Lecture 19:

Basics and Applications of

Reverse Phase Protein Arrays-II

Welcome, to more course on applications of interactomics, using genomics and proteomics technologies. As we started discussing about, reverse face protein arrays and a Dr. Ramesh Ummanni a gift scientist, probably do 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, spotting methods and signal detection methods. So, let us have Dr. Ramesh Ummanni, to continue his lecture now.

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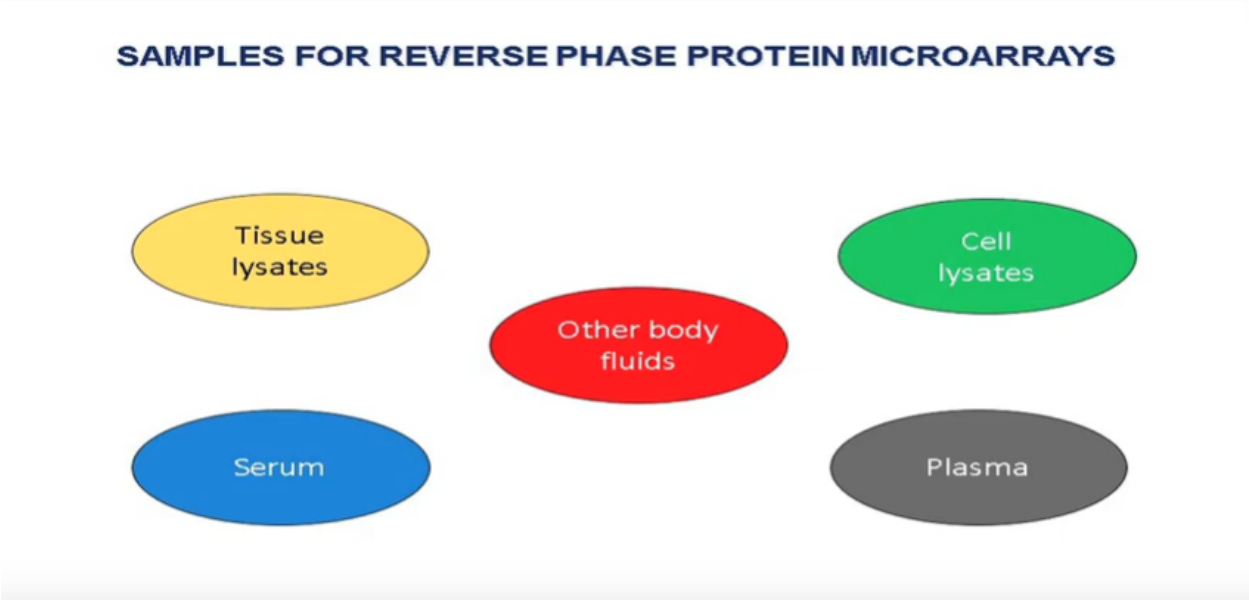

First with samples, there is no limitation any sample can be analyzed by RPMS, or any sample which can be analyzed by Western blot can be analyzed in RPMA, only thing is you have to follow certain methods to prepare your clean sample to make it ready, for RPMA approach.

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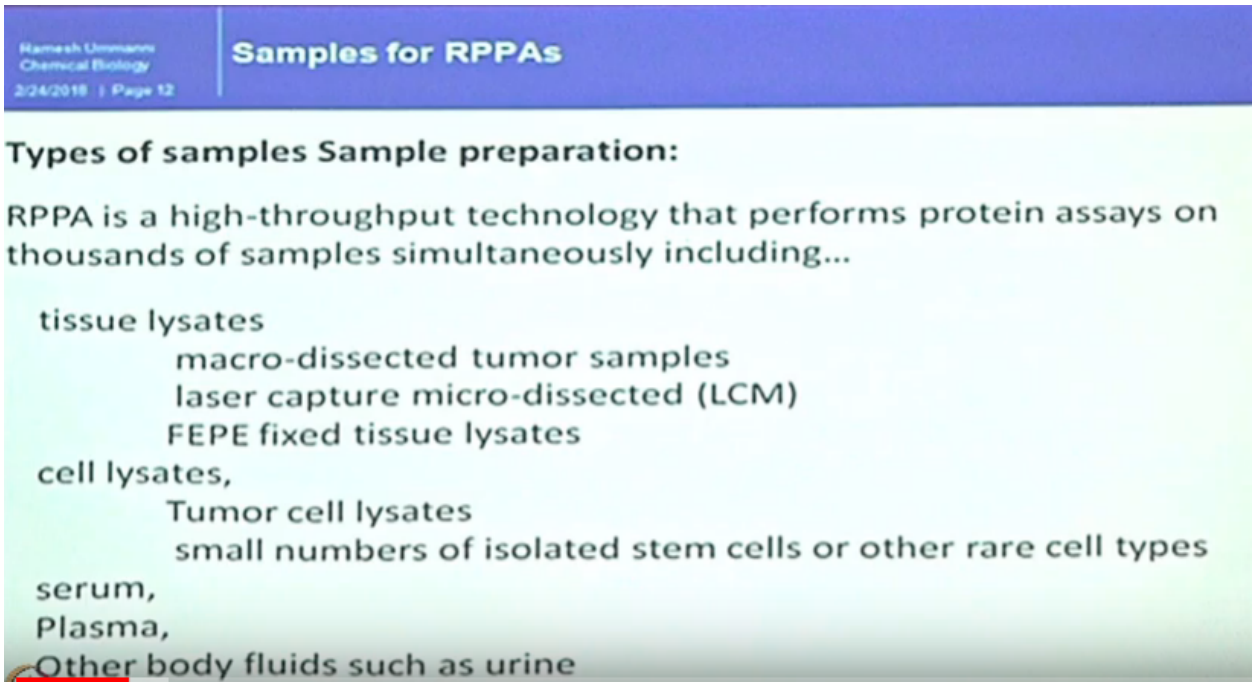

Is a high-throughput technology, that performs protein assays, on thousands of samples simultaneously including tissue lice it's, just micro this, macro dissected tumor samples there at least you will have a chance to get some microgram level proteins, some couple of micro milligram level proteins and then laser capture micro dissection, LCM there you end up with very, very low quantity of sample you cannot perform a number of estimates to validate a number of targets and PFA fixed tissue lice it's also you can do. so, there are separate methods are available, to make the protein extracted from formalin-fixed tissues to use for RPMS, sometimes those samples cannot be analyzed by Western blotting approach you see smearing effect, which can be avoided here, the cell I said particularly our tumor cell lysate every one of you might be handling some of the cell lines in the lab. So, the license can be prepaid and small numbers of isolated stem cells are 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 lead, 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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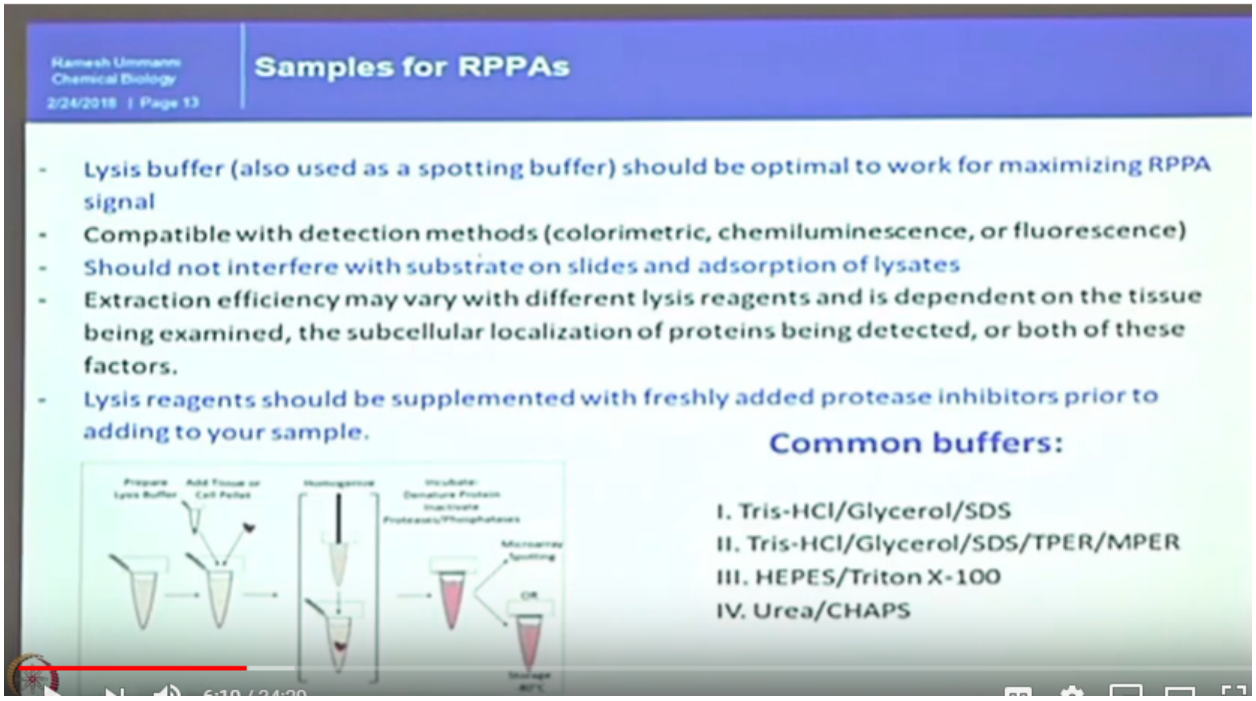

