LECTURE-1

GENERATING PROTEIN MICROARRAYS: FOCUS ON NUCLEIC ACID PROGRAMMABLE PROTEIN ARRAY

TRANSCRIPT

Welcome to the proteomics course. In today's lecture we will talk about generating protein microarrays and we focus on nucleic acid programmable protein array also known as NAPPA. So as we know the microarrays provide high throughput versatile platform for large scale analysis of functional proteins. The traditional cell-based expression methods which have been used in protein microarrays, they generate proteins in heterologous system such as E. coli but there are several challenges posed by these systems for protein purification as well as maintaining the protein integrity and functionality, protein storage all these pose several challenges. So to overcome these obstacles posed by the traditional cell-based expression methods, there are various cell-free expression based systems have been generated. In the last lecture we discussed about cell-free expression methods and how different types of protein microarray platforms have been generated by employing cell-free expression based systems.

So in the previous lecture we discussed about various types of cell-free expression based protein microarrays we talked about protein in situ arrays, Halo-tag arrays, DNA array to protein arrays, and multiple spotting technique as well as NAPPA. Although each one of these techniques offers several unique advantages but NAPPA approach has proved and overcome many limitations and it is applied for very high density arrays. So today, I will focus on NAPPA arrays. Just to refresh you NAPPA arrays which we discussed in the previous lecture.

As you can see in the slide you have a microarray slide and each feature which I have highlighted in the expanded view contains four features one of that is cDNA containing GST tag then you have bovine serum albumin (BSA) protein, capture antibody such as

anti-GST and BS3 crosslinker. So in NAPPA approach plasmids encoding the target protein are fused with an affinity tag and those are fixed to the array surface. The array surface is activated by addition of cell-free expression system which could be wheat germ expression system or rabbit reticulocyte lysate. Rabbit reticulocyte lysate is more preferred for eukaryotic mammalian protein expression so the target proteins are expressed and immobilized in situ and they are detected by using a universal anti-tag antibody so in this slide I have shown you the anti-GST tag and anti-GST antibody so if you have GST tag you can use anti-GST antibody if you have flag tag, you can use anti-flag antibody if you have mic tag you can use anti-mic antibody so these universal tag strategies can work out depending on what tag you use you can use corresponding antibodies for the same.

Little bit overview of cell-free expression based systems different type of protein microarray platforms which we have discussed, today we will discuss more on how to make protein microarrays by using NAPPA approach. I will walk you through with a work-flow, step by step how various parameters one need to monitor. I will talk to you about each step for example cloning and plasmid preparation, aminoacyl-silane coating of the glass slides. What type of printing parameters one need to ensure for good printing quality then in the NAPPA approach one need to ensure that DNA printing is of good quality as well as protein expression is good so all those quality control checks as well as different type of control features one need to have on these arrays so that during one type of application one can make sure that they are relying on good data so we will walk through all this process in the work flow.

So what are the requirements to assemble protein arrays so in today's lecture when we will discuss about making protein arrays that is quite similar for most of the cell-free expression based protein microarrays and largely it can be applied for all type of protein microarray platforms but it will be more in the context of NAPPA. So first of all, we need a repository of the expression ready clones which should be in the flexible cloning system. We have talked in the previous lecture about recombinational cloning which provides very high quality master-clones. Now those master-clones can be easily

transferred to the subcloning different type of expression systems. So if you have different vectors you can transfer those masterclones. Then you need a pipeline for very quick purification of DNA constructs and how to array or print them on the chip surface. You need robust printing chemistry as well as since you are aiming for the high throughput experiments you are aiming for thousands of features printing on these arrays so you need high throughput capability at every step. So in general when we talk about NAPPA protein arrays in the work-flow, we will talk about first of all what is the array design and the experimental planning required for performing these arrays.

