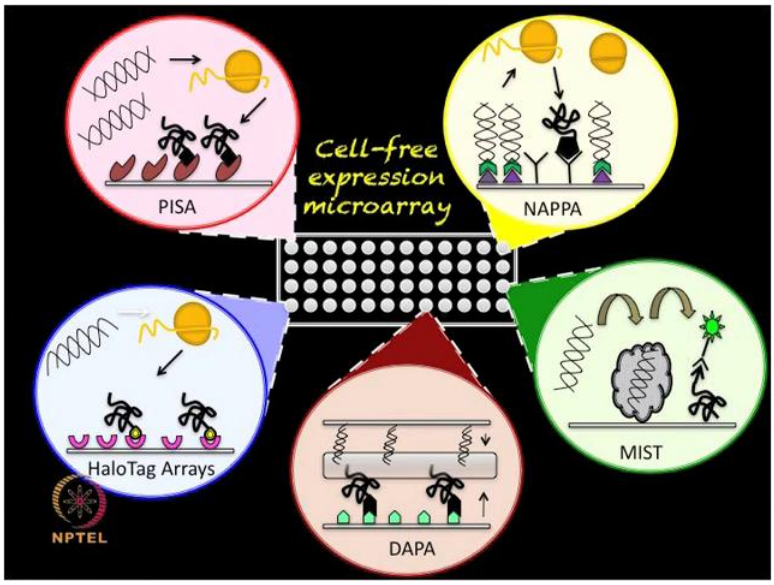
Proteomics: Principles And Techniques
Prof. Sanjeeva Srivasatava
Department of Biosciences and Bioengineering
Indian Institute of Technology, Bombay

Lecture No. #30
Generating Protein Microarrays:
Focus on Nucleic Acid Programmable Protein Array

Welcome to the proteomics course in today's lecture,we will talk about generating protein microarrays and we focus more on nucleic acid programmable protein arrays also known as NAPPA.So, as you 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 for protein microarrays they generate proteins in the heterogonous system such asintarsia collide. 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 challenge.

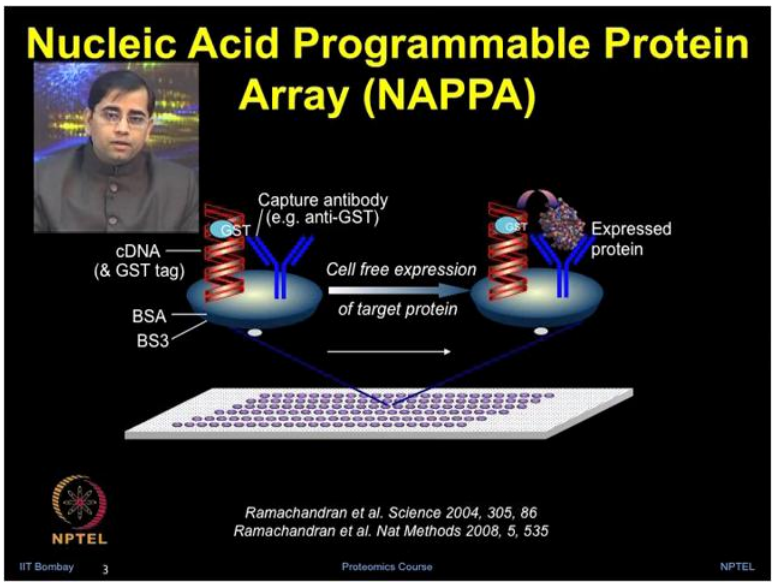
So, to overcome these obstacles posed by the traditional cell based protein purification methods, there various cell free expression based system have been generated. In the last lecture we discussed about cell free expression methods and how different type of protein microarray platforms has been generated by employing cell free expression based systems.

(Refer Slide Time: 01:48)



So, in the previous lecture we discussed about various type of cell free expression based protein microarrays, we talked about protein in situ arrays,halotag arrays, DNA array to protein array, multiple spotting technique, as well as nucleic acid programmable protein arrays. Although, each one of these technique offer, several unique advantages but, NAPPA approach has proved and overcome various limitations and it is applied for very high density arrays.

(Refer Slide Time: 02:41)

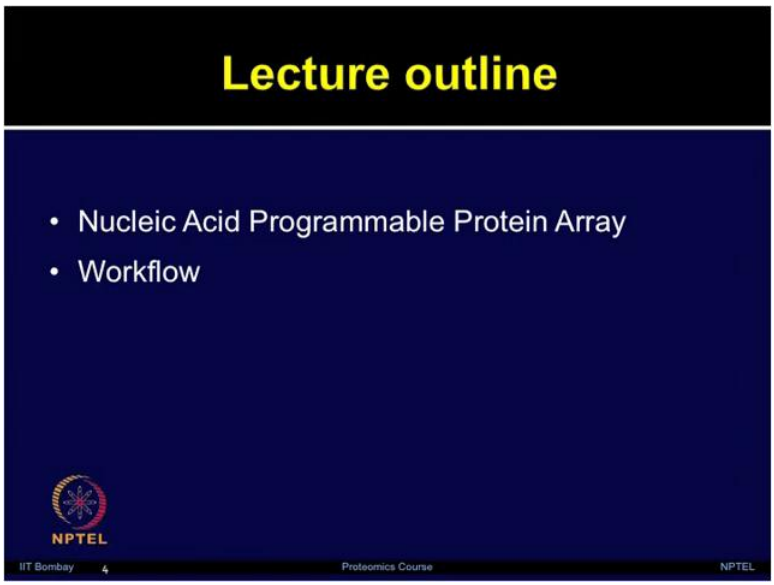


So today I will focus more on NAPPA arrays just to refresh you about nucleic acid programmable protein arrays, which we discussed in the previous lecture. As you can see in the slide, you have a microarray slide and each feature, which I have highlighted in the expanded view contains four features one of that is cDNA containing GST tag, then you have bovine serum albumin BSA protein, capture antibody such as anti GST and BS3 cross linker.

So in NAPPA approach plasmid, inter coding the target proteins or fuse with an affinity tag those are fixed with a array surface. The array surface is activated by the addition of a cell free expression system, which could be v germ expression system or rabbit reticulocyte lysate. Rabbit reticulocyte lysate is more preferred foreukaryotic 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 andanti 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 myc tag, you can use antimyc antibody. So these universal tag strategies can work out depending on what tag you use, you can use corresponding antibodies for the same. So,let us little bit overview of cell free expression basedsystems, different type of protein microarray platforms, which we have discussed.

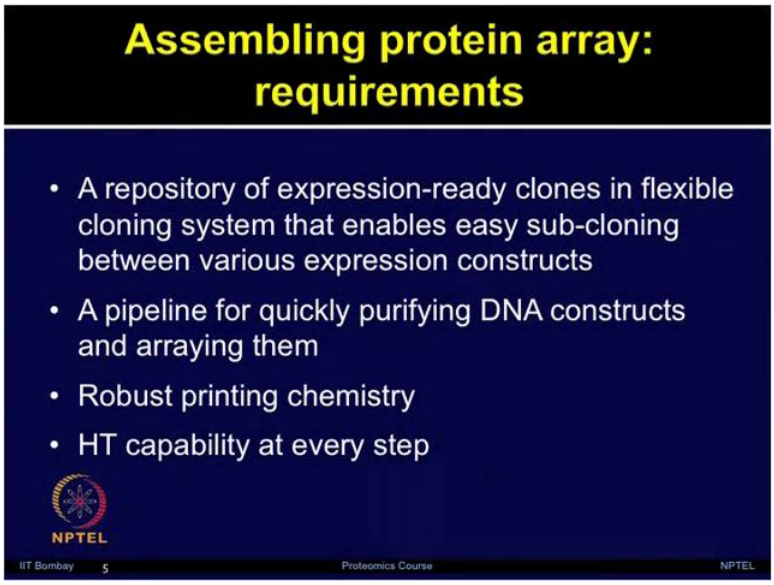
(Refer Slide Time: 04:33)



Today, we will focus more on how to make protein microarrays by using nucleic acid programmable protein array approach. I will walk you through, with the workflow a step by step, how various parameters one need to monitor. I will talk to you about each step for example, cloning and plasmid preparation, aminosilane coating of theglass slides, what type of printing parameters one need toensure 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 what different type of control features one need to have on these arrays, so that during various type of application, one can make sure that, they are relying on the good data. So we will walk through all these process in the workflow.

(Refer Slide Time: 05:42)

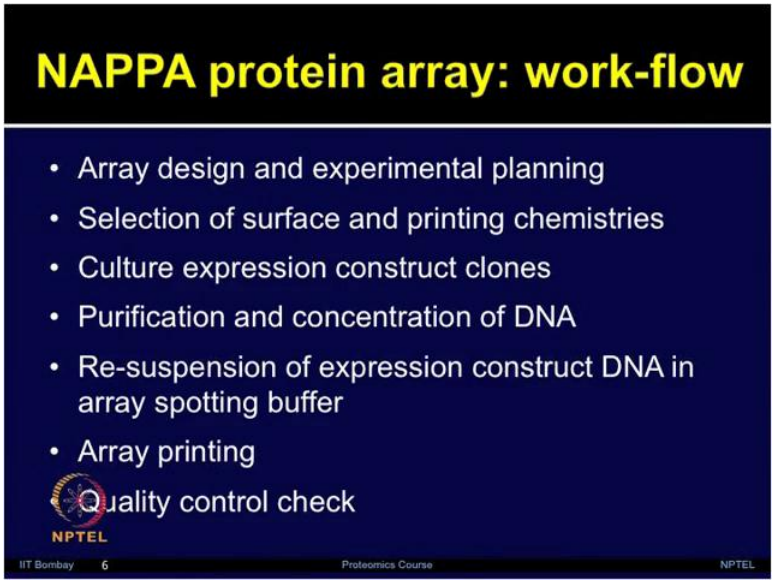


So, what are requirements to assemble the protein arrays? So in today's lecture when we will discuss about making protein arrays, that is quite similar for most of the cell free expression based protein microarrays and largely it can be appliedfor all type of protein microarray platforms but, it will be more specificin 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 recombination cloning which provides very quality master clones.

Now, those master clones can be easily transferred to the sub cloning different type of expression systems, so if you have different vectors you can transfer those from those master clones, then you need a pipeline for very quick probe 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 aimingfor 1000s of feature 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 workflow, we will talk about first of all, what is the array design and the experimental planning required for performing these arrays?

