Experimental Biotechnology
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Module-VII
Immunological Techniques
Lecture-31
Antibody-Antigen Interaction (Part 3)

(Video Starts: 00:23) (Video Ends: 00:59) Hello everybody, this is Dr. Vishal Trivedi from department of biosciences and bioengineering IIT, Guwahati. And in this particular module, we were discussing about the different types of the immunological tools, which are mainly been based on the interaction between the antigen and the antibodies. So, what we have discussed so far is as follows.

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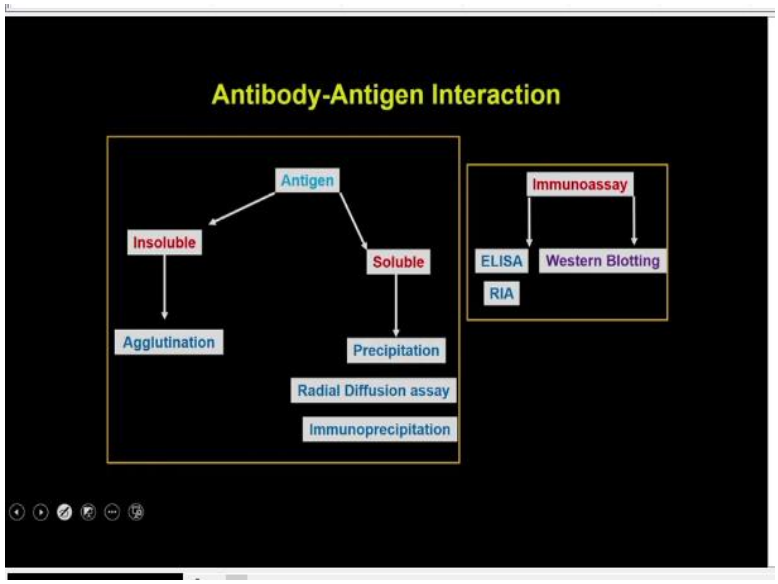

We discussed about, if the antigen is insoluble nature then you can be able to perform the agglutination reactions, if the antigen is insoluble in nature, then you can be able to perform the precipitation reactions or the radial immunodiffusion and in the previous lecture, we have also discussed about the immunoprecipitations. Apart from that, we have also discussed about the different types of immunoassays such as the Eliza, western blot and so, on.

So, with these tools, you can be able to perform different types of experiments and you can be able to answer many types of immunological questions or the questions which are related to the biology. Apart from that, there is a additional thing which you can also explore with the help of the immunological tools.

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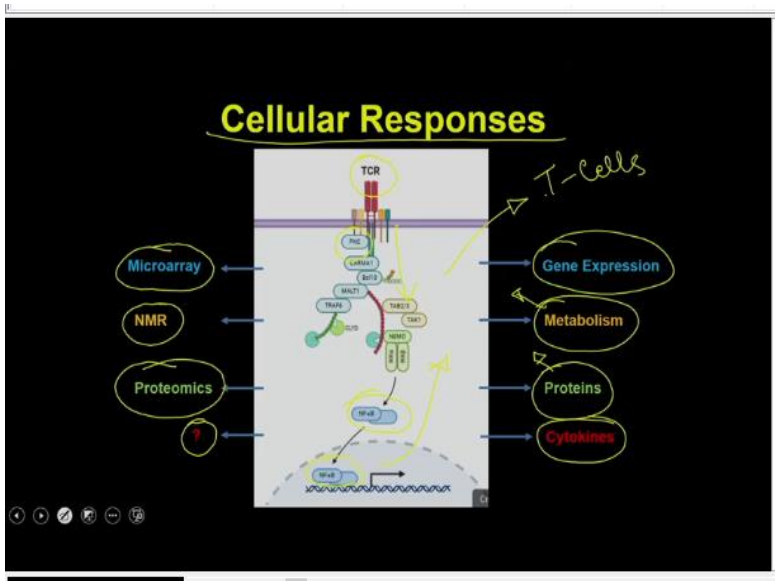

So, you can imagine that a cellular response of a immunological cells like macrophages or B cell or T cell, for example, in this cell in this case, it is the T cell which is actually responding to extracellular ligand and in response to that, it is actually driving a downstream signaling and it is involving all these signaling molecules and at the end, it is activating the NF kappa B transcription factor.

So, NF kappa B is going inside the nucleus and then it is activating its target genes. And in response, it is actually producing the different types of proteins, it is actually changing the transcriptional activities within the cell. And as a result, it is actually going to make a complete cellular response, whether this is going to be a humoral cellular response or to the cellular cell response.

Whether it is going to be a humoral immunity or the cell mediated immunity, but at the end, what you are going to see is that in the event of any such signaling, what will going to happen is that it is actually going to change the gene expression of that particular cell, it is actually going to change the metabolism of that particular cell or it is also going to change the proteins of the cell and if this particular cell is a immune cell.

For example, the T cells or the B cell, it is also going to change the cytokines secretion from this particular cell. So, if you have these particular 4 parameters, which you would like to monitor you have different types of techniques. For example, if you want to monitor the gene expression profiling, you can do that simply by using the microarray chips. If you want to

study the metabolomics or the metabolic changes, then that can be done simply with the help of the NMR techniques.

And if you are interested to see the changes within the proteins, then you can be able to do with the proteomics, but what would be the technique if you would like to study the complete cytokine profiling of a cell under a particular cellular response. So, to this we have the multiple options. One of the easiest way is that you can perform the Eliza's.

