Interactomics: Basics and Applications

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Lecture – 26
Basics and Applications of Reverse Phase Protein Arrays – II

As we started discussing about Reverse Phase Protein Arrays and Dr. Ramesh Ummanni, a guest scientist provided you an overview of how to use this technology for various applications.

So, today Dr. Ummanni will continue his lecture and he will talk to us more about this novel technology reverse phase protein arrays. In today's class, you will learn about how to choose the correct samples for the experiment, what are the methods for sample preparation selection of antibodies a spotting methods and signal detection methods. So, let us have Dr. Ramesh Ummanni to continue his lecture now.

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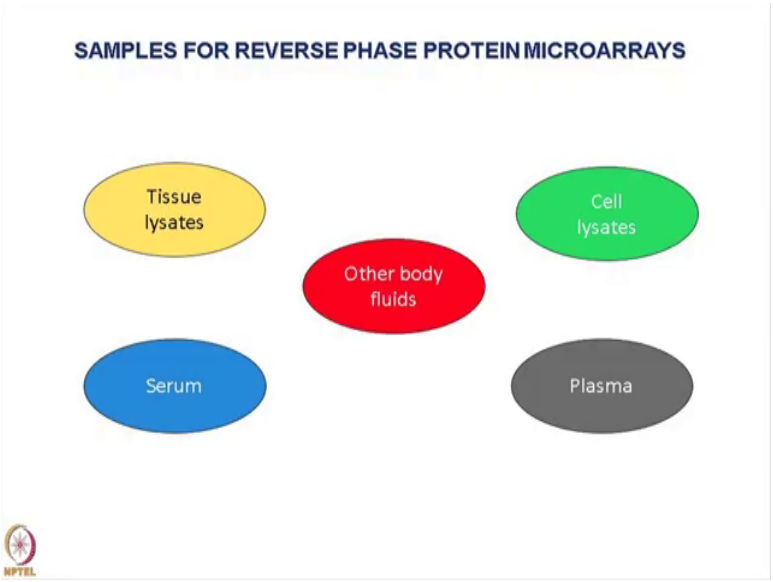

First is samples. There is no limitation any sample can be analyzed by r p m s or any sample which can be analyzed by western blot can be analyzed in r p m a. Only thing is you have to follow certain methods to prepare your clean sample to make it ready for r p m a approach.

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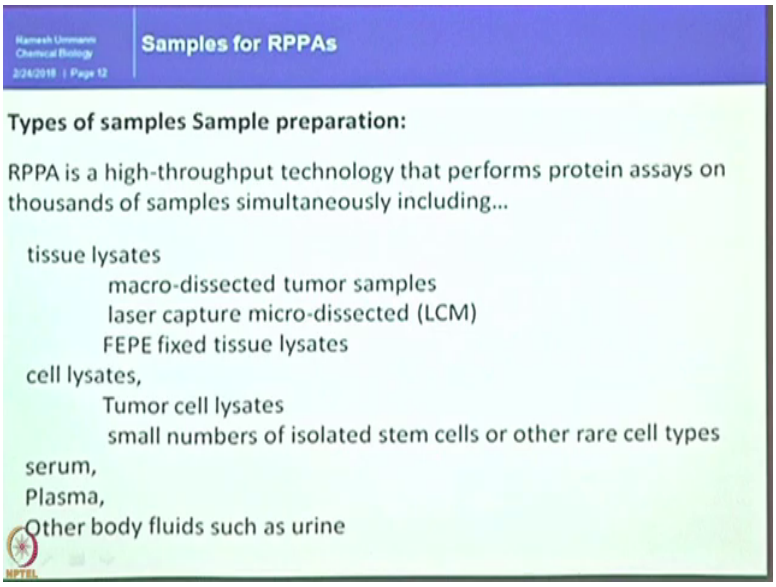

Is a high throughput technology that performs protein assays on thousands of samples simultaneously including tissue lysates which has micro macro dissected tumor samples there at least you will have a chance to get some microgram level proteins, some couple of micro milligram level proteins and then laser capture microdissection LCM there you end up with very very low quantity of sample you cannot perform n number of western blots to validate n number of targets. And FEPE fixed tissue lysates also you can do.

So, there are separate methods are available to make the protein extracted from formalin fixed tissues to use for r p m a s. Sometimes those samples cannot be analyzed by western blotting approach you see smearing effect which can be avoided here. The cell I said particularly have a tumor cell lysates every one of you might be handling some of the cell lines in the lab. So, the lysates can be prepared.

And small number of isolated stem cells or the other rare cell types. So, now stem cell biology is a very very attracting word but they will deal with always thousands of cells not even millions of cells we do millions of cells in cancer research. Serum also can be spotted the plasma or any body fluids such as cerebrospinal fluid or urine can be directly printed on the lysate by following specific methods.

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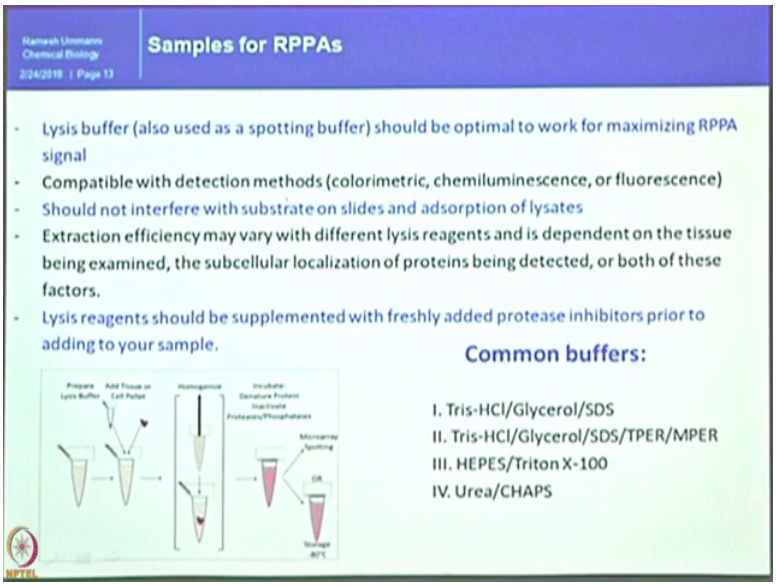

So to print the samples how to prepare the samples it is very simple cartoon here can take a lysis buffer put the tissue of rest and homogenate and spin down the tube and take the supernatant and go for printing or can be kept in a minus it go home and come back tomorrow. This is a way, but it is not that as simple as just preparing. There are multiple steps has to be taken care. So, you have to plan experiment and take all the parameters into consideration before executing the experiment.

