Immunology
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Lecture No -27
Tools and Techniques (Contd.,)

Welcome to the tools and techniques of means immunology that we are using. In last class we were discussing about immuno diffusion. So we purify antigen you inject antigen to animal we have some antibody with hopes of antibodies they are initially and then we check the see presence of antibody in serum with a very simple technique you just need petri plate agarose and your antigen and antibody to see the precipitate in line.

And we also discuss about why precipitant line is found what the logic of this precipitate line is why the how diffusion work? But that can tell you that the antibody production is going on but it cannot tell you exactly how much amount or quantitation is there. So if you want to quantitate any antigen or antibody what we do is normally? So normally what we are going to do this we are going to use a very popular technique of many of you might have heard about that in short it is called Eliza.

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In short it is called Eliza, Elisa stands for what **(())(01:44)** I will write it is enzyme linked immunosorbant I just wrote purposefully this is bigger immunosorbent assay enzyme-linked

immunosorbent assay that technique I mean this technique is very, very popular and routinely used for many labs in the pathological lab for detection of many things like hormone detection, quantification. So these particular techniques I am just not going to go the experimental details because it will take a lot of time and you may not need at this stage.

But just going to tell you how you can do that? What you can do with this experiment? We can do first detection. What we detect? We can do the detection of this detection of what either antigen or antibody we can detect both. Again I am telling you if you would need to do anything for antibody you need to have antigen and if you would want to know something about antigen you have to have antibody against that antigen.

So either 1 you can do and second mostly have been very importantly what you can do is you can do estimate you can estimation you can do. So estimation of either antigen or antibody you can do what is the principle? All the principle in general principle of all immuno techniques are antigen binds to antibody or antibody binds to antigen they interact. So their interaction we are going to detect. So what we do here.

What we do I mean Eliza? ELISA we do is actually we take care well that is a plastic well normally you can do it in cube also. But normally we use 96-well plate we also call it microtiter plate. So microtiter plate also or either 1 this is same thing what it is there, it is there are 12 well 1 2 3 up to their 12 and there are 8 rows. See in 1 row there are 12 and at the top of the plastic it is written 1, 2 and then gradually 12, 1 2 and this side in the column wise it is written A B C so then you have 8.

So total is 8 times 12, so it is a 12 into 8 is equal to 96 well plate. So 12 in each row and there are 8 in 12 in each so and there are 8 rows. In 1 row there are 12 wells and if I see this well big way if I I am just using this part. So if I see 1 of the world like this big what I will see is this well this you have to bide you have to call like there are many 96-well plate for different purpose. So we have to go when you are buying you have to play Eliza plate.

What is the difference between other plate because you can grow cell also in this 96 well. This is the format 96 well is a format but you have to buy 96 or Eliza plate. What is there? This is in the case of Eliza this well bottom for the wall of the well is coated with polyvinyl chloride. What is the purpose of this poly vinyl chloride because it can adds up protein because you know antigen is mostly protein that we are working with an antibody is definitely a protein.

So if antibody or antigen is a protein and PVC what the role of PVC because antigen antibody can attach to it or absorb to it. So what we do? So this plate is or the well is PVC coated. Now if I want to see that in a given solution if antibody is there or not what I will do is first I will add some pure antigen here. So analytic antigen solution antigen solution antigen solution I will add so if I fill this antigen if I fill this with antigen solution and keep it for some time what will happen?

Suppose the antigen color is red antigen is say like this or make it circle it will my life will be easy. So then if you keep this antigen solution everywhere it is everywhere antigen is fill up. So there are a lot of antigen molecules here and if you keep it after certain time what will happen? After certain time means maybe 1 hour maybe overnight depending on how much antigen you have what will see is?

We will see that antigen is attached to it the bottom of the well and the wall of the well. So antigen is attached with the wall you cannot see anything you just have to believe that antigen is attached. Then every step I am not going to repeat because I am not going to discuss about the total techniques like how exactly to work what is the problem I will just tell you the principle. So every time you is you use or we use any reagent next step is you have to wash out.