So, to print the samples, how to prepare the samples, it's very simple cartoon here, can take a lysis buffer, put the tissue of rest and homogenate and spin down the tube and take the supernatant and go for printing or cane kept in them - say - go home and come back tomorrow. This is a way but, it's 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 chosed? 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 the RPPS, signal. So, it should not interfere, with the visualization of signals using any labeling system you follow the secondary antibodies like IR labeling, HRP label or alkaline phosphate label, are directly SCI 3 a sci-fi label. And it should be compatible with the detection methods, like a calorimetric Emery luminescence and fluorescence methods, should not interfere with substrate on solids and absorption of light sites, see for example you might have performed washing steps, what if the buffer is not compatible? And washed away along with the proteins spotted in the glass light, end of the day you will have a red face no signals. So, definitely should not interfere with the substrate, on the slides in this case nitrocellulose substrate and should not interfere with odds of the lysate, I mean that will 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's say for example if you are taking a prostrate tissue. which will have a prostrate some stones will be there, then you need a stringent buffer, let's say for example your lysing the cell lines, simple Ripper 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 phosphates 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. In my X, 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 is work, it will not work. So, you have to optimize please keep this in mind.

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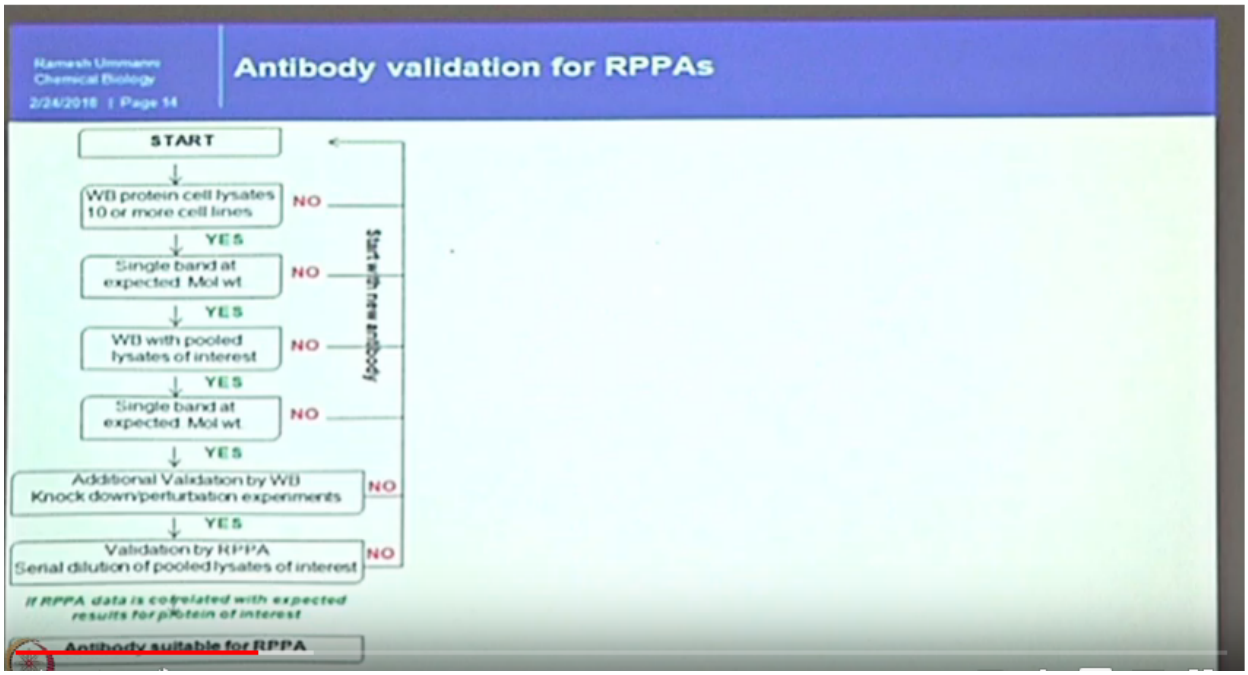

Then second point, highlighted was the essential requirement is the good quality antibodies. So, here you need to spend at least, some couple of months, two years, to establish antibody panel in your lab. At least we could we could establish I mean with my soup postdoc supervisor, we could establish one hundred and ninety antibody panel in one year, from three thousand antibodies purchased from the market. You can see that, two thousand eight hundred plus antibodies just went into disturb enough 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.

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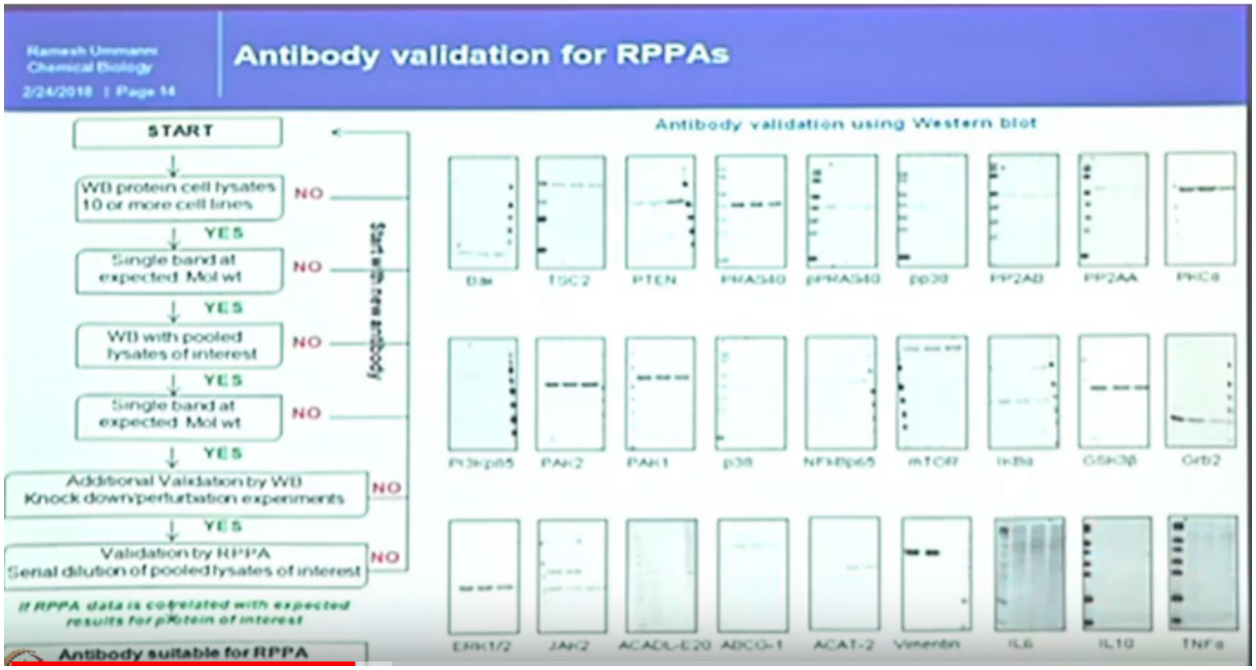