(Refer Slide Time: 07:26)



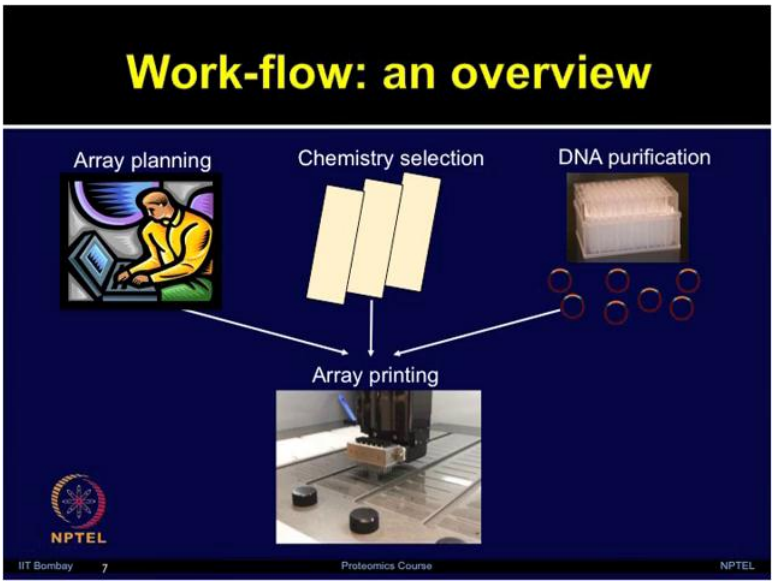
So, unlike other proteomic technique where one starts with unknown things and also after doing lot of experiments, then only they can identify the proteins by using mass spectrometry in the protein microarrays people are already aware, what type of contents are printed on the chip surface to start with and those contents has to be very well planned if you have a specific objective in your mind you want to study protein reactions, you need to have some good positive controls for the protein interactions, as there are some negative controls. You want to identify some biomarkers for example, you are looking for serum screening, you need to have again some good quality control spots, such as human I g g's or mouse I g g's.

So, lot of thought process goes into the designing the experiment for the microarrays, as oppose to what one do in the other type ofproteomic techniques. Then, what type of surface one need to select, what printing chemistry need to be used and since we are talking here about the self free expression base system, you need the DNA to start with, so how to express those and construct those clones. Now, once you have done the culturing, then you need to purify and concentrate DNA, almost all the molecular biology labs perform these experiments on day to dayroutine and it is very straight forward to do the DNA extraction DNA concentration etcetera. But, when we talk about doing the experiments in the high throughput, then it poses several challenges because the quality of all your features has to be very good.

So, various type of quality control checks, various type of observations one need to keep in mind for doing the simple experiments like even DNA purification and concentration. Then once your DNA is prepared, then you need to mix that with a 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 proteins by using the this cDNA.

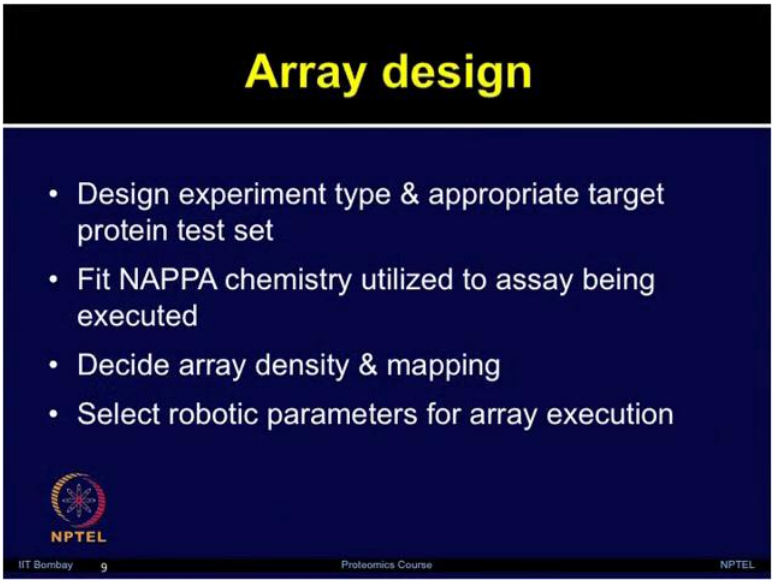
Now, array printing how to ensure that printing quality is good and then different types of quality control checks are required. So, this has overview one need to do the experimental planning, how to design those arrays, what type of all the features are going to be printed on the array surface, one need to design these experiments very thoughtfully, what is the features going to be printed on this arrays.

(Refer Slide Time: 10:19)



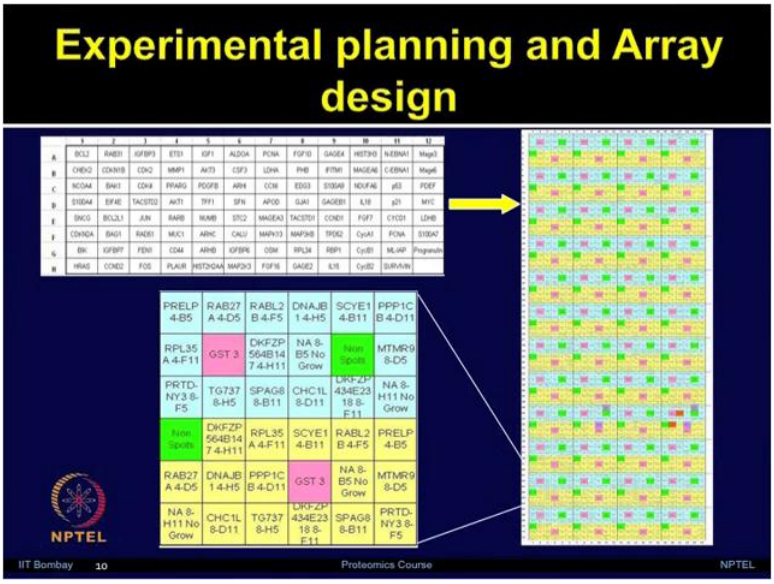
What type of chemistry need to selected, how to purify the DNA and once you have the slide which is functionalized, that is ready you have the DNA in the printing mix then, all you need to do print those on the large number of slides and you have chips ready for various applications. So,let us start with the Array design. First of all, as I mention lot of thought process goes even before starting this experiments, one need to do literature review, one need to look for their experimental objective very carefully even before moving forward for any of the printing steps.

(Refer Slide Time: 11:00)



So, design experiment type and appropriate target protein test set, once your test set works fine then, one can actually expand that for the large number of 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.

(Refer Slide Time: 11:36)



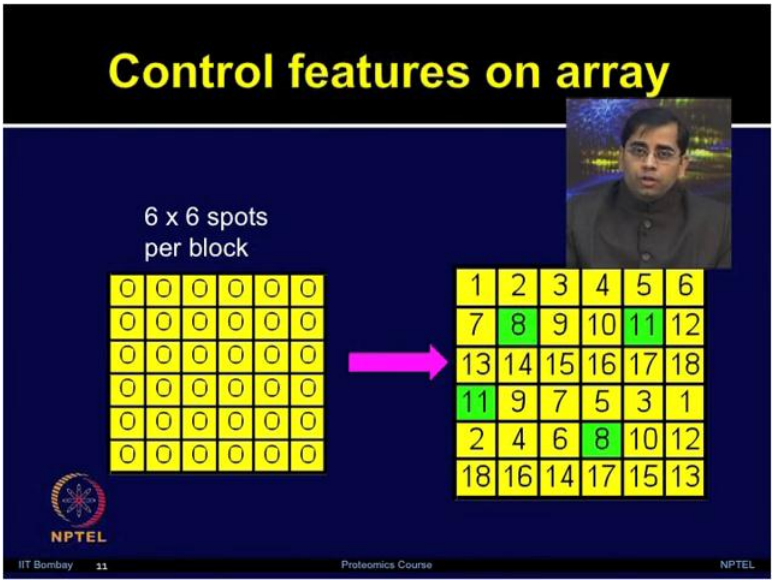
So, all this steps has to be very well planned in advance. So, for example, I have show you few insets here, the very top panel in the slide shows different genes which you would like

to make on the 96 well plates while doing the DNA prepping, so first of all you have the clones and now you want to do the DNA prepping, you know in each well for example, a one what gene is going to be there, for example, BCL2 then in the H12 you have left it blank, now G12 similarly, like you have all the wells defined genes are inserted there.

Now, each one of this plate is only giving you 96 features, you need to have multiple blocks of 96 well plates, then you need transfer those into the 384 well plates, so that four of these plates can be transferred to 1384 well plate and now that 384 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 a very high density map, how different type of features can be printed on the chip, all these things have to be designed in fact prior to doing any experiment.

Now, each spot need to be printed in the duplicates, also you need to ensure that your control features are split throughout the array surface. For example, if you see the bottom panel, once which are highlighted the red, those are showing the GST proteins, then in the green they showing the non spots, so these spots are printed in the duplicate and they are spread on the array surface not in the adjacent to each other, so that you can look for the duplicate results, how close they are.

(Refer Slide Time: 13:46)

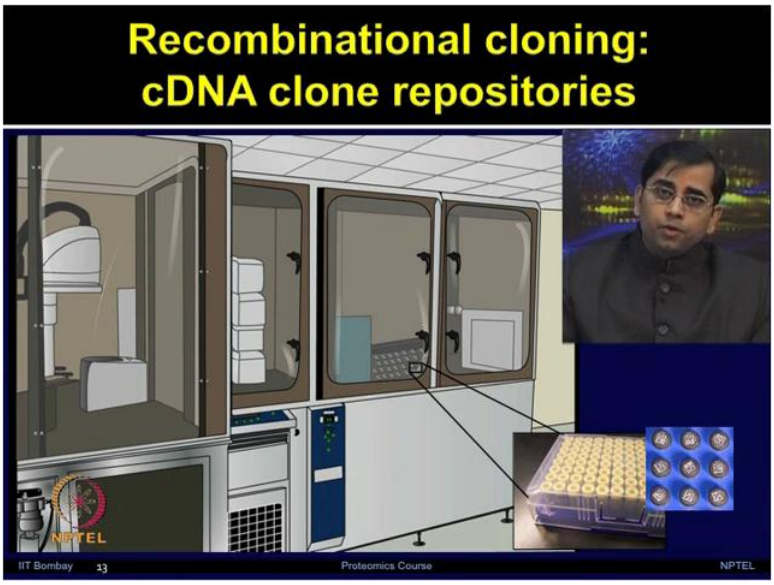


So, this is an expanded view here of a 6 by 6 spots per block, so these are the small blocks and obviously on the big array you have several features, yellow one shows the genes and

green one shows the control features. So for example, here one can use four different type of control features, non spot, no DNS control, a mouse I g g, GST protein, human I g g, ebna etcetera as I mentioned, it all depends on the objective of your experiment and then suitable controls can be incorporated in the array map.

