Basics of Biology
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Module V: Molecules of the Life (Part-II)
Lecture - 23
Proteins (Part-IV)

Hello everyone, this is Doctor Vishal Trivedi from Department of Biosciences and Bioengineering IIT Guwahati and what we were discussing, we were discussing about the living organisms. And in this context, we are going to discuss another aspect where we are actually going to detect the protein into the biological fluid.

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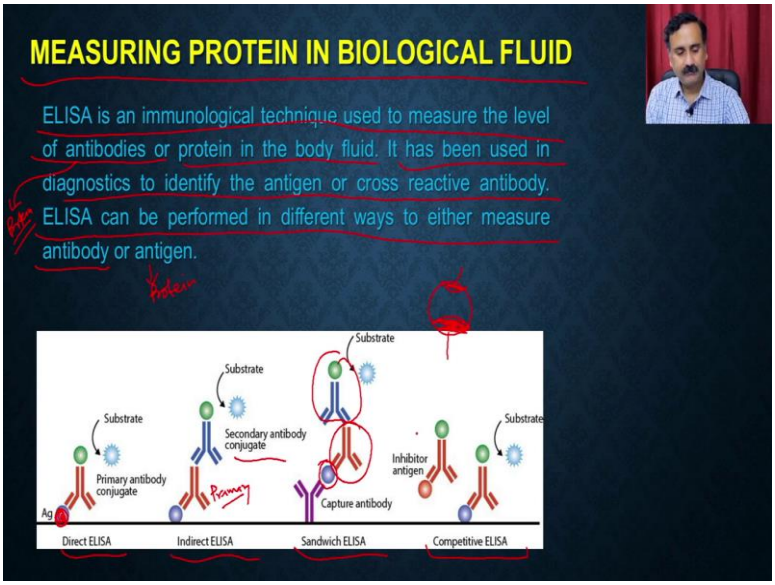

So, how you can be able to measure the protein in the biological fluid, ELISA is an immunological technique used to measure the level of antibody or a protein in the biological fluids. Remember that the antibodies are also been made up of the protein molecules. So, you can actually detect the proteins also into the biological fluid, it has been used in the diagnostics of identifying the antigen or the cross reactive (())(1:42). ELISA can be performed in different way to either measure the antibody or to the antigen or to the protein.

So, you can have the direct ELISA, you can have the indirect ELISA, you can have the sandwich ELISA or you can have the competitive ELISA, what you can do is in the direct ELISA, the direct ELISA, you are going to have the protein on coated onto the surface and then you are actually going to add the primary antibody and that primary antibody is going to coupled to an enzyme and so, the substrate is going to be converted into an edible product.

Same is true for the indirect ELISA, indirect ELISA is same except that you are going to have the 2 antibodies you are going to have the primary antibody and then you are going to have the secondary antibodies.

And the secondary antibody is going to couple to the enzyme. So, that is how the signal what you are going to see is some secondary antibody rather than the primary antibody and that is why this is called as indirect ELISA. Then we have the sandwich ELISA where you are actually going to keep one antibody which is going to use for capturing the protein which you are interested to measure and then to this protein you are going to have the primary antibody which is also going to be directed against this particular product, but to the different region.

For example, you have a ball. So, in this region can be used by the capture antibody and this region can be used by the primary antibodies, and then we can have the secondary antibodies to get the signal. Similarly, we can have the competitive ELISA also and that competitive ELISA also is going to use the inhibitory antigens.

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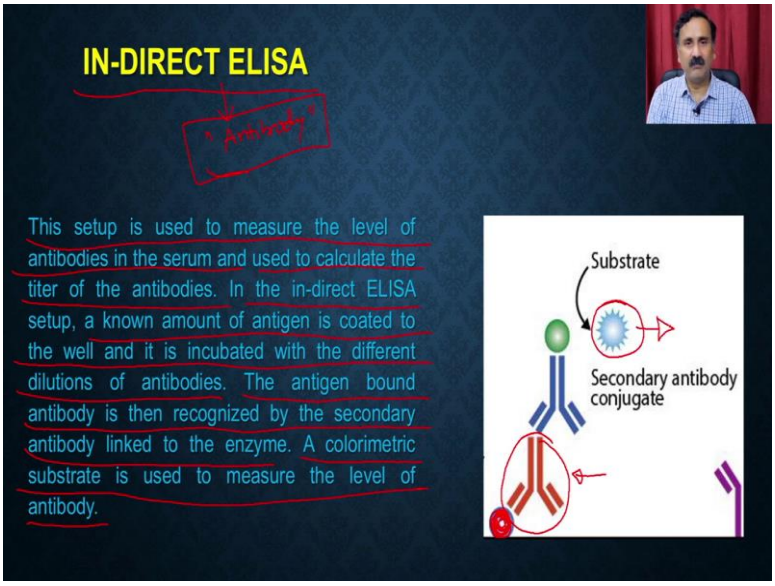

So, let is first discuss about the indirect ELISA, indirect ELISA is used to measure the level of antibody into the biological fluid or antibody what you are going to develop into an organism. This setup is used to measure the level of antibodies in the serum and used to calculate the titer of the antibody. Titer means the level of antibodies. In the indirect ELISA setup, a known amount of antigen is coated on to the well and it is incubated with the different dilutions of the antibodies.

The antigen bound antibody is then recognized by the secondary antibody leading to an enzyme. A colorimetric substrate is used to measure the level of antibody so this is what you are going to have. First you are going to code the protein of your interest which you are interested. So, this is going to be coated onto a plate and then you are going to add the biological fluid where the antibodies are present and which you are interested to measure. And then to this antibody, you are going to add the secondary antibody and then you are going to add the substrate and that is actually going to give you the colored product and that color product you can measure by the help of the spectrophotometer.