Because these solutes I mean the solution you added was full whole well was full of that. So what will happen some will let us here some will let us here but all lot of solution will be there so you have to wash with just phosphate buffered saline. Normally we use PBS phosphate buffered saline you can type this PBS and see what is the composition is sodium chloride phosphate because it is a saline after all.

So what is the composition so it is not important here. So we wash with PBS so what will happen all unbound and extracting will be washed out. So now when washing is done when washing is complete this is the case. Now in this stage there are computing we call it blocking I am not discussing here just see what is happening in this in this stage if suppose the antibody is; let me change the color green.

So now I will add in after this washing is done I will add antibody solution that may be directly serum or diluted. So now if that serum has antibody against your antigen what will happen they will bind here wherever antibody antigen is there they will bind; is depend now I am just making it saturated. So if antibody amount is well enough what will happen that will bind all the antigen available. If you have less antigen I mean less amount of antibody maybe some antigen will be free it is not going to bound.

So now in this case I assume that all antigen is covered by antibody molecule even after that you will give it for some time was out so this is now the condition. You cannot see that yeah because it is colorless. Now what happened 2 possibility now when we have to detect the antibody whether antibody is there or not because basically we are going to see the just a plastic. So to do that whether antibody is really attached to it first thing is the antibody solution I added it may or may not have antibody there that is what we are going to test.

Second if it is there, how much it is there? Both we can; so then we have to detail. So detection I am telling you first the simpler way though it technically it is little more difficult suppose this antibody solution that antibody what I can do is I can make some I can make some attachment with it. What is this attachment? So I make the antibody in such a way or purify the antibody and modify the antibody such a way I attached 1 enzyme. I attach one enzyme with this antibody I attached one enzyme with this antibody clear.

So this antibody these enzymes begin colorless you cannot see. So when I am attaching the adding this so every antibody has this enzyme everybody every antibody was attached to it this enzyme that is why the name and the enzyme linked is coming. This name enzyme linked sorry

the named enzyme linked is coming because the antibodies attach to enzyme every time. So then this enzyme also you cannot see.

So when antibody I add actually I added enzyme linked antibody. So even enzyme is linked and this enzyme what we use here this is generally horse radish peroxidase. Name is very common there many enzyme in the world not every enzyme we can use there are certain point for that. But the enzyme you use horse radish peroxidase. It is actually peroxidase is the enzyme and horseradish is the source which is a plant material it is no horse it is a horseradish is a plant there is no animal business though its name is horse.

Another is alkaline phosphatase why these 2 enzyme because this enjoy are very stable in room temperature you do not need any special incubator for that you can do the whole thing in just room temperature. They are very stable they are active and not only that both the enzyme has a very good property because they have this enzyme has a substrate commercially available which is colorless. This enzyme has a substrate which is colorless.

But they can give a product which is colorless which is colored. So they have a substrate which is colorless but their product is colorful not only that they have a product which is water-soluble. So, that means they have a water-soluble colored product that is why these 2. They are very stable in room temperature they can work they have a substrate colorless and colorful product. So why this is important because what we do is after adding that we add this substrate here and incubate in solution.

What will happen after certain time maybe 10 minutes, 15 minutes, 20 minutes you see the color is developed so what will what we will see actually, we will see that the well which was nothing that solution suppose this solution is green that solution is green and that then whole solution will be like green here whole thing you will be green and depending on depending on how much I mean so now the question is this is the whole technique.

So you is mobilize the enzyme antigen then you add your serum and see them contain antibody which is attached with enzyme and then if you add the substrate it will give color and color

solution because it is water-soluble. Now the question is if there is no antigen you did not know antibody will bind. If no antibody no enzyme is there no color that means. If I am working suppose I am taking the antigen whether some solution has antigen or not so in that case antibody is known antigen is unknown.

