Comprehensive Molecular Diagnostics and Advanced Gene Expression Analysis

Prof. Aritri Bir

Dr. B.C. Roy Multi-Speciality Medical Research Centre

Indian Institute of Technology Kharagpur

Lecture 53 : Immunoassay & Luminex Multiplex Assay

Namaskar. Welcome back to the NPTEL lecture series Comprehensive Molecular Diagnostics and Advanced Gene Expression Analysis. We are at our 11th module dealing with molecular diagnostics in medicine. In today's class, we are going to discuss Immunoassay and Luminex Multiplex Assay. Now today we are going to discuss broadly different types of immunoassay, the very basic principles and concepts of immunoassay and Luminex Multiplex Assay. They are its principle and mechanism.

Now in immunoassay, the assay is basically based on the reaction of antigen and antibody, antibody which is targeted specifically for that antigen. So, definitely immunoassay because one antibody is specifically designed for that targeted and target antigen, it is sensitive as well as specific. So, in a typical immunoassay what we want to know? We want to know whether a specific analyte in a sample is present or not. If present, what is the quantity of the sample? So, against that analyte, we basically develop one antibody.

So, of course, the analyte here behaves as an antigen to which there is a specific antibody degenerated and based on this antigen antibody reaction, there is identification of this specific targeted analyte. Now majority of that specifically designed antibodies are monoclonal antibodies. What are the different types of immunoassay? Immunoassay can be competitive or non-competitive based on that there are different types of immunoassay types like radio immunoassay, enzyme immunoassay, fluorescence immunoassay, chemiluminescence immunoassay. So, these are the different types based on the reporter molecule. Now, what is the reporter molecule? Let us see.

So, this is the very basic principle where there is one immuno where the immunoanalytical reagents are mixed and incubated, then analyte which is an antigen is bound to the antibody forming one immune complex. So, here this immune complex we cannot see in our naked eye. So, what we need? We need some identifiable tag or marker or reporter. Those reporters basically give is giving the reading for that immune complex and that reporter or label can be radiation for radio immunoassay, can be

fluorescence, can be enzyme and based on their signal we are going to measure the immune complex or the bound immune complex or the free immune complex. And this is how we can get a quantitative estimation of our targeted analyte.

Now, let us see once again how these labels are actually helping in identification. Coming to non competitive immunoassay, non competitive immunoassay is one antibody excess immunoassay. So, here what in the sample in the reaction mixture what we are having? We are having one fixed amount of antigen, a variable amount of unlabeled antibody and fixed amount of labeled antibody. So, once again we are having fixed amount of antigen, then variable amount of unlabeled antibody and fixed amount of labeled antibody. Now, what happens? The unknown analyte is our targeted analyte fine.

In the sample those unknown analyte is there are there and here we are mixing our labeled antibodies. So, the unknown analyte is captured by the labeled antibodies. Now, if we add unbound if in the mixture what happens after binding with this unknown analyte and the labeled antibody what we are having? We are having some free labeled antibody. So, those free labeled antibodies are basically washed off and what we are measuring? This bound part. So, the intensity of the signal what we are getting is now only from the bound antibody, antibody bound to the target.

So, the intensity of the signal is basically now directly proportional to the amount of unknown analyte. So, the concentration is directly proportional to the signal what we get. So, this is the non competitive immunoassay where there is no competition fine. Now, this competitive immunoassay by its name it is evident. So, they are there is one competition.

So, competition between fixed amount of antibody, fixed amount of labeled antigen and unknown quantity of unlabeled antigen. So, who are competing? The labeled and unlabeled antigen are basically competing for the targeted amount of antibody. So, what we are having? We are having some antibody which is fixed over a separation matrix over a matrix we are fixing the antibody. Then we are having unknown amount of sample, unknown amount of analyte in our sample and of course, in the sample there is no labeling. So, this is the unlabeled one and along with that we have designed the same analyte and labeled them fine.

So, these two are basically competing for binding over this antibody site. So, that is one competitive immunoassay. Now, the competitive immunoassay can be homogeneous or can be heterogeneous. So, in homogeneous assay what is happening? The unlabeled analyte in the sample basically competing with the labeled one. The amount of labeled unbound analyte is now measured.

So, again so, this is our antibody, this is our labeled analyte and this is our labeled analyte, this is our unknown unlabeled analyte. So, consider initially what we are having? We are having signals for these three. Now in a react in a mixture when there is no target analyte, this is our target analyte we want to measure. Now, when we are having no target analyte all these three will be bound here. So, they are fixed over the separation matrix.

If we wash it in the washed part or in the washed elute there will be no labeled antibody no labeled antigen. So, we are not getting any signal, but if there are some analyte which is present in our target sample what will be happening? They will compete here, they will compete here and these two will be replaced from the antibody binding site. So, in the washed elute what we will be getting? We will be getting for signals from these two. So, this is how homogeneous competitive immunoassay, we are basically measuring the labeled unbound analyte in the mixture fine. And of course, they will be proportional to the target analyte because the amount of target analyte will be attached that very much amount will be replaced from that very much labeled analyte will be replaced and we are measuring that.

So, they will be proportional. In the heterogeneous competitive immunoassay what happen? The unbound analyte this part we are basically washing off. So, this part we are discarding what we are measuring? We are measuring the bound part fine. So, this is the heterogeneous competitive immunoassay. So, the less signal we are getting the more analyte is present in the sample in case of heterogeneous competitive immunoassay.