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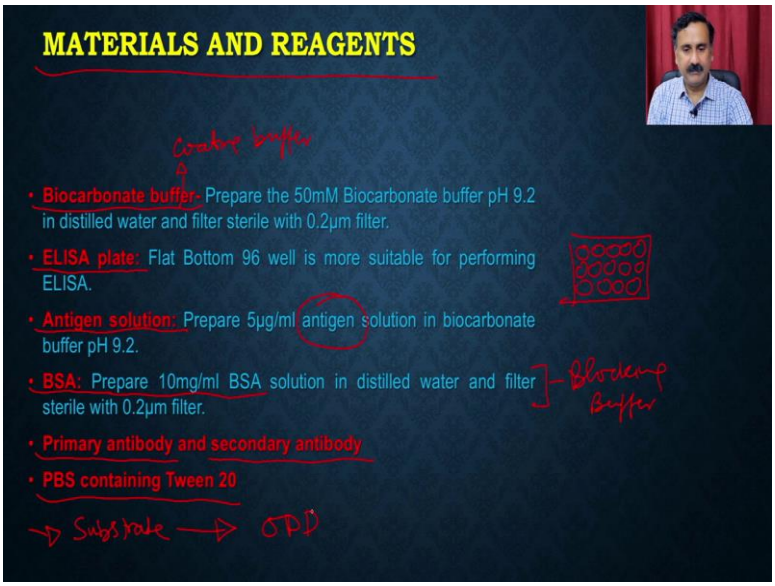

Now, what are the things you require if you want to perform this particular indirect ELISA, you require a bicarbonate buffer which is also called as a coating buffer. So, you also require a coating buffer which is called as the bicarbonate buffer. Then you require the ELISA plate. So, you require a 96 swell ELISA plates, so in the 96 well plate ELISA plate you have the 96 vessels or the wells so that you can use for the different types of reactions. Then you require the antigen solution, which means you require the protein solution which you are going to coat onto the this 96 well plate. Then you require the blocking buffer.

So, you require the BSA, which you are going to use to prepare the blocking buffers. So, this is you are going to use for preparing the blocking buffers, then you require the dilution of the primary antibody and then you also require the preparation of the secondary antibodies and then you require the PBS containing Tween 20. And lastly, you also require the substrate, substrate which you are going to use for detecting. So, what are the substrate what do you use is the OPD.

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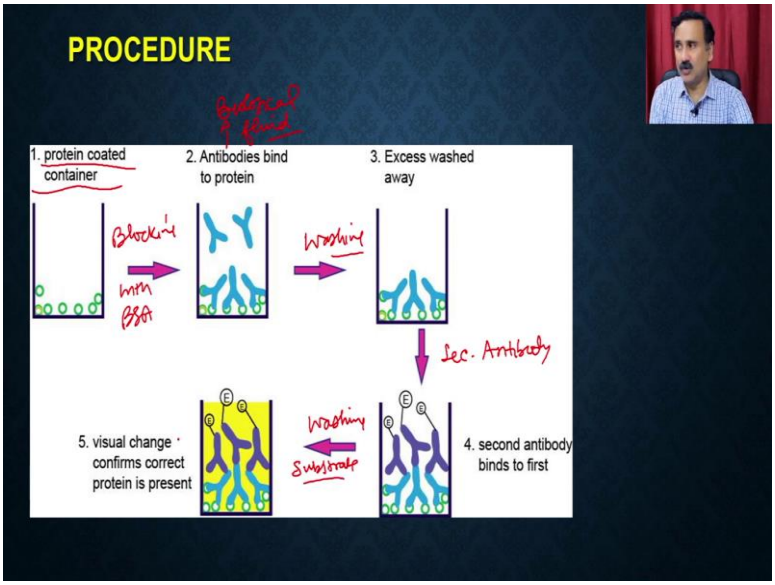

This is what you are going to do as for the procedure, so, first you are going to take the well, you are going to coat the protein of your interest or the antigen against which you are interested to measure the antibodies. So, you are going to make the code then you are going to do the blocking. So, in between you are going to do the blocking with blocking buffer which contains the BSA. And that is how all the areas are going to be blocked, then you are going to add the antibody which are going to be present into the biological fluid.

So, from where you are actually going to interested to measure the antibodies. And then the antibody is going to bind to these particular antigens. And that is how you are actually going to and then the excess amount of the unbound antibodies, you are going to do the washing. So, you are going to have a washing step where you are going to use the PBS, 2020.

And then once a wash access washing is over, then you are going to add the secondary antibody and the secondary antibody will not going to bind to the, this protein but they are also they are going to bind to the primary antibodies. And then you are going to do a washing and once the washing over then you can actually add the substrate and that is how you can be able to get the blue color and that blue color you can be able to measure using the spectrophotometer.

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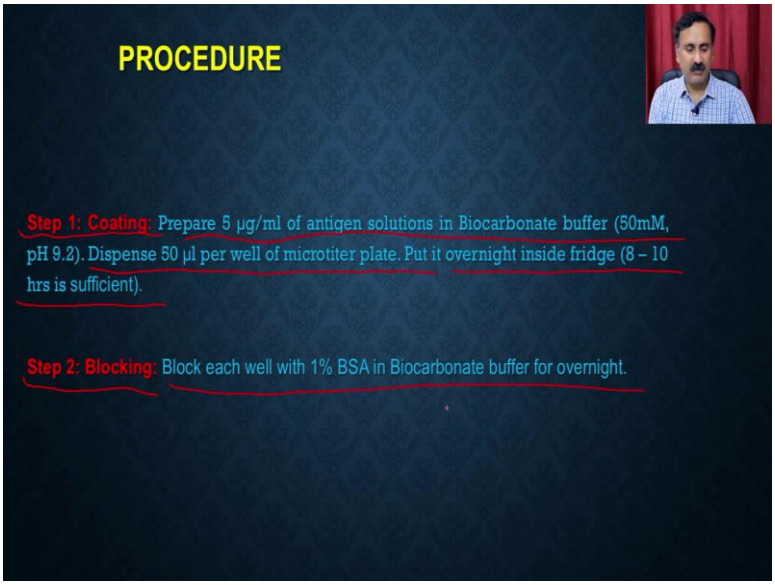

