Analytical Technologies in Biotechnology
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Module - 7
PCR, DNA Sequencing and ELISA
Lecture - 3
Enzyme linked immunosorbent assay (ELISA)

In previous 2 lectures of this module, we have discussed about two important techniques; one was polymerase chain reaction or PCR, and another was the automated DNA sequencing methods. And both these techniques are very important and widely used in different areas of biotechnology.

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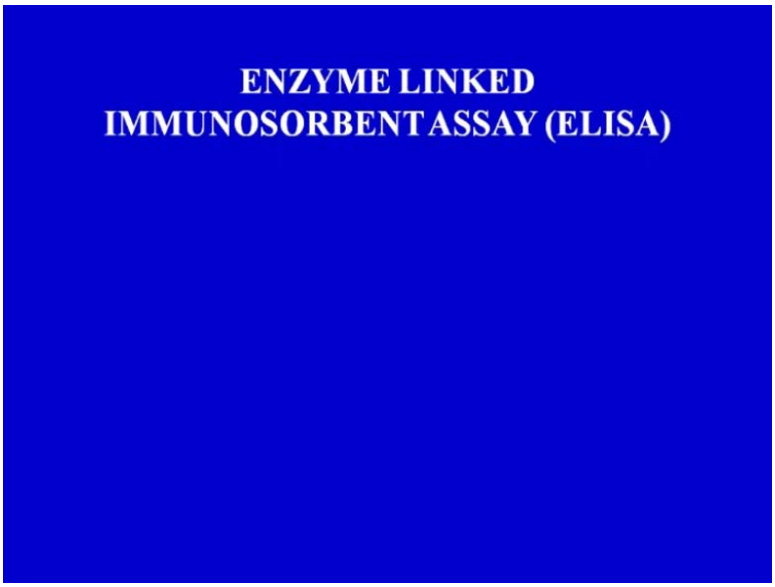

In this lecture we are going to discuss about another important technique that is Enzyme Linked Immunosorbent Assay or we can in short call it ELISA. Now, ELISA is a very popular wet lab method, which is in analytical method in biochemistry labs and ELISA is a fundamental tool of clinical immunology also. Based on the principle of antigen antibody interaction, this test allows for easy visualisation of results and can be completed without the additional concern of radioactive material use.

Now, if you can recall, we have discussed about radioimmunoassay earlier in radioisotope techniques, and in this regard the ELISA has almost replaced the

radioimmunoassay because, it is as sensitive as RIA or radioimmunoassay. But, the concerns of radioactive hazard are not there in this technique, ELISA is an immune assay in which one reactant is immobilized on a solid phase, and the signal generator or reporter is an enzyme.

So, here like it says enzyme linked, so enzyme is the signal generator here, and the fundamental principle of ELISA is the use of an enzyme to deliver a signal that, a particular antigen antibody reaction has occurred and to what extent this interaction has occurred. So, this particular antigen antibody interaction could be in terms of qualitative or quantitative terms, it could be evaluated. Now, enzymes are highly specific and they are catalytic properties can enhance a non enzymatic reaction manifold, further more enzyme signals unlike those of say radionuclide in RIA or fluorescent compounds, increase with time by continuing to turn over more substrate.

So, all ELISA configurations mostly are composed of three components, one is the capture system, the analyte and the detection system. Now, in each the analyte must be the one in limiting amount, and both the solid phase capture reagent and the detection system must be present in functional molar axis. Enzyme linked immunosorbent assay or ELISA these are plate based assays, and designed for detecting and quantifying substances, which could be like peptides, proteins, antibodies, hormones or other analyte as well.

And ELISA involves the stepwise addition and reaction of reagents to a solid phase bound substance, through incubation and separation of bound and free reagents using washing steps. So, an enzymatic reaction is utilized to yield colour, and to quantify the reaction through the use of an enzyme labelled reactant. The most crucial element of the detection strategy is a highly specific antigen antibody interaction, this one the ELISA was originally first described by Engvall and perlmann, in 1971.

And the method enables analysis of protein samples, immobilized in microplate wells using specific antibodies, a number of enzymes have been employed for ELISA, as reporter or signal generators. And they include like alkaline phosphates, horseradish, peroxidise, beta galactosidase and so on, and these assays approach the sensitivity of RIA'S, and have the advantage of being safer and less costly. So, before we move further let me give you, an overview of this technique on your screen or at.

So, like we said it is first thing is, it is based on antigen antibody interaction, so analyte acts as an antigen, and one has to have a specific an antibody against that antigen for a particular epitope. So, antigen antibody interaction takes place and there is an enzyme linked to the antibody, which could be primary antibody or secondary antibody. And finally, a substrate is added, which is converted to product by the linked enzyme and the signal is generated, which could be seen or it could be quantitated also.