So, here you can see ELISA or enzyme linked immunosorbent assay is basically example of one very common example of immunoassay where the reporter or label is one enzyme that reporter enzyme is taking part in a reaction which is giving some color. So, color or fluorescence visible color or fluorescence. So, when the antibody or antigen bound enzyme takes part in the reaction. So, this product is a colored product or this product can fluoresce to give a signal. So, based on that we can quant identify and quantify different types of analytes.

Now this ELISA can be direct ELISA where what we do? This is our separation matrix the matrix which can capture or hold the antigen or antibody of our interest. Now over this separation matrix antigen are basically sample antigen are added. Now those sample antigen are basically captured here. Now what happened? Now if we add some antibody which are tagged with the enzyme they will directly bind with the antigen and will give the signal. So, this is our direct antigen antibody bound immune complex mediated signal.

Now there can be indirect ELISA here you can see there is no competition this is uncompetitive one. Now indirect ELISA is another example of non competitive immunoassay where once again antigen is added then antibody is added. Now previously in the direct ELISA what we did? We directly measured this antigen antibody or immune complex, but here instead we are using another secondary antibody secondary antibody is tagged with the enzyme. This basically helps in signal enhancement and also it makes the experiment more specific. Then sandwich ELISA can be there now the sandwich ELISA here the antigen is basically sandwiched between two antibodies.

So, what is done? Initially the antibody is captured over the surface then addition of antigen they are basically add they are forming the immune complex then once again another antigen antibody we are adding and that antibody once again is attached over the antigen. So, the antigen is occupied by two different antibody and sandwiched in between them. Now the second antibody is having the level or tag which is giving the signal. All these are basically non competitive ELISA or immunoassay whereas, competitive ELISA here you can see what we do initially we basically incubate the antibody with the antigen. So, here you can see there is there is antigen antibody bound immune complex fine antigen antibody antigen and antibody they are bound to each other.

Now in the matrix previously what we have done there are antigen captured over the matrix and one antibody is basically tagged here. Now if we add our immune complex here you can see these are two different materials fine. So, if we add our sample they basically will replace this and will be bound. So, basically what is there? There is one antibody antigen complex. Now in the sample with the antigen there is antibody previously bound.

Now these antibodies can be labeled. Now if this complex is bound here what will happen? They will be attached here replacing this tagged antibody. So, we can get signal from here or if we add one secondary antibody which is antigen and labeled tag in that case also we can get one indirect measurement of competitive ELISA. So, these are the different types of immunoassay we can follow these are the different processes of immunoassay rather. Now based on that there is one Luminex multiplex immunoassay which is basically following the same sandwich principle of the ELISA where magnetic micro particle based immunoassay method is followed it can quantify multiple around 100 types of biomarkers in a single study in a very low sample volume.

So, that is why it is one multiplex type of immunoassay. So, what is there? There is one magnetic micro particle beads or color coded microspheres and they are internally dyed with different proportion of red and infrared fluorophores and that has a distinct

spectral range. So, basically in the bead so, different beads are having different spectral range. So, in this from this plate or strip in different in this strip has different spectral signature in different region because in different region different types of beads are present. Now antibodies which are specifically designed to our desired analytes are coupled to unique bead regions and are incubated with the sample.

Now what will happen? In this bead there is antibody to that antibody if there is antigen binding is there we can identify them. How we can identify? So, after this binding we wash off the unbound materials. Now the bound sample is now incubated with a mixture of biotinylated detection antibodies and streptavidin phycoerythrin reporter. So, we add another detection antibody which has a reporter that is streptavidin phycoerythrin reporter. Now using the Luminex instrument we now excite these beads from those region from the beads which are basically captured which has basically captured different antigens different targeted analyte they are having this streptavidin phycoerythrin reporter.

Now phycoerythrin one is one fluorescent molecule. So, under Luminex in instrument they can fluoresce. So, what will happen? From different those specific bead which are basically have identified their specific analyte they will fluoresce. So, the specific region of the bead of the plate or the strip will fluoresce. Now based on that we can detect the presence also via the getting the magnitude of the signal we can also quantify the analyte.

So, this is how multiplex immunoassay can be done. So, here you can see. So, you can see different regions are there. So, in different regions different types of beads are presents all those different types of beads number 1 beads, number 2 beads, number 3 beads, number 4 beads all those beads are having their specific spectra which basically is a signature for them. So, if we are getting spectra from this region we can say that this number 2 analyte is present and this number 2 analyte is a different one because here is a different antibody is attached to a different molecule.

So, this is how based on different type of spectra we are getting from a strip we are saying we can claim that this analyte is present and in how much quantity it is present that also can be measured via measuring the magnitude of the spectra. So, this is Luminex multiplex assay. Now what is the importance? Based on this signal different types of parameters can be checked. So, around 100 types of biomolecules can be checked simultaneously in this Luminex multiplex assay. So, in the summary we have learned that immunoassay is basically one antigen antibody based reaction which is identified by some reporter or labeled molecule that can be radiolabeled, that can be enzyme linked, that can be fluorescent linked, fluorophore linked and then there can be homogeneous and heterogeneous type of assay and also Luminex multiplex assay is

basically different Luminex bead based assay and multiple type of biomarker can be assessed there.

So, that is one multiplex type of assay. So, these are the references. Thank you.