysis and can also be analyzed using the mass spectrometry in alternatives of to this is that if you use the radioactivity or if you use the radioactively labeled protein from the lysate, then you also can perform the auto audiogram to check the presence of a

particular protein or you can just do auto radio gram and that actually is going to give you the pattern of the proteins.

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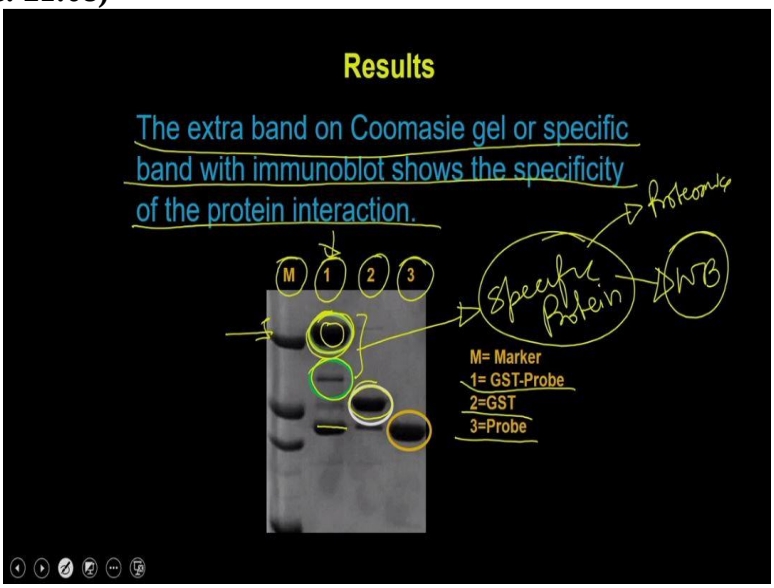

Let us see how the results look like. So, in the results, you are going to see an extra band on the coomassie or the specific band with the immunoblot showing the specificity of the protein. So, what you can see this is one of the representative images from the literature and what you see is that this is the marker, then the number 1 is actually the GST probe, which is actually the full the options, then the step 2 is the GST, which is actually the GST alone, and then the step 3 is actually the probe alone.

So, what you can see is, this is actually the band of the probe, what is present in reaction number 1 and this is the band of the GST and what you can see is that these 2 bands which are present in this full reaction are actually be a specific proteins which are interacting with the bait protein or the pro protein. So, these are the specific proteins can be identified by multiple methods either you can go with the proteomics approach, which means, you can just simply do the isolating this protein from the gel and then you do the (())(22:19) and downstream all the proteomics steps.

And then you ultimately can do the mass spectrometry and identify or you can actually do a western blotting with a specific antibody, which actually you can say simply because if you know the molecular weight of this protein, then you can say what will be the molecular weight of this particular protein and that is how you can be able to identify that particular protein. So, this

is all about the pull-down assays or the immunoprecipitation assays, where you are using the antibody as all the affinity columns for recovering or to isolating a specific antigen from the cell lysate.

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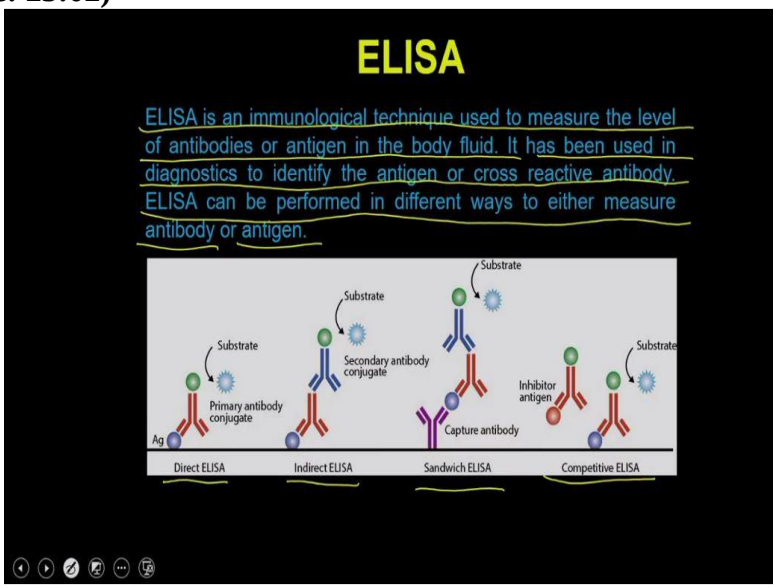