So unlike other proteomic techniques where one starts with unknown things and also after doing after lot of experiments then only they can identify the protein by using mass spectrometry. In the protein microarrays people are already aware what type of contents are printed on the chip surface to start with and those contents has to be very well planned. If you have a specific objective in the mind you want to study protein-protein interactions you need to have some good positive controls for the protein interactors as well as some negative controls. You want to identify some biomarkers for example you are looking for serum screening, you need to have again some good control spots such as human IgG or mouse IgG so lot of thought process goes in the designing the experiments for the microarrays as opposed to what one do in the other type of proteomic techniques then what type of surface one need to select, what printing chemistry one need to use and since we are talking here about the cell-free expression based system you need the DNA to start with so how to express those and construct those clone. Now once you have done the culturing then you need to concentrate DNA. Almost all the molecular biology laboratories perform these experiments on day to day routine and it is very straight forward to do the DNA extraction etc. but when we talk about doing the experiments in high throughput then it poses several challenges because quality of all your features has to be very good. So various type of quality controls and various type of observations one need to keep in mind for doing the simple experiments even DNA purification and concentration. Once your DNA is prepared then you need to mix it with proper printing mix. In this case we are talking about NAPPA

arrays so we need to have those components which are required to make the protein by the cDNA. Now array printing, how to ensure the printing quality is good and then different type of quality control checks are required.

So just an overview one need to do experimental planning, how to design those arrays, and what types of features are going to be printed on the array surface. One needs to design these experiments very thoughtfully, what are the features which are going to be printed on these arrays, what type of chemistry needs to selected, how to purify the DNA, and once you have the slide which is functionalized that is ready, you have the DNA in the printing mix then all you need to do print those on the large number of slides and you have chips ready for various applications.

So let's start with the arrays design first of all. As I mentioned lot of thought process goes in starting these experiments one need to do literature review, one need to look for experimental objectives very carefully even before moving forward for the any of the printing steps. So design experiment type and appropriate protein test set. Once your test set works fine then one can actually expand that for the large number of the features. Now, fit NAPPA chemistry utilized to assay being executed. Decide what type of array density you are planning to use and how you are going to map that. Then what are the robotic parameters one need to use for doing these experiments. So all these steps has to be very well planned in advance so for example I have shown you few insets here. The very top panel in the slide shows different genes which you would like to make on the 96 well plate while doing the DNA prepping so first of all you have clones and now you want to do the DNA prepping you know in each well for example A1 what gene is going to be there for example BCL2 then in the H12 you have to left it blank now G12 similarly like you have all the wells defined genes are inserted there. Now each of these plates is giving you 96 features you need to have multiple blocks of 96 well plates. Then you need to transfer those in the 384 plate so that four of these plates can be transferred to 1384 plate and now that 384 plate well plate and multiple 384 well plates are used for doing this printing. So now on the right hand side I have shown you the array map which shows just very high density map, how different type of

features can be printed on the chip. All these things have to be designed prior to the experiment. Now each spot needs to be printed in the duplicates also you need to ensure that control features are spread throughout the array surface. For example if you see the bottom panel once which are highlighted with the red those are showing the GST proteins. Then in the green they showing the known spots. So these spots are printed in the duplicates and they are spread on the array surface not adjacent to each other so that you can look for the duplicate results how close they are. So this is expanded view here of a 6X6 spots/block. So these are the small blocks and obviously on the bigger array you have several features. Yellow ones show #### and green one shows the control features. So for example here one can use four different type of control features known spot, no DNA control, a mouse IgG, GST protein, human IgG etc. as I mentioned it all depends on the objective of your experiment and then suitable controls can be incorporated in the array map.

So let's talk about cloning and plasmid preparation. In the last lecture, we talked about recombinational cloning so researchers have started to create such collection of cDNAs by using recombinational cloning which allows for the rapid transfer of DNA fragments from one vector to another in frame and without any mutation. I have shown you earlier that how these clones are stored these master clones which are similar to the gold quality, now those are collected in these repositories. We have several good repositories including at Horward institute of proteomics, at Arizona University. MCG clones and there are several other commercial repositories as well so these clones are sequence verified they are usually gate-way or creative vectors which can be transferred to the other suitable vectors easily. So one need to do the cloning and DNA extraction before performing the printing steps. So first of all, one need to do bioinformatic selections of all target cDNAs if you are focusing on the a particular cancer or a particular disease obviously you have to think of all the genes which could be involved in the disease as well as different type of control features. So one need to do lot of research and bioinformatics analysis prior to what type of genes need to be printed on the chip surface. Then once you have selected the gene of interest you need

to design the primers and use PCR to amplify those clones. Gel purify the right size band and then move them in the entry construct then you need to transform in the E. coli, select the right transformant colony and then obviously you would like to use those colonies for the long term it is good if you can make glycerol stock of the same and then you store it for the long purpose.