So, in that way this is also one of the critical point. So, this critical point basically highlights what kind of buffer can be choosed not all proteins in all samples can be solubilized by single buffer. This is a major limitation in array approach.

So, lysis buffer should be optimal to work for maximizing RPPAs signal. So, it should not interfere with the visualization of signals using any labeling system you follow either secondary antibodies like i r labeling h r p label or alkaline phosphatase label or directly (Refer Time: 03:56) three or (Refer Time: 03:57) label.

And it should be compatible with the detection methods like a colorimetric chemiluminescence and fluorescence methods should not interfere with substrate on solids and absorption of lysates. Say for example, you might have performed washing steps what if the buffer is not compatible and washed away along with the proteins spotted on the glass like end of the day you will have a red phase no signals.

So, definitely should not interfere with the substrate on the slides in this case nitrocellulose substrate and should not interfere with adsorption of the lysate I mean; that means, attachment of the or immobilization of your protein sample. Extraction efficiency may vary with the different lysis reagent and is dependent on the tissue being examined.

Let us say for example, if you are taking a prostate tissue which will have a prostate some stones will be there then you need a stringent buffer. Let us say for example, your lysing the cell lines simple reaper buffer is sufficient or emperor buffer is sufficient.

So, lysis reagent should be supplemented with fresh layer and protease inhibitors of course, if we wanted to understand the post translational modifications and you need to have the inhibitor cocktails like phosphatase inhibitors and also some of the protease inhibitors not to degrade the protein of interest.

So, with my experience at least I worked with these four different buffers definitely these four different buffers behaved entirely different with the different samples. Believe that any one

buffer will help is not true. So, in my hands we had a different experience with the different buffers and different tissues and different cell lines.

So, again one of the volunteer sitting back was emphasizing that this method is always to be optimized in your own lab in your own hands in your own setup. So, this there is no hard and fast rule that so, on. so teacher explained this concentration it will work, it will not work. so you have to optimize.

Please keep this in mind.

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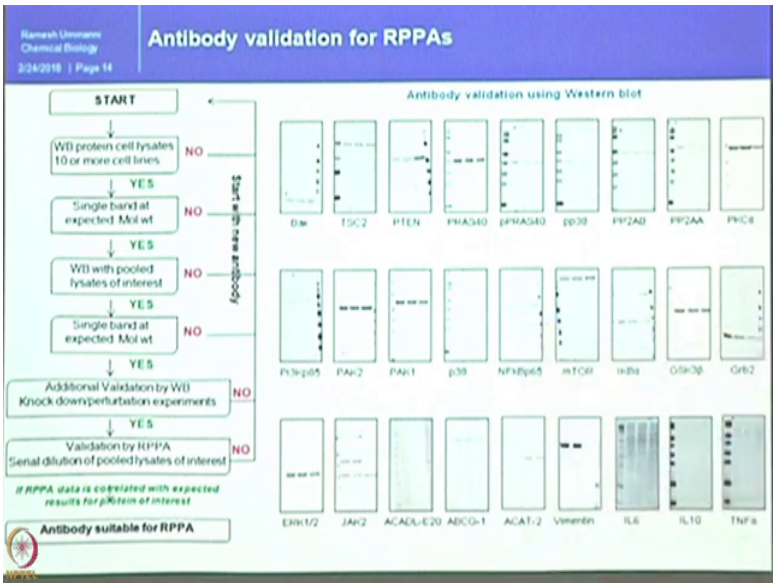

Then second point highlighted was: the essential requirement is the good quality antibodies. So, here you need to spend at least some couple of months to years to establish antibody panel

in your lab. At least we could we could establish I mean with my post docs supervisor we could establish 190 antibody panel in 1 year from 3000 antibodies purchased from the market.

You can see that 2800 plus antibodies just went into dust bin of that German Cancer Center at that time. So, now, those antibodies we are repeatedly purchasing and we are using it. But this effort one year effort helped in the long run definitely how it is I will highlight you here.

Our approach for antibody validation is like this starting point take that western blot through western blotting approach take ten different cellulite sites or from more cell lines front and different lines. And then do western blot if the antibody gives a single band say yes go to next step and then you pull the lysates from ten or more different cell lines run a single line; that means, in a complex mixes also antibody has a capacity to recognize the protein of interest without any non specificity.

If it gives a single band it will go to the additional validation steps by western blot using knockdown experiment and stimulation experiments and if it is and; if it is yes then it will go to the validation by RPPA. In RPPA, we will validate by serial dilution of the samples whether the antibody is able to detect the serial dilution the two fold tenfold dilation properly or fivefold dilation properly.

If it is yes then this correlated is if the RPPA data is correlated with expected results from protein of interest then we declare that it is useful for RPPA. And also every antibody will get a three coats: go ahead, use with caution and not suitable. These are the three different words in RPPA community we keep on hearing. So, antibody they right code. So, its like a go no go then use caution these are the three words we exchange by emails.

You can see that these is a like a pen we used to run these kind of western blots several hundreds to thousands for validation purpose. You can see that some of the bands are very nicely giving single bands some of them are like a nonspecific bands and here you can see that this is completely crap we cannot use them. And mostly with my experience I am not as again

brand ambassador for any antibody companies here at least I had a good experience with cells signaling very bad experience with Santa Cruz.

So, I you see I do not get offended please, just it was my experience I am sharing with a very lighter node with the student community here.

So, you can see that if you have a single band here directly if it is yes then we will proceed to this pipeline here. So, once we reach this step we need to follow additional steps this kind of antibodies we will grab them first step itself we do not spend any time and we will directly buy the another antibody.