Now, let us move on to the immunoassays. So, one of the immunoassay is the ELISA, ELISA is an immunological technique used to measure the level of antibodies or antigen in the body fluid, it has been used in diagnostics to identify the antigen or the cross reactive antibodies, ELISA can be performed in different ways to either measure the antibody or the antigen. So, ELISA can be performed as a direct ELISA, indirect ELISA sandwich ELISA as well as the competitive ELISA and all these ELISAs are actually designed to either measure the level of antibodies or to the measure the level of antigen. So, let us discuss about each and every ELISA in detail.

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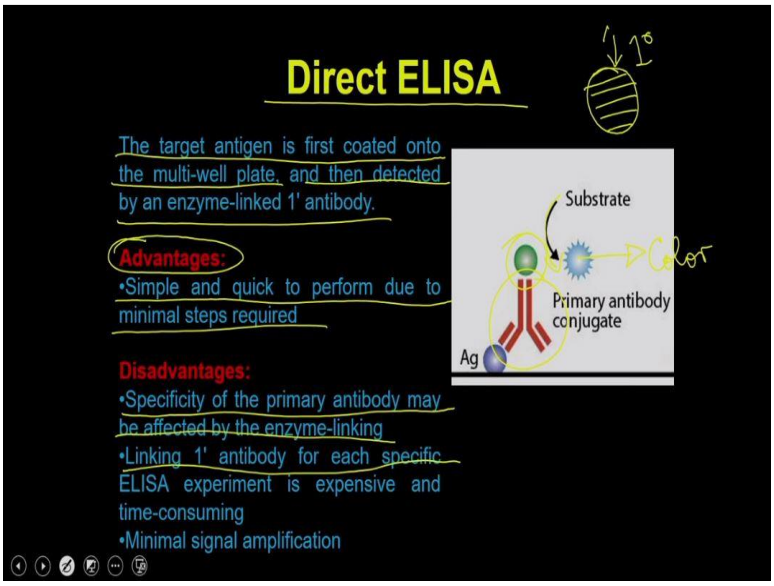

So the direct ELISA in the direct ELISA the target antigen is first coated onto a multi well plate and then you are detecting that by an enzyme linked primary antibody which means you are going to take a well, you coat it with the antigen what you are interested to identify and then you are going to add the primary antibody which means you are going to add the antibody which is actually coupled to an enzyme and then what you can do is you just add the substrate and that substrate is going to give you the color or substrate is actually going to give you some readouts.

It is simple and quick to perform due to the minimum steps required. So that is the advantage of the direct ELISA. But the direct ELISA solely depends on the specificity of the primary antibodies, which means there is no control. So that is why if there is a any problem with the specificity of the primary antibody, it is actually going to give you the false positive results. That is why the specificity of the primary antibody may be affected by the enzyme linking the primary antibody and that is why it is very, very troublesome to perform.

Because once you have the primary antibody you have to do a coupling reactions to that the enzyme is going to be coupled to the primary antibody and in that process some time, it actually affects the specificity of the antibody itself. The second thing is because it does not have the amplification step because, if you so, the signal is going to be as proportional to the amount of primary antibody bound to the antigen.

So, if you have a monoclonal antibody for example, then it is actually going to interact only with the single epitopes what is present onto a particular antigen and in that case, the signal is going to be less very low compared to if you are using a polyclonal antibody. And so, if the signal is low, it actually directly going to affect the sensitivity of the as well.