our approach for antibody validation is like this, starting point take that western blot, through western blotting approach, take ten different cellulite sites are from more cell lines run ten different lines and then do western blot, if the antibody gives a single band say yes, go to next step and then you pull, the live sites from ten or more different cell lines, run a single line, that means in a complex mixtures 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's and if It is yes, then it will go to the validation by RP PA, in RP PA we will validate by serial dilution of the samples, whether antibody is able to detect, the serial dilution the twofold, tenfold dilation properly or fivefold dilation properly, if it is yes then this correlated is, if that 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 it 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 write code. So, it's like a go, no-go, then use caution these are the three words, we exchanged by emails. You can see that these is a like a pain, 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 band 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'm not as again brand ambassador for any antibody companies here, at least I had a good experience with cell signaling very, bad experience with Santa Cruz. So, I don't, get offended please just it was my experience; I'm sharing with a very lighter node with the student community here. So, you can see that, if you have a single band here, directly 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 will grab the first step itself, we don't spend any time and we will write by the another antibody.

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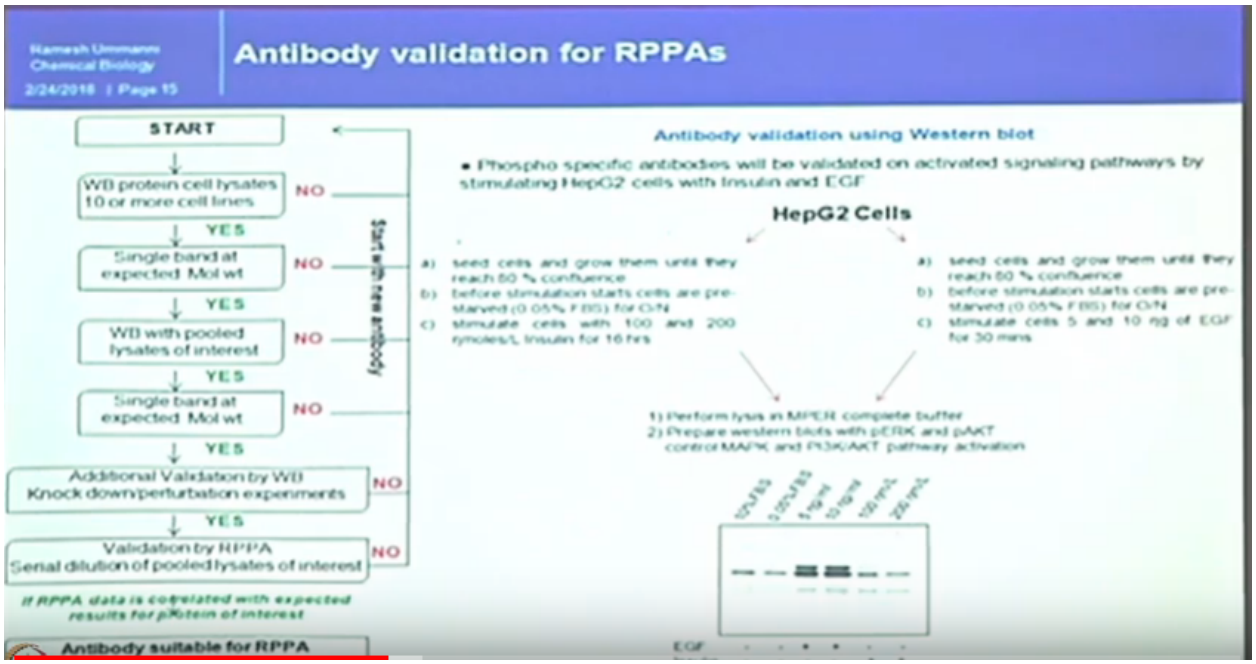

So, next round of validation is that again, value using Western blot, we take a suitable cell line and stimulate them with X Factor's, where your protein of interest gets modulated? you know it, prior information already and you look, for this known information in the Western blot results, in this experiment 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 Katie and phosphorylation of earth protein and those phosphorylation, 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. So, with this approach it is first then it will go into the next line.

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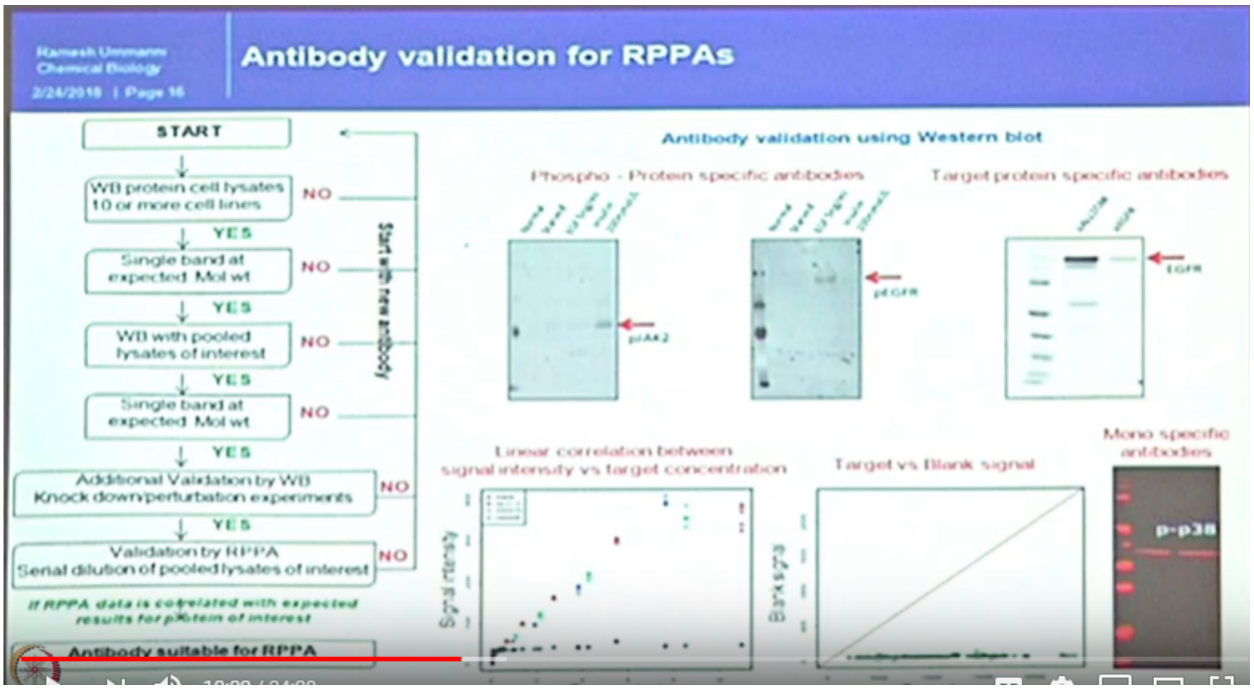

Then we will not leave that, story there itself again we take those antibodies and these phosphor specific antibodies, choose the different cell line, whether they are behaving in the same way or not. So, here can see that with the insulin, pass photo phosphorylation is going up with EGF, only EGF for is going up. So, again these are the mutually inclusive, exclusive garbage in, garbage out approach basically to tell you in a very crude manner. So, once we have this, we will not leave the story there again, we have now SARNA, available for all, the proteins expressed in the Orpheum, we by the SARNA, 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 SARNA agnus, EGFR and our antibody is detecting the down regulation of EGFR. So, it has passed through the this step here. Not on our Britta, duration experiments. So, after this then we validate them by reverse phase protein area we simply take very, pool of lies a small pool of lies, it's print on the glass lights and use this antibody and probe it see that, whether it is really detecting the signals are not. So, when you plot the results like concentration, versus the 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 don't get a linearity rate. So, then we have to drop the antibody or disturb 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 do passes these two filters we have a like mono specific antibodies, in this example I am showing phospho p38, these antibodies go for RDPA approach.