So one solution is given, suppose this water bottle so I am saying this water bottle has some antigen I would like to check. So come contamination is there so what I can do is I can take a little bit of this water add this in that tube in that well incubate it for a certain time and then the antigen I am looking for I am saying that this water contains some typhoid bacteria. So do not give me a challenge there is typhoid bacteria so that means some type of antigen will be there.

So if I take that solution isolate that bacterial extract and add it if and some of us I know that this water is completely pure there is no bacteria I would like to check. What I can do is, I can take that water extract the protein add it so then repeat this thing whole thing and the antibody I will give auntie typhoid antibody clear. So if this water has the antigen, it will this red thing will come and if this red thing comes.

And then your antibody will bind and at the end I will see the color if color develops then I am right that means this particular water that I was showing you has the typhoid bacteria. But if there is no color developed after repeated experiment that means there is no antigen. No antigen means there is no antigen in the water, clear. So this way it can tell you that whether this particular well has antigen or not same way if antigen is fixed I know antigen is pure on definitely antibody will you can test.

So if antigen I am sure it is there if serum or antibody solution which is unknown if I add it and give the color that means you have antibody in your solution. If there is no antibody that means no color so either one we can figure it out whether antigen is there or not then at one thing you have to is supposed to be known. If antigen is known antibody you can measure this antibody is known antigen you can measure.

Now depending on how many antigen is there the intensity of color will change because more antigen more antibody more enzyme that means more color will develop. So, one more enzyme can have multiple reactions. Same way you may have 10 molecule of and did you know how I mean let's see how many 1 2 3 4 5 6 3 3 3, 9 antigen molecule I have. And so now if your solution has 5 antibody molecules so then what will happen.

Then it will happen then suppose this is not there this is not there this is not there only say this 5 will be there. So if these 5 is there what will happen automatically your green part will be less. Less green solution so depending on the intensity also you can tell how much antigen or how much antibody is there because spectrophotometer measures this. So you have measured the spectrophotometer, the measure the intensity of this solution everything is standard substrate is standard everything is commercially available so you do not have to do much ok.

So that way the Elisa can tell you the amount how you can tell the exact amount? You just have to do a standard curve I am sure you did some DNA estimation or protein estimation in biochemistry lab ok in that what you do? First you did a standard of known amount. So what you have to do is you have to do basically you have to make multiple well where you have to give known amount imagine say 1 microgram then 2 microgram then 4 microgram then 8 microgram known amount of that suppose I am measuring the antigen.

So known amount of antigen if you give what will happen every once and then make a plot this is OD versus this is concentration or amount of antigen. This is the antigen concentration. So in one microgram in this one you will get somebody plot it 2 microgram you will get some 4 microgram you will get some 8 will get some so what will happen you will get a straight line. It may go with through the origin it may not go through the origin.

Then in one solution this one this is say unknown that you do not know this is the unknown that you want to measure. What you can do is you will get somebody say X that X OD I am taking another color. For, so that X OD suppose come here this is known standard this is called standard curve. So the unknown OD come here what I can do is this is the OD and if you go back from the slope also you can calculate.

If you go back whatever the concentration you will get here this is the concentration of antigen here. So that is how we can affect very closely measure the amount of antigen present in a given solution and most of the hormone we measure now in pathological laboratory who use Eliza. You might have heard the HIV detection is through Eliza the primary screening whether it is there or not Eliza test is so covered, so that is how it is.

So if anything is there you will get color if it is not there no color. So having color means it is there. So this is more or less the primary part that what is there.