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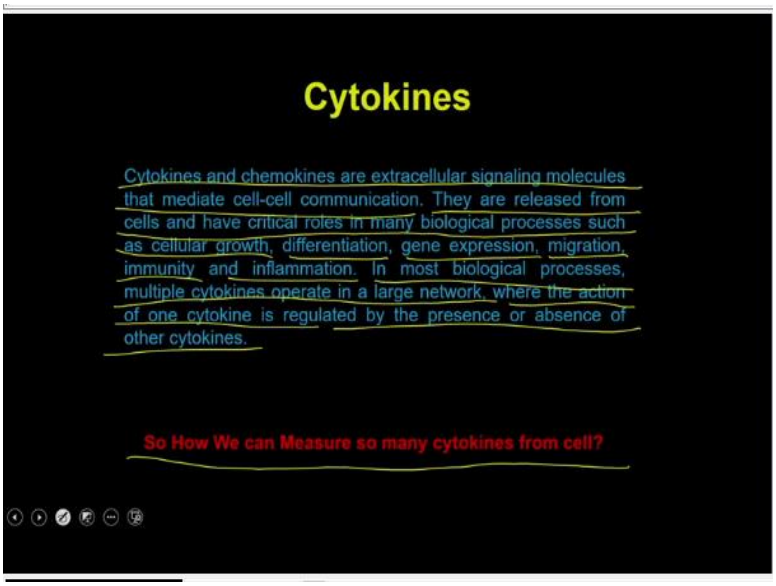

So, I think in the previous lecture also we discussed about the different types of Eliza what are available for you to explore and**.** So, the cytokines are actually the signaling molecules. So, cytokines or the chemokine are the signaling molecules that mediate the cell to cell communications, they are released from the cells and have the critical role in many biological processes such as the cell growth, differentiations and gene expressions.

Migrations, immunity and the inflammations. In most biological processes, the multiple cytokines operate in a large network, where the action of one of the cytokine is regulated by the presence or the absence of the other cytokines, this means, the cytokines are actually very, very versatile in nature. So, they can be secreted from one cell, they can go to the 5 more cells, and then that is how they can actually be able to make a very, very complicated network where the one cytokine is regulating the secretion as well as the action of the multiple cytokines.

So, what is the option what we have to measure these many cytokines from a single cell that to at a particular moment. So, if you remember in our previous lecture, we discuss about the different types of immunological tools, what you can actually use to measure the antibody or the antigens.

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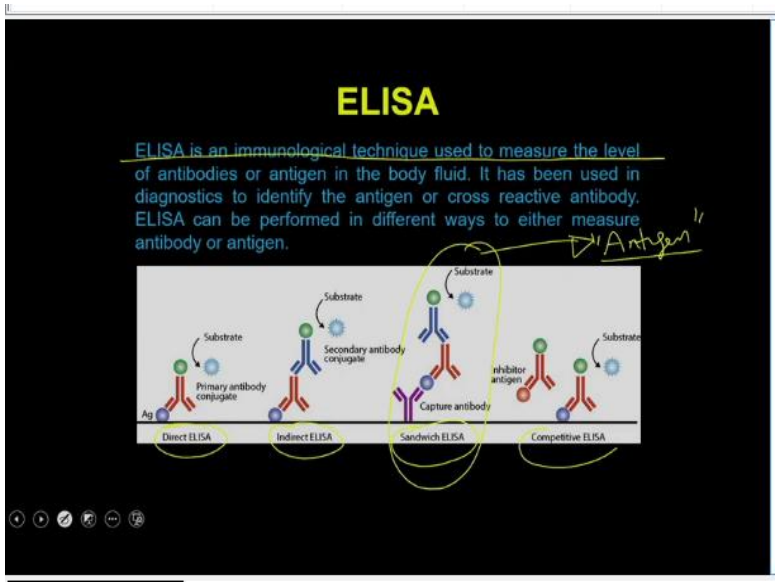

So, Eliza is one of the immunological tool that what we have discussed and we have discussed about the different types of Eliza like the direct Eliza, indirect Eliza, sandwich Eliza as well as the competitive Eliza. So, if you remember that the sandwich Eliza is something what you can use to measure the level of the antigen. So, sandwich Eliza can be a potential immunological tool, which actually you can use to measure the level of the cytokines.

But the number of cytokines are very, very big. So, if you perform the sandwich Eliza for the individual cytokines, it is actually going to be a very, very laborious work, and on the other hand, it is actually going to give you it is time consuming and as well as it is actually going to cost you a lot of money. So, instead of doing a sandwich Eliza, the people have developed the cytokine arrays, where you can be able to measure the level of cytokines at a particular moment from a cell.

So, whether you use the cells or the cell supernatant or whether you use the cell lysate or whether you use a tissue, you can be able to measure the different types of cytokines. So, how the cytokine array works.