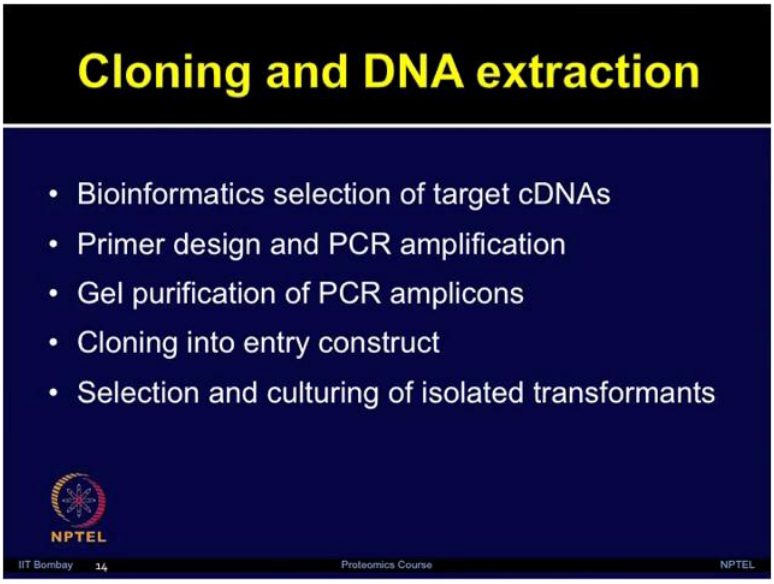
So, let us talk about cloning and plasmid preparation. In the last lecture, we talked about recombination cloning, so researchers have started to create such collection of cDNA's by using recombination cloning, which allows for the rapid transfer of DNA fragments from one vector to another in frame and without any mutation.

(Refer Slide Time: 14:48)



Now, I have shown you earlier that how this clones are stored, this master clones which are almost similar to the gold quality, now those are collected in these repositories, we have several good repositories including at Harvard institute of proteomics at Arizona university MCG clone and there are some other commercial repositories as well, so these clones are sequenced verified, they are in theusually gateway or creatorvectors, which can be transferred to the suitable vector very easily. So, one need todo the cloning and DNA extraction, before performing the printing steps. So, first of all one need to the bioinformatics selections of target cDNA's.

(Refer Slide Time: 15:42)

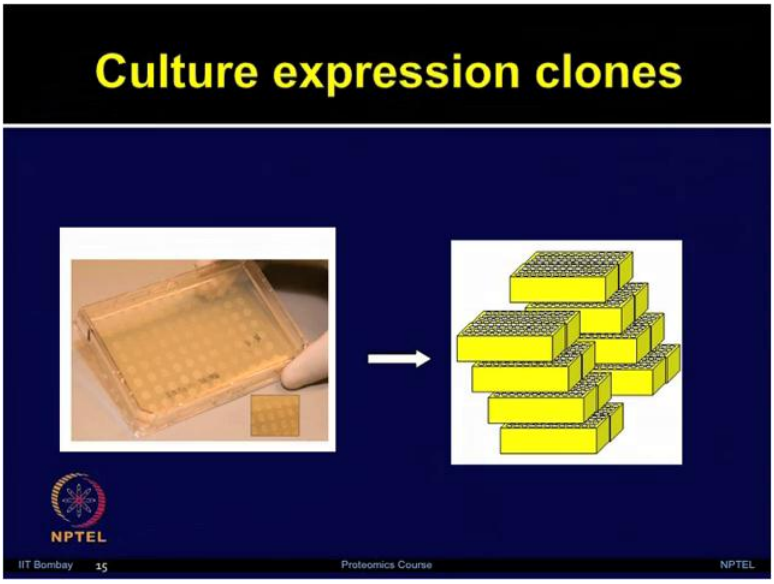


If, you are focusing on a particular cancer or a particular disease, obviously you have to think of what are all the genes which could be involved, in that disease, as well as different type of control features, so one need to do lot of literature search and bioinformatics analysis prior to looking for what type of genes need to be printed on the chip surface.

Then, once you have selected the genes of interest, you need to design the primer and use the PCR to amplify those clones, gel purify the right side bands and then move into the entry construct, then you need to transform that in Escherichia coli, select the right transform at colony and then obviously you would like to use those colonies for the long term, so it is good that, if you make the glycerol stock of the same and then you store it for the long purpose.

So, the DNA preparation process is very similar to whateverybody performs in the regular molecular biology lab, only difference here is that things and strips have to be automated and has to be in the more high throughput manner because, if you are doing the tubes you are doing 5 or 10 genes you cannot achievethe 20,000 density.

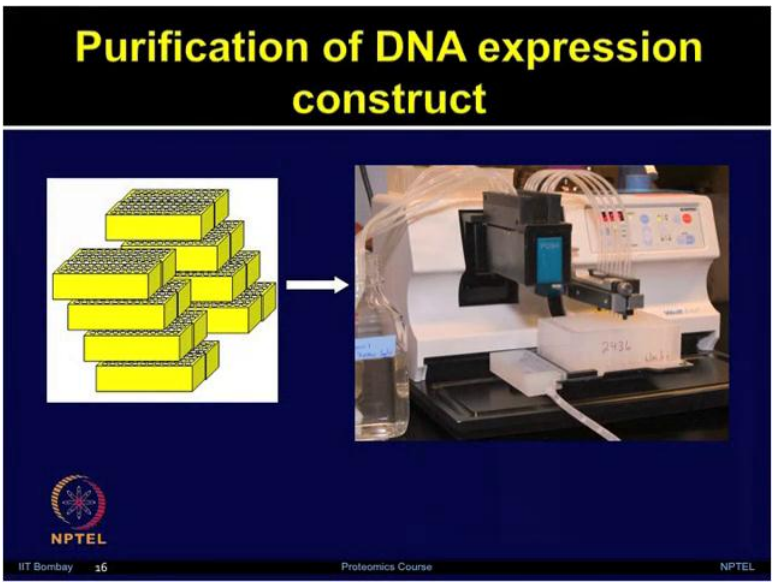
(Refer Slide Time: 17:03)



So, all this steps need to be automated for example, I have shown on the left slide the inoculated liquid cultures, on the plate l b 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 yourself, there is a good possibility of introducing various type of errors.

(Refer Slide Time: 17:39)

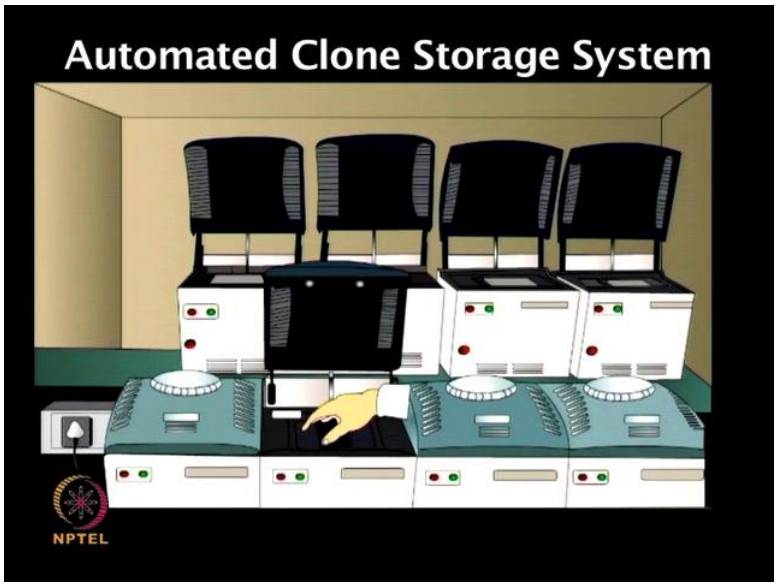


So, lot of these steps are automated, by using different type of liquid handling system, so can transfer the proper buffers, buffer solution 123 by using different type of liquid handling system and robotic platforms. Just an image here to show that different type of robotic arrays and platforms can be used for performing these experiments.

(Refer Slide Time: 18:07)

So, you can do the prepping in 96 well plate formats, so lysate can be loaded directly on to the DNA binding plates by using this type of robots. I will explain you the 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 the clone cDNA's representing the target proteins.

(Refer Slide Time: 18:53)

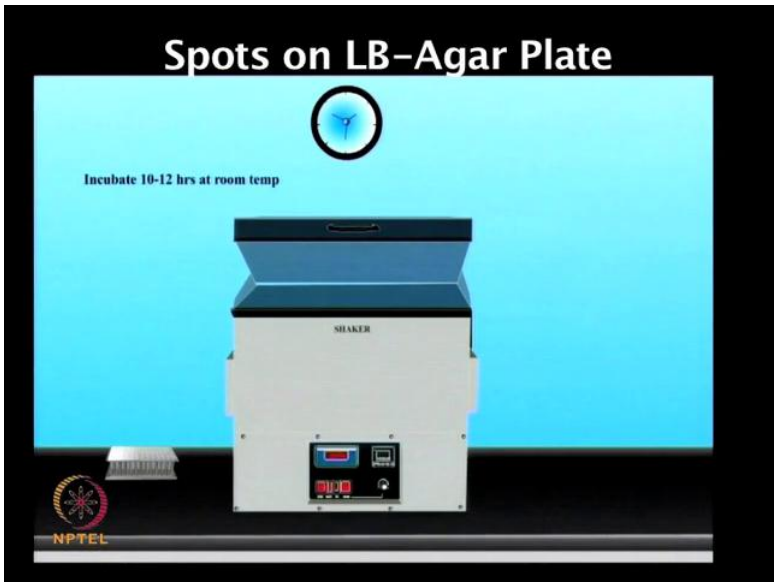


The cDNA clones are stored in the repositories, in minus 80degrees, all the genes should befully sequenced, the full length open reading frames, captured into a recombination based cloning system.

Genes of interest can be easily transferred into any expression vector, by simply adding the appropriate recombination enzymes. This process is highly efficient and enables the transfer of many genes, simultaneously into different expression vectors in a single step, in frame and without mutations, by using recombination cloning approach, the coding sequences can be transferred into any expression vector, in simple overnight reaction, after the cloning is done, perform the spotting of the glycerol stock under l b agar plate.

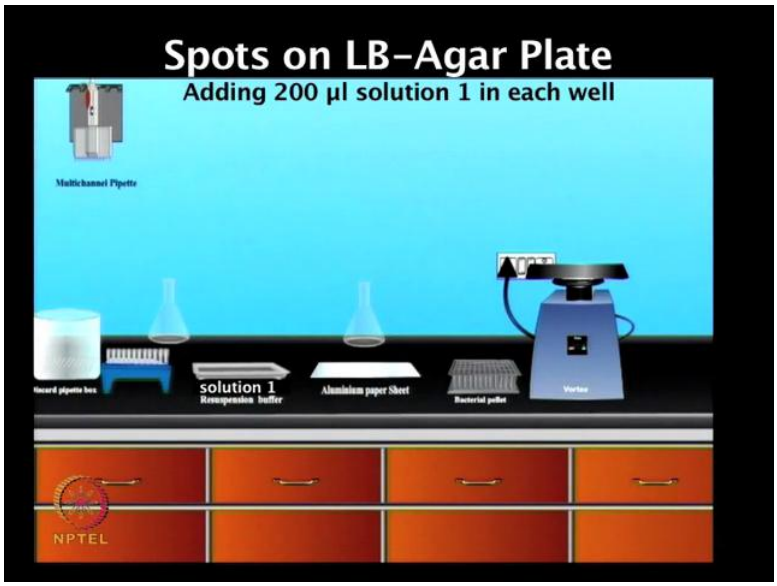
So, transfer the bacteria from the glycerol stocks to an l b agar plate that contains appropriate antibiotic,you can use that either using multi channel pipe tents or using liquid handling systems, its preferred to do this in 96 well plate format, so that you can avoid any miss labeling of the samples.