So DNA preparation process is very similar to what everybody performs in the regular molecular biology lab, only difference here is that things and steps have to be automated and has to be in high throughput manner because if you are doing with the tubes, you are doing five or ten genes. You cannot achieve 20000 density so all the steps need to be automated for example I have shown on the left slide that inoculated liquid culture on the LB agar plate and then those can be used for inoculating large number of blocks for doing the DNA extraction. Now all these steps need to be modified in such a way that you have less manual intervention. If you use lot of pipetting by yourself there is a good possibility of introducing various type of errors. So lot of these steps are automated by using different type of liquid handling system. So you can transfer proper buffers- solutions-1, 2, 3 by using different type of liquid handling system and robotic platforms.

Just an image here to show the types of arrayers and platforms can be used for performing these experiments so that you can do the prepping in 96 well format so lysate can be loaded directly on to the DNA binding plates by using these type of robots. I will explain you cloning as well as plasmid preparation in the high throughput manner by showing you this animation.

Automated clone storage system-

In order to program the NAPPA arrays, first of all we will require access to cloned cDNAs representing target proteins. These cDNA clone are stored in the repository in -80°C. All the genes should be fully sequenced; the full-length ORFs captured into a recombination based cloning system. The genes of interest can be easily transferred into any expression vector by simply adding the appropriate recombinational enzymes.

This process is highly efficient and enables the transfer of many genes simultaneous into different expression vectors in a single step *in frame* and *without mutations*.

By this approach, the coding sequences can be transferred into any expression vector in a simple overnight reaction. After the cloning is done perform the spotting of the glycerol stock on the LB agar plate -

So transfer bacteria from glycerol stocks to an LB agar plate that contains appropriate antibiotic. You can use that either using mulitchennel pipetting or using liquid handling systems, prefer to do it in 96 well plate format so that you can avoid any mis-labeling of the samples.

So allow the bacteria to grow overnight at 37°C before inoculating the liquid medium so as shown here you can use multi-channel pipette or liquid handling system. So first of all you need to prepare the culture blocks and add the liquid broth in 96 well plate formats. You need to ensure that you have added the antibiotic solution in the each well.

Once culture blocks are ready then inoculate the liquid cultures from the bacteria grown on Agar plates similarly you can fill the all the wells.

Cover the deep-well block with a permeable seal, ensure the sealing is proper.

Incubate the cultures on a shaker for 24 hours at 37°C, at 300 rpm. Ensure the shaking is uniform.

After these blocks have completely grown the bacteria then we need to pellet the bacteria by centrifugation for 15 minutes at 2000–2500 rpm. While doing the centrifugation, make sure that centrifuge is balanced. So once centrifugation is complete then the bacterial pellet will be in the well and you need to remove the supernatant, discard the tips and once you have completed that for all the wells, now you are ready to perform DNA plasmid preparation.

Isolating DNA plasmid

So remember your basic molecular biology and you need to prepare solution 1, 2 and 3 for doing the plasmid preparation.

Add 200 **µ**l of Solution 1which is resuspension buffer that contains 50 mM of Tris at pH 8, 10 mM of EDTA and .1 mg/ml of ribonuclease. So you need to add the resuspension buffer to each well so remember in the last step we have made bacterial pellet of those culture blocks and now you can add 200 **µ**l resuspension buffer in each well. After adding the buffer you can close the plate by adding a cover slip.