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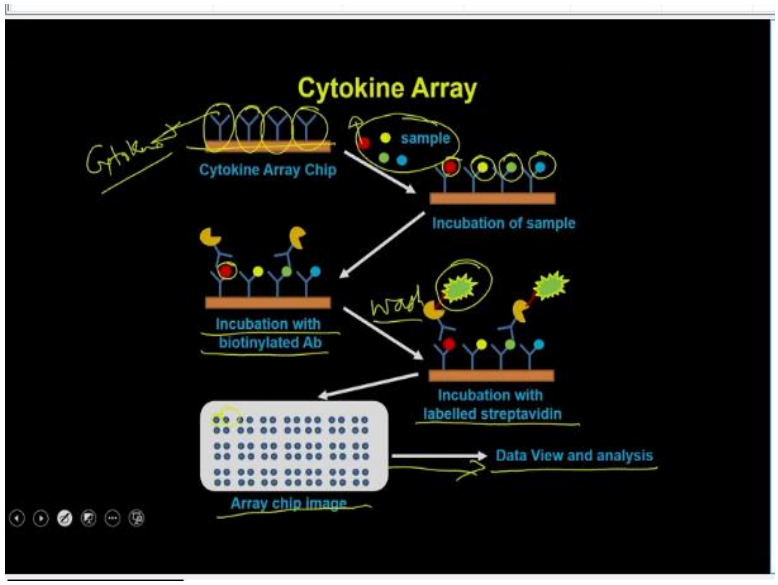

So, in a typical cytokine array, what you have is you have a membrane on which the antibodies are being immobilized. So, these individual antibodies are directed against a particular cytokines like. So, one antibody is for a single cytokine and you have multiple antibodies, and then what you are going to do is once you add the samples, these samples are the different cytokine molecules.

So, they will go and bind to their respective antibodies. And once you are done with these, then you can do a washing and then you are actually going to add the biotinylated antibodies, these biotinylated antibodies are also monoclonal antibody and they will be also against this particular cytokines. So, you are actually going to have the multiple monoclonal antibodies which are biotinylated.

And all these multiple antibodies are going to bind to its respective cytokines. And ultimately at this stage again you are going to wash and then you are actually going to add the streptavidin which is actually linked either with a fluorophore or it is linked to an enzyme and that actually will allow you with the to do the reactions to visualize the samples and what we are going to see on to the array chip, that you are going to see the individual spots glowing up after the reactions.

Either they will going to give you the fluorescence signal or they are going to give you the 09:15 chemiluminescent array in some cases you can also use the X ray films to acquire the image or acquired the data from these cytokine array chips. And ultimately what you can do

is you can just capture the image and you can be able to do the analysis of this particular signal. So, the question comes how cytokine array is different from the sandwich Eliza.

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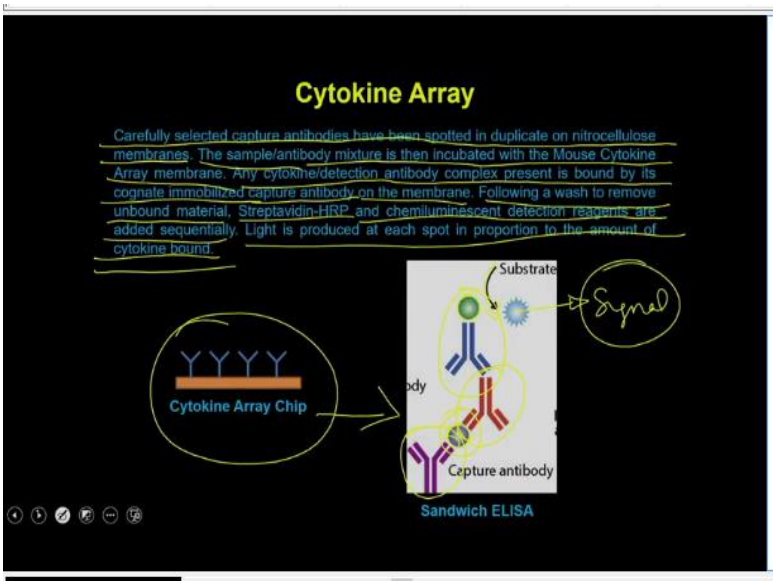

So, in the cytokine array, the carefully selected capture antibodies are being spotted in duplicate onto a nitrocellulose membrane. The sample or the antibody mixture is then incubated with the cytokine array membrane, any cytokine detection complex present is bound by its cognate immobilized capture antibody onto the membrane following a wash to remove the unbound material.

Streptavidin-HRP and chemiluminescent detection reagents are added subsequently, light is produced at each spot in proportion to the amount of cytokine present. So, this is actually a cytokine array where you have a membrane where the antibodies are being immobilized and these antibodies will bind the cytokines which are present into your sample. That cytokine array and the complete idea of doing a cytokine array is very much similar to the sandwich Eliza except that in this case, you are using the different antibodies.

And as well as you are actually it is just like that you are performing the 40 or 50 Eliza reactions at the same time, but the only issue is that the antibodies are immobilized on to the nitrocellulose membrane and you are using 2 antibodies just like a sandwich Eliza. If you remember in a sandwich Eliza also we have a capture antibody, which actually going to capture the antigen from the reactions.

And then you are actually adding the additional monoclonal antibody which is actually going to recognize the antigen, but not with the same epitope it is going to recognize with the different epitope and then you are actually adding the secondary antibody which is coupled with the enzyme and then you are adding the substrate and that actually is going to give you the signal.

So, it is exactly the same that cytokine array is nothing but the sandwich Eliza which is you are performing but it is present onto a thin sheet of membrane instead of doing it in the well. Let us see how to perform this.