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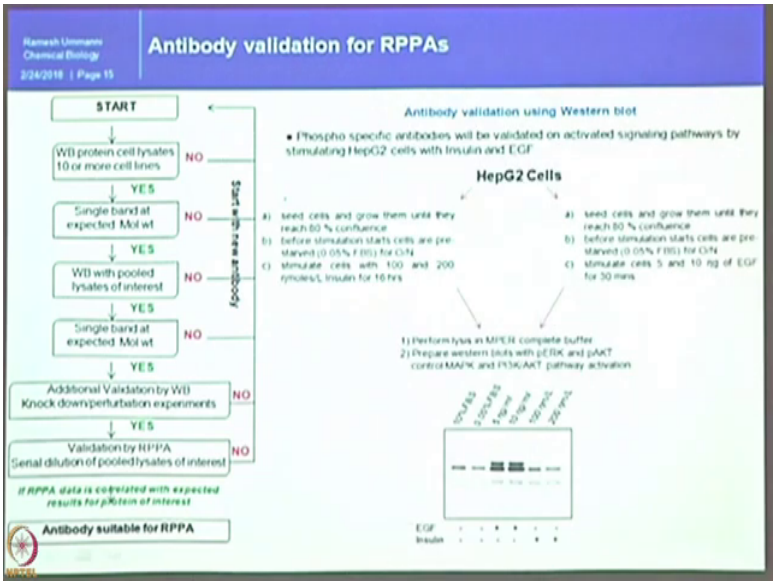

So, next round of validation is that again using western blot we take a suitable cell line and stimulate them with some kind of factors where your protein of interest gets modulated you

know it prior information already. And you look for this known information the western blot results and.

In this experiment what we have done is we have taken HepG2 cell line and we stimulated with EGF and insulin. These two factors will stimulate the phosphorylation of a k t and phosphorylation of erk protein and those phosphorylations have been observed or not. These are also mutually exclusive you can see that EGF is stimulating here phosphorylation insulin is not doing. So, this kind of approach we follow this is one example I am showing you.

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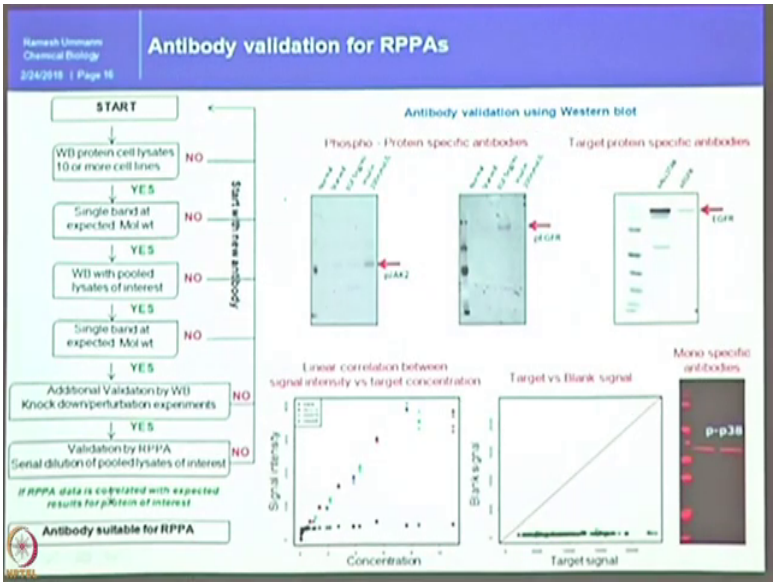

So, with this approach it is passed then it will go into the next line. Then we will not leave that story there itself.

Again we take those antibodies and this phosphos specific antibodies choose the different cell line whether they are behaving in the same way or not. So, here you can see that with the insulin phosphor z to phosphor j correlation is going up with EGF only EGF four is going up.

So, again these are the mutually inclusive exclusive garbage in garbage out approach. Basically, in to tell you in a very crude manner. So, once we have this we will not leave the story there again we have now s a r n a available for all the proteins expressed in the orpheum we buy the s a r n a down regulate them treat to transfer to the cells, make the lysis run them on a western blot whether this antibody is really detecting the down regulation or not.

So in this case we have given to the cells s i r n a ignace EGFR and our antibody is detecting the down regulation of EGFR. So, it has passed through the this step here knock down or perturbation experiments. So, after this then we validate them by reverse phase protein array approach. We simply take very pool of lies a small pool of lysates print up on the glass slides and use this antibody and probe it see that whether it is really detecting the signals or not.

So, when you plot the results like concentration versus signal intensity. So as increasing the concentration so the signal is going up blank signal is at the base level the signal intensity is fine here.

And also to see that the detection levels are really in the range of detectable or not. So, target signal versus blank signal if the target signal is very near to blank signal there is no point we dont get a linearity rate. So, then we have to drop the antibody at this step after reaching here. If we see no here then we have to go here again.

So, this is a problem after this process if we passes these two filters we have a like mono specific antibodies in this example I am showing phospho p 38 this antibody is go for RPPA approach.

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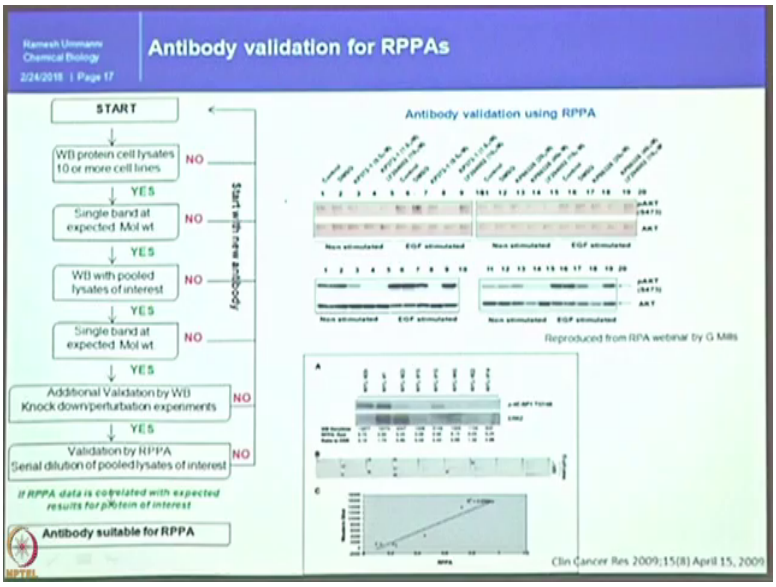

The antibody is approved for reverse phase protein array approach we also need to do one more step. You need to see the correlation between western blot and RPPA results. Because many of the why I am emphasizing this was that one of the RPPA conference many people did not believe that RPPA is better than the western blotting. So, that was the reason.