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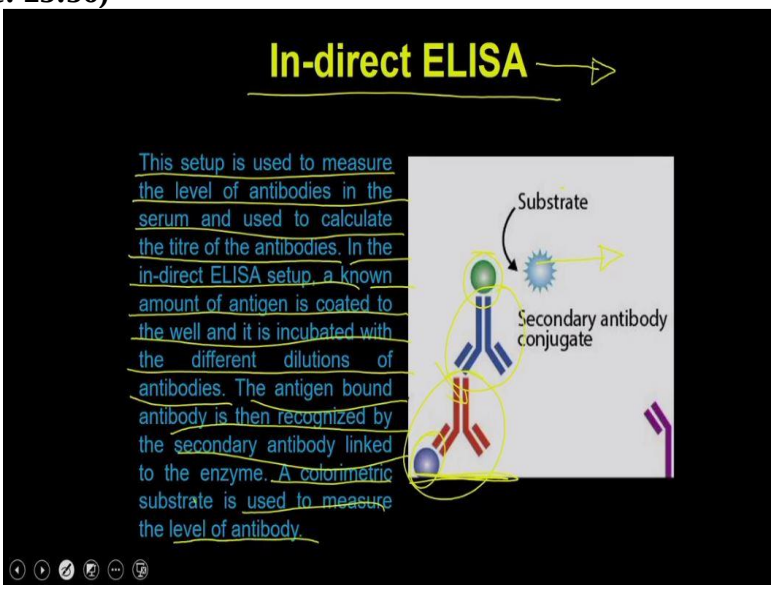

Then you have the indirect ELISA. So, in the indirect ELISA, what you are doing to do is you are actually coating the surface with the antigen then you are coating the primary antibody to detect the antigen and then you are adding the secondary antibody to detect the primary antibody and then the secondary antibody is having the enzyme and then you are actually adding the substrate and that substrate is getting converted into a product.

And that product is giving you either the color or the readable readouts and that actually can be done. So, in the secondary. So, this kind of indirect ELISA is being used either to measure the level of antigen as well as to measure the level of antibodies. So, this setup is used to measure the level of antibodies in the serum and use to calculate the titer of the antibodies in the indirect ELISA setup a known amount of antigen is coated onto the well and it is incubated with different dilutions of the antibodies.

The antigen bound antibody is then recognized by a secondary antibody linked to the enzyme a colorimetric substrate is used to measure the level of antibodies. So, let us see how to perform the indirect ELISA.

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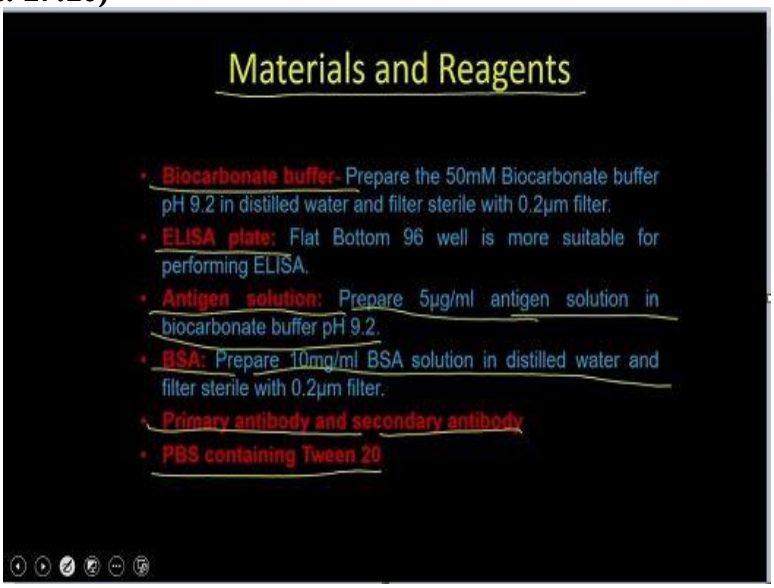

And what are the materials you are required. So, the materials what you require is the bicarbonate buffer which you require for coating then you require the Elisa plate that is what you require for coating the antigen then you require the antigen solutions and so, you prepare the antigen solution of 5 microgram per ml in the bicarbonate buffer pH 9.2 then you require the BSA which is actually going to be for a blocking and then you require the primary antibody and the secondary antibody and then you require the PBS which contains the Tween 20.