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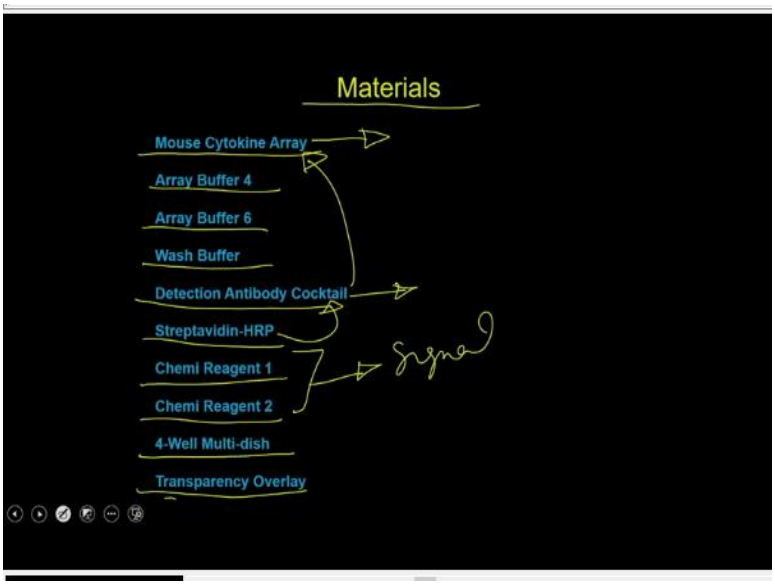

So, to perform the cytokine array, what you need is you need the cytokine array from a particular company you require the buffers, different types of buffers, then you require the Bosch buffers, you require a detection antibody, detection antibody is also going to be the monoclonal antibody directed against a set of cytokines for which the array is also been designed.

Then you need a streptavidin-HRP complex that actually is going to recognize the detection antibody and then you require the chemi agents to generate the signal. So, this you required for generating the signal and then you require the well or dish where you are going to do the incubations with the cytokine arrays or you also require a cover. So, you will protect the evaporation and other things.

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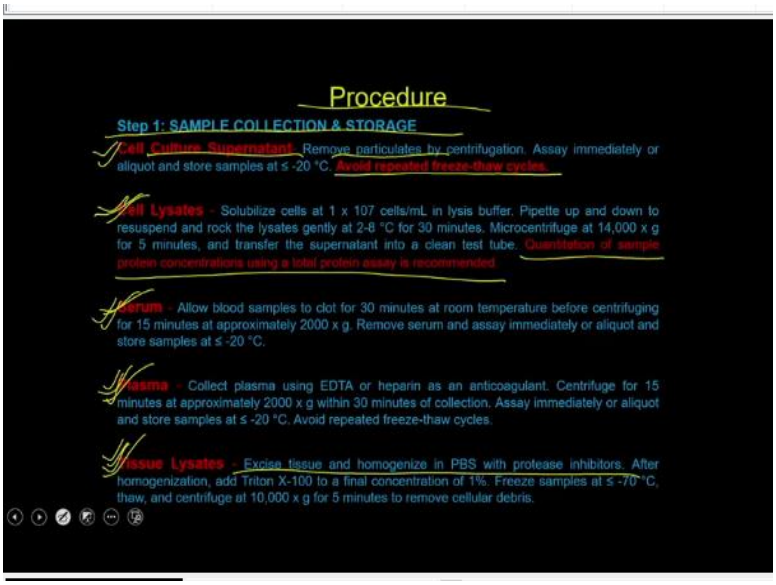

Then how to reconstitute the different types of reagents. So, in the step 1, or what is the procedure. So, in step 1 you are going to prepare the samples and you are going to have the multiple types of sample like cell cultures, cell lysate, you can have the serum, you can have the plasma and you can have the tissues and the processing of all these samples are different except that you have different depending on the type of the contaminating agents what is present and how to process.

For example, in the case of cell culture supernatant you do not need to do anything except that you remove the particulate matter and then you are simply can use these supernatant into the cytokine array assay. The only thing that you have to avoid is you have to avoid the repeated freeze thaw cycles. Similarly for the cell lysate you might have to you know pass through the cell lysate through by 0.2 micron filters and other kinds of things.

So, that there will be no interfering agents present and then you have to do protein estimation. So, that will not going to add lot of cytokines and very little cytokines otherwise the detections are going to be not perfect. Similarly, the similar kind of thing what you have to do for the serum as well as the plasma and the tissue analysis is also being done with a set of protocols, which you have to follow.

And ultimately what you are going to get by following any of these processes is you are going to get a mixture of the proteins and where your cytokines are present. And then the idea is that you should use that particular cell mixture or the protein mixture immediately or you just keep it on a - 20, you avoid the freeze thaw cycle and then also you should do the protein

estimations. So, that you should not see a lot of variability from your results from one batch to another batch.