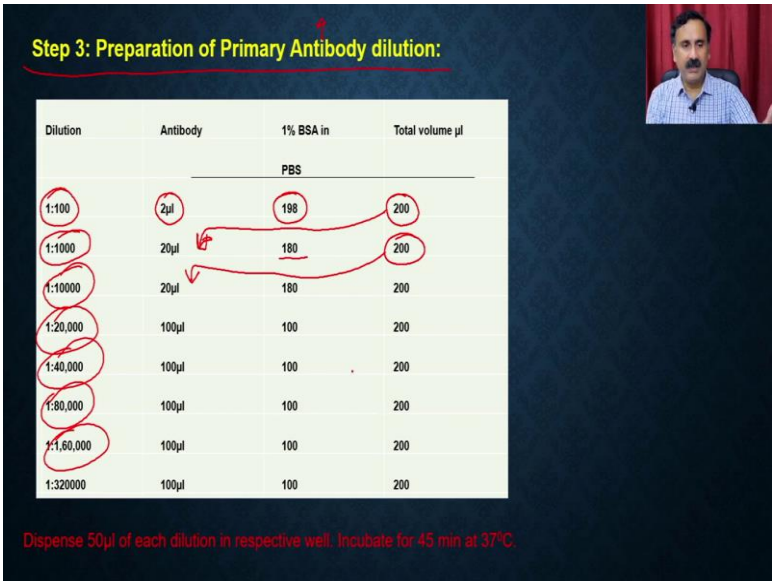

The procedure, procedure is the step one, you have a coating, so you can prepare a 5 microgram of antigen solution in the bicarbonate buffer and then you dispense the 50 microliter per well of the microtiter plate, put it overnight inside the fridge hour 8 to 10 hour is sufficient. Then step 2, you are going to do the block for block with the 1 percent BSA which you are going to prepare in the bicarbonate buffer for overnight, then you are actually going to prepare the dilutions.

So, the antibodies which you are going to get from the biological fluids, you can actually make the dilutions. So, you can make the one is 200 dilutions and 2000, 10,000, 20,000, 40,000, 80,000 and something like that. And this is the scheme through which you can do, so

you can take the 2 microliters, you add it to 198 microliters, that is going to give you the 200 microliters.

From these 200 microliters you can take the 20 microliters, and then you can add the 180 microliter and that is how you are going to get the 2 microliters back. Again, you take this and you can just add here, and that is where you can continue like that. And that is how you are going to get the serial dilution of the complete curve.

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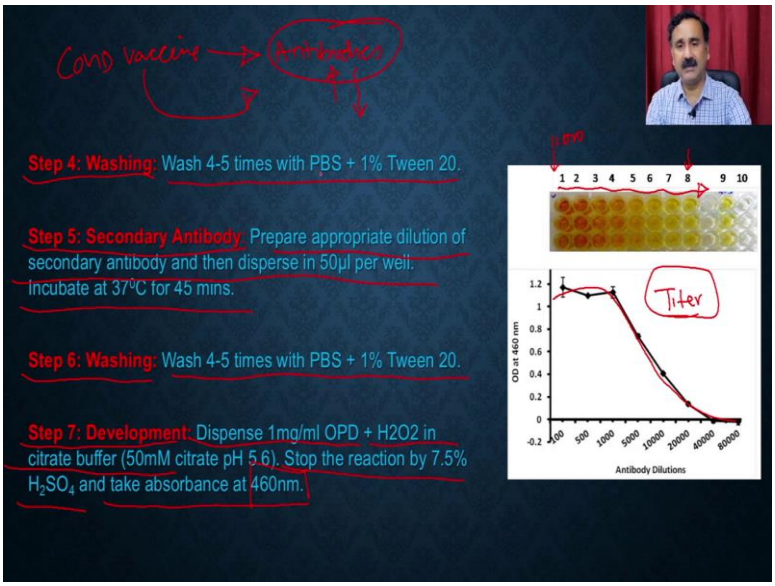

Then what you are going to do is you are going to do the washing, and that washing is you are going to add like 4 to 5 times you are going to do the washing with the PBS plus tween 20. And then you are going to add the secondary antibody. So, you are going to prepare the appropriate dilution of the secondary antibody and then dispersed it in 50 microliter per well, incubate at 37 degrees Celsius for 45 minutes.

And then you are going to have the step 6, which is going to be washing so wash 4 to 5 times with PBS. So, after every antibody, you are going to do a washing and then ultimately you are going to do the deployment. So, you are going to dispense 1 milligram per ml OPD plus H2O2 in a citrate buffer. And then you stop the reaction by the 7.5 percent sulfuric acid and take the absorbance at 460 nanometers.

So, what you see here is that these are the different dilutions of the antibody and you see the here you are getting the very intense signal because here the antibody 1 is to 100. And then you keep going it like so the by the antibody dilutions are and reducing and that is how the reactions are also reducing. So, if you plot this you are going to get a curve like this. And

using this curve, you can be able to calculate the titer of the antibody what you have into this particular biological fluid.

And depending on this titer, you can be able to say whether the antibody level is pretty high or low. For example, if you go and go for a vaccination, if you go for a COVID vaccination, if you are interested, if you are interested to see whether the antibodies follow COVID are being developed or not, then you can actually do like this, what you can do is you can just take the COVID virus and you can just coat that particular virus into these wells, and then you add the some amount of the blood from your body and that is that will contain the antibodies and that antibodies you can do under the indirect ELISA.

And it is actually going to tell you what will be the level of antibodies present. If the level is antibody is pretty high, this tag will say that the vaccine is working and it is actually going to give you a very high level of antibodies. So, that is just a simple example to explain you that what will be the utility of this particular type of tool. Now, apart from that, you can also measure the direct proteins.