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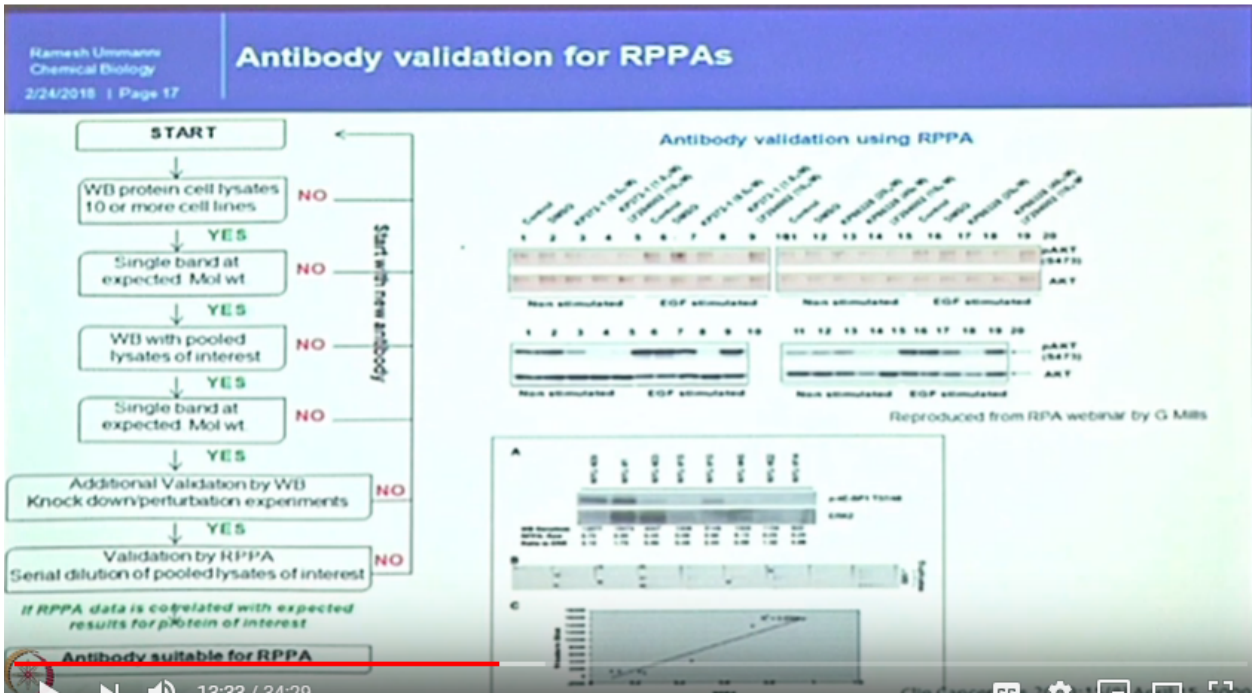

The antibody is approved for reverse phase protein area approach, you also need to do one more step you need to see the correlation between Western blot and our to be results. Because many of the pipe assessing this was that one of the RPPA conference many people didn't believe that our RPPA is better than the Western blotting. So, that was the reason so this expert 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, I mean this group got an males from MD Anderson they spotted see really diluted tumor lysis and did western blots and normalize the data this outcome here. So, now we have the antibody suitable for reverse phase protein array approach.

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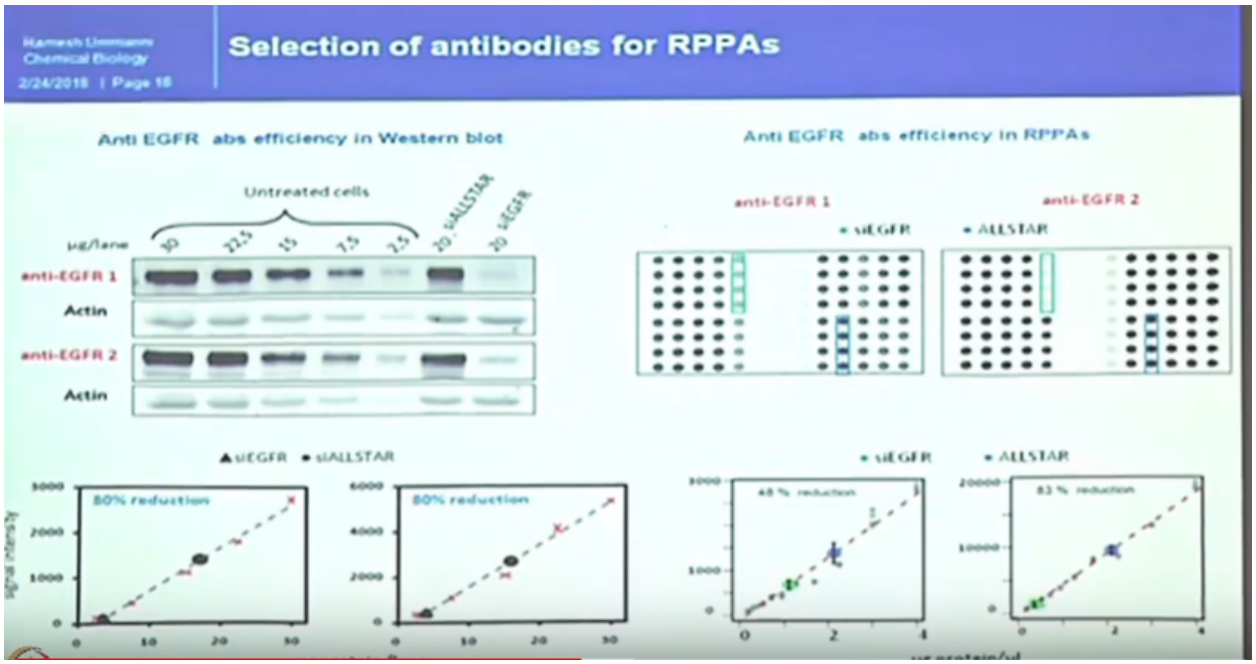

So, at this point, to educate you in a different way not all, antibodies against one target will behave the same way. Let's say for example if you search cell signaling website, for a EGFR or phosphor EGFR antibody target will have a 10 different antibodies with the different catalog numbers, that means there is there from different clones, at different policy, different monoclonal antibodies and not all the antibodies behave in the same way. How it is? Now, you look at this picture. So, it comes in one lysate we used, for non regulation of easier from the same lysate we saw we did a Western blot in a serial dilution 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 result shows that eighty percent down regulation happened for EGFR after adding SARNA. This data is completely wrong, how it is? When you did RPPA with the same samples, you can see that this is anti EGF, for one antibody detecting the lysate, where EGFR is down regulated and here is also control samples in this case this is antibody one, antibody two, when you look at the correlation, this first antibody detects 48 percent down regulation, second antibody says eighty-three 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 not able to conclude or not able to infer any meaningful date. So, this is what? Again all antibodies cannot detect the single protein, in the same way like for example in this slide.