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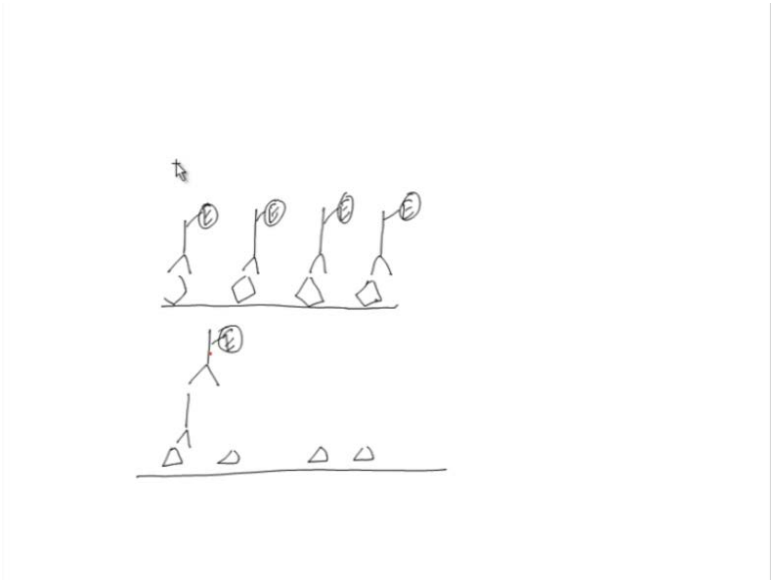

So, how it is done is for example, on a surface, a solid surface which could be plate or a well, microtiter well or any other simple thing, were there could be different methods of capturing the analyte. It could be either, it could direct say, antigen could be directly placed onto the surface and then antibody interacts with surface. So, and this antibody contains enzyme here, so what is done first thing is to immobilize the analyte here, so we have immobilized this analyte here.

And then the interaction of antibody with the analyte, this antibody could be having enzyme directly linked with or there could be other method, were that is called indirect ELISA, rather than primary antibody having the enzyme linked in here. There could be another antibody, which could have enzyme linked to it, so when you add substrate there will be colour or certain other signal, which be generated and could be counted.

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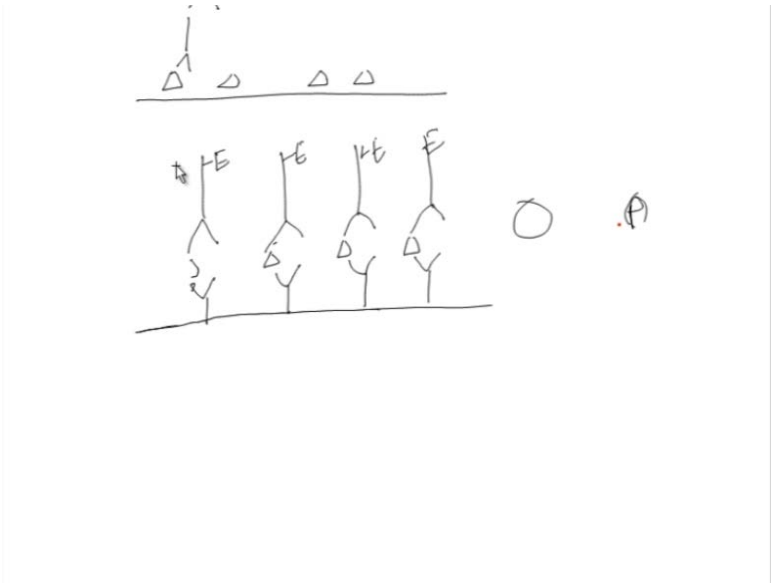

Now, there could be another method that rather than immobilizing the antigen directly onto the surface, sometimes it could be not so good actually, because of specificity reasons. There could be another method were what can be done is that, rather than directly putting antibody there, antigen there or analyte there first an antibody could be immobilized onto the surface, and then the antigen could be antigen could be put in which specifically interacts with that particular antibody. And other substances or other molecules will not interact, and they could be washed off then afterwards.

The next antibody with enzyme could be put in here ((Refer Time: 08:41)), and this is like kind of where the analyte is not directly being immobilized, but it is immobilized through an antibody, which is directly immobilized onto the surface. And then another antibody for is comes in the picture with enzyme linked to it, now when you add substrate to it, then substrate will be converted to product and product gives a particular colour, and so that could be quantitated or it could be measured in like, just for qualitative purpose.

And we will give you the idea about that particular analyte, in both qualitatively or quantitatively. So, this is a very basic phenomenon of ELISA or enzyme linked immunosorbent assay.

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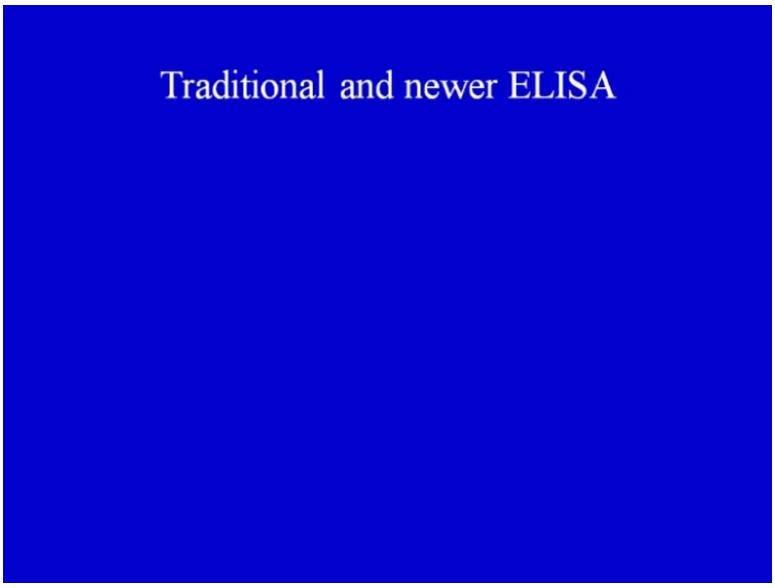

So, there are two different types of ELISA methods, one is traditional and other are new ELISA methods. Now, traditional ELISA typically involves chromogenic reporters and substrates that produce some kind of observable colour change to indicate the presence of the antigen, or we can call it analyte. So, these are simple or you can sty traditional ELISA method whereas, in contrast to traditional ELISA methods, newer ELISA like techniques utilize fluorogenic, electrochemiluminescent or real time PCR reporters to create quantifiable signals.