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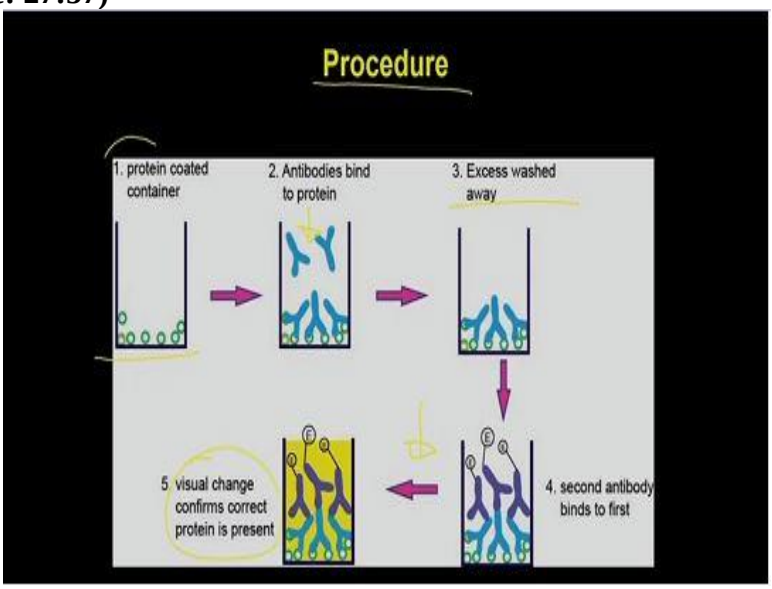

So, in a procedure, what you are going to do first you are going to do the coating of the antigen onto the plate dialyzer plates, then you are going to incubate that with the primary antibody, then you are going to remove you are going to do a washing step to remove the unbound antibodies,

then you are going to add the secondary antibodies then you are going to do a washing to remove the excess antibodies and then you are going to add the substrate and that actually is going to give you the colors. Let us see how to perform these steps.

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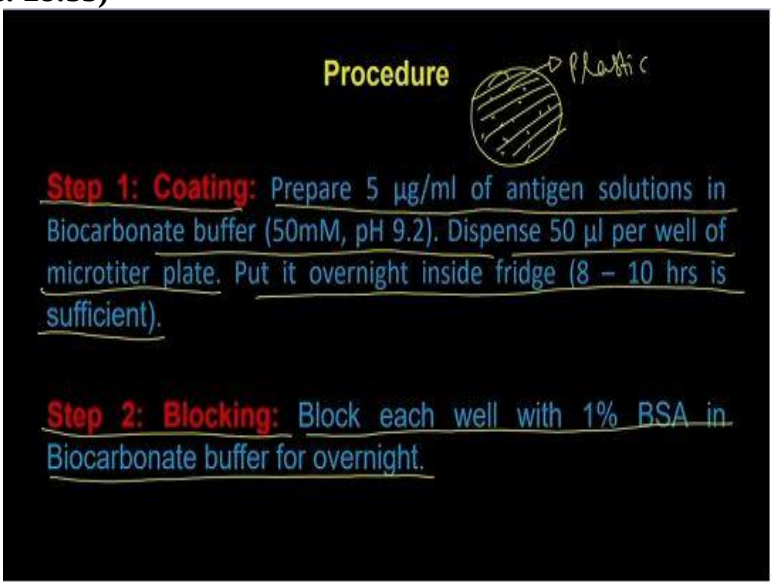

So, in the step 1 you are going to do a coating of antigen. So, you prepare the 5 microgram per ml of antigen solutions in a bicarbonate buffer dispense the 50 microliters per well of the microtiter plate put it for overnight inside the fridge and that is sufficient for the antigen to coat to the microtiter plate then you are going to do a blocking step. So, in the step 2 you block each well with the 1% BSA in a bicarbonate buffer for overnight. This blocking step is required because when you are coating the antigen, you are actually coating the antigen in a well.