(Refer Slide Time: 20:27)



So, allow the bacteria to grow overnight at 37 degrees, before inoculating that into the liquid medium, so as shown here you can use a multi channel pipette or you can also use the liquid handling system. So, first of all you need to prepare the culture blocks and add the liquid broth in 96 well plate format, you have to ensure that you have added the antibiotic solution in each well, once culture blocks are ready then, inoculate the liquid culture from the bacteria grown on the agar plates. Similarly, you can fill all the wells, then cover the deeper block with the permeable seal, ensure that sealing is proper and then incubate the cultures on a shaker, you can grow the culture at 37 degrees at 300 rpm,ensure that shaking is uniform. After these blocks have completely grown, the bacteria for the overnight or 10 to 12 hours of incubation then we need to pellet down these blocks.

(Refer Slide Time: 22:51)

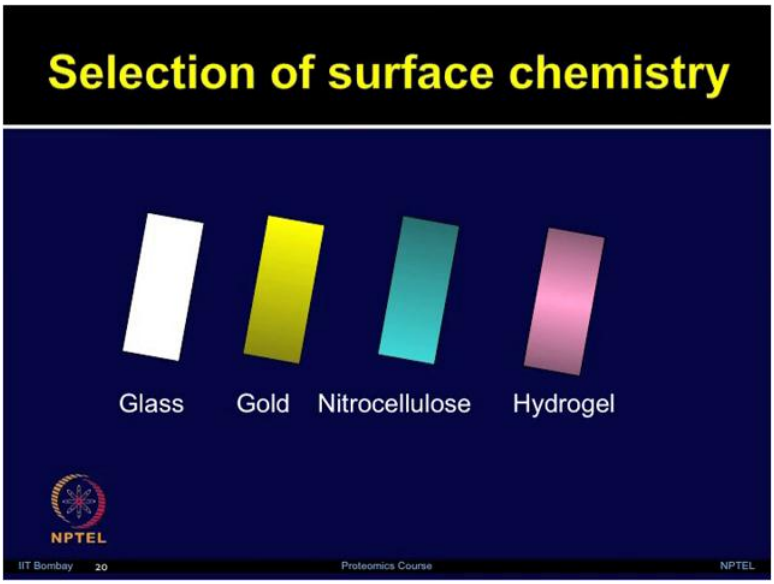


So, you can centrifuge these blocks for 15 minutes at 2000 to 2500 rpm, while doing centrifugation, ensure that centrifuge is balanced, so once centrifugation is complete then the bacterial pellet will be in the well and then you need to remove the supernatant. So, remove the supernatant from these bacterial cultures, discard the tips and once you have completed that, for all the wells then you are ready to perform the Dena plasma isolation. So, remember your basic molecular biology and you need to prepare the solution 1, 2 and 3 for doing the plasmid preparation. So, at 200 microlitre of solution one, which is recess mentioned buffer that contains 50 mill molar of tripsthat contains p h 810 mill molar of e d t a and 0.1m g per m l of ribonucleic.

So, you need to add the recess as mentioned buffer, around 200 microlitre, to each well. So, remember in the last step, we had made the bacterial pellet from those cultural blocks and now you can add this 200 microlitre of recess mentioned buffer in each well after adding the solution then, you can close the plate by adding a cover slip. Once, the seal is placed then you need to vortex it, one can also do it by pipetting up and down or doing the vortexing few times, in similar way add 200 microlitre of solution 2 to each well solution 2 contains 0.2 normal NaOH and one percent of sodium codicil sulfate, this is lyses solution, after adding this solution, again you need to repeat the same process of adding 200 microlitre of solution 2 to each well seal the plate and then invert the plate few times.

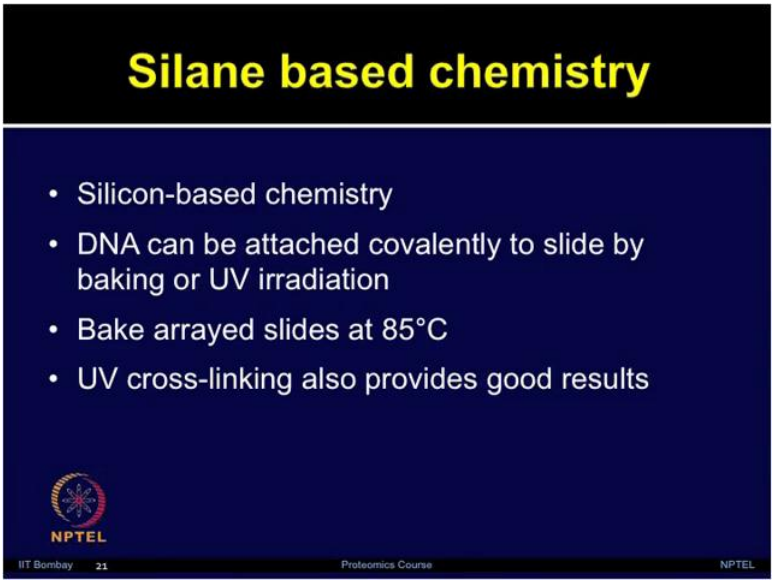
Once, solution 2 is added make sure that, you invert the plate few times, now add 200 microlitre of solution 3 to each well, similar to what we have seen earlier, the solution 3 is the neutralizing solution, which contains 3molar of potassium acetate at pH5.5, now centrifuge the block, at maximum speed for 20 minutes, at the room temperature, once centrifugation is complete, then you need to discard these 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. So, let us talk about Aminosilane coating here, so one can use different type of surface for example, glass goldnitrocellulose hydrogels etcetera.

(Refer Slide Time: 28:05)



The different type of properties, one need to look for, while selecting the right surface obviously, cost is one of the major factor, when you are talking about experiments such as biomarker discovery, you need to use 100 of chips for screening, 100s of patients. So, if you have glass slide which is of low cost, then that will be more affordable for largeexperiments. So, gold is obviously very high cost so as nitrocellulose and hydro gels, the reactivity for the glass is moderate, so as nitrocellulose and hydro gels but, gold has low reactivity, absorption to surface that is low in the case of glass and gold it is high in case of nitrocellulose and hydro gels. Now, compatibility for the mass spectrometry, that is only applicable in case of gold surface. Now, these surfaces can be derivative by using different type of chemistries, including amino groups or trioester.

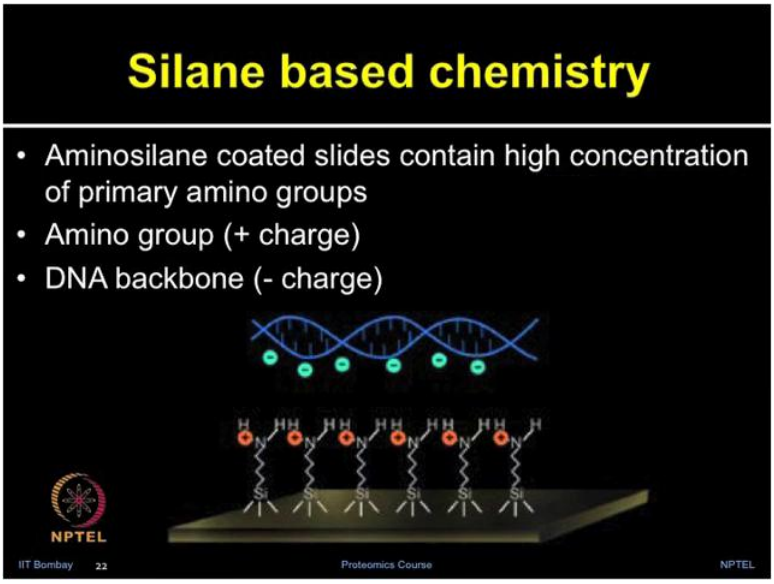
(Refer Slide Time: 29:16)



So, let us focus more on the Silane based chemistry. We want to talk about printing the DNA on the chip surface, for using cell free expression based NAPPA protein microarrays. So, silicon based chemicals, they contain two types of group the inorganic groups such as, alkoxy groups like methoxy or ethoxy or oregano functional groups such as, amino epoxy viral etcetera.

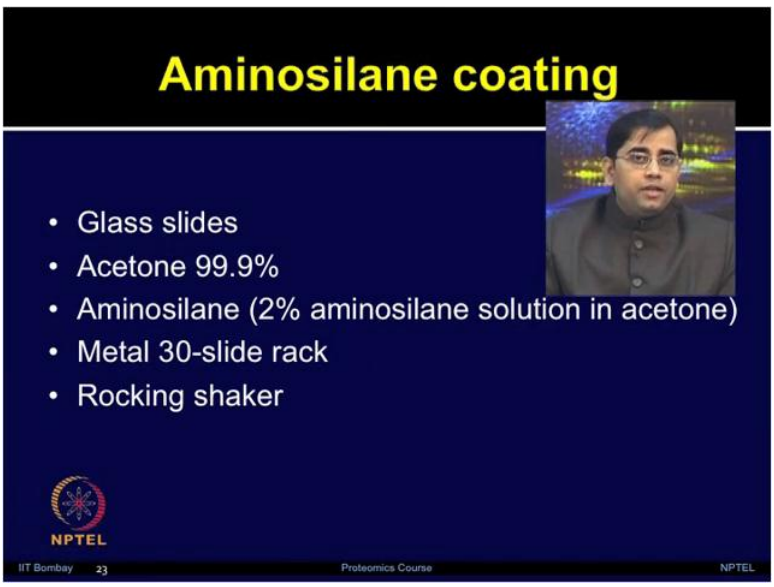
So, the DNA can be attached covalently on the glass surface by baking or UV radiations, one can bake the arrayed slide at 85 degrees also UV cross-linking is a popular method which also provides good result for DNA mobilization Aminosilane coated slides, they provide high concentration of primary amino groups, availableat the chip surface.

(Refer Slide Time: 30:15)



Now, why we need to use this chemistry, so amino group provides the positive charge and how that will help, one placed in the contact with the neutral aqua solution, the groups becomes positive charge, now DNA backbone that provides, the negative charge, so DNA backbone will form the multiple ionic interactions, as you can see in the slide with positive charged, amino surface coating. For performing Aminosilane coating in the lab, one need to have the Glass slides, Acetone, Aminosilane usually 2 percent, Aminosilane solution in acetone, works best then, you need metal slide racks and you need rocking shaker.

(Refer Slide Time: 30:55)



In this slide, I have shown you various steps involved in performing Aminosilane coating, on the left side a rocking shaker is there, in which a glass tray contains metal rack, containing 30 slides, on the right side in the inset, you can see those 30 slides are placed in the metal rack, which is submerge in the Aminosilane solution.