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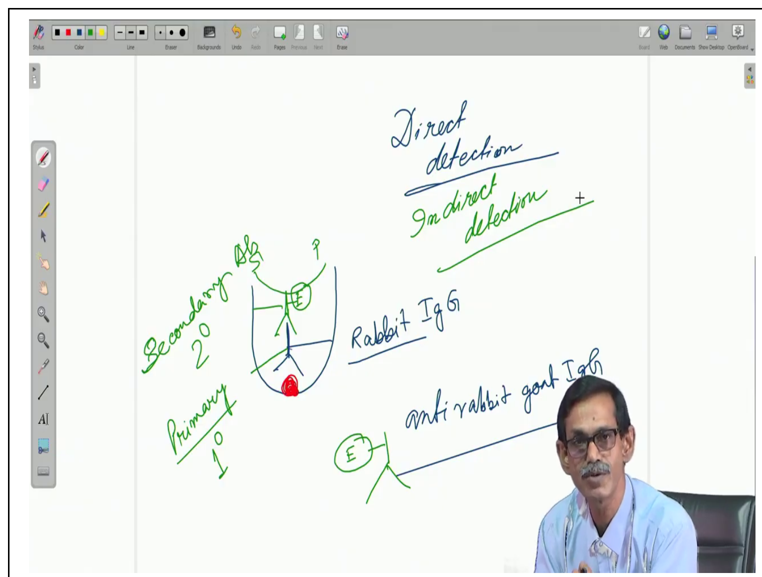

So in this case what we have is in this well we had antigen I am just now drawing one just for easy. You had antigen then we add antibody which was leveled with the enzyme but we can do second. This is when the antibody the first antibody is attached with enzyme we call it direct detection it is called direct detection. But sometimes it is not always possible because you isolate the antibody the serum labeling it with enzyme it may or may not be possible in all the labs.

So there is no problem for that also what you can do is you can buy anti antibody that is why I was talking about antibody and TDO type in a previous class so you can buy. Suppose this is the antibody which is raised in rabbit, rabbit antibody. So I can buy anti rabbit suppose this is rabbit IgG you are you erase the antibody in rabbit and after booster first booster is mostly IgG. So you

can buy and anti rabbit say goat IgG that means good rabbit IgG was injected in goat and antibody assessed.

So if this is the antibody if this color is say red no not red say green. So if this color of this antibody is green where it will bind it will bind to this antibody because it is raised that way. So this antibody you can buy commercially it is commercially available you can buy anti rabbit, anti human and anti goat, anti Mouse whatever antique antibody one you can buy commercially and not only the only antibody you can buy anti rabbit goat IgG attached to it enzyme.

What enzymes whether you would like HRP or alkaline phosphatase up to your choice no problem because both are commercially available. And it mostly what happened actually it depends which lab you are working what is their availability. There is my lab HRP is working so if you join in my laboratory it will work with HRP but generally it is same but definitely there are certain advantage disadvantage of both the enzymes but that is not very important for estimation I mean in this guy I am for this class.

So this enzyme linked second area this is called so this 1 called primary antibody which is my major interest this is primary antibody also designated like one degree and this is called this is called secondary antibody. Secondary antibody or 2 degree, so these secondary antibodys which is enzyme linked same. Now just 1 step extra and 1 hour extra then this is same now if you give substrate it will give product you will get color.

And these kind of using secondary antibody to do Eliza is called indirect detection indirect detection first one is direct because the primary antibody was labeled with enzyme this is second and this is actually the simple principle of Eliza.