So, you are going to have the different antigen molecules, but in between the antigen molecule this this space is empty which means like the plastic is empty and this plastic can easily provide the attachment site for the primary antibodies. So, before you add the primary antibody, you have to fill this whole cavity or fill with this whole well with our proteinaceous solution so that you actually going to cover the whole surface. So, the blocking is not going to block the epitopic sites on to the antigen but it actually going to block all the remaining surfaces what is available in the well.

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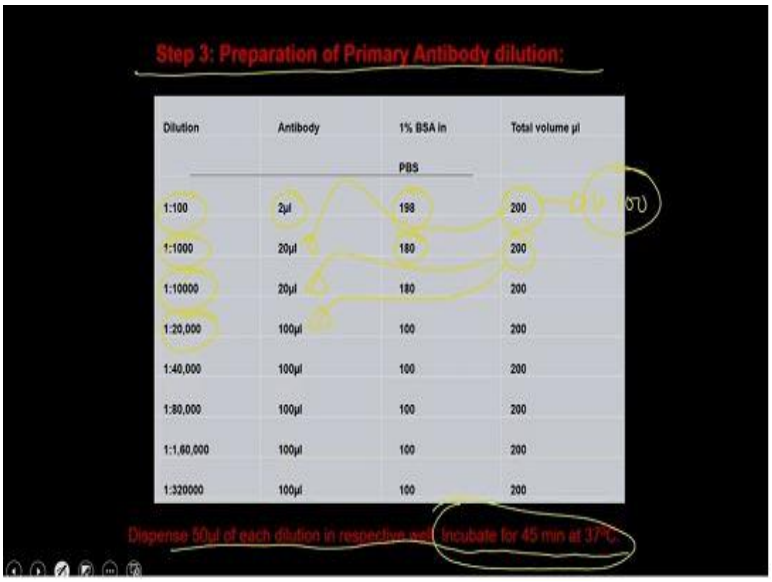

Now was once your blocking is over then you can prepare the primary antibody dilutions. So, you can make a dilution of 1 is to 100, 1 is to 1000, 1 is to 2000 like that, so what you have to do is you have to take this 1 and 20 microliters of original serum and then you mix it with 198 microlitre of PBS that actually is going to give you a 200 microlitre 1 is to 1000 diluted sample then you have to do a serial dilution like that, and that actually is going to give you a serial diluted antibody stock and that actually can be used in a subsequent step for the incubations.

Then you dispense the 50 microliters of each diluted antibody stock into respective well, and then incubated for 45 minutes for 37 degrees Celsius.

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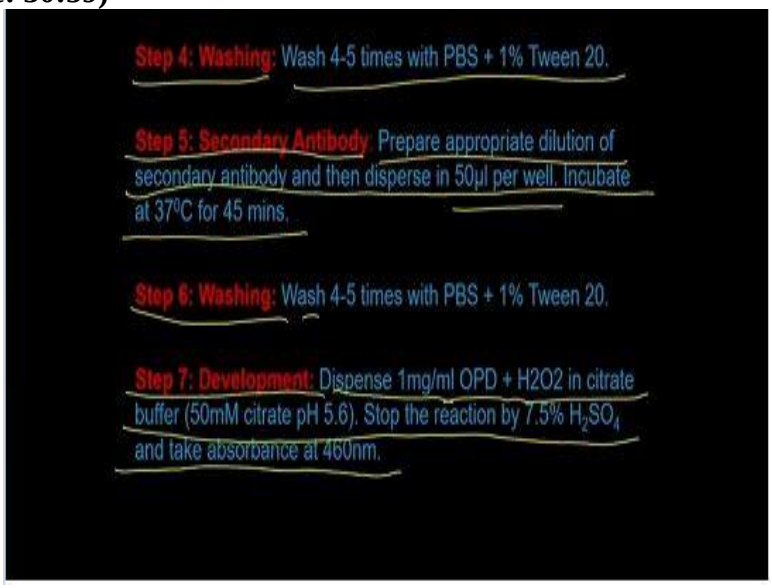

Then in the step 4, you are going to do a washing so in the washing for 4 to 5 time with PBS containing tween 20. And then step 5 you have to add the secondary antibodies. So, you have to add the appropriate concentration of the secondary antibody, and then you dispense the 50 micro liter per well and incubate in 37 degrees Celsius for another 45 minutes. In the step 6, again, you have to wash for that the access layer secondary antibodies can be removed.