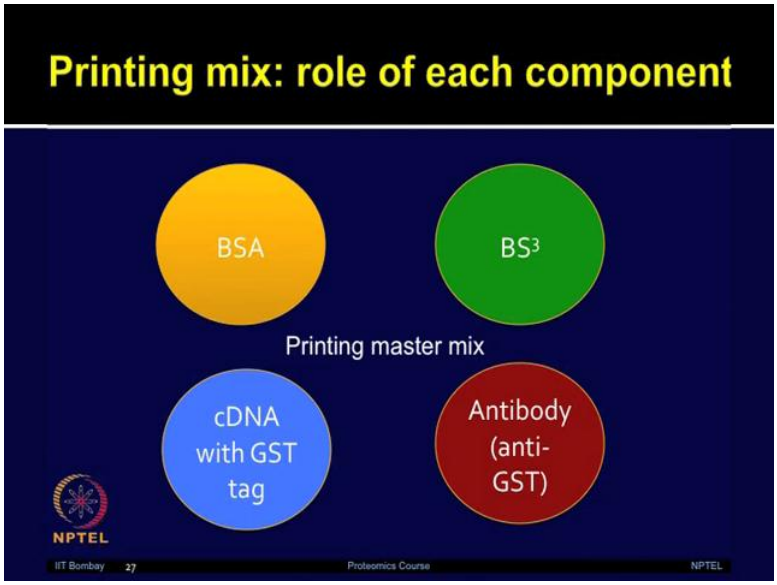
(Refer Slide Time: 31:16)

At the bottom panelsof the right side, I have shown that these slides, can be either centrifuged or dried with the compressed air and then those are ready for further printing purpose.

(Refer Slide Time: 31:55)

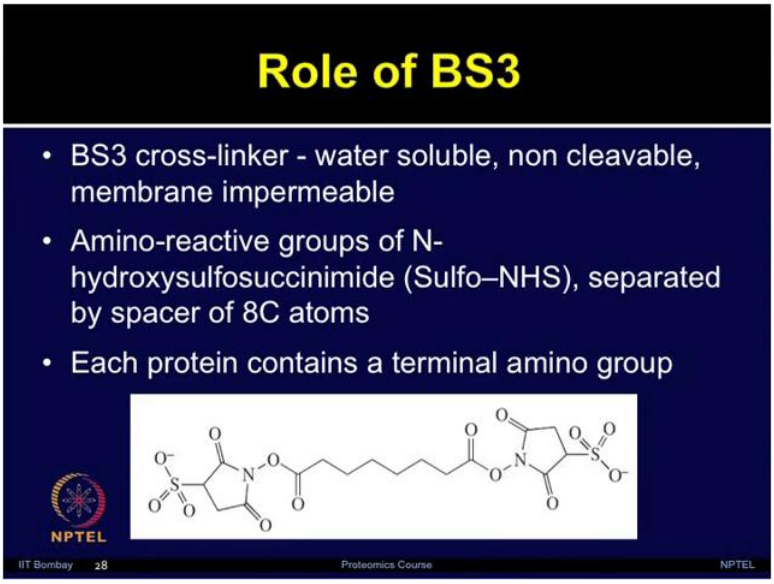
So, let me show you this animation, how to do the Aminosilane coating, prepare 300 m l of Aminosilane coating solution, which is 2 percent of Aminosilane reagent in acetone, place the slides in metal rack, treat glass slides in Aminosilane coating solution for around 15 minutes in glass box on shaker, rinse with acetone and followed by brief rinsing with Mill-q water, after washing steps are done then, you can spin dry on the speed rack. So, now let us talk about printing arrays, you have DNA ready as well as, you have the glass slide, functionalized that is also ready, now you need to think about how to print those features on these strip surface.

(Refer Slide Time: 32:54)



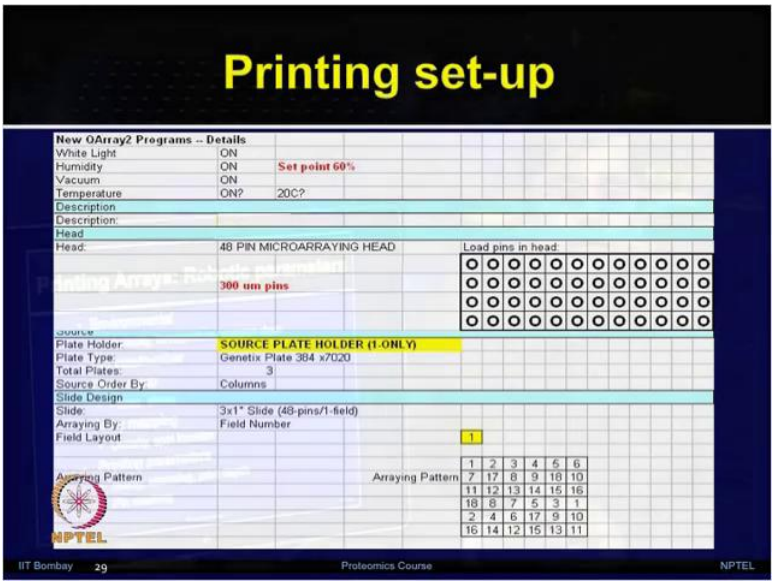
So, first of all you need to make a master mix or printing mix and in case of NAPPA as I mentioned earlier, you have four features BSA, BS 3, the cDNA containing the GST tag as well as, an antibody which is anti-GST.So, let me briefly explain you, the role of each of these four component, so BSA dramatically improves the DNA binding efficiency, BS 3 BSA and capture antibody they are coupled to the amine coated glass surface, where the activated ester terminated homobifunctional cross-linker, the cDNA contains GST tag or you can introduce any other tag, thenyou 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 four components ready then, you are ready to perform the printing.

(Refer Slide Time: 34:18)



So, what is a role of BS 3 cross-linker, it is water soluble, non-cleavable and membrane impermeable, the amino-reactive groups of an hydroxysulfosuccinimideSulfo-NHS are separated by the spacer of 8 carbon atoms and each protein contains a terminal amino group.

(Refer Slide Time: 34:34)

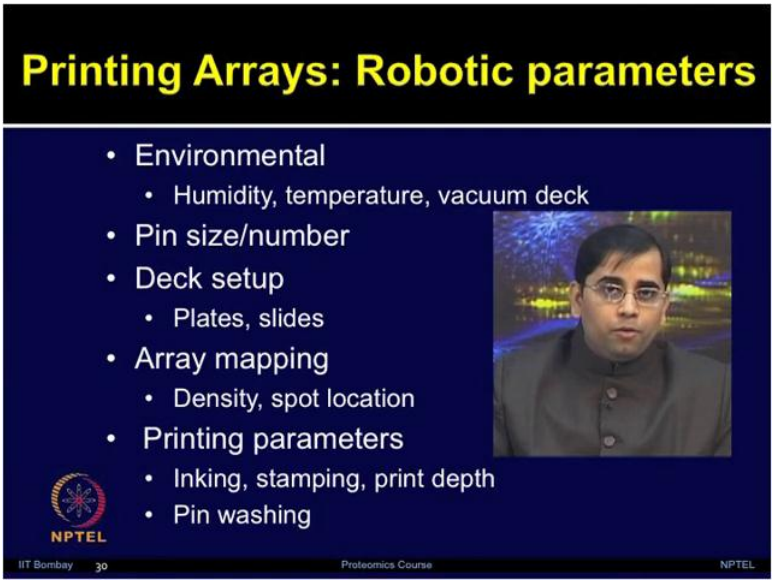


Now, once you have these components ready, you are ready to perform the printing. So, on the software's you first need to define various type of parameters, you need to define what type of prints you are going to use for example, one can useeven 48 prints for printing

several feature simultaneously or even go as low as 1 or 4 prints, which could be of different type of microns like 300 microns, 150 microns depending on what type of density you want to achieve, you definitely need to ensure that there is good humidity, maintained throughout the experiment.

So, at least 60 percent of humidity should be there, you need to define the source plate which you are going to use for printing whether, you have 1 plate or you have multiple plates and what type of arraying pattern, these 48 means have to follow, all those things have to be written in the programs on the software and then once you place the plates 384 well plates, containing the master mix, those can be used for printing purpose.

(Refer Slide Time: 35:59)

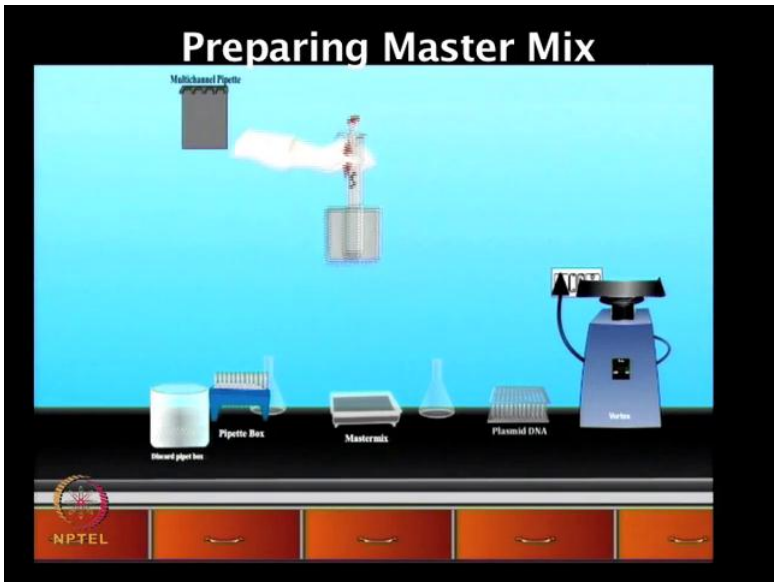


Now, one need to ensure different type of robotic parameters are in place for example, environmental conditions such as humidity, temperature, vacuum check all these things need to be ensure before you place your plates for printing. The pin size and the number of pins again depending on the density which you want to achieve, how you want to set up the deck for printing, you want to use the plates, you want to use the slides, how many number of slides you want to use, again it depends on different type of arrays which are commercially available, one can use large number of slides, array mapping that is already you have planned experimentally before hand, what density you want to achieve, where each of your genes are going to be located on the map.

Now, 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 issufficient,so how the pin washing is performed and the you definitely like to track and log all of this detail so that if during a large printing anything goes wrong, you can actually troubleshoot and know that at which step things have gone wrong.

So, printing good quality arrays is very challenging but, once you have the good quality arrays in your hand, then you can perform different type of experiments.So, let me show you this animation, to explain you the procedure master mix preparation as well as, printing procedure.