Now, these new reporters can have various advantages, including higher sensitivities and multiplexing, but since like in technical terms if you say, the newer assays or newer techniques here are not strictly ELISA techniques. Because, as ELISA says it is a enzyme linked immunosorbent assay, these are not really enzyme linked, but they employ other methods for signal generation and quantification, and they are attached to or linked to non-enzymatic reporters here. But, however, this general principle you can say is almost similar or and so they are grouped in you can say larger category as ELISA techniques.

So, traditional ELISA is simple enzyme linked, but other newer techniques also utilizes other non-enzymatic reporters. Now, there could be many variants of ELISA technique have been developed and used in different situations, but most of the techniques depend on the same basic elements. So, the basic procedure as such is very much similar in all

different kinds of techniques, so first thing to be done is the coating or capture, like I showed you just before, first thing is to put the antigen or the analyte on the solid surface.

Now, these are it could be a plate like I said, it could be microplate wells or certain other surface, now coating can be done or capture can be achieved either directly or indirectly. So, either you can directly immobilize the analyte or antigen onto the surface, and the surface could be like I told to you, it could be polystyrene microplate wells or lot of other surfaces could be there, or it could be in direct immobilization of antigen can also be performed. Then once you have coated or you have put in your material analyte there, then plate blocking is the next step in most of the techniques.

What is plate blocking here, plate blocking is the addition of irrelevant like a protein, or other molecule to cover all unsaturated surface binding sides of the microplate wells. So, many times like casein or other certain other proteins BSA or some other protein could be utilized or other molecules could also be utilized to cover up all unsaturated surface binding sides, for non specific signal or otherwise and this is done or this is called as plate blocking; so that is the next second step in ELISA procedure. Third one is the probing or detection now, here it is incubation with antigen specific antibodies that affinity bind to the antigens.

So, when antigen is immobilized plate blocking has been done, then it will be incubated or the antigen specific antibodies will be added to the plate and they will bind, because of higher affinity to the antigens. And then finally, signal measurement or generation is done where detection of the signal generated via a direct or secondary tag on the specific antibody. So, it is the enzyme, which is linked on their and when you add substrate a coloured product is formed that is the signal generation.

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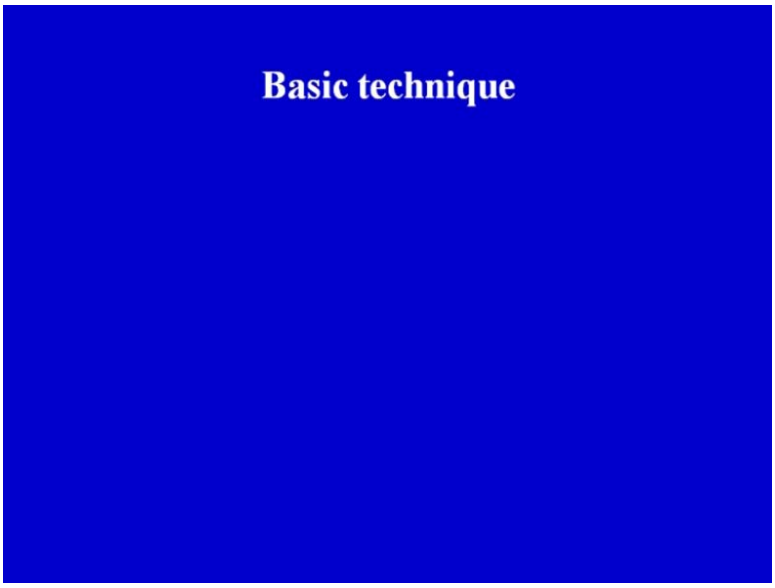

Now, very basic techniques in a typical assay designed to detect an antigen in a complex protein mixture, the antigen is immobilized either by a direct absorption or via an antibody adsorbed to the wells of the microplate. So, either you can directly put in antigen on the surface or one can also put antibodies for that particular antigen and then, that is indirect absorption can also be performed.

Now, the plate is blocked like I said, and the antigen is probed with the specific detection antibody, the detection antibody may be directly labelled that is with a single generating enzyme or a fluorophore or it may be a secondary probe, which an enzymed or fluoro labelled secondary antibody, that is antibody against the primary antibody. So, for enzymatic detection the appropriate enzyme substrate is added, so we will be discussing about these different forms of ELISA techniques as we go along.

So, as enzymatic detection the appropriate substrate for that particular enzyme is added, and then signal will be absorbed which is proportional to the amount of antigen in the sample. Now, washing between steps ensures that only specific or high affinity binding events are maintained to cross signal at the final step, so that specificity is maintained here.