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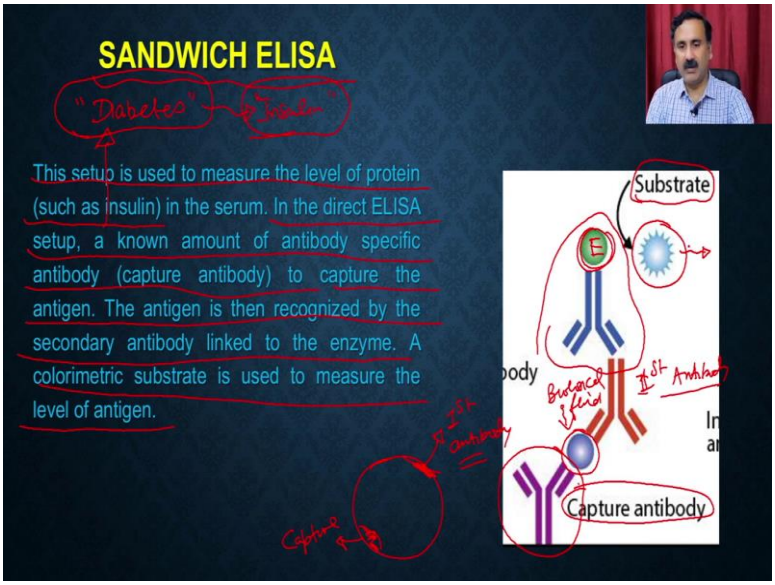

How you can be able to measure the direct proteins, this setup is used to measure the level of protein such as insulin. So, that the level of insulin is important to measure in especially in the case of if you are expecting a person to be infected or person to be suffering from the diabetes. So, in that case, you want to see whether the insulin production is happening in the body or not. So, for that, you can actually whether the insulin, how you can do that? You can do that simply by the ELISA setup, a known amount of antibody specific.

So, you can use the sandwich ELISA in that and where you can actually be able to use the capture antibodies and to capture the antigen and then the antigen is then recognized by the secondary antibody linked to the enzyme and a colorimetric substrate is used to measure the level of antigens. So, this is what you are going to do.

In a sandwich ELISA you are going to use the 2 different antibodies, one is to capture antibodies which you are going to use. So, capture antibody you are going to coat onto the valves and then the capture antibody is going to bind the particular protein what is present into the biological fluid. So, this is going to be present in the biological fluid.

For example, the insulin so and then you are going to detect this with the help of another antibody. So, this is going to be called as the primary antibody. So, this primary antibody is going to detect using the different region of the protein. For example, you if you have a ball, this area is probably can be used by the capture antibody. So, this can be used by the capture antibody, and this area can be used by the primary antibody. So, that is why they will not going to recognize the same area, they are going to recognize the different area of that particular protein.

Once the primary antibody is going to bind, then you actually can put the secondary antibody and that is how you are going to get the signals because the secondary antibody is going to have the enzyme and that enzyme is going to catalyze the reaction. So, you can just put a colorimetric subset, and it is actually going to give you the signal and that signal you can measure using a spectrophotometer.

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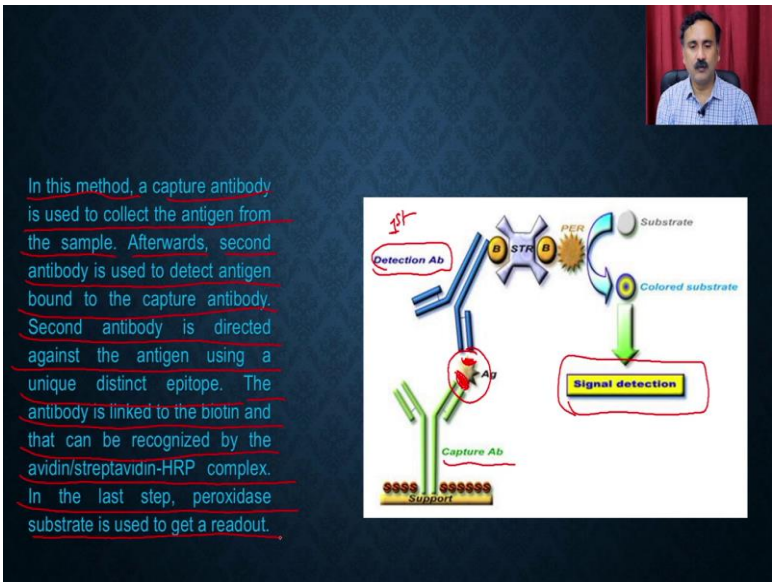

So, let us say how you can be able to perform the sandwich ELISA. So, this is what I have just discussed so far, that you have a capture antibody which you are going to use for the antigen, but the area of antigen is going to be different. So, in this case, this area is going to be identified by the capture antibody, whereas this is the area which is going to be recognized by the detection antibody or the primary antibody.

And then this primary antibody is going to be recognized by the either secondary antibody or it can be recognized by the detection systems. And that is how you are going to utilize the substrate and it is going to give you the signal and that signal you can measure using the spectrophotometer.

So, in this method, a capture antibody is used to collect the antigen from the biological sample. Afterwards, the second antibody is used to detect the antigen bound to the capture antibody and 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 the streptavidin HRP complex and in the last step peroxidase substrate is used to get the readouts.

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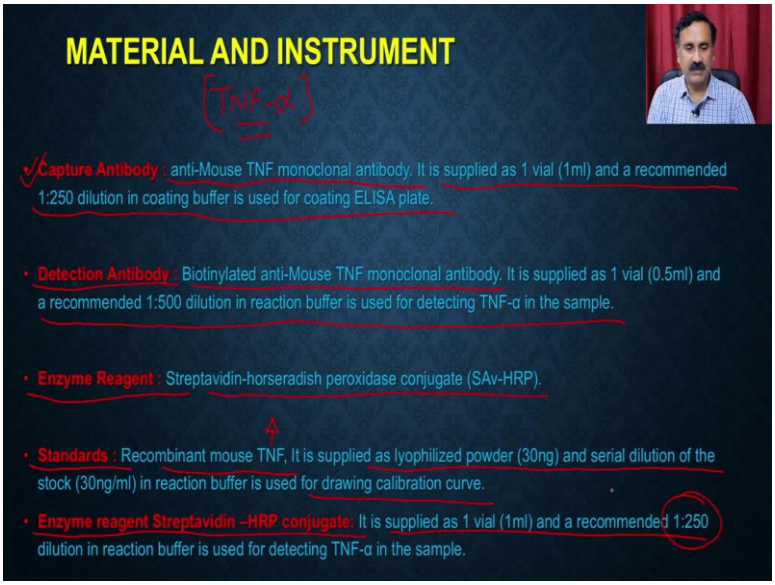

Now, if you want to perform this what are the things you require? You require the capture antibody, so capture antibody is for example, in this case, we are showing you the measurement of the TNF alpha. So, in this case, you are going to use the antivirals, TNF alpha monoclonal antibody. And when you purchase the kit, it is going to supply it as the 1 ml vial and it is recommended to use at 1 is to 250 dilutions in a coating buffer.