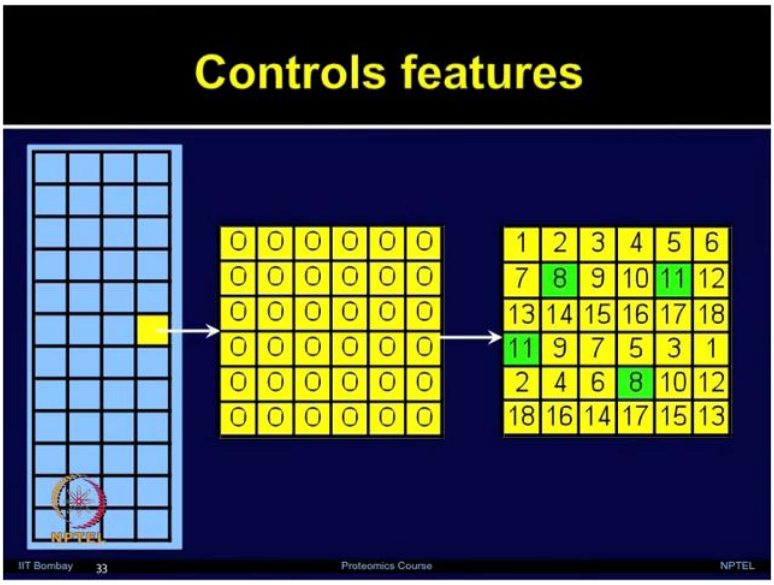
(Refer Slide Time: 37:34)



So, prepare enough master mix, so that you can do printing for large number of arrays, the master mix contain DNA, polyclonal GST antibody, BSA and BS 3 linker for detailed recipe of each of these reagent you can follow the publication manuscript by Rama Chandra natal 2008 published in nature methods, once master mix is prepared, you can mix it well and then transfer 20 microlitre of master mix, to the block containing plasmid DNA, once the master mix is added to all the wells in the block, then it would shake the plate for 15 minutes, for printing you will require 384 well plates, now you need to transfer your master mix containing plasmid DNA from the 96 well plates to the 384 well plate. Again, these steps can be performed either, using multichannel pipettes or by using liquid handling systems, most master mix is prepared and transferred into the 384 well plate, we can

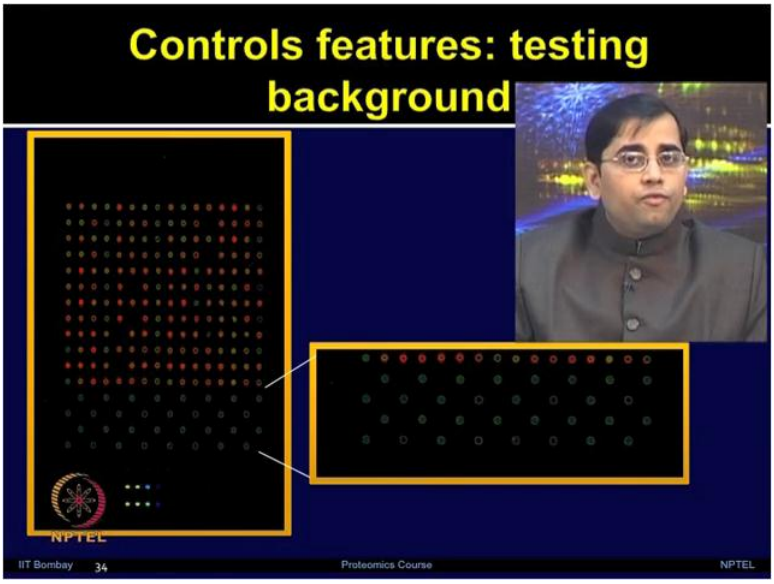
perform the printing step, so we can use these microarrays to print the DNA and the master mix on Aminosilane coated glass slides, first you need to ensure that pins are washed thoroughly, you can wash with ethanol and water and during entire printing procedure we need to ensure that, humidity is maintained at 45 to 60 degrees, one can use different type of pin heads and different type of microarrays for printing the chip.

(Refer Slide Time: 41:03)



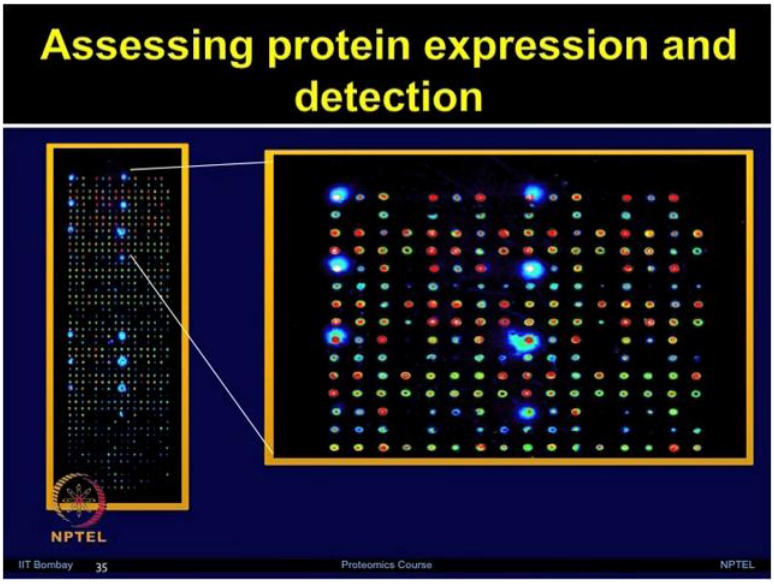
Now, this master mix can be printed on the chip, so what are different types of control features you need to put on the array. Let us have a brief discussion on that, so as briefly mentioned earlier, you need to ensure that different type of control features are spread across on the array surface and these could be mouse I g g, no DNA, non spot Edna human I g g's etcetera.

(Refer Slide Time: 41:25)



Now, to test the background you need to have the certain control features, now since we are talking about NAPPA arrays we are talking about the clones containing the GST tag. So, it is a good idea to have the different type ofspots with the GST alone, so you can have the purified GST in the dilution series, so that you can use that for doing the calculation for protein expression later on.

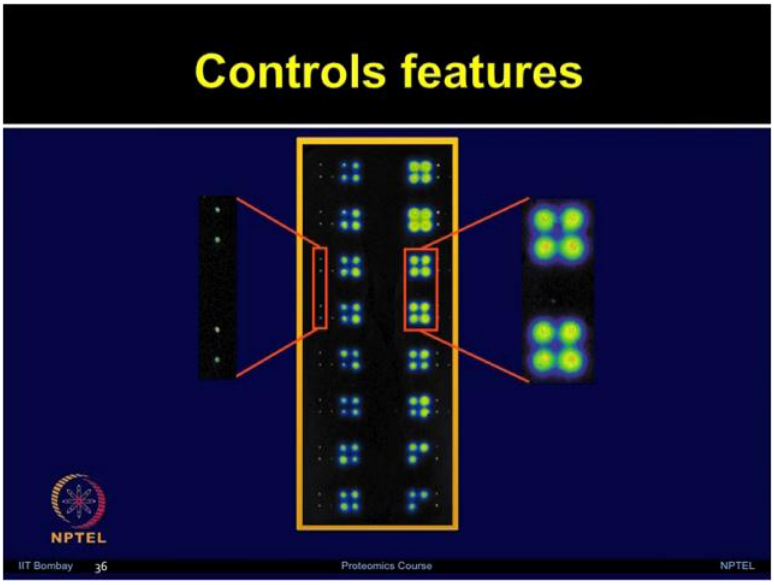
(Refer Slide Time: 41:57)



Now, if you are looking for different type of immunological response, biomarkers discovery etcetera, you need to ensure that I g g is printed at the varying concentration, to assess the

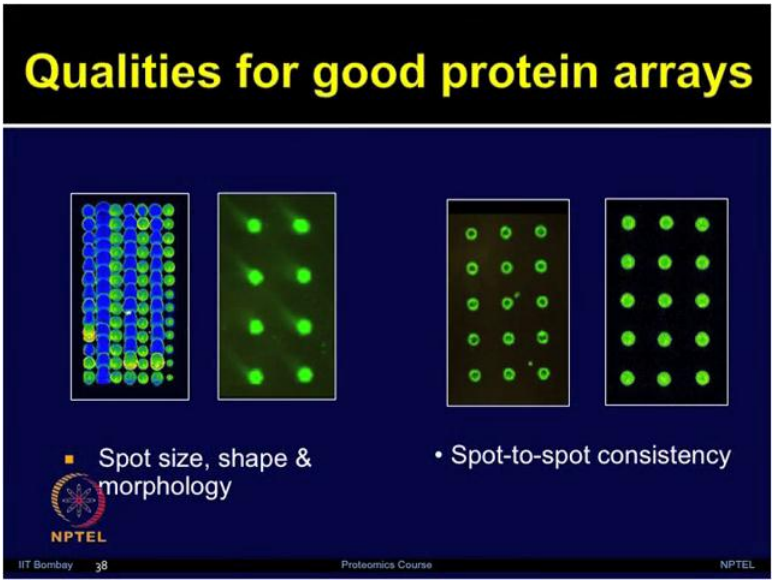
immunological responses, such as I mentioned for skinning human biomarkers, it can also used for the statistical analysis later on by the biostatistician, so that different type of protein expression can be normalized.

(Refer Slide Time: 42:26)



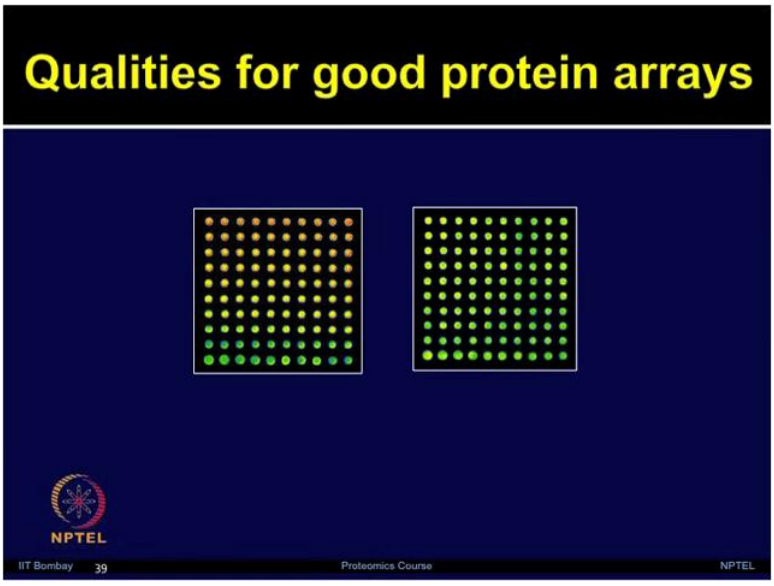
So, another control feature and the QC check here shown on the slide, same gene printed four times in the blots repeated and then negative controls are used to give an idea for the background. Now, what is the different qualities one need to keep in mind for good protein arrays? Obviously, you like to know the spot size, spot shape and morphology those have to be uniform, so spot to spot consistency has to be there, positional consistency has to be there for the large number of slides sample loading, sample integrity as well as, different type of controls replicates and positional flexibility all those need to in place, for good quality protein arrays.

(Refer Slide Time: 43:11)



This shown here,one can actually encounter a different type of problems, while printing these arrays, so shown on the left is spot size shape and morphology, shown on the right is spot to spot consistency and many times these type of a printing issues do come, while performing these high through put experiments, so one need to ensure that all these parameters are a properly quality control checked, again we are looking at the spot morphology.