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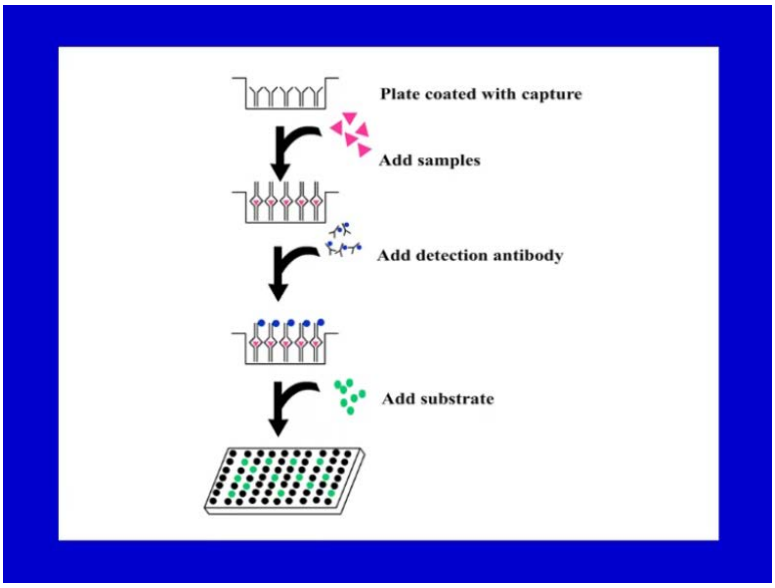

Now, very simple way like I have shown you this also figure here shows, this very simple method of ELISA. Now, here these antibodies are immobilized on a surface or this is capture antibody you can call, and this is well of a plate microplate, then your sample that is antigen is added. And since, these antibodies are against the antigen, they will bind to these antibodies, the nonspecific binding will be very much less and then also plate blocking will be done, then at detection antibody that is the antibodies where either enzyme or other reporter is attached. And then finally, substrate is added and colour development takes place here, this is the plate and then it could be directly read on a spectrophotometer which can accommodate these plates here. So, that is how the simple technique or basic technique of ELISA works.

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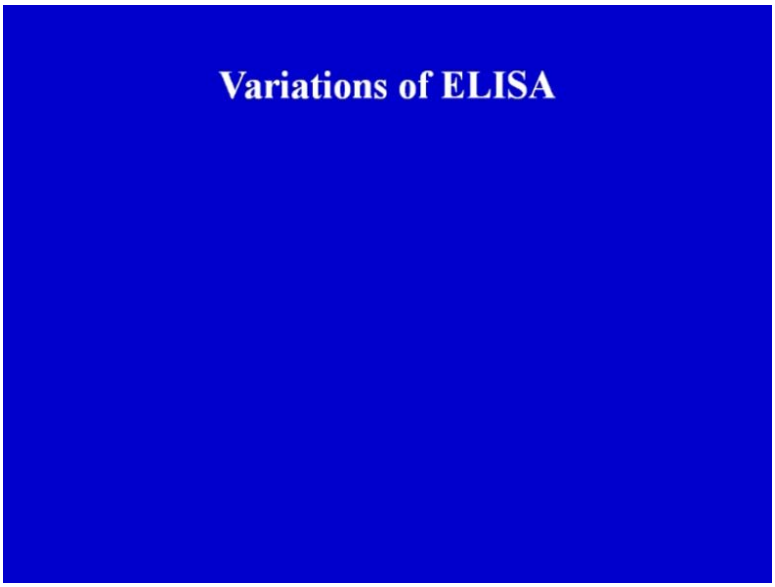

Now, there are lot of variations of ELISA methods, there are number of variations which have been developed, allowing qualitative detection or quantitative measurement of either antigen or antibody. Now, each type of ELISA can be used qualitatively to detect the presence of antibody or antigen, alternatively a standard curve based on known concentration of antibody or antigen is prepared. And they form the basis for determining the concentration of the unknown sample, if you could recall in radioimmunoassay also, a particular curve was drawn through the standards. And then unknown concentration could be calculated or determined through this standard curve, we said there are lot of variations here, let us discuss those different types or different forms of ELISA technique.

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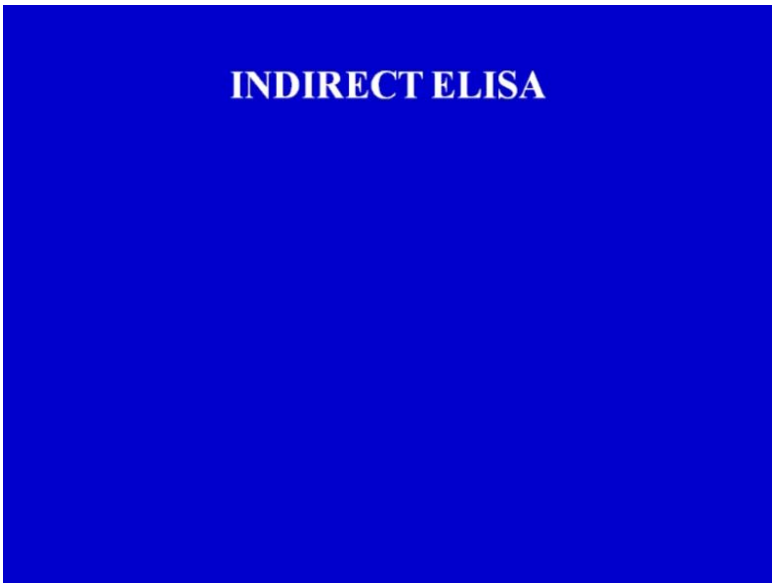

One ELISA technique is indirect ELISA, now indirect ELISA here antibody can be detected or quantitatively determined with this particular technique. So, serum or some other sample containing primary antibody is added to an antigen coated microtiter well, and allowed to react with a antigen attached to the well. So, here like you have antigen already attached to the well and there is an antibody, which interacts with these antigen. Now, after any free antibody that is primary antibody we call or antibody one its washed away.