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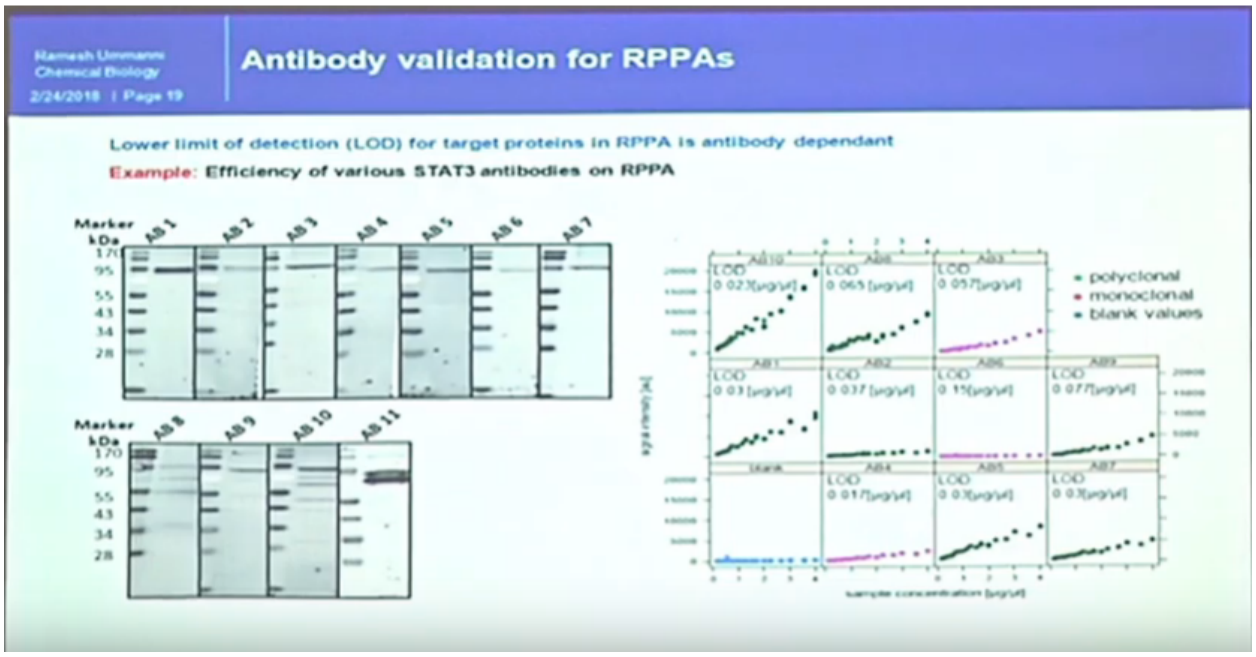

You can see this stat3 antibody, different start three antibodies, detecting the Stat3, three in the same sample in different manner. You can see LOD, this limit of detection some numbers are written here, definitely I think I will circulate the slides also to you, if you can't, note the numbers you can go through later on. So, this is green is a polyclonal, red is a monoclonal, blue is a blank values, can see that so different signals are going in a different scale along the y-axis. Okay? 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 particularly phosphoproteins, very few copies will get phosphorylated then that total protein. Right? So, in that way we should be very cautious in choosing the antibody.

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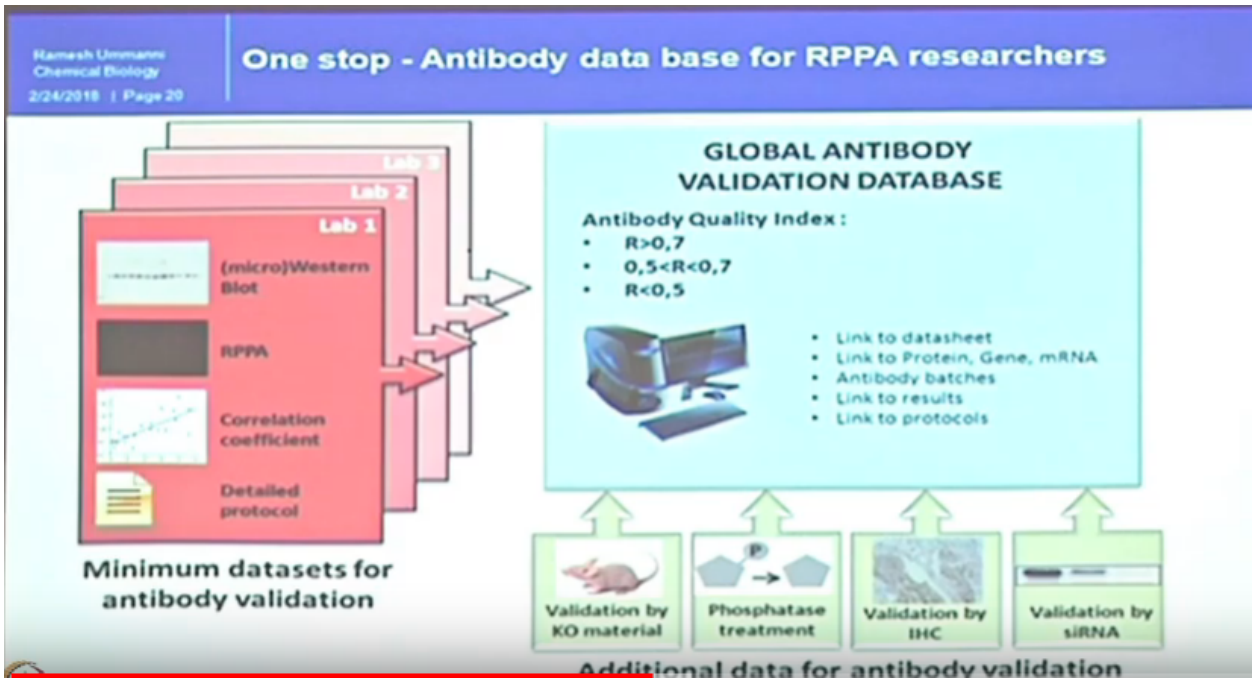

So, all in all, I would like to say that, if one stop database is available for antibodies which are approved for RPPA approach will be an ideal situation. So, the community or the society is working towards that they have formed a consortium ,with the different labs and all the labs will share the data and they will they wanted to fit 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. So, this is now in the process.

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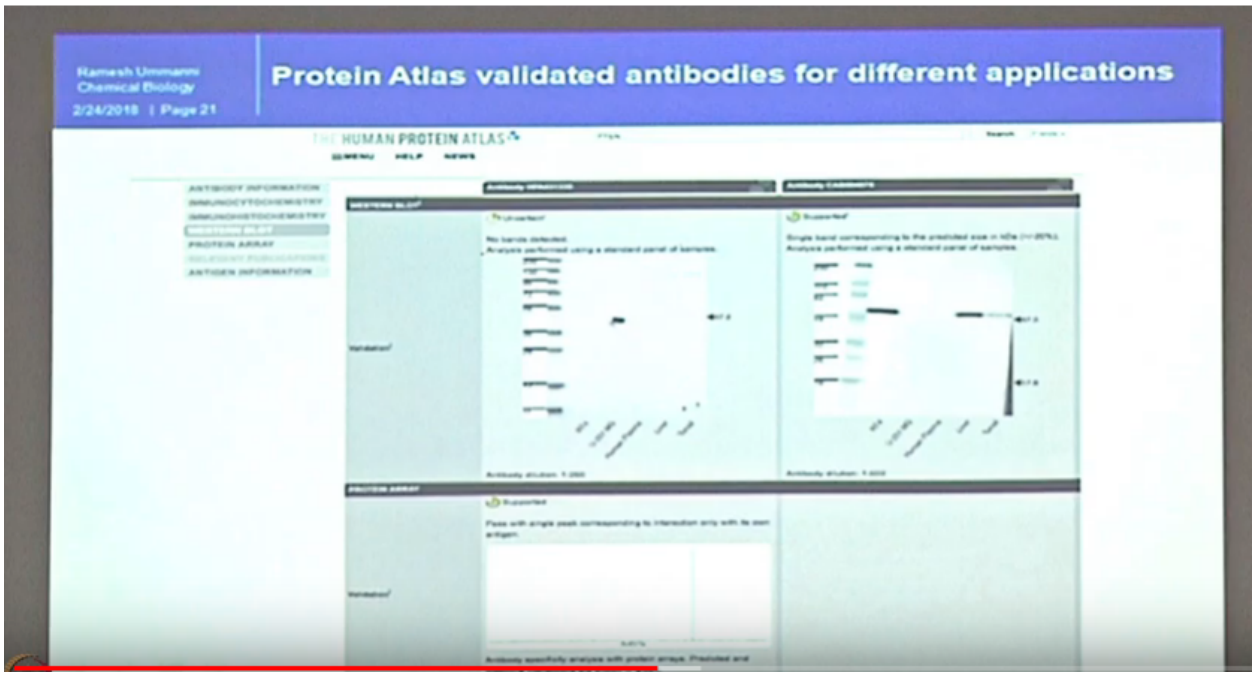

But, another approach the protein at last company, is included in their catalog, of validation steps is by RPPA also. So, if you look at the data sheet of any antibody, if you purchase then there will be a different form of applications like compatible for Western blotting, immunohistochemistry, immunofluorescence or not.