So, this paper was published in Clinical Cancer Research where the RPPA data is very well correlated with a western blot data. So, the same sample set the RPPA data is generated compared with the western blots generated from the same sample set.

So, then it is clearly understood that the community accepted that the RPPA data will correlate with a western blot data. So, that it is useful to validate large number of proteins in a smaller quantity of samples.

So, here we have spotted the I mean this group go down means from M.D. Anderson they spotted the serially diluted tumor lysates and did western blots and normalize the data this outcome here. So, now, we have the antibodies suitable for reverse phase protein array approach.

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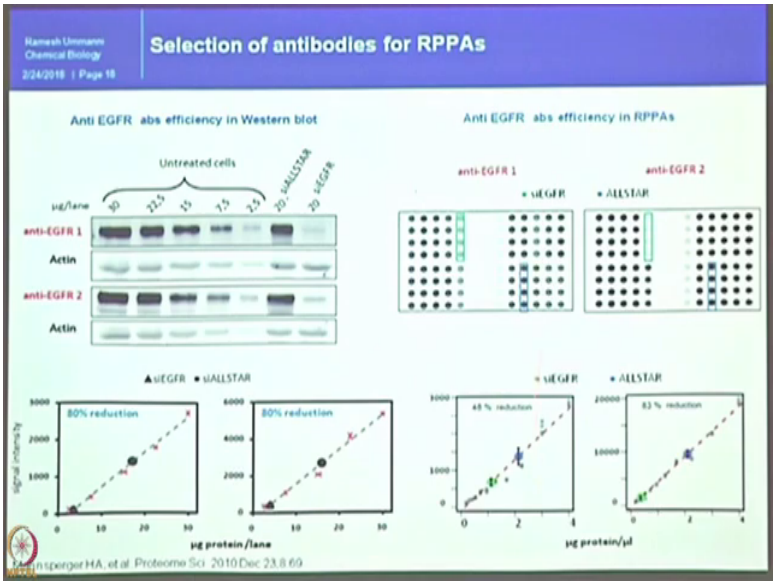

So, at this point to educate you in a different way not all antibodies against one target will behave the same way. Let us say for example, if you search sale signaling website for a EGFR or phosphor EGFR antibody target will have a ten different antibodies with the different catalog numbers. That means, there is there from the different clones or different poly different monoclonal antibodies and not all the antibodies behave in the same way how it is.

Now, you look at this picture. So, in one lysate we used for non regulation of EGFR from the same lysates we did a western blot in a serial dilation manner and used two different

antibodies to detect the signals here. Two different antibodies nicely showed the down concentration dependent signals for EGFR and also western blot results shows that 80 percent down regulation happened for EGFR after adding s a r n a.

This data is completely wrong. How it is? When you did RPPA with the same samples you can see that this is a anti EGFR 1 antibody for detecting the lysate where the EGFR is down regulated and here is also control samples in this case this is antibody 1 and antibody 2.

When you look at the correlation this first antibody detects 48 percent down regulation. Second antibody says 83 percent down regulation.

Now again question mark.

So, when we deal with this antibody related approach or antibody based approach we should be very very cautious in choosing the antibody. Otherwise end of the experiment we will not able to conclude or not able to infer any meaningful data. So, this is what. Again all antibodies cannot detect the single protein in a same way.

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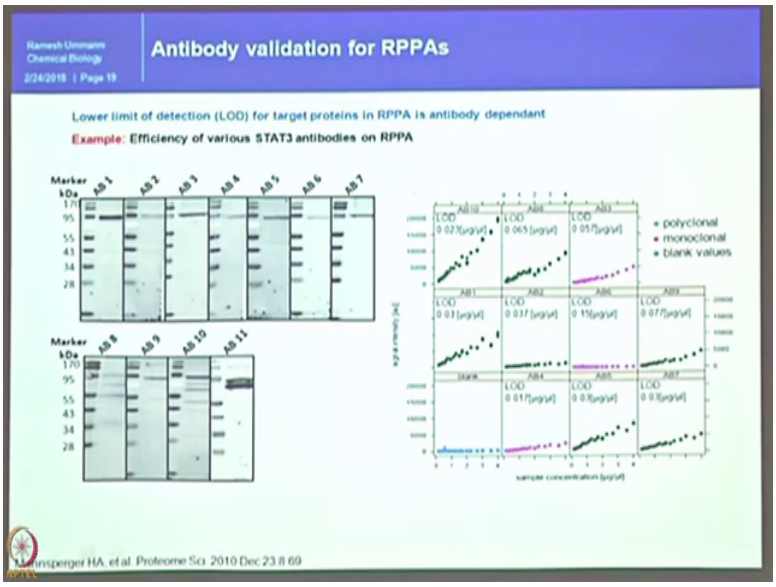

Like for example, in this slide you can see the STAT3 antibody different stat three antibodies detecting the stat three in the same sample in different manner, you can see LOD this limit of detection some numbers are written here.

Definitely, I think I will circulate the slides also to you if you cannot note the numbers you can go through later on.

So, this is green is a polyclonal red is a monoclonal blue is a blank values you can see that. So, different signals are going in a different scale along the y axis ok. So, you also need to consider if the protein is very very less abundant in your samples you should use more efficient antibody. If it is highly abundant you may not worry much. If it is really low abundant protein

or particularly phosphoproteins very few copies will get phosphorylated then the total protein right. So, in that way we should be very cautious in choosing the antibody.