And the presence of antibody bound to the antigen is detected by adding an enzyme conjugated, secondary anti isotype antibody which binds to the primary antibody, so this could also be called as double antibody system. Now, any free antibody that is secondary antibody then is washed away, after that a substrate for specific for the enzyme is added. Now, the amount of coloured reaction products that forms is measured by specialized spectrophotometric plate readers, which can measure the absorbance of all of the wells of a 96 well plate in seconds actually, they could do it very fast, and whole plate could be done.

The enzyme acts as an amplifier even if only few enzyme linked antibodies remain bound, the enzyme molecules will produce many signal molecules. So, within common sense limitation the enzyme can go on producing colour indefinitely, but the more primary antibody is present in the donor serum, the more secondary amount antibody and

therefore, more enzyme will bind and the faster colour will develop. So, there is a method a procedure to evaluate all this, to measure the colour development and quantitative information can be derived, it is not that its indefinitely done in here.

The indirect ELISA a major disadvantage of this particular indirect ELISA is that, the method of antigen immobilization is nonspecific, because you are directly immobilizing the antigen, when serum is used as the source of test antigen. All proteins in the sample may stick to the microtiter plate well, so a small concentration of analyte in serum has to compete with other serum proteins, when binding to the well surface. So, this might be a problem, because if there is a sample it is not a pure antigen, then lot of other things might also bind to the well.

This problem is quite well settled in sandwich ELISA or it called which provides a solution to this problem, by using a capture antibody specific for the test antigen to pull it out of the serums molecular mixture. Now, indirect ELISA is a method of choice to detect the presence of serum antibodies against human immunodeficiency virus, causative agents of AIDS. In this assay recombinant envelope and core proteins of HIV are adsorbed and solid phase antigens to microtiter wells, as solid phase antigens to microtiter wells. Individual infected with HIV will produce serum antibodies to epitopes on the viral proteins. Now, generally serum antibodies to HIV can be detected by indirect ELISA within 6 weeks of infection, so this could be a very good technique.

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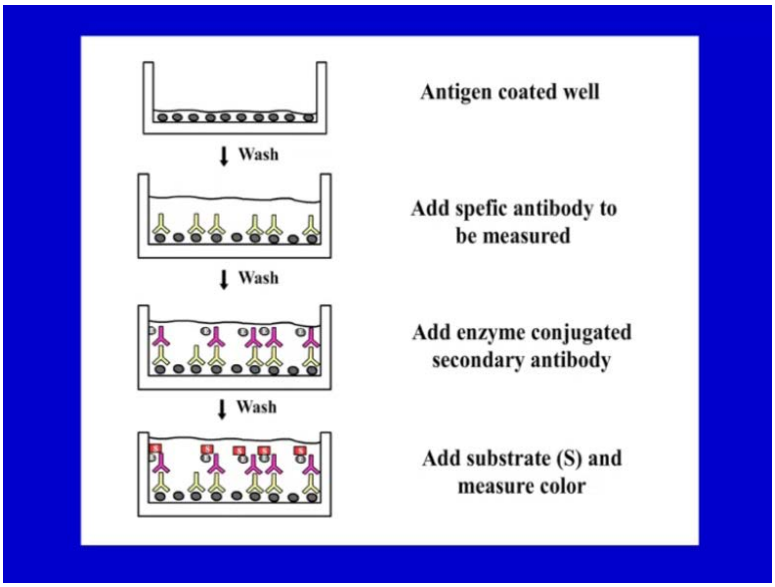

So, if you can see on your screen in indirect ELISA first thing is antigen is coated, like I said antigen might be in very small quantities in the whole of the sample, if that is so then other proteins will also be coated in here. Now, once antigen is coated in here, then specific antibodies are added and then, specific antibody extra antibody will be washed off by washing. Then secondary antibody which is this one is added, which is enzyme conjugated, then substrate is added and colour production takes place or signal generation takes place, which could be measured, so this is indirect ELISA.

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Like we were discussing, the problem of non specificity or other proteins binding could be solved by sandwich ELISA, and sandwich ELISA plate is coated with capture antibody. And sample is added to it and any antigen present will bind to the capture antibody, so other things could be washed off they will not bind of course, a plate needs to be blocked. So, that nothing else binds in there, then afterwards detection antibody is added and it binds to the antigen. Enzyme linked secondary antibody is added and binds to the antigen enzyme lit secondary antibody is added, and binds to the detecting antibody. So, it could be like detecting antibody, could be directly attached to the secondary one, to the enzyme or there could be a secondary antibody with enzyme linked, then substrate is added and converted by enzyme to the detectable form.

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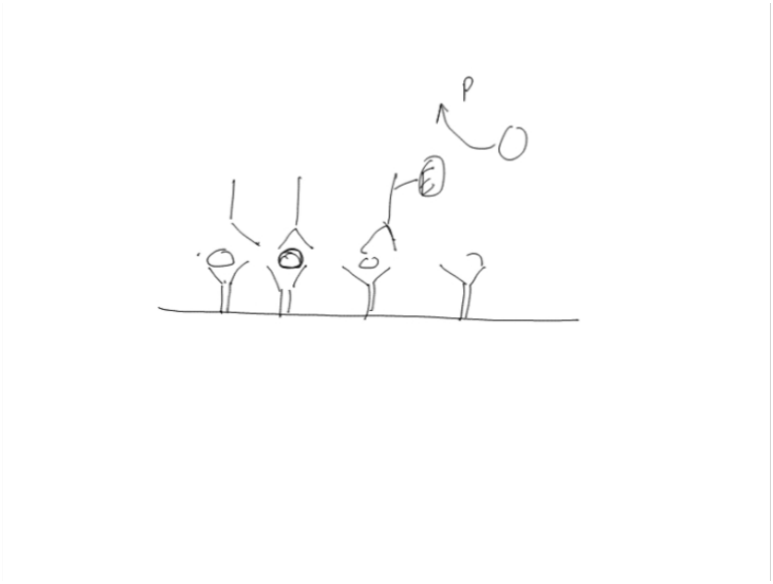

Let me explain the sandwich ELISA on your screen here, in sandwich ELISA what is done is, first thing is an microtiter wells or other surfaces, the first thing is to put in antibody. Now, these antibodies the first thing is that antibodies will bind to the antigen, in next step the antibodies will be binding to the antigen, and then secondary antibody which is enzyme linked will be bound at here.