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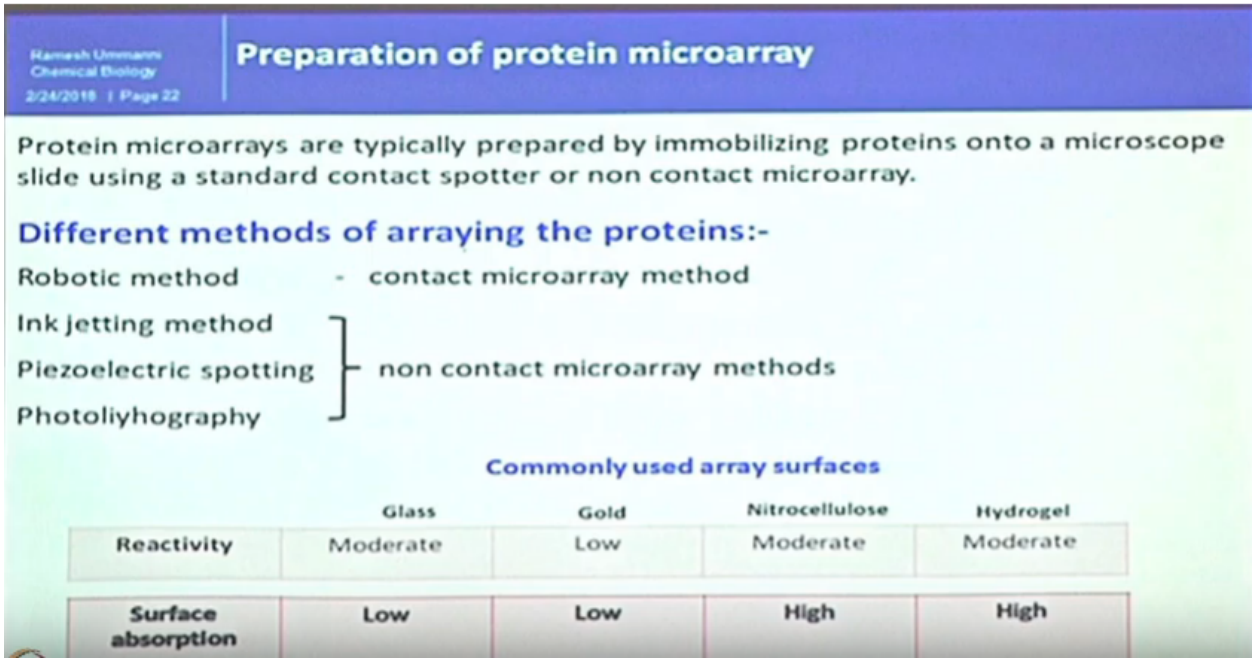

So, this company included another validation, point that it can be used for RPPA or not. So, if the single peak is the, that means it is useful for RPPA, approach they also have the catalog, indicating that whether

it is go, no-go or use with caution for RPPA approach, miss 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 don't have, instrument to print and show you all. So, definitely it is similar strips, exactly same steps it followed as Sanjeva**,** 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 on, non-contact spotters. So, there are different methods are available, one is a robotic method, which is a contact micro or a 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 photo layout graphic. 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, 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, nitro cell is moderate and hydrogel is moderate. So, compared to this nitrocellulose is better, because gives low background and low fluorescence while detection methods are applied. So, that's the reason most problem most of the people are using, nitrous illness coated slides whom I know, at least my brothers 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's why I prefer to use nitro cellulous coated slides. So, that nitro cellulous coated slide looked like a white layer, on the glass slide you might Rodin 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 nitro cellulous coated coating will be there, that's it. There's a difference once you have a glass like.

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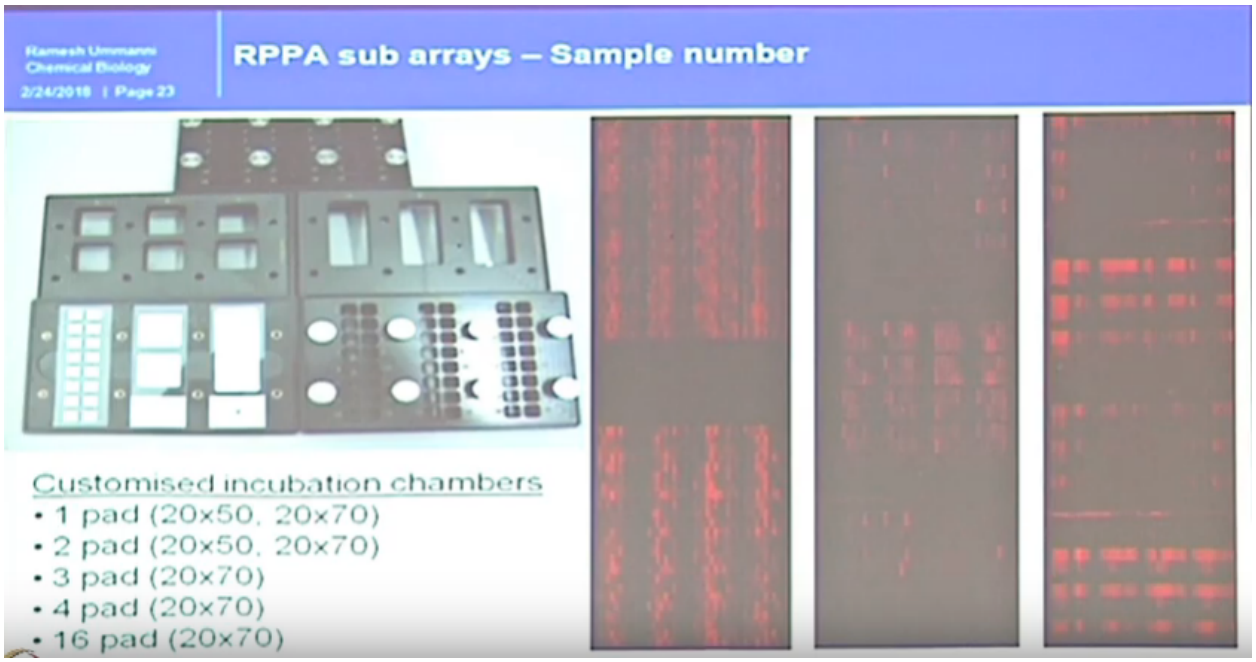

So, then you can decide the array, one slide can accommodate two thousand samples, will you wait until two thousand samples, will be accumulated from different studies, not necessary .Right? So, then you can design the array into the miniaturized fashion, it is quite easy and possible this chambers, or customized, in our lab now over are available in the market. So, one pad means, it is like this. So, you can use one antibody, for the entire slide for to thousands, if you have only thousand samples, you can divide them into two groups. So, thousand here, on thousand here you can use two antibodies on each slide and if you, very less number of samples you can also divide the chamber into 16 different chambers, glass layer into 16 different chambers. So, that you can put 16 antibodies in one glass sided, with maybe 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 two pads, here are the three pads, here are the four pads, 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, it suggests like any kind of miniaturizing your array you can cast a custom-made array.