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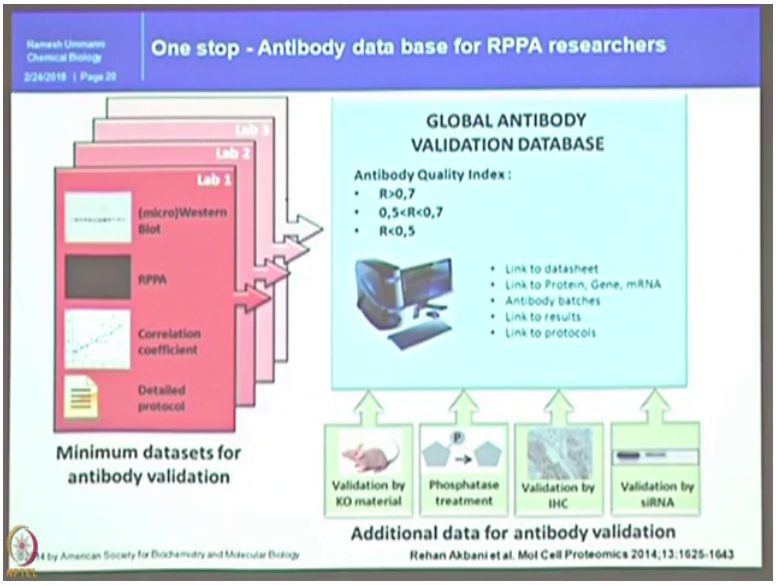

So, all in all I would like to say that if one stop database is available for antibodies which are approved for RPPA approach will be a ideal situation. So, the community or the society is working towards that they have formed a consortium with the different labs and all the labs will share the data and they will they wanted to feed it in the computer. So, this data is accessible to open anybody, then they can use the antibody information from which company which catalog number and so on then they can use for RPPA approach directly.

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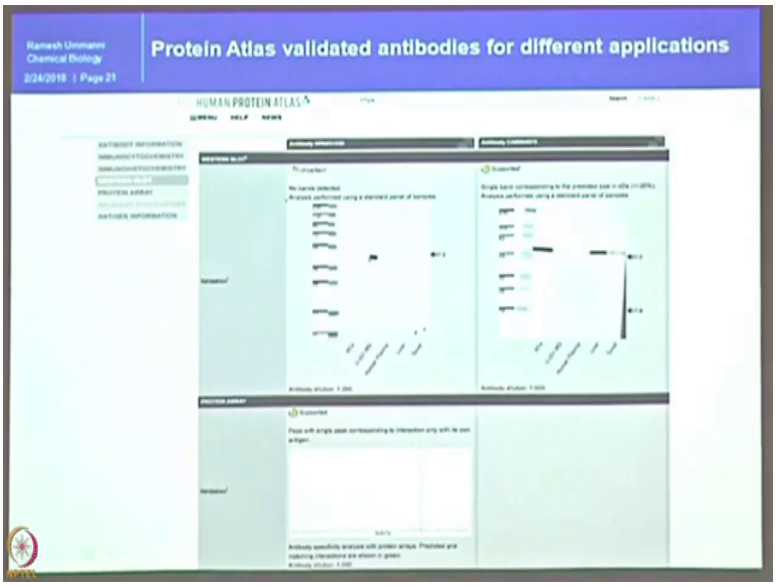

So, this is now in the process. But another approach the protein atlas company is included in their catalog of validation steps is by RPPA also. So, if you look at the data sheet of any antibody if you purchase then they will be of different form of applications like compatible for western blotting immunohistochemistry immunofluorescence and all.

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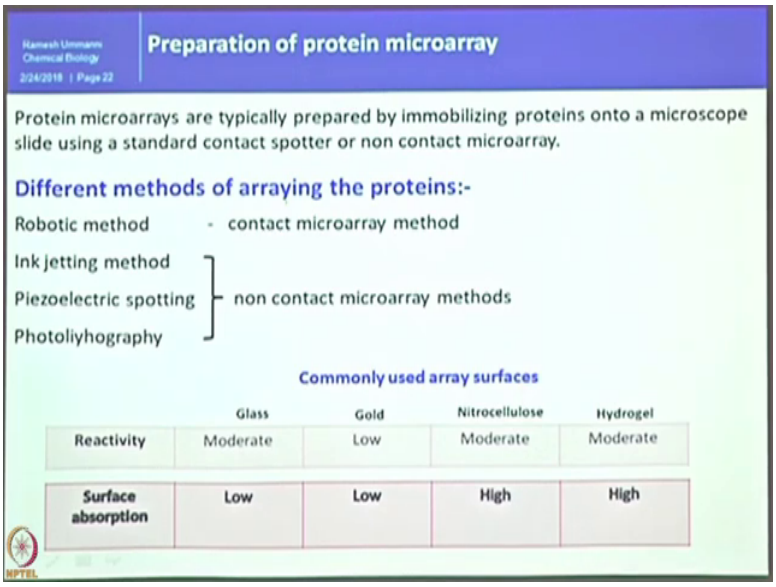

So, this company included another validation point that it can be used for RPPA or not. So, if this single peak is there that means, it is useful for RPPA approach. They also have the catalog indicating that whether it is go no go or use with caution for RPPA approach this protein atlas.

So, next is a preparation of protein micro array. Here actually, it is important to show you how to print and everything since I do not have an instrument to print and show you all. So, definitely it is similar steps exactly same steps it follow as Sanjeeva explained in the morning, the glass slide with the nitrocellulose membrane in white color and the sample is printed by a small pin from the machine. So, there are different methods are available to print.

So, protein microarrays are typically prepared by immobilizing the proteins onto a microscope slide using a standard contact spotter or non contact spotters. So, there different methods are available one is a robotic method which is a contact micro array method.

Contact microarray method means needle will go and touch the membrane and disperse the liquid in that. So that sample will not dispersed in a big size of the spot it will accommodate sorry small area on the membrane will accommodate the larger quantity of sample.

These are the remaining three methods are the non contact methods, inkjet method, piezoelectric spotting and photoliyhography. So, maybe many people know about the inkjet method it will spray the sample near to the glass slide. So, there is a chance of big wider spots than the concise spots. So, that you can accommodate less number of proteins on one slide compared to the contact now contact microarray methods.

And also you can use different wide array of substrates. But in RPPA approach we need a matrix coated glass slides. So, this matrix can be anything glass directly or gold and nitrocellulose or hydrogel. So, every substrate or every surface has its own advantages and disadvantages. So in this case so glass is moderate reactivity towards the substrate and gold is low, nitrocellulose is moderate and hydrogel is moderate.