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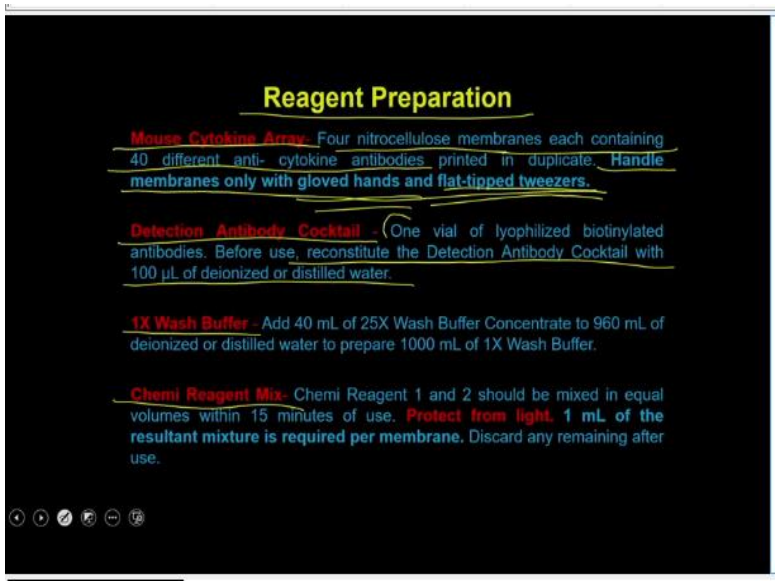

So, the reagent what you need to prepare a region is that you have to prepare the cytokine array. So, you are going to get the nitrocellulose membrane which actually contains the cytokine antibodies, the only thing what you have to do is because these are very sensitive cytokine membranes. So, you should not handle the membranes only with the gloved hand and you use the tweezers rather than the hand or the gloves.

So, you use the gloves and as well as you use the tweezers, then you have to prepare the detection antibody. So, what the company is going to give you, it is going to give you a concentrated stock and then you have to simply dilute the concentrated stock and reconstitute with the help of the buffer for table provide or you can use the distilled water, then you have to prepare the wash buffer, you have to prepare the chemi reagents.

And all these you are going to use or you are going to prepare as per the instructions what the company is going to give you in the leaflets.

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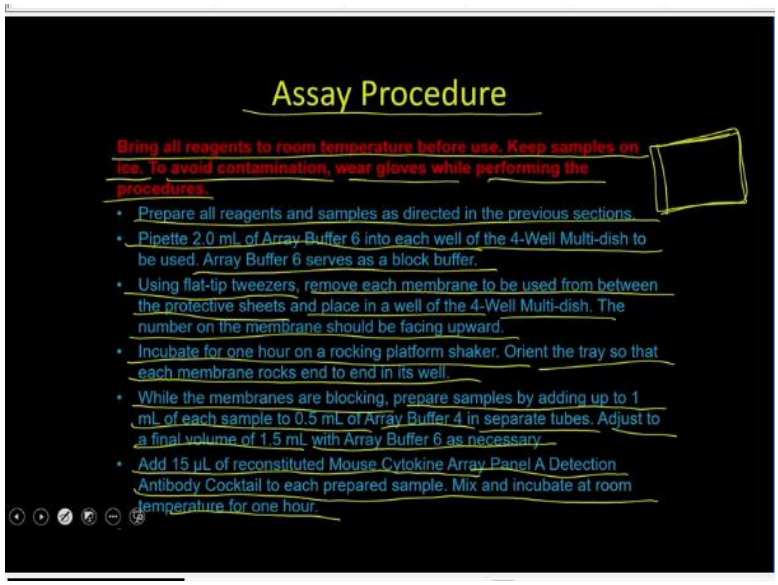

And then how to perform the assays. So, bring all before you start this process you have to ensure that all the reagents are on the room temperature and then you incubate them and your sample is on the ice, to avoid decontamination you have to wear the gloves while performing the procedures. Because you know that the human hands are also contains the different types of is made up of skin.

So, skin cells are very fragile. So, when you are doing all these procedures, the skin cell also can actually be peeled off and they can also be able to contaminate your sample and that actually is going to give you the variability. That is why you have to be wear the gloves in case. So, that you should not add the proteins from your body. First you prepare all the reagent and as we discussed.

Then you are actually going to do the you know you have to wet the buffer and then you have to use the assay buffer 6 as a block buffer. Then you have to use the flat-tip tweezers and remove the each membrane to be used between the protective sheets and place it in a well or for well multi dish, the number on the membrane should be facing upwards. So, the membrane when you are it has been kept in a between the 2 sheet of polythene.

And that has to be removed and then this membrane has the some printed material and that printed material should be facing upward. Then you incubate this with the with your sample on a rocking platform and orient the trays. So, that each membrane rocks end to end in swell. While the membranes are blocking prepare the sample by adding 1 ml of each sample, 2.5 ml of array buffer 4 in the separate tubes.

Adjust to the final volume of 1.5 ml with the array buffer 6 as necessary. Then you add 15 microliters of reconstituted mouse cytokine array, detection antibodies, cocktail to each prepared sample, mix and incubate for room temperature for 1 hour.

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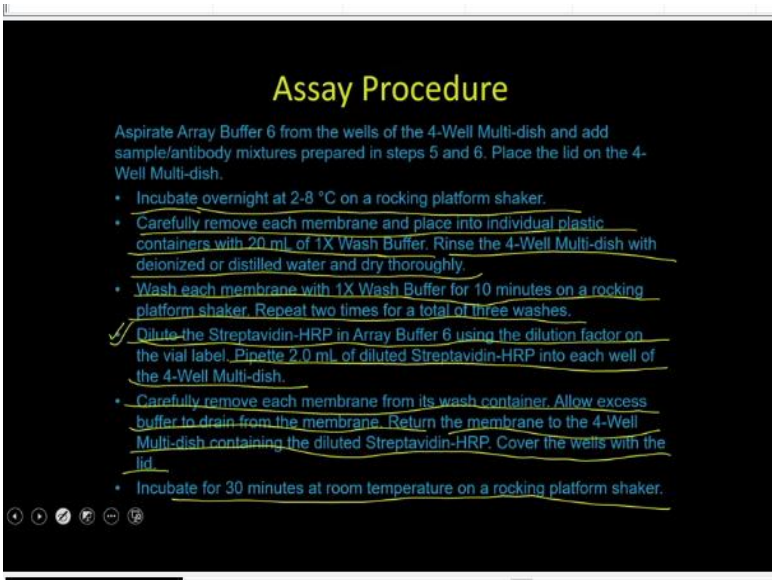