In similar way add Solution 2 in each well. Solution 2 contains 0.2 N NaOH and 1% (w/v) SDS (sodium dodecyl sulfate). This is lysis solution. After adding this solution again you need to repeat the same process of adding 200 **µ**l of Solution 2 to each well, seal the plate and invert it few times. Once solution 2 is added make sure that you invert the plate few times.

Now add 200 **µ**l Solution 3to each well. Solution 3 is neutralization solution which contains 3 M potassium acetate at pH 5.5.

Centrifuge the block for 20 minutes at maximum speed.

Once centrifugation is complete then you need to discard the supernatant by dumping the block over a waste container.

So once you are ready with the clones, you have the good DNA preparation.

Now you need to make the slides, you need to functionalize the glass slide surface for printing purpose. One can use different type of surface for example glass, gold, nitro-cellulose, hydrogels etc. there are different type of properties one need to look for while selecting the surface. Obviously, cost is of the major factor when you are talking about experiment such as biomarker discovery you need to use hundreds of chips for screening hundred of patients. So if you have glass slide which is of low cost then that would be affordable for large experiments. So gold is obviously very high cost so as nitrocellulose and hydrogels. The reactivity for the glass is moderate so as the so as

nitrocellulose and hydrogels but gold has low reactivity. Absorption to surface is low for gold and glass, it is high is high in the case of nitrocellulose and hydrogels. Now compatibility in the case of mass spectrometry is only in the case of mass spectrometry. These surfaces can be derivatized by using different type of chemistries including amino groups or thiol ester. So let's focus on the silane based chemistry, we want to talk about printing the DNA on the array surface for using cell-free expression based NAPPA microarrays. So Silicon based chemicals they contain two type of groups inorganic groups such as alkoxyl groups like methoxy or ethoxy or organo-functional groups such as imino, epoxy, vinyl etc. so the DNA can be attached covalently on the glass surface by baking or UV radiation. One can bake the arrayed slides at 85°C, UV cross linking is also a popular method which also provide good result for DNA immobilization.

So, aminosilane coated slides, they provide high concentration of primary amino groups available at the chip surface. Why we need to use this chemistry? Amino groups provide a positive charge. How that will help? When placed in contact with the neutral aqueous solution, the groups become positively charged. The DNA backbone provides a negative charge. So DNA backbone will form the multiple ionic interactions as you can see in glass slide, with positive charged amino group coating.

For performing aminosilane coating in the lab, one needs to have the glass slides, acetone, aminosilane .Using a 2% aminosilane solution in acetone works best. Then you need a metal slide rack and you need a rocking shaker.

In this slide, I have shown you the various steps involved in performing aminosilane coating. On the left side, a rocking shaker is there in which a glass tray contains a metal rack containing 30 slides. On the right side on the inset you can see, those 30 slides are placed in the metal rack which is submerged in the aminosilane solution. On the bottom panel on the right side I have shown that the slides can be either centrifuged or dried with the compressed air and those are ready for printing purpose.

So let me show you this animation, how to do the aminosilane coating. Prepare 300 ml of aminosilane coating solution which is 2 % of aminosilane reagent in acetone. Place

the slides in metal rack. Treat glass slides in aminosilane-coating solution for 15 minutes in glass box on shaker. Rinse with acetone, followed by brief rinsing with milliQ water. After washing steps are done, you can spin dry in speed vac.

So let us talk about printing arrays. You have DNA ready as well as you have glass slides functionalized, that is also ready. So now you have to think about how to print these features on chip surface.

So first of all you need to make to make a master mix or printing mix. In case of NAPPA as I mentioned earlier, you have 4 features: BSA, BS3 crosslinker, cDNA containing the GST tag and an antibody that is anti-GST. So let me briefly explain the role of these components. BSA dramatically improves the DNA binding efficiency. BS3, BSA and capture antibody they are coupled to the amine coated glass surface where the activated, ester terminated homo-bifunctional crosslinker, the cDNA contains GST tag or you can introduce any other tag. Then you need to have the capture antibody so that the expressed proteins can be captured with the antibody to a c-terminal GST tag on each protein.

Once we have these 4 components ready, then you are ready to perform the printing.