So, compared to this nitrocellulose is better because it gives low background and low fluorescence while detection methods are applied. So, that is the reason most of the people are using nitrocellulose coated slides whom I know may be at least others are using hydrogel methods as well.

So, surface absorptions very low with the glass, gold is very low and these nitrocellulose and hydrogel are very high. That is why, I preferred to use nitrocellulous coated slides. So, that nitrocellulous coated slide look like a white layer on the glass slide you might have done thin layer chromatography in your masters right. So, exactly this thin layer is formed by silica gel in the thin layer chromatography in this case nitrocellulous coated coating will be there that's it. That is a difference.

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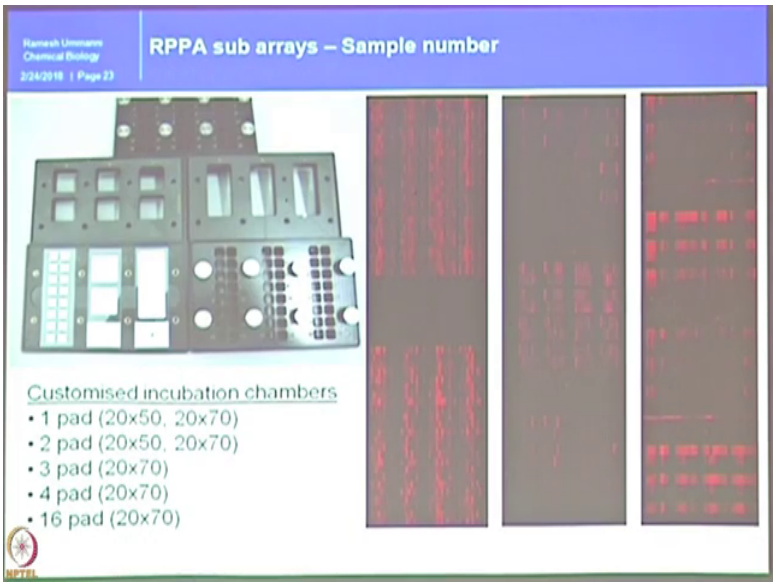

Once you have a glass slide. So, then you can decide the array. One slide can accommodate 2000 samples will you wait until 2000 samples will be accumulated from different studies, not necessary right. So, then you can design the array into the miniaturized fashion it is quite easy and possible these chambers are custom made in our lab. Now are available in the market.

So, 1 pad means it is like this. So, you can use 1 antibody for the entire slide for 2000 samples. If you have a 1000 samples you can divide them into two groups.

So, 1000 here and 1000 here you can use two antibodies on each slide. And if you have very less number of samples you can also divide the chamber into 16 different chambers glass slide layer into 16 different chambers. So, that you can put 16 antibodies in 1 glass slide it maybe a couple of hundreds of samples.

So, in that way you can miniaturize your assay. So, in this case I will show you the examples here is the 2 pads here are the 3 pads here are the 4 pads. You can see this is the same sample set with the two different antibodies, same samples with three different antibodies, same samples with four different antibodies that's it.

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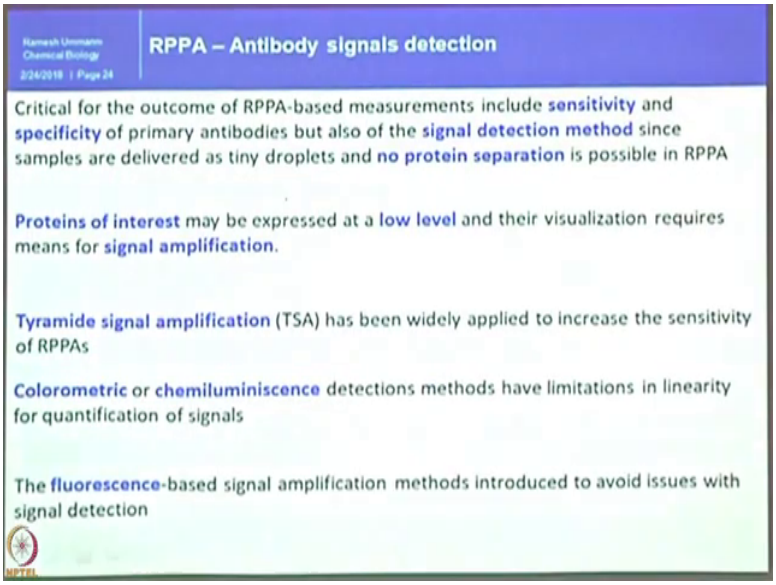

It is just like and kind of miniaturizing your array you can cust custom made array. So, once you have the array printed your protein of depending on the protein of interest you wanted to analyze and you can add antibodies and then probe and further next steps to be processed.

So, after primary assume that in the RPPA also now you printed you added the primary after blocking and washing and everything. Here also nitrocellulose membrane empty spaces need to be blocked by using the blocking buffer. We use rockland buffer which is a low fluorescence. and after blocking your primary antibody hybridized. Then the second after

washing to remove the excess antibody excess primary antibody bound to the membrane then you will add the secondary antibody.

While choosing the secondary antibody you should be also very cautious. So, this choosing the antibody for detection is very critical and the outcome of the RPPA is based on the sensitivity and specificity of the primary antibodies. So, you should worry about the sensitivity and specificity but also the signal detection method since the samples are delivered as a very very tiny droplets. And no protein separation is possible in RPPA at least in western blot.

So, your proteins are separated into small different bands it is like a partial purification way. But here we are not following any separation methods right. So, the protein of interest may be expressed at a very low level and their visualization requires signal amplification.

So, if the abundancy of the protein is very less if your primary antibody is also very weak then still you have a chance to correct it in the secondary antibody detection method as I said.

So, how we can do that we will see.

Tyramide signal amplification method has been widely applied to increase the sensitivity of the reverse phase protein arrays. And calorimetric and community relations detection methods have very very high limitations in terms of linearity for quantification of signals.