Then you incubated the overnight in a rocking platform shaker, carefully remove each membrane and place it into the individual plastic container with 20 ml of 1X wash buffer. Rinse the 4 well multi deionized or dry thoroughly. Wash each membrane with 1X wash buffer for 10 minutes on a rocking platform. Repeat 2 times for a total of 3 washes, then you are going to do a detection.

So, with this you are going to do a detection of the sample. So, now your membrane is ready to be detected by the streptavidin-HRP system. Then you pipette the 2 ml of diluted HRP mixture into the each well of the 4 well multi dish. Then you carefully remove each membrane from its wash container, allow the excess buffer to drain from the membrane. Return the membrane to the 4 well multi dish container, the diluted listen that.

Covered the well with the lid, incubate for 30 minutes. So, that the streptavidin-HRP complex will go and bind to the biotinylated antibodies.

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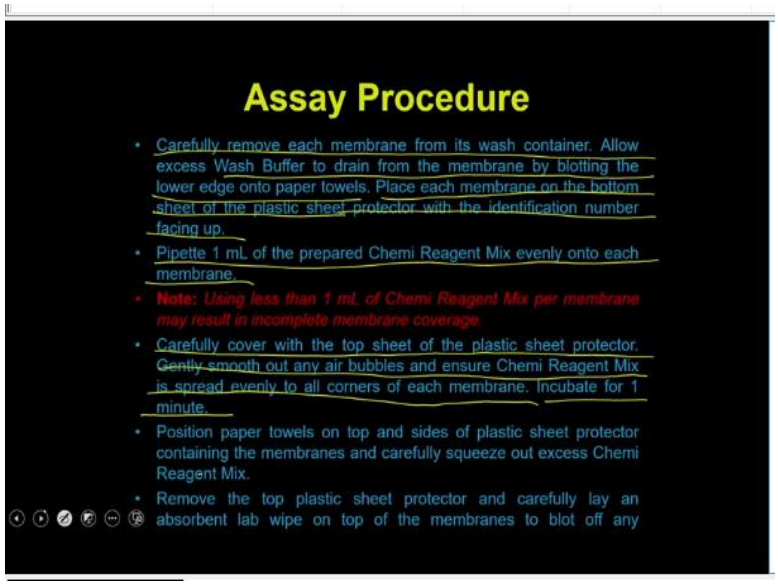

And then you remove the membrane from the each well allowing the excess wash buffer to drain off by blotting the lower edge onto the paper towel. Place each membrane onto the bottom sheet of the plastic sheets protector with the identifying number facing up. Then pipette 1 ml of the chemi reagents on to the each membrane and then carefully cover with the top sheets of the plastic paper protector.

Gently smooth out any air bubble and ensure that you are going to spread evenly to all corners of the each membrane, incubated for 1 minute and then you are going to acquire the image. Because once you add the chemi reagents you have to make for the 1 minute. So, that the substrate what is present in the chemi reagents are going to be processed by the enzyme and then it is going to give you the signal and then you have to capture the image.

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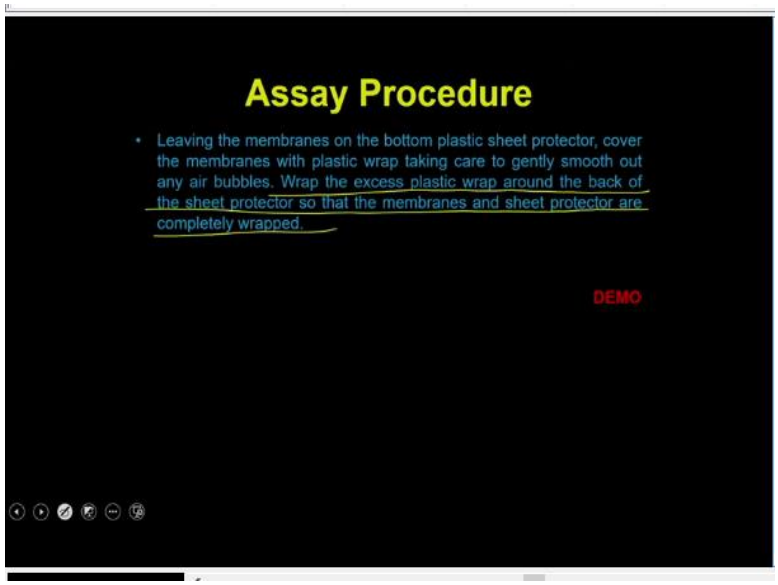

So, then you are going to wrap the excess plastic membrane around the back of the sheet protector. So, that the membrane and the sheet protectors are completely wrapped. So, this is all about the protocol what you have to follow and I would like to take you to my lab where the students are actually going to show you how to use the cytokine array to measure the different types of cytokines.