Now, this antibody may contain enzyme or may be another secondary antibody could be utilized, but most of the time this detection antibody contains enzyme linked in here. Afterwards substrate will be added and substrate will be converted to product and colour development is takes place, so it is called a sandwich ELISA, because this particular one is sandwiched this antigen or analyte is sandwiched between the two antibodies. So, this is called sandwich ELISA, this the problem of like small amount of antigen being directly coated onto the surface.

The problem were other molecules might also bind, and for binding surface this small amount has to compete that problem could be solved here, because there is a capture antibody which is a specific for that antigen.

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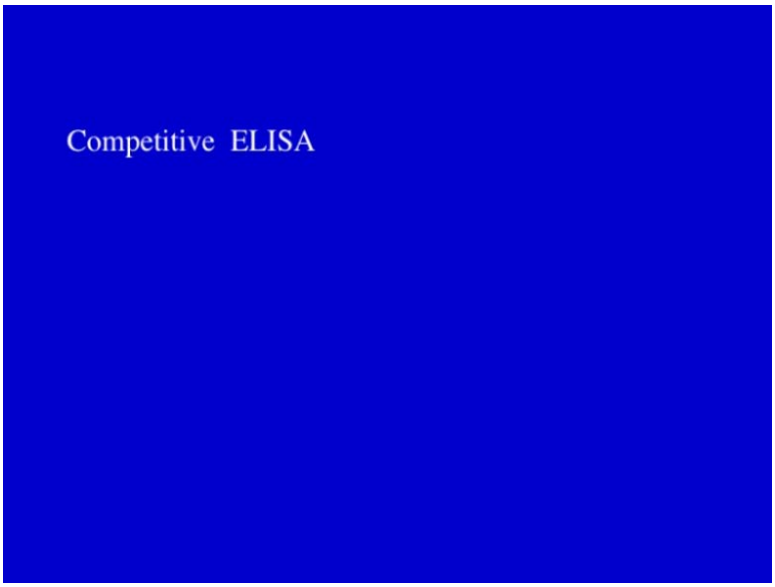

Now, there is another form of ELISA technique that is competitive ELISA, the competitive ELISA unlabelled antibody is incubated in presence of it is antigen. So, first thing is done is that there is a unlabelled antibody in free solution, it is incubated in presence of its antigen, now this bound antigen antibody complex are then added to the antigen coated to well. So, first thing what is done that antigen antibody complex is made, which is outside the well, then it is added to the antigen coated well, the plate is washed and unbound antibody is removed.

Now, secondary antibody specific to the primary antibody is added and the second antibody is coupled or conjugated to the enzyme, substrate is added and remaining enzyme will elicit a chromogenic or fluorescent signal. So, for competitive ELISA higher the original concentration of antigen the weaker the eventual signal, so depending on that since it is called competitive, because there is a competition in here.

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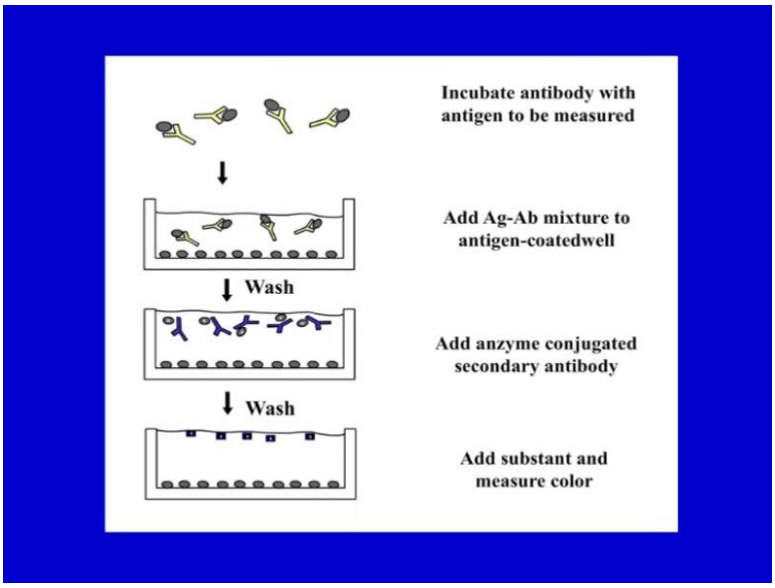

If you can see on your screen, so first thing is incubate antibody with antigen to be measured, so there will be like binding here of antigen and antibody complex formation. There is a antigen, which is coated in the well and this complex is added to that well, then interaction will take place where it is not shown here clearly. So, there will be a competition between the well coated antigen and the one which is bound, and there will be some antibodies which will be binding to this depending on the concentration.