See for example, to get into your mind that what is the linearity means. If the if you use a fluorescence method the detection limits between 10 to 15 microgram. If you use calorimetric or chemiluminescence method if the signal gets saturated after 25 microgram you cannot have a proper quantification between 10 to 50 microgram range. But infrared detection system will have a very nice linearity between lower to high level of high level of abudancy of the proteins. the.

fluorescence based signal amplification methods have been improved introduced to avoid issues with a signal detection. I will explain you one method.

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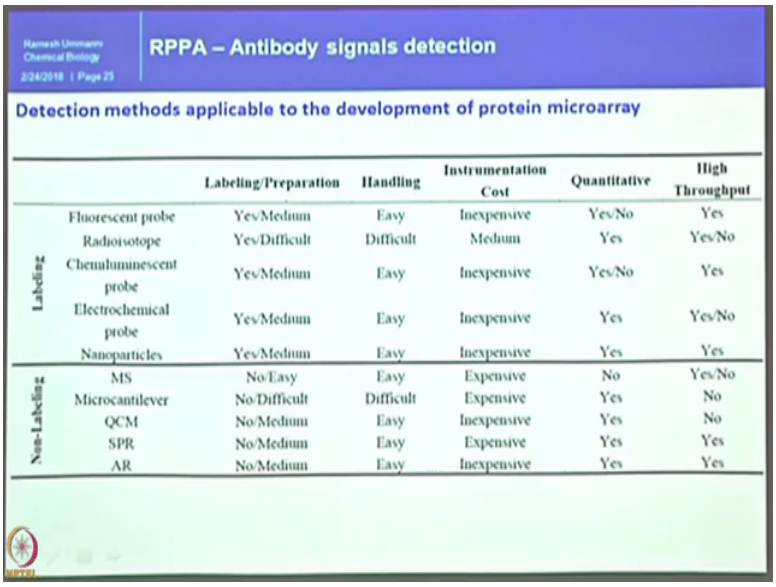

		Labeling/Preparation	Handling	Instrumentation Cost	Quantitative	High Throughput
Labeling	Fluorescent probe	Yes/Medium	Easy	Inexpensive	Yes/No	Yes
	Radioisotope	Yes/Difficult	Difficult	Medium	Yes	Yes/No
	Chemiluminescent probe	Yes/Medium	Easy	Inexpensive	Yes/No	Yes
	Electrochemical probe	Yes/Medium	Easy	Inexpensive	Yes	Yes/No
	Nanoparticles	Yes/Medium	Easy	Inexpensive	Yes	Yes
Non-Labeling	MS	No/Easy	Easy	Expensive	No	Yes/No
	Microcantilever	No/Difficult	Difficult	Expensive	Yes	No
	QCM	No/Medium	Easy	Inexpensive	Yes	No
	SPR	No/Medium	Easy	Expensive	Yes	Yes
	AR	No/Medium	Easy	Inexpensive	Yes	Yes

So, there are different methods are available or applicable to development of the protein microarrays it is again non labeling and labeling. So, I do not want to get into explain more about the labeling and non labeling methods on differences between these two.

You can simply go through here and you can already see that labeling methods have labeling methods have little less usefulness in RPPA approach than the non labeling approach. So, nowadays, some people are using surface plasmon resonance as well.

So what is a signal amplification method.

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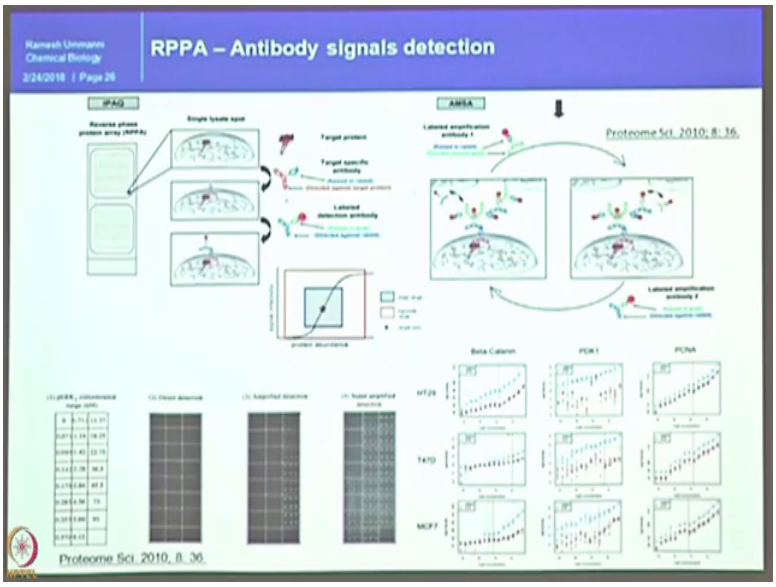

You can see that simple spot is there am I right primary antibody and labeled secondary antibody is added then you got a signal output. So, if you do not see any signal in this approach still you have a way to get it out. This is called amsa method a m s a method which was developed by one of the Phd student in my post doc supervises lab (Refer Time: 25:53) Mansperger.

Amsa means antibody mediated signal amplification and you have a spot here can clearly see that you can have a spot here this is a protein of interest and first antibody is added. Once the primary antibody is detected let us say for example, assume that it is a rabbit the secondary antibody will be added anti rabbit raised in a goat ok. Then again we will select the third antibody which is raised against the goat in rabbit this is again.

So, now imagine that one label molecule became two label molecules. One molecule of infrared floor is attached with the one round of secondary to secondary antibody we will have two fluorophores there. Then again if you repeat this for ten times ten up to three or four antibodies five antibodies one protein target it attached to five different signaling molecules then obviously your signal will be increased by five times.

So, this reiteration step will increase how it is. For example, in this exam in this cartoon you can see that the first direct detection you do not see any spot here in the amplified detection first round of amplification we can start we can look at some of the spots in the right corner. After two rounds to three rounds of amplification then we can see almost all the spots in all the blocks here. So that means, we are increasing the binding of labeled molecule to the protein of interest by repeating the signal amplification methods. So, only thing is cleverly we have to choose the what kind of antibodies to be used in the cycle. Primary, secondary and tertiary, tertiary to quaternary.

So, this is the kind of cycle we have to repeat it. So, this is a reference if you wanted to go through how it is done here and you can clearly see that this red line is shifted from x axis towards to top. So, this shift of signal indicates that sensitivity has been increased the signal detection has been increased. So, that we can visualize or we can detect the low burn and proteins in a large pool of proteins in the lysate present. This is a way.