(Refer Slide Time: 43:47)



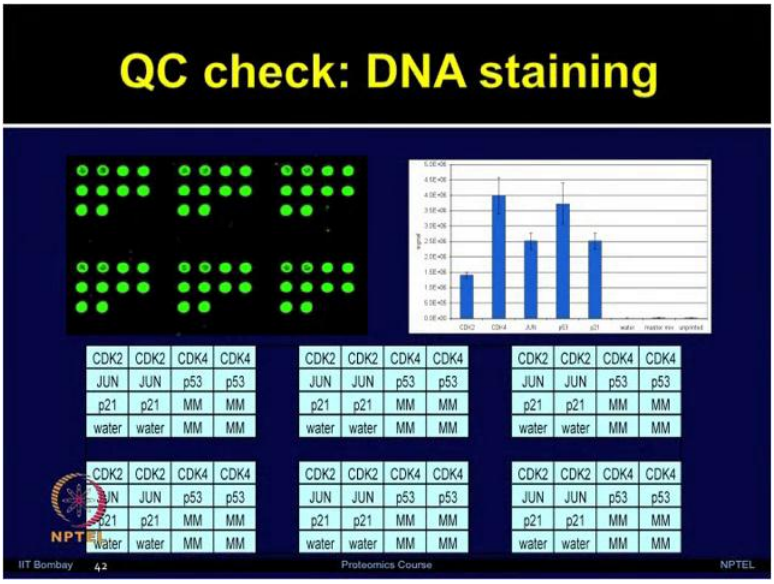
And the uniformity of this spot, one column think of various type of parameters which could be useful for example, spotting solutions, spotter capability, maintaining the temperature, maintaining the humidity conditions, keeping the dust free environments etcetera. Now, printing the quality arrays, that is very useful you need to have the precise liquid handling systems, as I mentioned that each step can be automated, for the same you need to do the very straight tracking of the entire process.

(Refer Slide Time: 44:13)

So, that you can troubleshoot, if anything goes wrong. It is a good idea to build d t l log history for each step, starting from the cloning, DNA preparation, printing and etcetera. Now, if you have done this step by step optimization and evaluation, there is less chances of making any errors because, on the high through put approaches when we are talking about 1000s of feature, it becomes very essential that everything has to be tracked throughout and logged in detail in the log books. Now, let us talk about we have done the printing, we have ensured that our printing has very good quality, now can we use these NAPPA arrays for doing thefurther biological experiments.

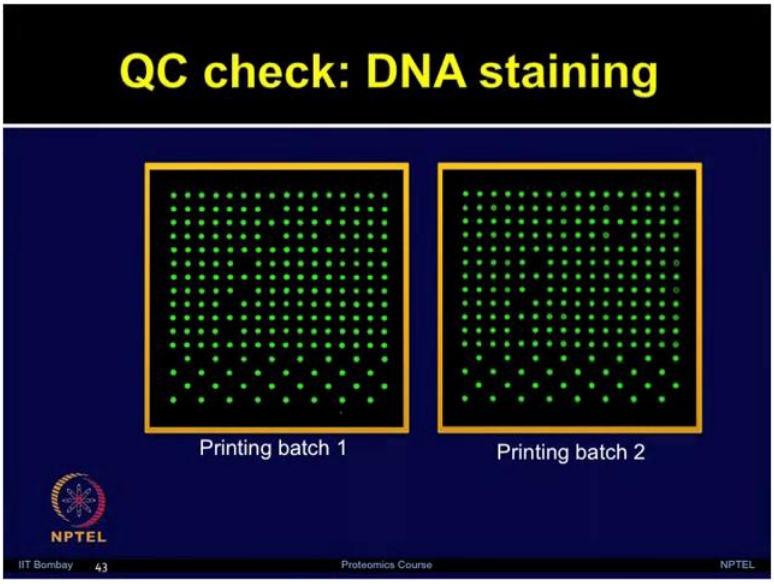
So, first of all since we have printed the DNA we need to ensure that DNA printing is good, so to assess that one can use the Picogreen staining and next once the DNA quality is good then, one need to ensure that protein can be expressed from those DNA, by using in vitro transcription and translation system, so the protein expression need to be quality checked.

(Refer Slide Time: 45:32)



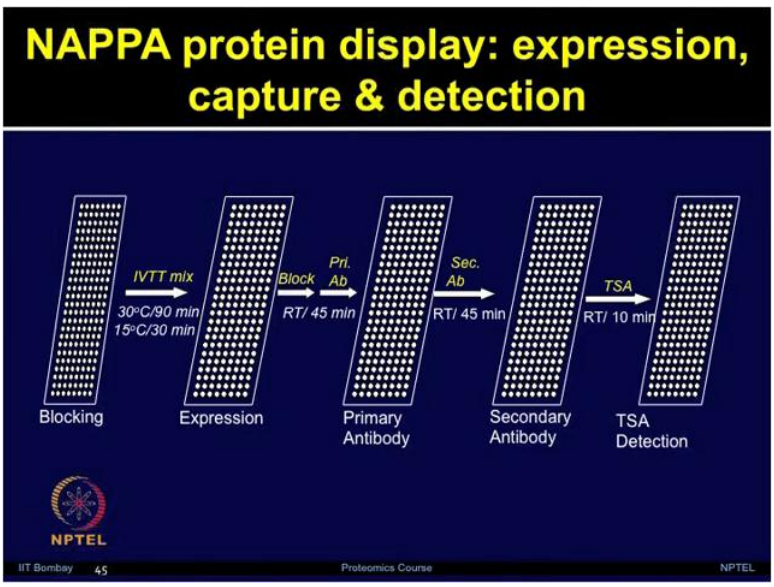
So, as shown in the slide, a very small test array, which I used to teach in the cold spring harbor to the students and these are actually made by those students. So, in the bottom you have a map,where only five genes are there CDK 2,CDK 4, Jun p 53 and p 21 each one of those genes are printed in the duplicate, then you have master mix and you have water a very simple proof of concept test array, now only 5 genes should have the DNA, so if you do the Picogreen staining to observe the DNA quality then, as you can see o the upper panel, those green spots are lighting up showing that DNA is printed properly, one can use the different type of statistical tools for analyzing, how good the DNA is printed on the chip surface, which is shown on the graph in the right side.

(Refer Slide Time: 46:37)



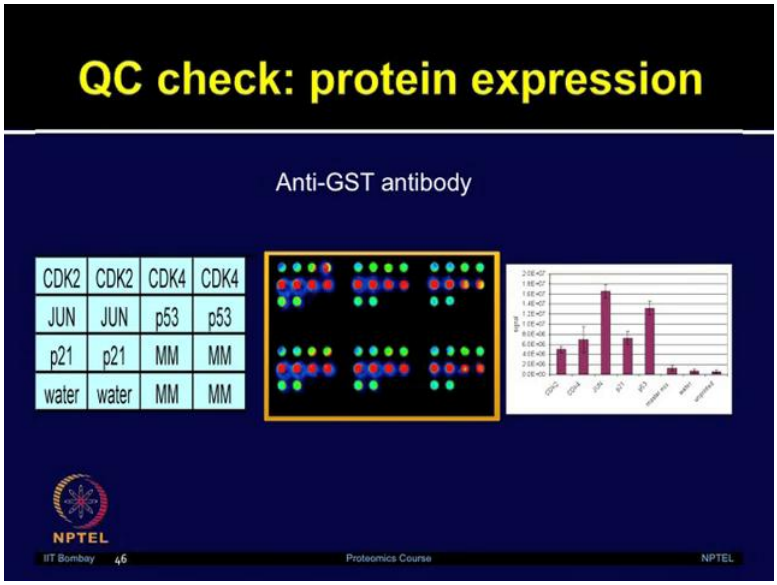
Now, different batch to batch variation need to be ensured because, if you are printing 200 slides 100 in each batch, you need to ensure that all the batches can be used for one experiment and those data can be compared, so always printing batch to batch should be ensured for both DNA staining as well as the protein expression. Now, when the DNA is quality control checked then we need to move on to the protein expression testing. This is the schematic showing that, what are different steps involved in performing at a NAPPA based protein expression.

(Refer Slide Time: 47:08)



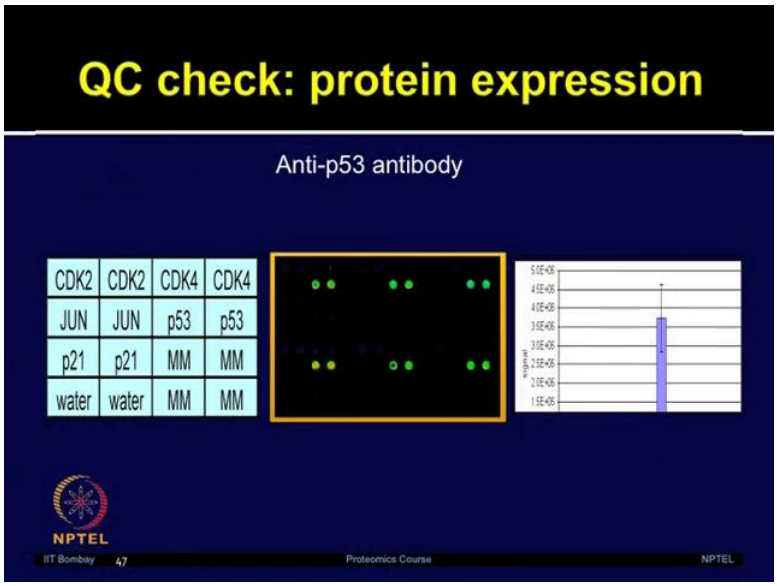
So, you have the slide, you need to block those then, use the in vitro transcription translation system, so that proteins are expressed and now you want detect the signal, so you can add the primary antibody and digest in this case and then followed by the secondary antibody containing either cy 3 or cy 5 labels or you can use the h r p based tar amide signal amplification system, TSA base systems.

(Refer Slide Time: 47:42)



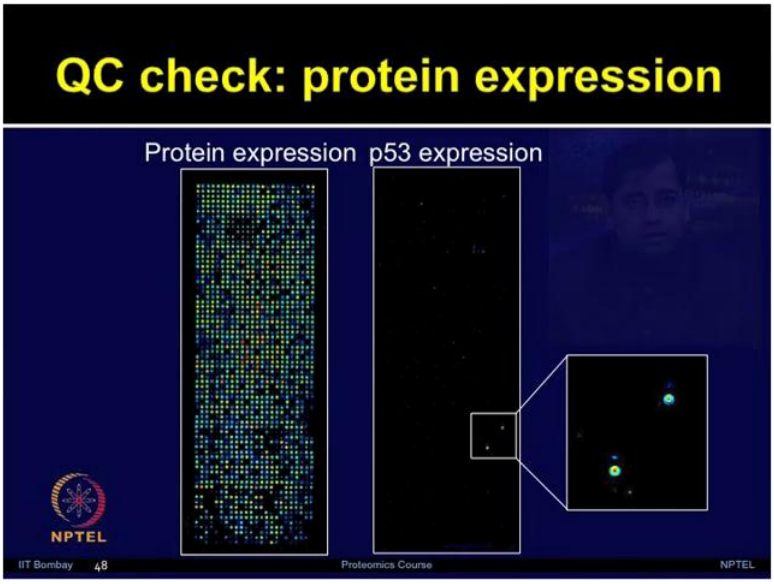
So, shown here is again a proof of concept,array map with only 5 genes and these proteinsneed to Q cied here, so one can use the anti GST antibody and as you can see only 5 proteins in duplicates are expressing where as master mix and water are blank and same can be plotted on the graph, which is shown on the right hand side. Now you have assess that you have good protein expression.