What is the role of the BS3 crosslinker? It is water soluble non-cleavable and membrane impermeable. The amino reactive group of a N-hydroxysulfosuccenimide (sulfo-NHS) are separated by the spacer of 8 carbon atoms and each protein contains a terminal amino group.

Once you have these components ready, you are ready to perform the printing. On the softwares, you first need to define various types of parameters. You need to define what type of pins you are going to use. For ex: one can use even 48 pins for printing several features simultaneously or even go as low as 1—4 pins. This could be of different kind of microns, like 300 microns, 150 microns depending on what type of density you want to achieve. You want to ensure that there is good humidity maintained throughout the experiment so atleast 60 % humidity should be there. You need to define the source

plate you are going to use for printing whether you have one plate or multiple plates and what type of arraying pattern these pins have to follow. All those things have to be written on the program in the software and then only once you place the plates, 384 well plates, containing the master mix, those can be used for printing purpose.

One needs to ensure that different type of robotic parameters are in place, for example environmental conditions such as humidity, temperature, vacuum check, all these things need to be ensured before you place your plates for printing. The pin size and the number of pins depending on the density you want to achieve, how you want to set up the deck for printing, you want to use the plate, you want to use the slides, how many number of slides you want to use, again it depends on the different kinds of arrays that are commercially available. One can use the different kinds of slides. Array mapping that you have already planned experimentally beforehand, what density you want to achieve, where each of your genes are going to be located on the map, what are different type of printing parameters such as inking, stamping, print depth and then you need to ensure that in between the printing, the washing is sufficient so how the pin washing is performed. You definitely need to track all of this detail so that during a large printing, anything goes wrong, you can trouble shoot and know at which step things have gone wrong. So printing good quality arrays is very challenging, but once you have the good quality arrays in hand, you can perform different type of experiments.

Let me show you this animation to explain you the procedure for master mix preparation as well as printing procedure. So prepare enough master mix so that you can do printing for large number of arrays. The master mix contains DNA, polyclonal GST antibody, BSA and BS3 linker. For detailed recipe of each of these reagents, you can follow the publication manuscript by Ramachandran et al, 2008, published in Nature Methods. Once master mix is prepared, you can mix it well and then transfer 20 ul of master mix to the block containing plasmid DNA. Once the master mix is added to all the wells in the block, you need to shake the plate for 15 mins. For printing you will require 384 well plates so now you need to transfer mastermix containing DNA from the

96 well plate to the 384 well plate. Again these steps can be performed using multichannel pipettors or liquid handling systems.

Once master mix is prepared and transferred into the 384 well plate we can perform the printing step. So we can use these microarrayers to print the DNA and the master mix on aminosilane coated glass slides. First you need to ensure that pins are washed thoroughly. You can wash with ethanol and water. And during the entire printing procedure we need to ensure that humidity is maintained at 45 to 60 degrees(??). One can use type of pinheads and different type of microarrayers for printing the chip. Now these mastermix can be printed on the chip.

What are the different types of control features you need to put on the array, lets have a brief discussion on that. As briefly mentioned earlier you need to ensure that different types of control features are spread across the array surface and these could be mouse IgG, no DNA, non-spot, EBNA, human IgGs etc. Now to test the background you need to have the certain control features. Since we are talking about the NAPPA arrays, we are talking about the clones containing the GST-tag. It will be a good idea to have the different type of spots with the GST alone so you can have the purified GST in the dilution series so you can have that for doing the calculation for protein expression later on.

Now, if you are looking for different type of immunological response, biomarker discovery etc, you need to ensure that IgG is printed at the varying concentration to assess immunological response, for example, the varying responses such as I mentioned for ___ human biomarkers. It can also use for statistical analysis that are run by the biostatistician so that different type of protein expression can be normalized.

Another control feature and QC check shown here on the slide, same gene printed 4 times in the blocks, repeated and then negative controls are used to give an idea for the background.