And with this demo, they will actually going to tell you each and every precaution what you have to take and what are the different steps what you have to follow. **(Video Starts: 20:41)** Today I am going to demonstrate how to perform a cytokine array. Cytokine array is nothing but a protein array where you can detect my full contacts from sampling. The samples can be from cell culture supernatant or tissue homeowners something or cell lysates.

You can use serum or blood also. So, I generally put behind this cytokine arrays carefully selected capture antibodies are spotted on a array membrane, when these capture antibodies comes in contact with the unlike they will attach, then again you have to provide a detection antibodies, which will form a complex with your analyte which is simultaneously detected with the chemiluminescence substrate.

So, today we are going to use R and D systems most cytokine (()) (21:46)**.** So, just turn, so I am going to open this cytokine panel, pour cytokine array panels, as you can see there are blue dots, each blue dot indicates capture antibody. So, what we have to do, first we have to block this index. So, in order to do that, we use a array buffer 6 dispense, we have made up array buffer 6 into each well into this 4 well panel.

So, the next thing is you have to place these arrays in this array buffer 6. So, carefully take out the membrane outside from that pouch into this film, this number what you can see it should face upward side. So, when I keep in array buffer 6, as you can see the blue color gradually disappears. So, that blur color appeared what we have to do is we have to cover this one and keep on a shaker for 1 hour at room temperature,

While the array blocking is going on, we have to prepare for samples, these are the cell culture supernatants. So, first we have to centrifuge to remove any debris. And out of that we have to take out 1 ml of sample and make it up to 1.5 ml using array buffer 4 okay. So,

already centrifuged. Now I am going to add array buffer 4 to samples. Once you added array buffer 4 to samples.

Next thing you have to prepare detection antibody cocktail by adding 100 microliters of distilled water to detection antibody. So, this I have prepared by adding 100 microliters you have to suspend properly. So, out of this you have to take out 15 microliter and add each sample. So, let us do this, here now we will keep this samples under rocking platform with constant shaking for 1 hour at room temperature.

After blocking the array amended we have to remove the black paper. Next, we how if detection antibody incubated with these samples. Now we have to introduce the samples into a members. So, I am going to pour all these into this membrane, for every sample we have to use separate tip . So, that we will not get any cross signaling. Once you enter this sample, carefully close the lid and keep at it at 4 degrees Celsius in a constant shaking for overnight.

So, after that we have to develop incubate with the streptavidin-HRP conjugated antibody then we will embed, but that will be later on, now we will keep this rocking platform overnight for 4 degree Celsius. So we have incubated overnight with sample and now we have to remove section from block, after removing sample solution, we have to add wash buffer to remove unbound antibodies.

Cover the lid and you how to keep 10 minutes on a rocking platform shaker, after washing here we remove the washing buffer from that, once you remove all the washing buffer you have to add streptavidin-HRP diluted solution to this tray. So, what you have to do you have to prepare diluted streptavidin-HRP as per the manufacturer's instructions, this is the streptavidin-HRP substrate.

As you can see dilute 1 is to 2000. So, I already prepared diluted solution, this is streptavidin-HRP diluted solution. So, after this 2, 2 ml you have to add to each array. So, I am going to add this one. So, during this process, do not let the blat dry, once you added this streptavidin-HRP keep this plates on a rocking platform for 30 minutes. So, after incubation with streptavidin-HRP now we have to wash at least 3 times using wash buffer.

Then we will develop the broth. So, these are washed and ready to develop. So, we will use the manufacturer provided chemi reagent agent. So, we have to mix a chemi reagent 1 with chemi reagent 2 in 1 is to 1 ratio, then we will add it to array, then we will see it in chemi data. So, you have to add chemi reagent on top of it, allow it to roll out over the blat, then you gently swipe to remove excess chemi reagent, just we will develop the sample.

So, while exposing you have to keep constant for every sampling to prevent any signal mismatch. So, these are the signal exposure only we are going to use normalization of the cytokines present in sample. So, as you can see here, this is the actual cytokine. What are the red dots are showing those are actually saturated pixels. So, we can remove that one simply. So, this is the cytokine array as we can see, each dot represents 1 cytokine and corners one. This corners one, these 3 represents a control dots which we can use for normalization.

And here 2 dots actually we cannot see, that is actually PBS 1. So, this is the cytokine array. During this process, sometimes we may not get signal or we may get excess signal. So, in that case, you have to look carefully into your protocol and all the buffers, whether you prepared right concentration of buffer. And so always use gloves. **(Video Ends: 35:15)** So, with demo, I am sure you might have understood the protocol what you have to follow and how to detect the cytokines into the using the cytokine array.