And then in the step 7, you are going to do a development so dispense 1 mg per ml OPD plus H2O in the citrate buffer. Stop the reaction by 7.5% sulfuric acid and take the absorbance at 460 nanometer. So let us see how the results comes.

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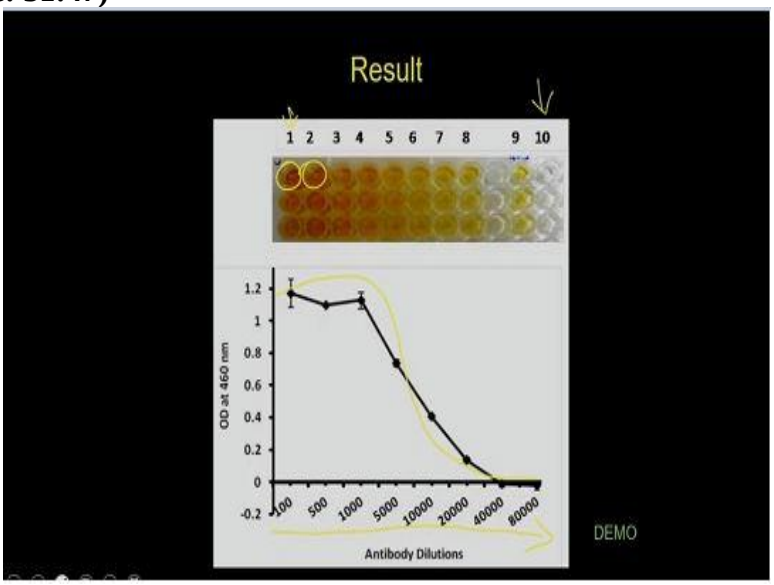

The results will give you like this where this is the highest antibody concentration and this is the lowest antibody concentration. So, what you see is a orange color reaction which is a color what you get when you do use the OPD and what you and if you plot these absorbance against the concentration of the antibody, what you see is that it is actually going to give you a sigmoidal curve and with us using this sigmoidal curve, you can be able to determine the titer of the antibodies.

So, this is all about the theoretical steps what we have discussed about the indirect Elisa. Now, to show you all these steps, I can I will take you to my lab to show you a small demo and with this demo, we are going to show you each and every step and then ultimately the students are going

to show you the development of Elisa as well as that will actually is going to be helpful for you to understand the whole process.

(Video Starts: 32:51)

In this video we will demonstrate how to use a pathogenic infections detection kit and what is the underlying principle of that. So mostly the detection kits, version immunoassay. So, what is an immunoassay in immunoassay, we will use specific antibodies like monoclonal antibodies against disease specific or pathogen specific antibodies antigen, then we will develop in the substrate. So, this will give some pasty color and that will be detected by spectrophotometer reading.

So, the steps include first step is we have to coat the plate polyamide chloride plate with the capture antibody following capturing of the actual antigen, disease specific antigen. For suppose in most of the cases in viral infection, it will detect the coat protein and in bacterial infection, the external (())(34:02). These kind of antigens it will detect. Once the antibody is coated on the plate, then we will incubate with the sample taken from patient in there it is saliva or serum sample.

After incubation, we will wash properly then again incubate with the primary antibody specific to that particular antigen. It is it mostly it should be monoclonal antibody, otherwise, that detection is nonspecific. In next step, after washing the unbound primary antibody, we will incubate with the secondary antibody that is septravidin conjugated to HRP. So, once the secondary antibody incubation is over, we will wash and that substrate solution mostly it is TMB or variants of TMB also available for enhanced chromophoric detection.

Then we will read the color development using spectrophotometer. These are the main steps. So, in step by step, now, we will demonstrate how to perform the immunoassay in order to perform a immunoassay we need the following materials. First we need polyamide chloride well plate 96 well plate which should be flat bottom, then the other materials we need is coating buffer which is bicarbonate buffer system that pH 9.6. So, once it is ready you are adjusted pH then we will dilute the capture antibody in the coating buffer.