Then you are going to have the detection antibody, so detection antibody is biotinylated, anti-mouse TNF alpha monoclonal antibody, and that is also going to be supplied and you have to use at 1 is to 500 dilutions. Then you require the enzyme reagent. So, enzyme reagent is a streptavidin HRP conjugate then you required the standard for you, in this case, you are actually required the standard recombinant mouse TNF alpha and that is also going to be supplied as the lyophilized powder and that also can be used to draw the calibration curve. Then you require the enzyme reagents stepped out in HRP conjugate that is supplied as the one vial and you can use the 1 is to 250 dilutions.

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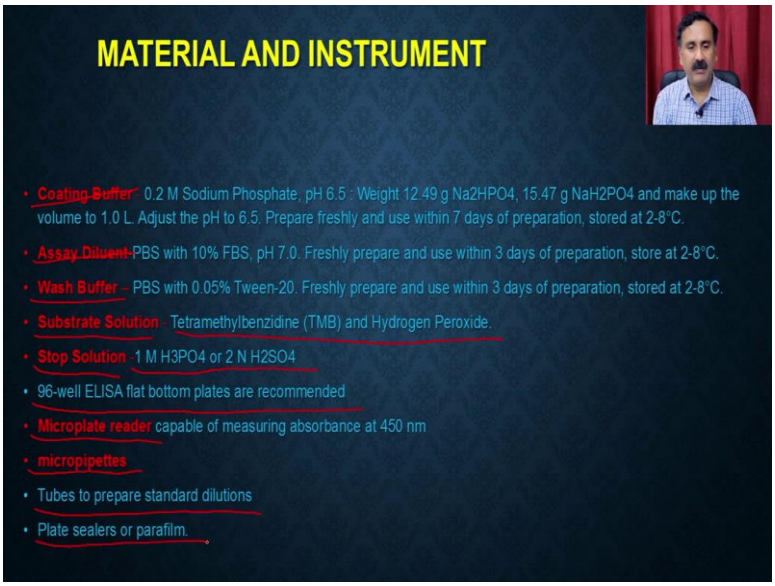

Then you require the coating buffer, required the essay dilutions, you require the wash buffer, you require the substrate solutions, so TMB is a substrate what we are going to use along with a hydrogen peroxide and you required the stock solution so, a stop solution is the phosphoric acid or the sulfuric acid then you require the 96 well plate and the microtiter plate reader and the micropipettes and the tube to prepare the standard dilution and as well as the plate sealer or the parafilms. How you are going to perform the procedures?

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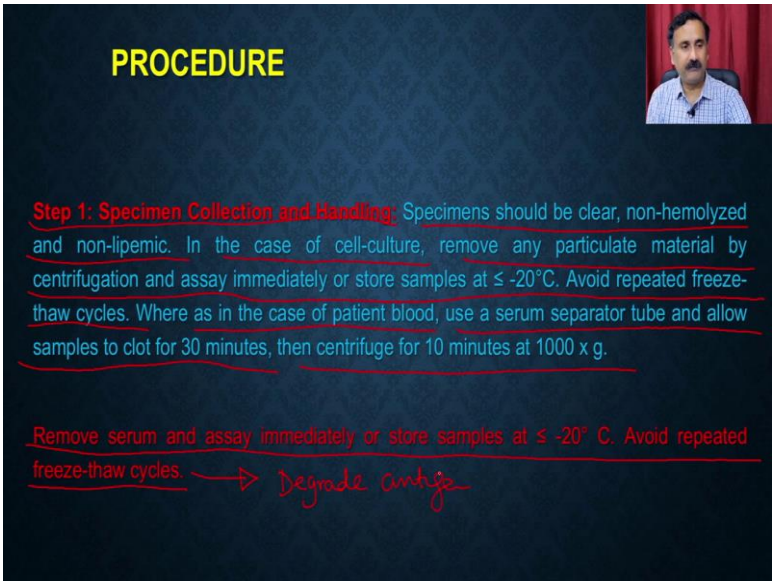

The procedure you are going to first prepare that you are going to collect the specimen. So, a specimen could be different types. It could be a tissue, it could be a blood, it could be the biological fluids or it could be cell also. So, depending on the cell, depending upon the tissue, you can actually be able to prepare the different, you can be able to prepare different types of procedures. So, a specimen could be clear non-hemolyzed and non-lipidomic. In the case of cell culture, remove any particulate material by the centrifugation and the assay immediately or store at minus 20.

Avoid repeated freeze thaw cycles whereas, in the case of patient blood use a serum separating tubes and allow the sample to clot for 30 minutes, then centrifuge for 30 minutes, 10 minutes at 1000 g. You can remove the serum and acid immediately or stop solution at minus 20. Avoid the repeated freeze thaw cycle.

So, this is very important that you should not thought the biological fluid on a multiple location which means if you require to remove the serum and other canes from the blood, you should do it and then you Ellicott it and then keep it at minus 20, so that it should not do the repeated freeze thaw because if you do the freeze thaw, it is actually going to degrade the antigen.