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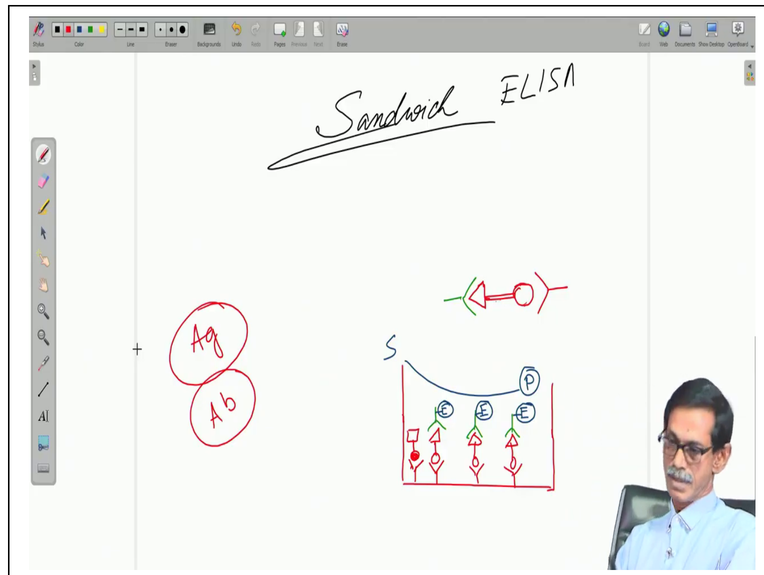

So dialyzer that we just discussed this is a straightforward Eliza either I mean you can do both or you can first immobilize antigen or you can immobilize the antibody then you can do but very common Eliza which is called sandwich Eliza that is what is this sandwich Eliza? You know sandwich that we eat so what is there to bread sandwich is to bread and in between there you can put whatever you like what is there in sandwich Eliza which same well same PVC coated well but it is little bit more specific why it is more specific? Say suppose there is one antigen has 2 different epitopes.

That now you know one is circular and another is triangular say it looks like that. So now if this is this is the and this is the kind of antigen here what will happen first and we are going to use 2 different antibodies one antibody can bind this and another antibody can bind this that is the sandwich. How? so first suppose I use this green antibody. So I immobilize everywhere I immobilize 3 antibodies there just to make life simple.

So here after this 3 antibody these they are also protein if you quote it will immobilize there then you add the antigen what will happen as soon as you add antigen right here as soon as you add antigen it will bind like this. So first you immobilize the antibody then you add antigen one of the epitope attached. So if by any chance there is a mistake or some nonspecific interaction it happened because it is a protein-protein interaction some nonspecific can always happen just to eliminate that to make it more specific I am going to use another antibody.

What antibody this red one so now this red one binds here. So this structure will happen so this 1 will happen if you see this if you see this, this kind of structure will happen. So then if this red which is coming second or if I draw this flat way so it will be better so one antibody here instead of making this one antibody here then like this. So then second antibody will come I just reverse this thing. So let me draw areas this just to avoid confusion.

So if this is the case the second antibody will bind it like this and this is your; this is the well where everything is happening. If this particular antibody the second antibody for example is enzyme-linked what is going to happen same thing same enzyme HRP or alkaline phosphatase and you give the substrate and you have you and then you have product here. Principle it exactly same in the same substrate same OD you can measure but what is this?

This sandwich analyze I will give you more specificity because you are using 2 antibody to detect one antigen so by any chance if one antigen one antibody non-specifically bind another will not. So suppose one antibody suppose in this case here in this case suppose this antibody bind something bind something like this but the other epitope is not triangular but square. So if this is the by mistake it binds the other one will not mind because the green one will not bind to this square.

So even in the primary time this thing happened the second antibody is not specific to this so it will not bind. So you can eliminate but if you do the single that all 4 will be detected. But here as we do the sandwich one will be eliminated because the second antibody will not recognize the antigen which is not really you want that will increase the specificity or it will give you more accurate result in comparison to single antibody.

Here also you can do direct indirect this is called sandwich Eliza is that clear. So here also you can have standard curve you can have this estimation on detection but normally sandwich a Eliza we use for accurate estimation is clear. So this is an Eliza test. Here what we can do we can measure antigen concentration we can detect antigen in a given solution we can measure

antibody concentration in a given cell oh sorry this we can measure antibody concentration in a given solution.

Or we can detect whether antibody is present in a given solution or not. So this is actually a Eliza what we call. So this is for today in next lecture we are going to discuss about immunoblotting. Immunoblotting is very similar principle but slightly different it is also known as western blotting. So see you in the next lecture.

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