Then add 100 microliters dispense each into this dispence. So, once the dispense is over, then we will incubate this plate at 4 degrees Celsius preferably, but we can incubate at room temperature also for 2 hours. If you are incubating at 4 degrees Celsius you have to keep it overnight otherwise 2 hours is enough. So, now we have coating buffer So, I have dispensed into reservoir then I will take the capture antibody. We have to see the dilution.

We have to follow manufacturer instructions for a dilution otherwise improper dilution make you false positive results also have shown no results. So, once it is over then we have to dispense 100 microliters into each plate after proper mixing. Once the material is dispensed into plate, we have to cover the this plate with the parafin or covering plate then we will incubate displayed for 2 hours at room temperature. Post incubation we have to remove the unbound solution.

So, we will remove that. Next, we will wash the plate with the TBST buffer. It should be pH 7.4. We have to wash at least 3 times properly then we will we are going to block with the blocking buffer which contains 3 percentage BSA in TBS. In next step, we will dispense blocking buffer into each well 100 microliters each. So, it will cover the nonspecific area where there is no capture antibody. So that the reaction will be reaction is specific to particular antigen. Blocking should be done at least 2 hours at room temperature or overnight at 4 degrees Celsius.

So here we have done it room temperature. Once the blocking is over we have to remove remaining blocking buffer and wash 3 times and incubate with the primary antibody. So I am going to do that in this step, we have to dilute the primary antibody with the acidic diluent or in blocking buffer then mix properly and dispense 100 microliter each into 96 well plate and incubate at room temperature for 2 hours. Once the primary antibody incubation is over, we have to remove unbound antibody and wash with the TBST for at least 3 times.

So, it will remove unbound primary antibody and in following step will incubate with the HRP conjugated secondary antibody and incubating it for 2 hours. After incubation with secondary antibody, we have to remove secondary antibody and wash thoroughly. So, after washing will incubate with the substrate solution, which is usually (())(41:46). So, we will dispense the

substrate solution in to wells. So, once the dispensing is over, we have to keep at room temperature for some time until the we can see a visible blue colour.

Once the reaction is over, then we have to stop the reaction with the 2 normal Hcl our sulfuric acid. After 15 minutes, if you observe it a plate, we can see the blue color intensity in some of the plates is very high and in some of the wells is very less. So, that means, whatever the wells it is giving intense blue color that means, the concentration of the antigen is very high. So, at this moment we have to stop the reaction otherwise, all the wells may turn into blue colour. So, we cannot identify positive sample versus false positive sample.

So, there may be some artefacts, so, that is why we have to stop the reaction using a 2 normal HCl or H2SO4. We will add 2 normal H2SO4 to stop this reaction. So, as we can see that blue color turned into yellow color. So, we have to read this in spectrophotometer at 540 nanometer and 450 nanometer to get absolute values of these things. So, this is qualitative purpose as well as quantitative purpose. Qualitative purpose in the sense if you are using samples from patients, you have to do it in triplicate.

And you can just identify whether an entity that particular person is disease positive or negative. And another the case for quantitative purpose you need to have a various varying concentration type creation sub, the particular antigen, which that disease causes. So, in this case, you have to dilute the tatigen in different concentration and you have to develope the assay in the same way developed here. So, you have to compare you have to plot a standard graph aganist, the concentration versus the observance we have taken.

From that value, you will come to know what is the unknown persons that antigen titration in this serum or saliva. So, that is why it is qualitative as well as quantitative method. With this, we can understand this method can also be applied for a prediction of various chemicals or various interests in blood, like drugs used for halucination purpose, and recreational drugs, and also some of the drugs used in the pharmaceuticals.

(Video ends: 45:29)

With this, I would like to conclude my lecture here and in the in the demo video, the student might have discussed in detail about the different steps related to Elisa and I hope this would be helpful for you to perform the assay in your lab. So, with this, I would like to conclude my lecture here. Thank you.