(Refer Slide Time: 48:04)



But, how specific that protein expression is, to ensure that protein's specific expression one can use the specific antibodies. For example, in this case we have p 53 also on the chip surface, so we can use anti p 53 antibody and as you can see in the middle panel, only one pair of the spots are lighting up, which shows that only p 53 protein can be detected, if you use the anti 53 antibody on the right side, the graph only one spot is showing the graph bar.

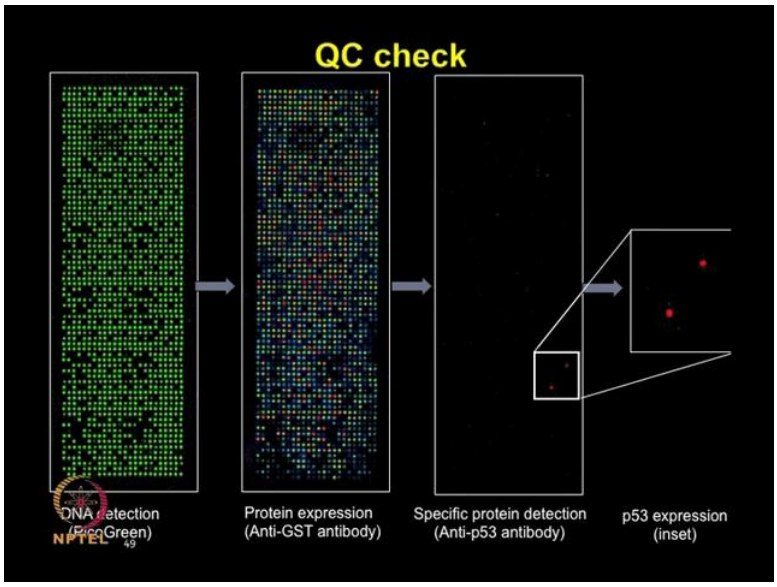
(Refer Slide Time: 48:39)



So, it shows that in a high density arrays similar type of quality control checks can be performed for the protein expression, shown in the left side p 53 expression or the protein

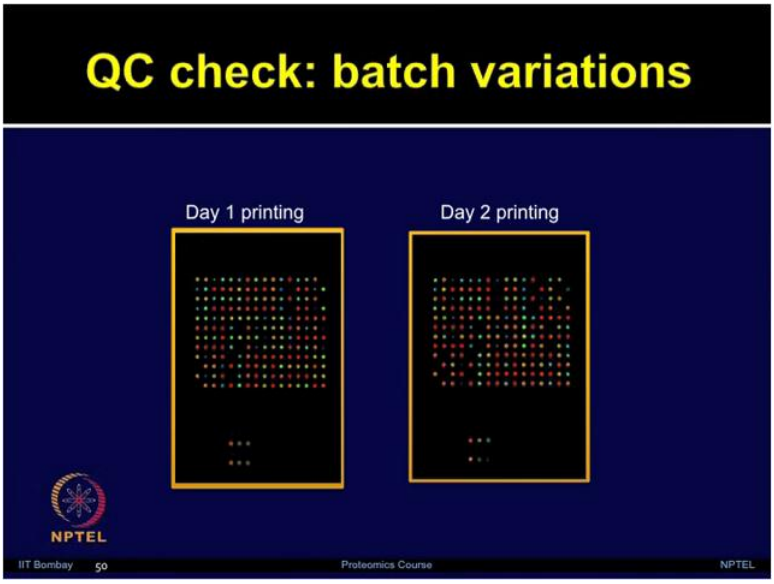
specific antibodies in the middle panel and the inside showing that how duplicate spots can be looked at, so those duplicate spots are for the p 53 proteins. This is an over view of the different type of quality control checks, which one need to perform during the whole NAPPA step.

(Refer Slide Time: 49:05)



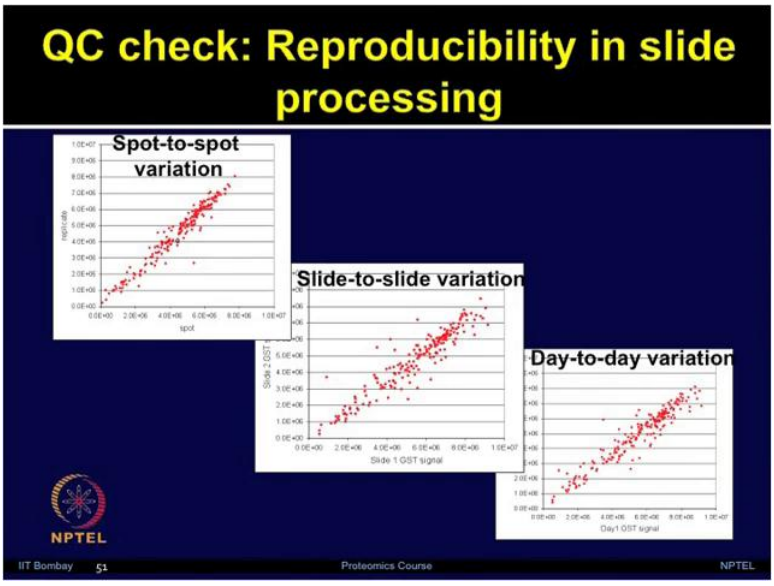
Before, you use these slides for doing any biological questions; you need to ensure that DNA is properly printed, shown in the left side in the slide Picogreen staining next to that is the protein expression, by using the anti GST antibody. Next, is the specific protein expression for example, using anti p 53antibodies and then looking at the inset for those duplicate spots, again one need to look for the batch to batch variation after the protein expression.

(Refer Slide Time: 49:38)



And, if you print the GST protein in the dilution series as shown side,which is printed in the bottom of the slide, those can be used for assessing how much protein is produce on each feature, so batch to batch c v, sample to sample coefficient of variation as well as replicate coefficient of variations should be calculated.

(Refer Slide Time: 50:01)



Now, once you have looked at day-to-day variations,and then also look at different type of other variation such as spot-to-spot variation, slide-to-slide variation and day-to-day variation, again plotted in this graph. It shows that NAPPA arrays are quite reproducible

and the r square is more than 0.95 in all the cases. So, let me show this animation for the quality control check, how to detect the DNA and the protein expression on these chip surface?

When the arrays are printed, then we need to perform the quality control checks, whether DNA printing was appropriate on these chips,the storage form of NAPPA microarrays is merely DNA, so in an activated array all following 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 superblock, block the chips with the superblock for an hour and after blocking is done, then we can use the Picogreen mix to place on the these printed slides.

So, let us first look at the NAPPA expression on the slide and then I will describe about scanning for both DNA printing and the protein expression at the same time. So, first you need to prepare the in vitro transcription translation mix, which contains t n t buffer, T7 polymerase, amino acid, RNA inhibitor and d e p c water, apply that on the slide which contains the hybrid well gasket and then seal the pores.

Now, this printed slide which contain plasmid DNA they have the in vitro transcription translation mix, shown in the red color here and the next step will be to incubate these slides for the protein expression step, so incubate the chips for 1.5 hour at 30 degrees for protein expression, followed by 30 minutes at 15 degrees, which allows the protein to bind on the anti capture antibody, after this incubation is done then, we need to wash the slides.

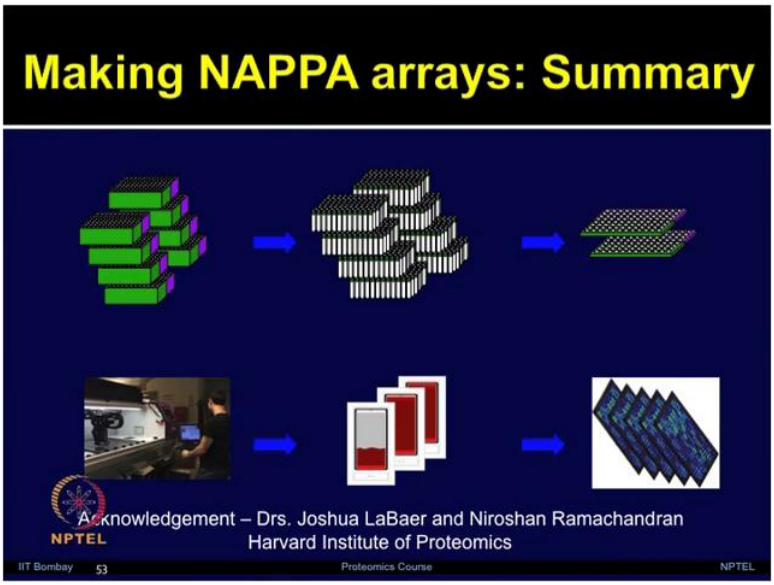
So, remove the hybriwell wash withmilk in p BS,for 3 times 3 minutes each, block the slide with milk or superblock at the room temperature, for an hour, after blocking is done then, we need to drain the excess liquid on a paper towel and then we are ready to perform the incubation with the primary antibody. 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, which is already expressed proteins are there and after adding the primary antibody, we need to incubate it for an hour at the room temperature, now place the cover slip 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, in this case anti mouse h r p and incubate again for an hour atthe room temperature, after secondary antibody incubation, we need to wash with p BS 3 times and then apply tar amide signal amplification system for signal detection, after adding the TSA solutionan incubation then, 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 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 of DNA detection or protein expression, now you need to scan the slides a different type of a scanners available including those ones which are automated and multi slides can be scanned in the automated way, first you can preview the scan and then you can adjust different type of settings to see the signals better, then you can look at each region in the zoomed in its view and look for the signal.

(Refer Slide Time: 57:18)



So, in summary today we discussed about, how to make NAPPA protein arrays, similar type of concept can be applied for other cell-free expression based systems as well, to make the NAPPA arrays one need to have the access for cDNA clones, one can do the recombination cloning or obtain the clones for from various repositories, they grow those cultures and make the good plasmid preparation, after that add the master mix containing BSA, BS 3 anti-GST antibodyas well as cDNA containing the GST tag, transfer these master mix from the 96 well plate to the 384 well plates, which can be used for preparing the array plates.

Now, these can be used on the microarrays which can use the printing, once printed these slides can be stored on the room temperature and then one need to use the different type of quality control checks, such as Picogreen staining for DNA detection as well as, adding the in vitro transcription translation mix for doing the protein expression, once these chips are ready and one has performed all the quality control checks then, these arrays can be used for different type of biological applications. So, in summary we talked about how to make NAPPA protein arrays but, same concept also gives you an overview of various type of inter cases, involved in making any type of protein microarrays. Thank you.