What are the different qualities one need to keep in mind for good protein arrays? Obviously you would like to know the good size and shape, these have to be uniform. So spot to spot consistency has to be there. Positional consistency has to be there for a large number of slides. Sample loading, sample integrity, as well as different types of controls, replicates and positional flexibility, all of those need to be in place for good quality protein arrays.

As shown here, one can encounter different types of problems while printing these arrays. Shown on the left is spot shape, size and morphology. Shown on the right is spot to spot consistency. Many times these types of printing issues do come while high throughput type of experiments so one needs to ensure that all these parameters are properly QC-checked.

Again like looking at the spot morphology and the uniformity of the spot,one can think of various types of parameters that could be useful, such as spotting solutions, spotter capability, maintaining the temperature, maintaining the humidity conditions, keeping the dust free environment etc.

Now, printing the quality arrays, that is very useful. You need to have the precise liquid handling systems. As I mentioned, each step can be automated for the same. You need to do that various step tracking of the entire process so you can troubleshoot if anything goes wrong. It is good idea to build a detailed log history for each step, from cloning, DNA preparation, printing etc. Now if you done the step by step optimization and evaluation, there is a less chance of making any errors, because in high throughput approaches when you are talking about 1000s of features, it becomes very important that everything has to be tracked throughout and logged in detail in the logbooks.

So now let us talk about we have done the printing we have ensured that our printing has very, very good quality so now can we use these NAPPA arrays for doing the further biological experiments? So first of all we have printed the DNA we have to ensure that the DNA printing is good. So to assess that, one can use the picogreen staining. Once the DNA quality is good then one need to ensure that the protein can be

expressed from the DNA using in vitro transcription and translation systems. So the protein expression needs to be quality checked.

So shown in the slide, a very small test array which I used to teach in the Cold Spring harbor to the students and these are actually made by the students. In the bottom, you have a map where only 5 genes are there, CDK 4, CDK 5 Jun, p53 and p21, each one of those genes are printed in the duplicate, then you have mastermix, then you have water. A very simple proof of concept test array. So only 5 features, 5 genes should have the picogreen staining to observe the DNA quality and you can see on the upper panel those green spots are lighting up showing that DNA is printed properly. One can use the statistical tools for analyzing how good the DNA is printed on the chip surface which shown in the graph on the _____.

Different dashed batch (?????) variation need to ensured because if you are printing 200 slides, 100 in each batch then you need to ensure that all the batches can be used in one experiment and those data can be compared. So always printing batch to batch should be ensured, for both DNA staining as well as the protein expression.

Once the DNA is QC checked, then you need to move on to the protein expression testing. This is the schematic showing that the different steps involved in performing NAPPa based protein expression. So you have the slide, you need to block those and then you use IVTT system so the proteins are expressed and then you want to detect the signal so you can add the primary antibody, anti-GST in this case and then followed by the secondary antibody containing either the Cy3 or Cy5 labels or use a HRP-based terramide signal amplification system (TSA based systems).

Showing again a proof-of-concept array map with only 5 genes and these proteins need to be QCed here. So one can use the anti-GST antibody and you can see that only 5 proteins in duplicates are expressing whereas mastermix and water are blank and same

can be plotted on the graph which is shown on the right hand side. Now you have good protein expression but how specific that protein expression is.

To ensure that specific protein expression, one can also use a protein-specific antibody .For example, in this case we have the p53 also on the chip surface and we can use Anti p53 antibody and as you can see in the middle panel, only one which shows that only 1 spot is lighting up which shows that only p53 can be detected. If you use the anti-p53 antibody, on the right side, the graph , only 1 spot is showing the graph bar. So it shows that in a high-density array, similar types of quantity control checks can be performed for the protein expression, shown in the left side, P53 expression or the protein specific antibodies in the middle panel and the inset showing that how duplicate spots can be looked at. So those duplicates spots are for the p53 protein.

This is an overview of the quantity control checks that one has to perform during the whole NAPPA procedure. Before you use these slides for doing any biological questions, you need to ensure that DNA is properly printed shown in the left slide, with picogreen staining. Next to that is the protein expression by using the anti-GST antibody. Next is the specific protein expression, for example using the anti-p53 antibody and looking at the iset for those duplicate spots.