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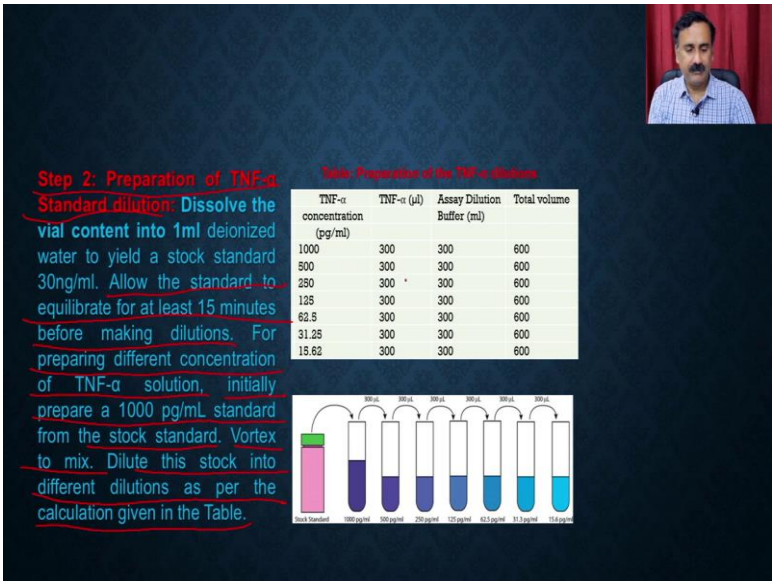

Then you can actually prepare the TNF alpha standard. So, this is what you are going to do. You are going to prepare the different types of dilutions, just now, I think we have discussed what we have discussed for the indirect ELISA, same way you can actually be able to prepare those different dilutions. Then once the different dilutions are ready, then you can allow the standards to equilibrate for at least 15 minutes before making the dilutions. For prepare in different dilutions of TNF alpha solutions. Initially, you could prepare 1000 picogram per ml standard from the stocks and then vertex to mix and dilute this stock into a different dilution as per the given table.

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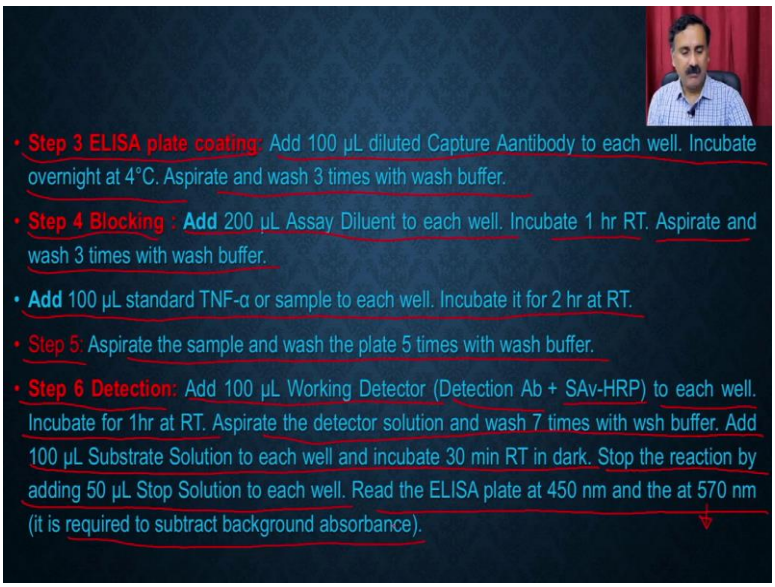

Then in the step 3, you are going to do the coating, so add the 100 microliter of diluted capture antibody to each well, the capture overnight at 4 degrees aspirate and wash the 3 times in the wash buffer. Then the step 4, you are going to do the blocking. So, add the 200 microliters assay diluted to each well incubate for 1 hour at room temperature aspirate and wash 3 times in the wash buffer. Then you add the 100 microliters of standard TNF per sample to each well and incubate it at 2 hours for room temperature.

And the step 5, you aspirate the sample and wash the plate 5 times in the wash buffer and the step 6, you are going to add the 100 microliter of working detection detector which means it is going to detect the antibody and it is going to be stepped out in the HRP complex to each well. Then you incubate that for 1 hour at room temperature, aspirate the detector solution and wash seven times with the wash buffer.

Add the 100 microliter of substrate solution to each well and incubate 30-minute room temperature in dark. Stop the reaction by adding the 50 microliters of stop solution to each well. Read the ELISA plate at 450 nanometer and add the 570 nanometer it is required, why we require to run it at 570 nanometer because it is required to subset the background absorbers.

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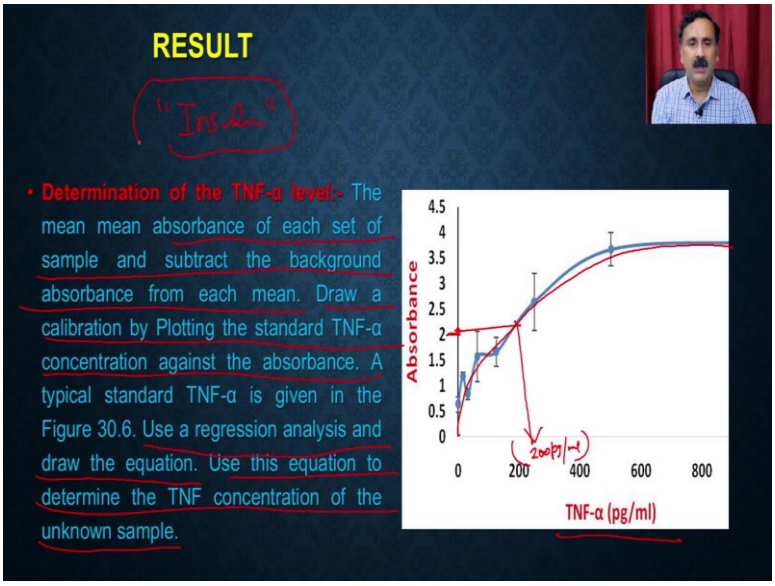

And what you are going to see is that you are going to see a pattern like this so you are going to see a curve of TNF alpha picogram per ml. Now, what you are going to do is suppose this is the curve what you have and suppose the biological fluid also you are going to run along with it then it is actually going to give you the absorbance. For example, if it gives the

absorbance at 2 then you actually can use this and you can say okay the Pico the amount of TNF alpha is 200 picogram per ml.

And accordingly, you can use, so this calibration curve you can use to detect the amount of TNF alpha into the biological fluid. 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 coefficient against the absorbance. A typical standard is given in this, use a regression analysis and draw the equations, use this equation to determine the TNF concentration of the unknown sample or unknown biological fluids.

So, this is what the calibration curve what you can actually use for detecting the level of TNF alpha into unknown sample, the same kind of calibration curve can be drawn for the other biological fluid for example, I said you can actually be able to detect the insulin. So, you can actually do the same kind of calibration even for the insulin molecule and therefore, you can be able to use that.