And also linearity you can clearly see that for example, here you can see this is a length. If we use infrared detection system you will have a this much distance to detect from lower concentration to higher concentration. If you use some of the other methods you will end up only in the between the middle of the graph here up to maybe half of the concentration of the protein can be detectable.

I am; I am trying to just get you get you into the imagination not able to show you the how signals can be amplified and all.

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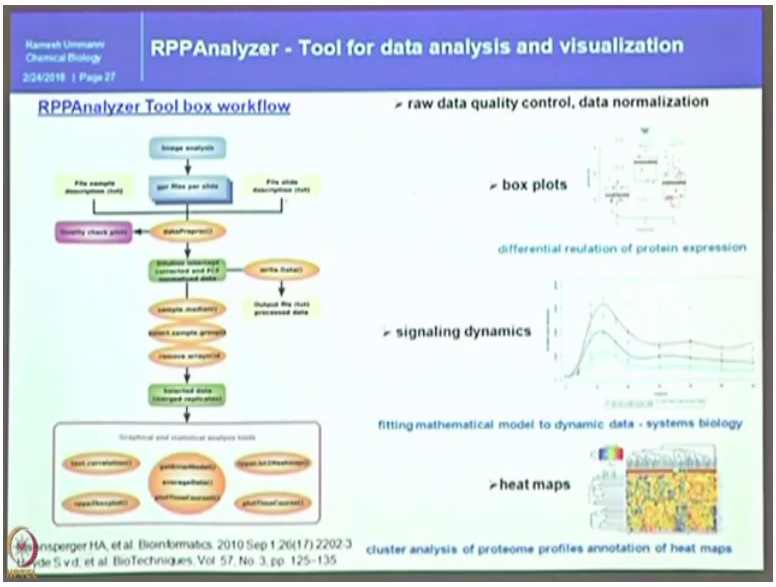

So, this is a way once we have the array once we have the signals here definitely we need a software's to analyze. Nowadays, RPPA pipe which is a kind of open source software has been available and some of the companies have developed soft special software's to analyze the RPPA data generated. But it will have a always commercial software's will have a lot of limitations you cannot play with the data according to your biological question to be asked.

So, keeping that in mind so that I am myself also involved (Refer Time: 29:26) Mansperger with cover lab developed a RPPA analyzer which is a open source platform deposited into the website then you can use. So, there are different steps available here after image analysis the data can be collected in the form of gpr files and you will also give the files sample description what kind of samples you printed there.

And slide description what kind of antibodies probed like a ten or twenty different antibodies signals a set of samples. And then you process it the processed data will be here and then automatically software look for quality controls means whether blank signal is really blank signal, the signal is really signal, what is the difference between blank signal to the real signal and also it will calculate the signal to noise ratio and if necessary to drop some of the arrays it will suggest what to drop, what to include then we can proceed further.

Then obviously once we have the quality control check we always try to print a pool of sample in a serial dilution method. So, then the serial dilution will tell that whether antibody is detected to the serially diluted samples the linearity is it and r value is point a, but at least above point nine when one is always ideal which will never achieved.

So, at least nine is the cutoff once we have this then we will go ahead with the analysis further is like is like a box plots time dependent and all. So, when you have the box plots like here you will see that whether the protein is up regulated or down regulated. And also you can understand the signaling dynamics.

Let say for example, your samples were collected for a period of time zero minutes to ten minutes time. So, from zero minute to ten minutes time how the protein expression is going up or going down. Simply you can see that the first few minutes signal is going up then started going down.

So, signal dynamics we can measure and also we can feed this information into mathematical model to make a kind of network or to make a platform or to devise a pathway regulation over the period of time how it is getting stimulated activated or inactivated and so on. Then obviously you can design or you can draw a heat map you can see the what are the different cluster of proteins are altering among the proteins I tested whether they have any relation or no relation.

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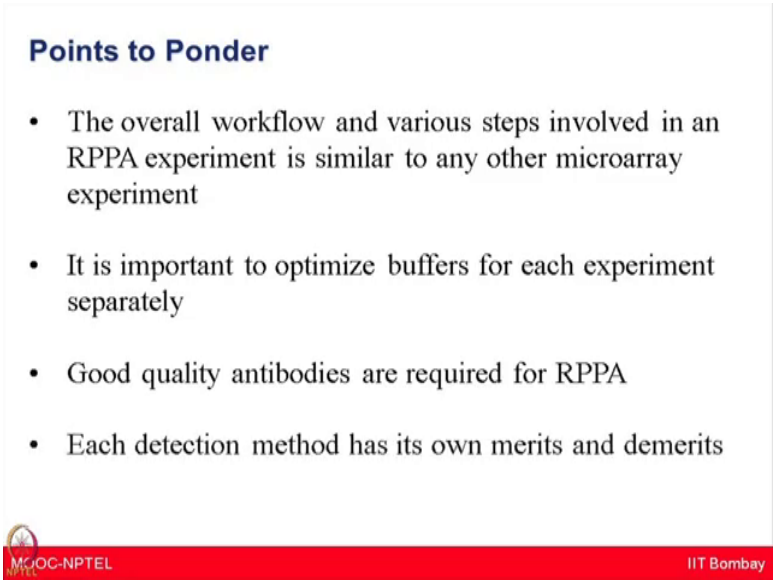

So, I hope today you have learnt about the importance of each and every step involved in RPPA workflow. You have probably now got a good glimpse understanding of how different buffers behave differently with different sample types. Their optimization is of utmost importance Dr. Ummanni also emphasized on how it is important to establish good quality antibodies.

And I must say this is one of the major issues in the entire proteomics community or the life science community to have the a good standard antibody availability and that is where the human protein atlas project along with several commercial companies have really provided the very high quality reagents which could be used for many applications including the one which we are talking today about reverse phase protein arrays.

Today you must have also learned about different detection methods and how each method has its own advantages and disadvantages. Dr. Ummanni further discussed about a m s a in detail.

In the next lecture, we will continue this interaction and talk about how to analyze reverse phase protein arrays microarray based dataset.

Thank you very much