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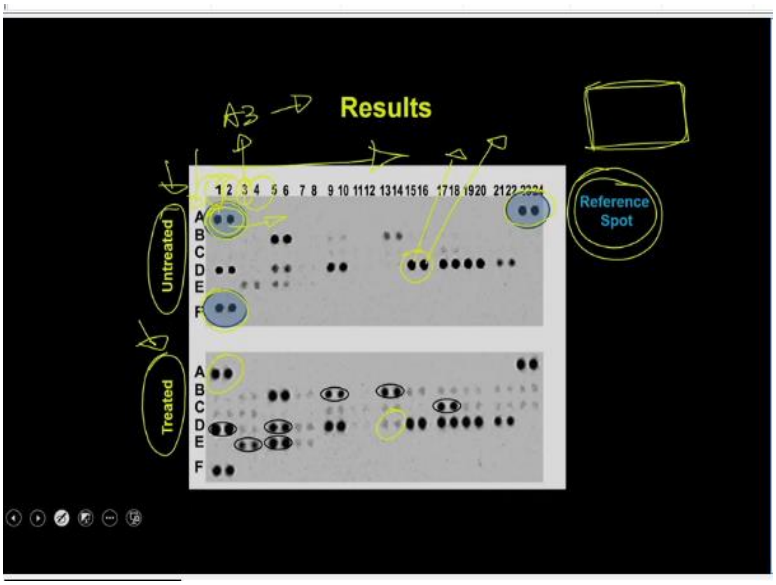

The result what you see after you are doing with this assay is the number of spots what you are going to see on the membrane. So, you can imagine that you have 2 samples, one is untreated sample, the other one is a treated sample. And what you are going to see is these

spots, what is there on to the corner, and these spots are actually the reference spots. So, what you have is you have a cytokine array sheet where they have given you that what is mean by the A 1 A 2 and all that.

So, what you see is that you have the numbering onto the left side, which is like A B C D like that, and you have the numbers on to this side. So, all these numbers are actually corresponding to the individual cytokines. For example, the like so A 3 is corresponding to something and similarly, the A 4 is corresponding to something. So, you know, which is part is corresponding to what.

And these are the references spots. So, these references spots are being used to align the membrane with the reference sheet. So, that you will know which spots is actually corresponding to what and reference spots are also been used for equalizing purposes. For example, if you are trying to compare this and versus this, then all the intensities has to be compared with each other using this as a equalizing factors.

So, how to analyze these results, so, what you are going to see is, you are going to see a different types of spots and what you have to do is you. So, for example, these 2 spots are a part of the pair like the 2 cytokines, once a single cytokine in duplicates. So, you have to measure the intensity of this particular spot. And similarly, you have to go spot into the treated sample also and then you have to measure.

And then by using these reference points, you have to calculate what will be the fold of multiplications I have to use to equalize this spot with this spot and then only you can be able to plot the intensity of this particular cytokine whether it is up regulating or down regulating.

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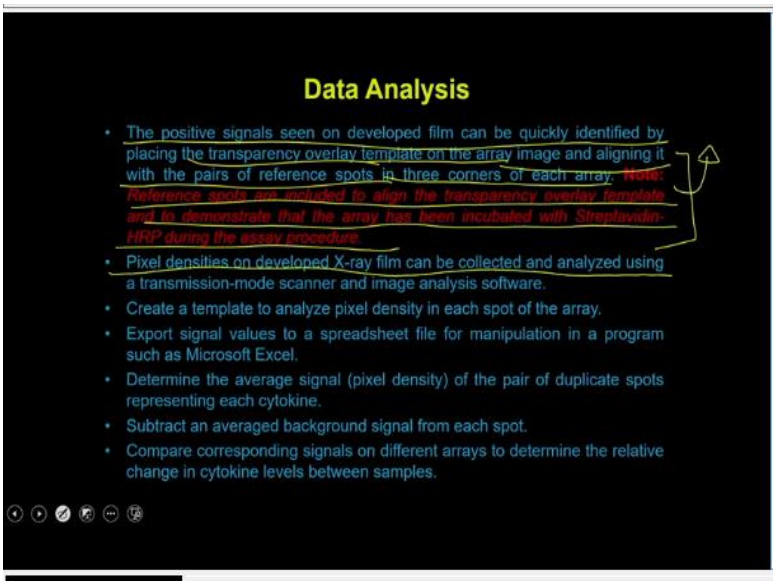

So, positive signal what you see on the developed film can be identified the placing on the transparent overlay template onto the array image and aligning it with the pair of references spots in the 3 corner of the each array, references spots are included to align the transparency and to demonstrate that the array has been incubated with the streptavidin-HRP during the assay procedure.

So, this actually references spots are being done simply because they are actually going to be a positive control that the nothing is wrong with the streptavidin-HRP system or detection system and you are actually getting the reactions work. But for example, in some cases if you do not get the results, then the reference point all definitely should give you the signal irrespective of whether the sample is giving you a signal or not.

Because the reference point does not have the primary antibodies, they are simply having the other antibodies and that is why the streptavidin will definitely go and bind to these points, then you have to measure the pixel intensity on the each spot and then you have to use any image analysis software to compare the pixel intensity of the untreated sample versus the treated sample, you have to use these references spot as the equalizing factors to multiply or to divide the signals.

And that is how you can be able to compare to know whether which signal is over expressing or which signal is down expressing. For example, if I show you that, this image that what you see is that this signal is actually not present in this particular untreated sample, but it is very

intense in the treated sample. Similarly, this signal is faint compared to the signal is very high.

And what you see is all other these in circled signal all these signals are pretty high compared to that, they are very, very weak in the in the untreated sample, which means these are the cytokines which are actually increasing when you are treating the cells with a particular with a treatment. This means these cytokines probably could have some role in driving the biological processes or they might have role in driving the immunological responses.

So, this is all about the different types of immunological tools what we have disused based on the antigen and antibody interactions, we have also discussed some of the immunoassays what you can also use to monitor the immunological reactions or you can also use them to answer some of the biological questions. So, with this I would like to conclude my lecture here, in a subsequent lecture we are actually going to discuss some more aspect related to experimental biotechnology, thank you.