So, to make you more familiar with this particular procedure, I have prepared a very small demo where my students (())(21:00) Manish is actually going to discuss the different procedures and different aspects what you have to do when you are performing the sandwich ELISA. So, let us see what the Manish is going to discuss with you in this particular demo video.

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Student: In this video, we will demonstrate how to use a pathogenic infections detection kit and what is the underlying principle of that thing. So, mostly the detection kits Watson

immunoassay, so, what is an immunoassay? In immunoassay, we use specific antibodies like monoclonal antibodies against a disease specific or pathogen specific antibodies antigen then we will develop in the substrate. So, this will give some positive color and that will be detected by spectrophotometer reading.

So, the steps include first step is we have to coat the plate (())(22:05) plate with the detail capture antibody following capturing of the actual antigen, this is specific antigen. For suppose in most of the here suppose in viral infection, it will detect the code protein and in bacterial infection, the external polysaccharides this kind of antigens it will detect, once the antibody is coated on the plate, then will incubate with the sample taken from patient either it is saliva, see that 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 not specific. Next step, after washing the unborn primary antibody, we will incubate with the secondary antibody that is septamin conjugated to HRP. So, once the secondary antibody incubation is over, we will wash and add substrate solution mostly it is DMV or variants of DMV also available for enhanced chromophoric detection. Then, we will read the color development using spectrophotometer. These are the main steps. So, it step by step now, we will demonstrate how to perform the immunoassay. In order to perform a immunoassay, we need the following materials.

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First, we need polyvinyl chloride may plate 96 well plate which should be flat bottom. Then the other materials we need is coating buffer which is bicarbonate buffer system with pH 9.6. So, once it is ready you are tested pH then we will dilute the capture antibody in the coating buffer then add 100 microliters dispense 100 microliters each into these wells. So, once the dispensing is over, then we 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.

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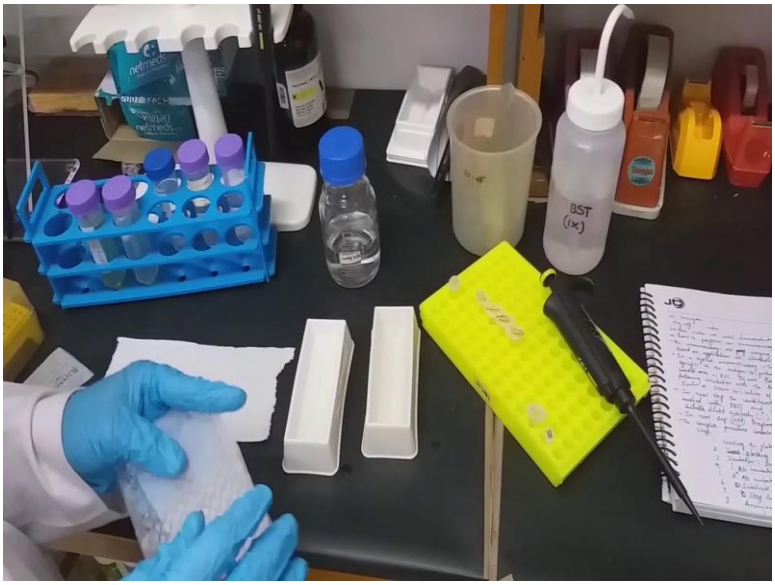

So, now we have coating buffer, so I have a dispensary to reserve well then I will take the capture antibody, we have to see the dilution we have to follow manufacturer's instructions for dilution otherwise improper dilution make you false positive results also, no results. So, once it is over then we have to dispense 100 microliters into each plate after proper mixing once the material is dispensed into plate, we have to cover the displayed with the parafilm or covering plate. Then we will incubate this plate for 2 hours at room temperature.

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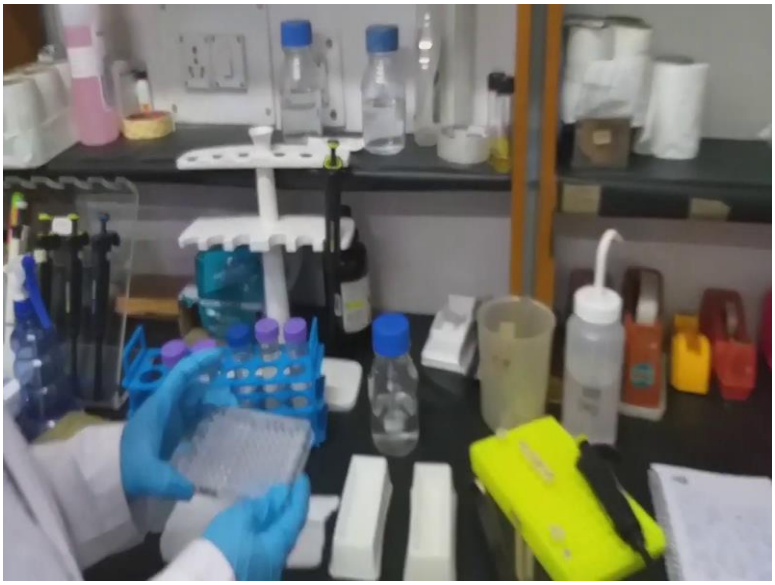