And then enzyme conjugated antibodies against the primary antibodies are put in and then finally, substrate is added and product formation takes place. So, like I said depending on in competitive ELISA, if higher is the original concentration of antigen. Then the binding to the well coated antigens should be less and eventually the signal will be weaker and vice versa, so this is how the competitive ELISA works.

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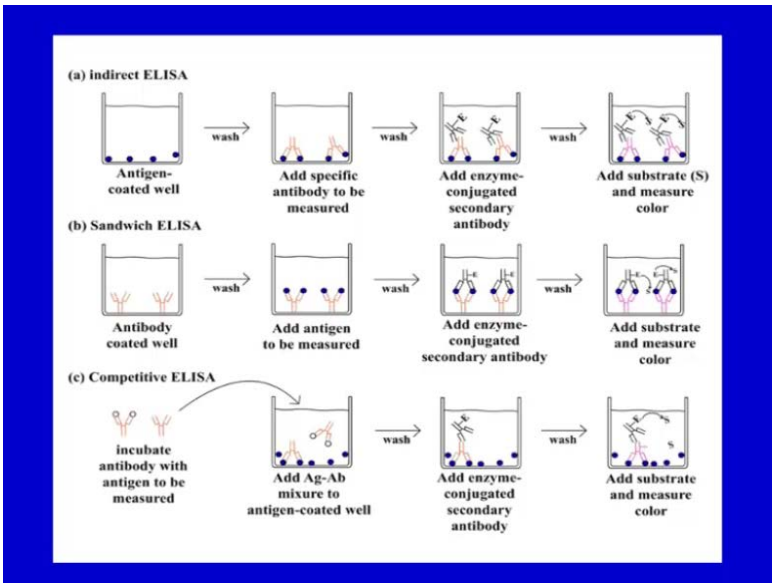

Now, in this figure all three techniques or different forms of ELISA are summarized in here, and as we can see that if you can see first is indirect ELISA. In indirect ELISA what we have discussed this the steps here, the antigen is coated here on the surface of the well. The first antibodies added which will bind to the coated antigen, which is specific antibody and needs to be measured, then there will be addition in the next step you will wash off any primary antibody which is not bound.

Then there will be addition of the enzyme conjugated secondary antibody and finally, the substrate addition and colour development, which will be measured through spectrophotometer or other methods. So, in indirect ELISA this is a very straight forward method, and sandwich ELISA rather than coating antigen directly, what is done first a capture antibody is coated on the well and then antigen is added, rather than coating the antigen directly.

Then afterwards a secondary antibody, which is enzyme conjugated antibody is added and then finally, substrate is added and colour development takes place and colour will depend on that how many enzyme molecules are there. So, in between like at every step washing has to be done to remove, the non binders here like say primary antibody or antigen is not bound which has to be washed off in this case, and this secondary antibody which is not bound to needs to be washed off. So, that extra signal is not there signal

comes from only the antibodies or enzyme, which is linked to the antibody, which is bound to the antigen here.

And then there is competitive ELISA where first antigen antibody interacts outside and forms the complex, then they are added to the antigen coated well and then there will be a competition, and there will be the antibodies will be washed off and finally, you will see the signal. And depending on whether what is the concentration in here, and what is known concentration you can determine the, you can quantitate. And in terms of qualitative terms you can evaluate or measure the signal and finally, the antigen concentrations, so this is three forms of ELISA techniques.

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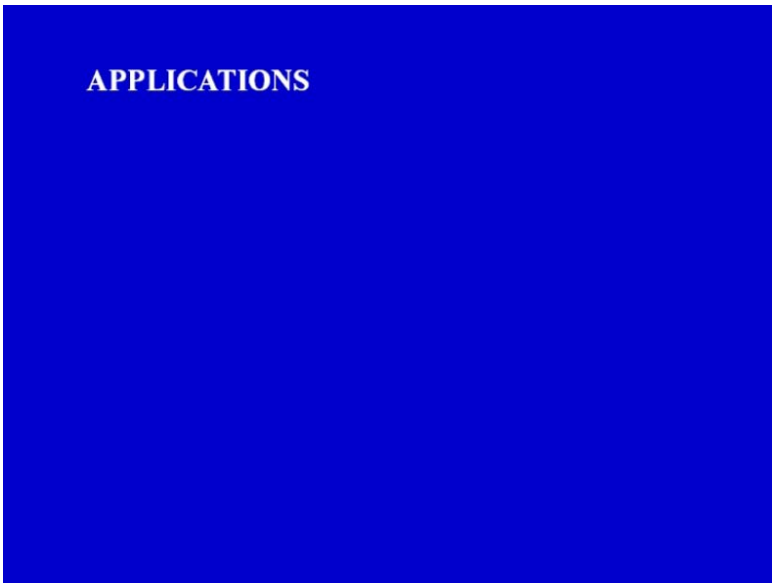

Now, there are lot of applications, like I said this is a very popular method, popular analytical method in biochemistry or in wet labs, and it also has applications is lot of like clinical diagnostics, it is quite popular and it is replaced RIA, because of the hazards of the radioactivity. So, because the ELISA can be performed to evaluate either the presence of antigen or the presence of antibody in a sample, it is a very useful tool for many different applications.

Like for example, determining serum antibody concentrations, like such as with HIV test or West Nile Virus test, one can determine the serum antibody concentrations. It has also found applications in the food industry, where in detecting say potential food allergens like in peanuts or walnuts or almonds, milk, eggs etcetera. So, it has lot of application in

food industry as well, food pathogens can also be detected here, ELISA can also be used in toxicological as a rapid presumptive screen for certain classes of drugs, it can be used for that.