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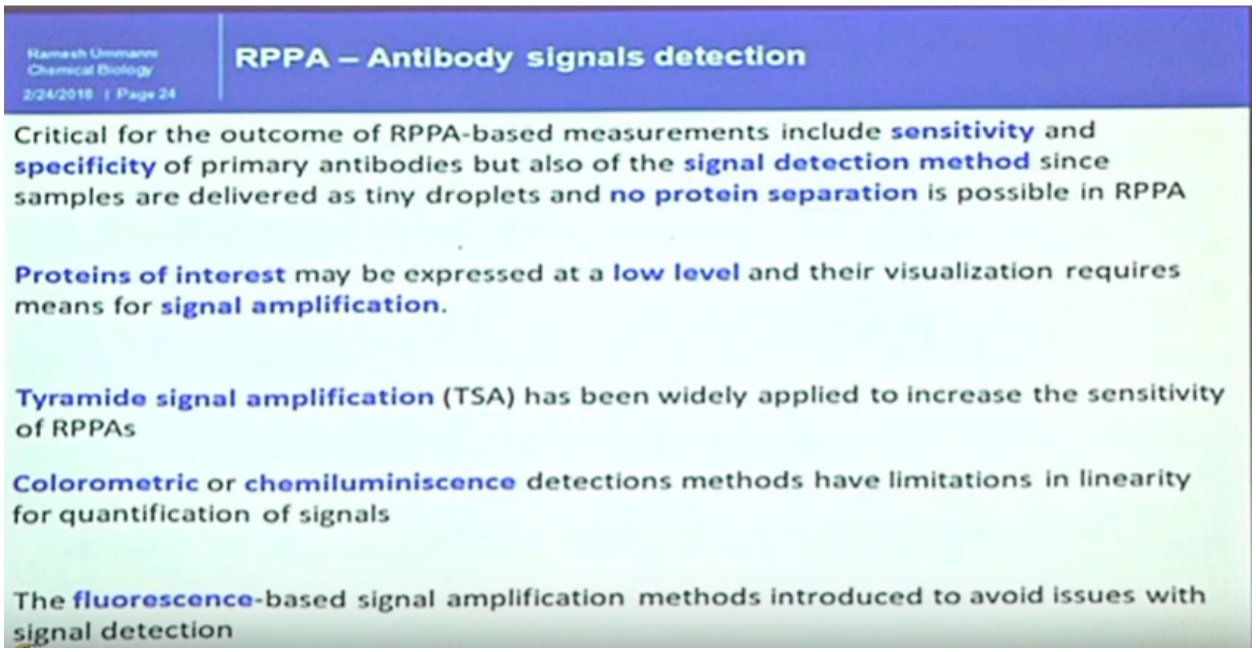

So, once you have the array printed, your protein of depending on the protein of interest you wanted to analyze and you can add antibodies and then probe and further the next steps to be processed. So, after primary assume that in the RPPA also, now you print it, you added the primary after blocking and washing and everything, you're 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's 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, on their visualization requires signal amplification. So, if the abundance 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 revolutions 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 the fluorescence method the detection limits between 10 to 50 microgram, if you use calorimetric or chemiluminescence method, if the signal gets rated 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 intensity of the proteins, the fluorescence based signal amplification methods have been improved introduced to avoid issues with the signal detection I will explain you one method.

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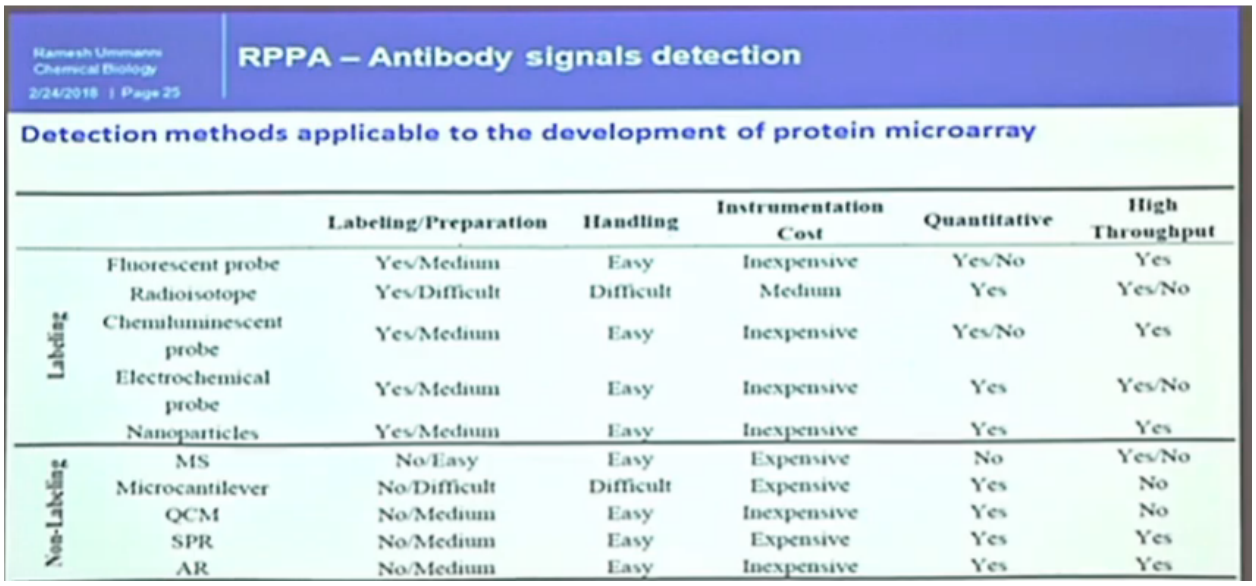

		Labeling/Preparation	Handling	Instrumentation Cost	Quantitative	High Throughput
Labeling	Fluorescent probe	Yes/Medium	Easy	Inexpensive	Yes/No	Yes
	Radioisotope	Yes/Difficult	Difficult	Medium	Yes	Yes/No
	Chemiluminescent probe	Yes/Medium	Easy	Inexpensive	Yes/No	Yes
	Electrochemical probe	Yes/Medium	Easy	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, different methods are available. So, applicable to development of the protein microarrays, here is again nonlabeling and labeling. So, I don't want to get into explain more about, the labeling and non labeling methods on differences between these two, can simply go through here and you can already see that, labeling methods have labeling, labeling methods have, little less usefulness in RPPA approach, then the non labeling approach. So, nowadays some people are using surface Plasmon resonance as well

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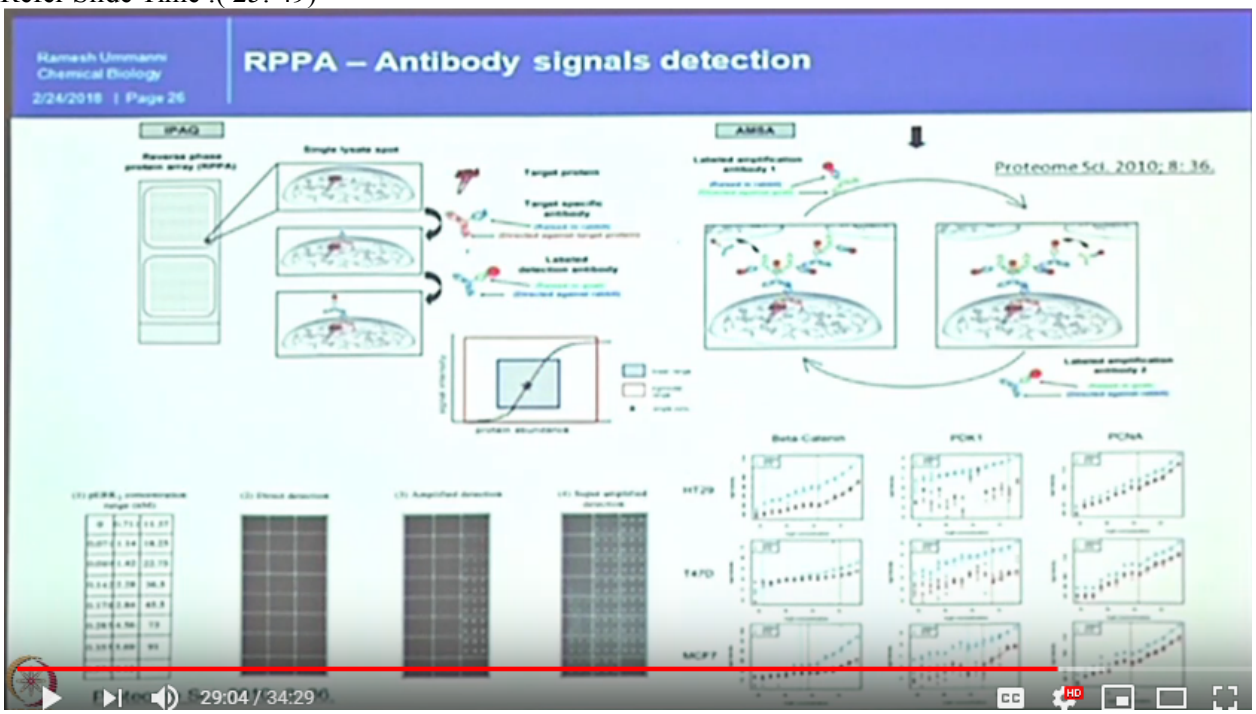