Again one to need to look for the batch to batch variation for the protein expression. If you print GST series as shown in the bottom side, which is printed in the bottom of the slide, those can be used to determine how much protein is produced on each feature. So batch to batch CV, sample to sample coefficient of variation as well as replicate coefficient of variation should be calculated.

Now, once you have looked at day-to day variations, then also look at different types of other variation such as spot-to-spot variation, slide-to-slide variation and day-to-day variation. Again plot it in this graph. It shows that NAPPA arrays are quite reproducible and the r^2 is quite more than 0.95 in all the cases.

So let me show you this animation for the QC check, how to detect the DNA and the protein expression on these chip surface. Once the arrays are printed, we need to perform the QC checks, whether DNA printing was appropriate on these chips. The storage form of NAPPA arrays is merely DNA. So in an activated array, all following possible reactions elapse in the solution and in the real time. Therefore restrictions caused by the instability of proteins occur very rarely. To perform the DNA staining, first prepare the picogreen stock solution. Dilute it in the milk or the superblock. Block the chips with the superblock for an hour. And after the blocking is done, then we can use the picogreen mix to place on these printed slides.

So lets first look at the NAPPA expression on the slide and then I will describe about scanning for both DNA printing and protein expression at the same time. So first you to prepare the IVTTM which contains TNT buffer, T7 polymerase, amino acids, RNAs inhibitor and DEPC water. Apply that on the slide which contains the hydriwell gasket and then seal the pores. Now this printed slide which contains the plasmid DNA also contains the IVTTM shown in red color here. The next step will be to incubate these slides for the protein expression step. Incubate these chips for 1.5 hours at __ for protein expression, followed by 30 minutes at 15C for protein to bind on the anti-GST tag antibody. After this incubation this done, then we need to wash the slides. So remove the hydriwell and wash with milk in PBS for 3 times, 3 minutes each.

Block the slide with milk or superblock at RT for an hour. After blocking is done, then we need to drain the excess liquid on a paper towel and then we are ready to perform the incubation with the primary antibody. So apply primary antibody, which is mouse anti-GST in this case, because we are looking for the protein expression and all the clones contain a GST tag.

So we need to add the primary antibody on the chip on which already, the expressed proteins are there. After adding the primary antibody we need to incubate for an hour at RT. Now place the coverslip so that liquid is uniformly placed and then we need to perform the washing steps after an hour. Proper washing is very important for all the

microarray experiments. Now we need to add the secondary antibody which is anti-mouse HRP and incubate again for an hour at RT. After secondary antibody, wash with PBS 3 times and add terramide signal amplification system for detection. After adding the TSA solution and incubation, we need to wash the arrays in water very quickly so that excess TSA is removed. Now we need to dry the slides with the compressed air. Or the drying step can also be performed by using centrifugation.

Now we will see animation for the scanning slides. Whether you have done the QC experiment for DNA detection or protein expression, now you need to scan the slides, using the different types of scanners available, including the ones that automated and multiple slides can be scanned in the automated way. First you can preview the scan and then you can adjust the different types of settings to see the signals better and the look at each region in the zoomed in view and look for the signal.

So in summary, today we discussed about how to make NAPPA protein arrays. Similar types of concept can be used for other cell free expression systems as well. To make the NAPPA to have the access the cDNA clones, one can do the recombinational cloning or obtain the clones from various repositories and grow those cultures and make the good plasmid separation and then add the mastermix containing the BSA,BS3 ,anti GST antibody and as cDNA containing the GST tag. Transfer this master mix from the 96 well plate to the 384 well plates which can be used to make the array plates. Now these can be used on the microarrayers which can do the printing. Once printed, these slides can be stored on the RT. After one has done the QC checks such as picogreen staining for DNA detection as well as adding the IVTTM for doing the protein expression. Once these chips are ready and one has performed all QC checks, these arrays can be used for different types of biological applications. In summary today we discussed how to make NAPPA protein arrays but same concept gives an overview of various types of intricacies involved in making any type of protein microarray. Thank you.