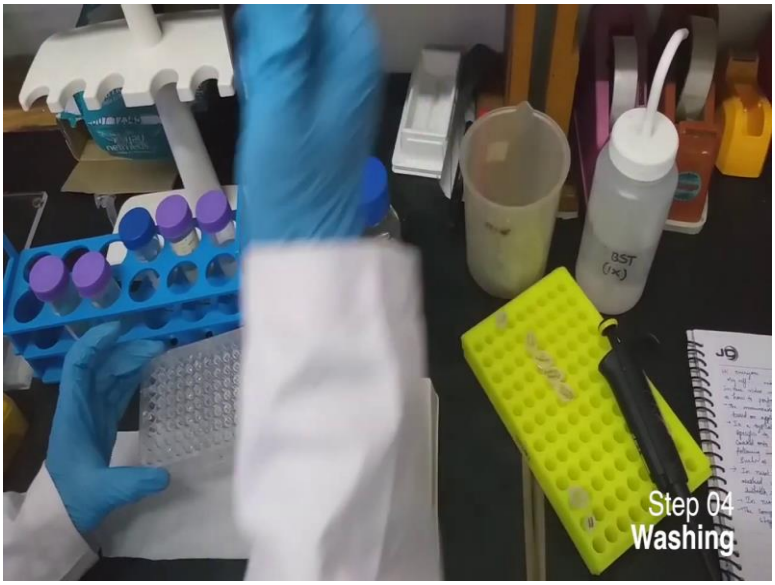

Post incubation how to remove the unbound solution. So, we will remove that, so next we will wash the plate with TBST buffer which should be pH 7.4. We have to wash at least 3 times properly then we will we are going to block with the blocking buffer which contents 3 percentage BSA in TBS. In next step, 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 needs specific to particular antigen just blocking should be done at least for 2 hours at room temperature or overnight at 4 degrees Celsius. So, here we have done at 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.

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In this step, we how to dilute the primary antibody with the acid… 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 tmp tetramethylpiperidine.

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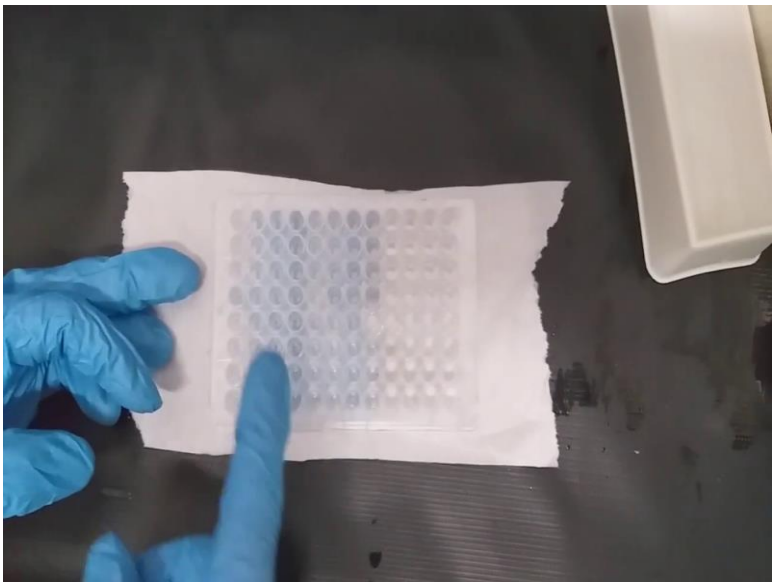

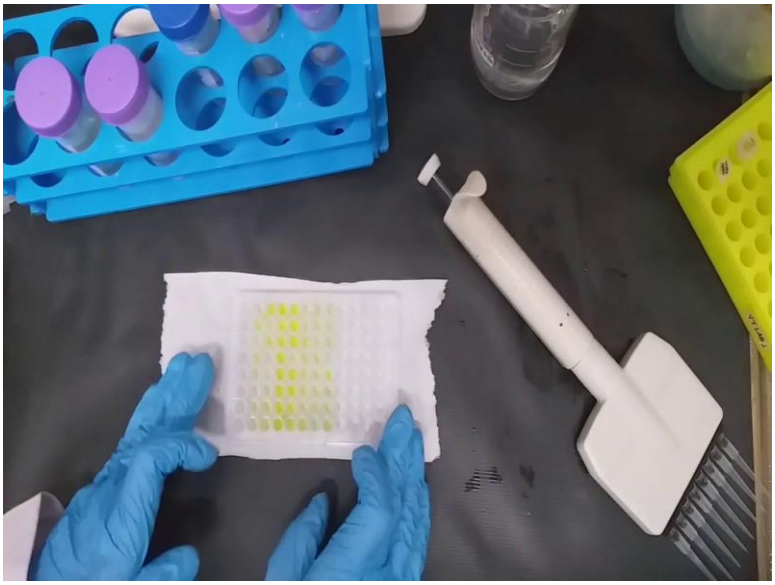

So, we will dispense the substrate solution into wells. So, once the dispensing is over, we have to keep at room temperature for some time until the we can see a visible blue color. Once the reaction is over, then we have to stop the reaction with the 2 normal HCl or sulfuric acid. After 15 minutes, if you observe it a bit, 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 intensive broker that means the concentration of the antigen is very high.

So, at this moment, we have to stop the reaction otherwise, all the wells will turn into blue color. So, we cannot identify positive sample versus false positive sample. So, there may be some artifacts. So, that is why we have to stop the reaction using a 2 normal HCl or H_2SO_4. We will add 2 normal H_2SO_4 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.

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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 at that particular person is, this is positive or negative. Now, another case for quantitative purpose, you need to have various varying concentration titration of the particular antigen which that disease causes. So, in this case, you have to dilute the antigen in different concentration and you have to develop the assay in the same way developed here. So, you have to compare you have to plot a standard graph against the concentration versus the absorbance we have taken.

From that value, you will come to know what is the unknown persons that antigen titration it is 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 prediction of various chemicals or various drugs in blood like drugs used for hallucination purposes and recreational drugs and also some of the drugs used in the pharmaceuticals.

Professor: In this demo video, the Manish has discussed many aspects related to the sandwich ELISA which you are going to use to measure the TNF alpha, I think he has taken an example of the TNF alpha, how you can be able to measure the TNF alpha into the biological fluid. And you can see that he has discussed also the different types of precautions what you should take when you are performing the measurements by this kind of sandwich ELISA

technique it is. So, with this, we have discussed about the detection of the biological proteins into the biological fluid, what we have discussed?

We have discussed about the different types of ELISA techniques, we have discussed about the direct ELISA, we discuss about the indirect ELISA, sandwich ELISA as well as the competitive ELISA. And we have discussed in detail about the indirect ELISA to measure the level of antibodies into the biological fluid and then we have also discussed about the sandwich ELISA how to detect the particular protein into the biological fluids.

So, with this a brief discussion about the different techniques to detect the proteins into the biological fluid. I would like to conclude my lecture here. In our subsequent lecture, we are going to discuss some more aspects related to living organisms. Thank you.