Like there were have been like ELISA test have been done to detect various kinds of diseases like for example, malaria or chagas disease or other diseases have been detected using ELISA technique. ELISA tests also are used as in say in vitro diagnostics, in medical laboratories, there are a lot of other uses of ELISA which includes like say detection of mycobacterium antibodies in tuberculosis, detection of say rotavirus in faeces, detection of say hepatitis B markers in the serum, antidote toxins of e coli and faeces. Likewise there are whole lot of detections, whole lot of applications of this particular method.

Now, there are like ELISA as very popular technique and very sensitive technique, it has lot of advantages and some disadvantages also. ((Refer Time: 34:35)) In advantages of ELISA these reagents are relatively cheap and have a shelf life ELISA is a highly specific and sensitive technique as enzymes are highly specific in nature. There is no radiation hazards like, radiation hazards in radioimmunoassay where radioactive substances are used.

The ELISA is very easy to perform and it has a quick procedure, it could be done very fast the equipment for ELISA is almost inexpensive, and it is widely available. ELISA can be used particularly in clinical terms for detection of variety of infections, and lot of toxicological studies of food borne pathogens or other like diseases, pathogens, viruses all these things could be easily detected. If you have the specific antibodies for those antigens, with lot of these advantages there are some disadvantages as well.

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Like measurement of enzyme activity can be more complex than the measurement of activity of some types of radio isotopes, this could be one disadvantage, but not all the time. Enzyme activity may be effected by plasma constituents, so these are like nitty-gritty's which needs to be standardized, when one has to do ELISA for particular technique. Like for example, plasma may contain certain things, which might be inhibiting the enzyme or which might lower down the activity of the enzyme and proper quantitation cannot be performed.

There are kits available commercially, but they are not cheap, so when one who needs to do with regular chemicals and other things like generating antibodies, one has to standardize the techniques and then only can be followed subsequently. So, these are different technique, different advantages and disadvantages of ELISA. So, this completes this particular section here to summarize, ELISA is one of the most popular techniques and has replaced RIA or radioimmunoassay to a large extent, it is a wet lab you can say analytical biochemistry technique.

And quite has applications in clinical diagnostics and other various areas, it is a very simple technique and we have discussed about different forms of ELISA, where we have discussed about indirect ELISA, sandwich ELISA and competitive ELISA. There are other forms which we have not really discussed in where other than enzymes, reporters

are other than enzymes or other chromogenic substrates, like time PCR's or other methods, which are not truly in a sense ELISA techniques.

But, since their general principle is same, general procedure of ELISA is four step procedure, where one is the coating of the antigen directly or indirectly that is adsorption of the antigen or analyte. Second is blocking the plate, so that anything else does not bind third thing is adding the antibodies, which are conjugated to enzyme and fourth step is signal generation by adding substrate, which is converted to product. So, this was about all about the technique of ELISA which is very important, which has lot of applications in areas of biotechnology; and it could be both qualitative as well as quantitative.

So, with this lecture, we have completed this section and also the course on analytical technologies in biotechnology. Now, in this course, we have gone through various techniques which are widely used and utilized by different branches, in various areas of biotechnology. We have started with this course with the microscopy technique, then we moved onto radio isotope technique, chromatography separations, electrophoresis technique, then centrifugation technique and spectroscopy.

And finally, in this section we have discussed last section PCR, polymerase chain reaction, DNA sequencing methods and ELISA. If you could recall we will just summarize all these sections which we have discussed, in microscopy we have gone through different various techniques of light microscopy and electron microscopy. In light microscopy we have discussed about various methods of generation of contrast, through say phase contrast microscopy or fluorescence microscopy or say polarization microscopy, and like say dark field microscopy likewise.

In electron microscopy we have discussed about scanning electron microscope and transmission electron microscope, then in radio isotope technique we have discussed about all three techniques, based on ionization that is GM counters, based on scintillations that is scintillation counters and autoradiography. Then in chromatography separations we have dealt about say, different kinds of chromatographic methods used widely like ion exchange chromatography, gel filtration chromatography, affinity chromatography and a gas liquid chromatography.

We have also dealt in all these sections about basic concepts in the beginning, to make understand these techniques, the basic principle of these techniques for to understand. Then from chromatographic techniques, we have moved on to electrophoresis techniques, where we have discussed about the basic concepts. And also agarose gel and polyacrylamide gel electrophoresis, and we moved on to the techniques like SDS page, isoelectric focusing. Then we have discussed about pulsed field gel electrophoresis, and sequencing gels etcetera.

Likewise in centrifugation methods we have discussed about basic concepts and various centrifugation methods, like preparative and analytical methods, we also discussed about types of centrifuges, types of rotors ultra centrifuges etcetera. And various types of rotors and care of rotors, we have discussed about in spectroscopy techniques, we have discussed about various basic concepts, and that is the interaction of matter with the radiations. And then we have discussed about UV viz, fluorescence, NMR, X-ray crystallography and then finally, mass spectrometry techniques.

And in this section that is last section we have discussed about the PCR polymerase chain reaction, DNA sequencing method and ELISA that is enzyme linked immunosorbent assay. I hope that this course on analytical technologies in biotechnology will certainly be helped to all students who will go through this, and will help in understanding the basic concepts, as well as understanding the certain techniques, which are based on these concepts. And will help in improving and understanding these techniques for application or for applying in your daily, like research routines and also in certain applications.

Thank you.