So, hot is a signal amplification method. You can see that, simple spot is there and at the primary antibody and labeled secondary antibody is added then you got a signal output. So, if you don't see any signal in

this approach, still you have a way to, get it out, this is called, ‘Enza Method’, AMSA method which was developed by one of the PhD student, in my postdoc supervisor slab, high command's Birgit Enza means, antibody mediated signal amplification. And we have a spot here, can clearly see that 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. Okay? Then again we will select the third antibody, which is raised again s the goat in rabbit, this is again. So, now imagine that one label molecule, became to label molecules, one molecule of infra-red florae attached, with the one round of secondary to secondary antibody will have to fluorophores there, then again if you repeat this for 10 times, 10 up to 3 or 4 antibodies 5 antibodies, 1 protein target, it attached to 5 different signaling molecules, then obviously your signal will be increased by 5 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 don't 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 2 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, abundant proteins in a large pool of proteins in the lysate peasant this is, the 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 at this much distance to detect from lower concentration to our 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 and I am trying to just get you, get you into the imagination not able to show you, the how signals can be amplified and out.

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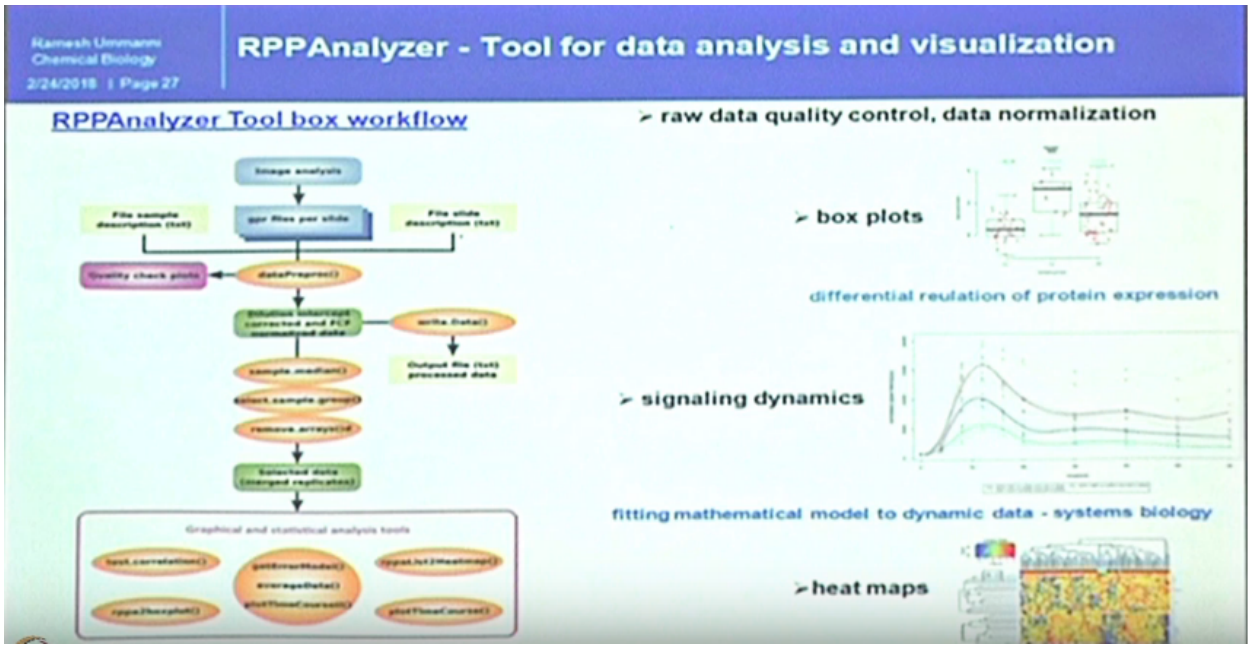

So, this is a way, once we have the array, once we have the signals here, definitely we need a soft ways to analyze nowadays RPPA pipe, which is a kind of open source software has been available and some of the companies have developed soft special, soft ways to analyze the RPPA data generated. But, 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 Heiko months Berger, with rule de Korff lab, developed a RPP analyzer which is the 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 slight description, what kind of antibodies proved? Like a 10 or 20 different antibody, 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 the serially diluted samples, the linearity is it and R value is point a but, at least above 0.9 mean 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, they look 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, for example your samples were collected for a period of time zero million 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 fit 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 can design or you can draw a heat map, you

can see that what are the different cluster, of proteins are altering among the proteins I tested whether there have any relation or no relation.

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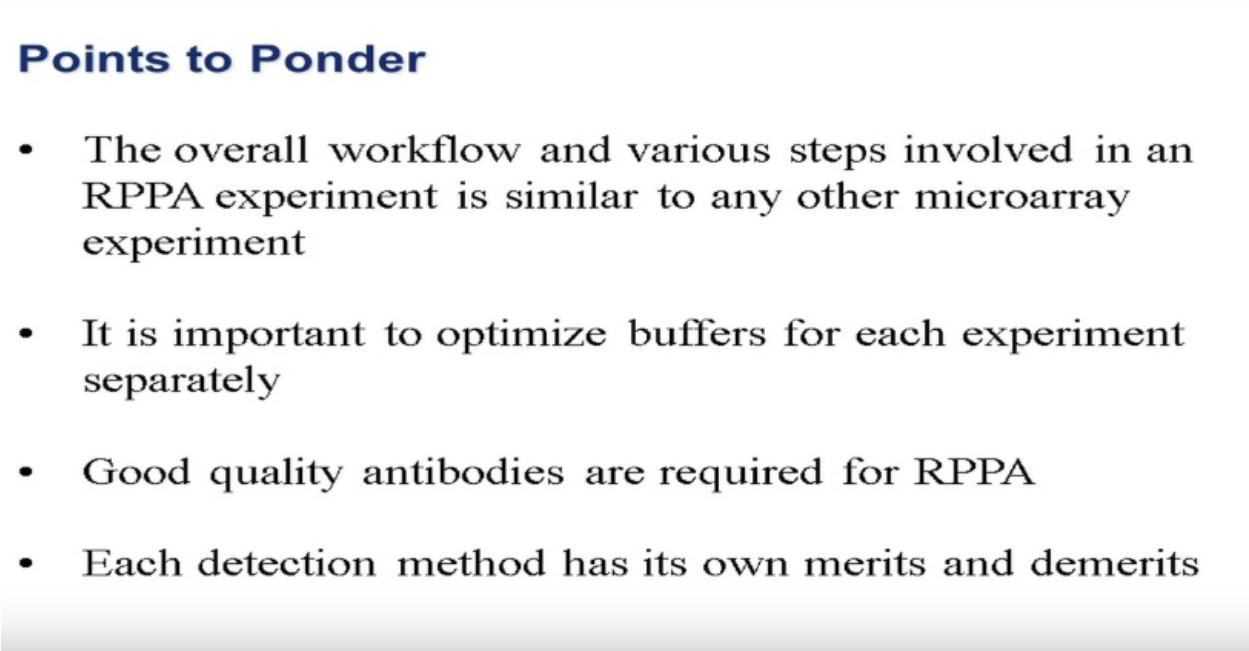

So, I hope today you have learnt about, the importance of each and every step, involved in RPPA workflow. You have probably know, what a good glimpse and understanding of how different buffets behave differently, with different sample types, the optimization is of utmost importance Dr.Ummanni also emphasized 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's 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, EMSA in detail. In the next lecture, we'll continue this interaction and talk about, how to analyze, reverse phase protein arrays, microarray based, dataset. Thank you very much.