Interactomics Basics and Applications
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Lecture – 05
NAPPA: Recombinational Cloning, Basic Workflow, Surface Chemistry, Printing and Assessment

Hello students. In the last few lectures we discussed about the progression of proteomics field, various milestones which we are made in the field of proteomics. We also tried to discuss where (Refer Time: 00:31) fits within the broad discipline of proteomics. And, then we have started discussing about the field of protein microarrays. I gave you an idea that protein content could be prepared using different manner; either the conventional way of protein expression and purification using heterologous system one could purify the protein and then print them on the chip.

Or, even one could use different type of antibodies for the arrays to be made or one could use only peptides and use the synthetic peptides to print on the chip or even you can use plasmid and cDNA for the printing on the chip. If you want to use nucleic acid contents for printing, you have to use cell free expression based system. In this slide I talk to you about different ways of making cell free expression based protein arrays. I talked to you about PISA: Protein In Situ Arrays, mist multiple spotting technique, DAPA: DNA Arrays to Protein Arrays and hello tag arrays.

I could not get time in the last lecture to talk to you about one of the most prominent technology of the cell free expression based arrays which is NAPPA or Nucleic Acid Programmable Protein Arrays.

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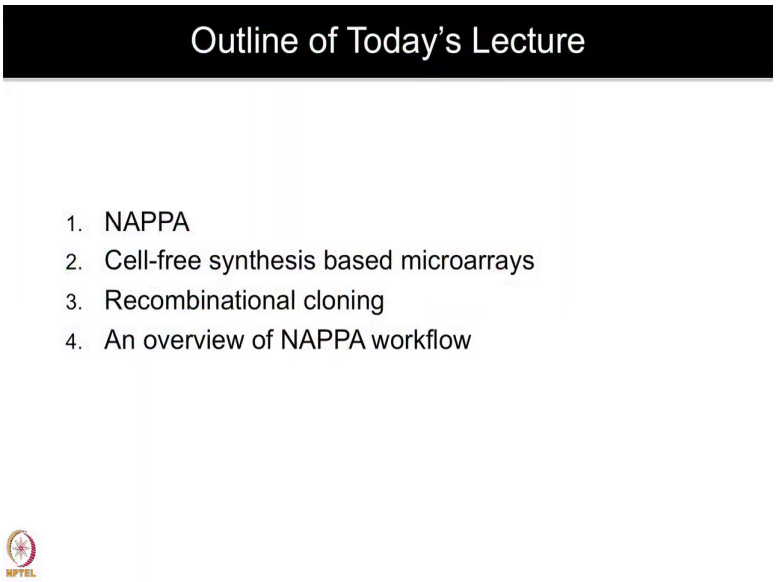

Today in the outline of the lecture, I will first talk to you about NAPPA technology, I will give you again brief concept and reminder for the cell free expression based system. I will briefly touch upon another concept which is recombinational cloning and then I will give you a workflow of how to perform the experiment using NAPPA technology.

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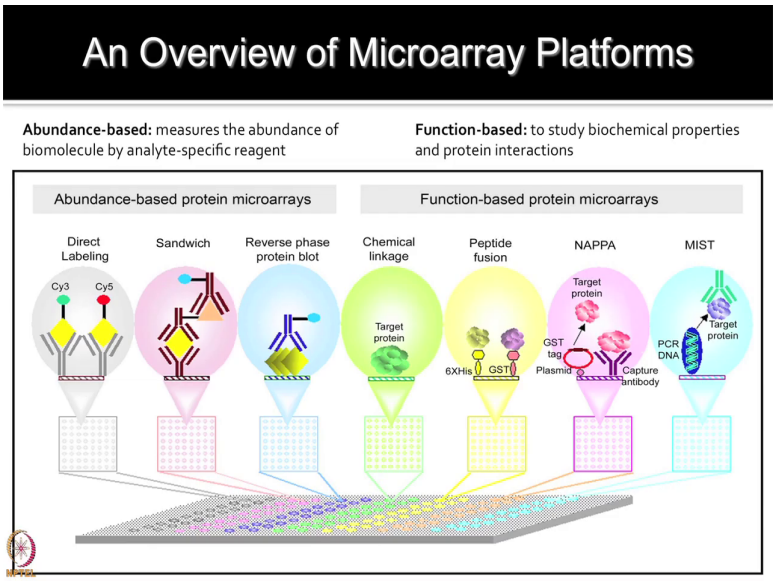

This is my overview slide which just to recall you from the previous lectures, we have talked about different type of array platforms from the abundance based protein arrays to the function based protein arrays which summarizes that you can use make the protein arrays using different type of contents from antibodies, proteins, peptides to the nucleic acid content.

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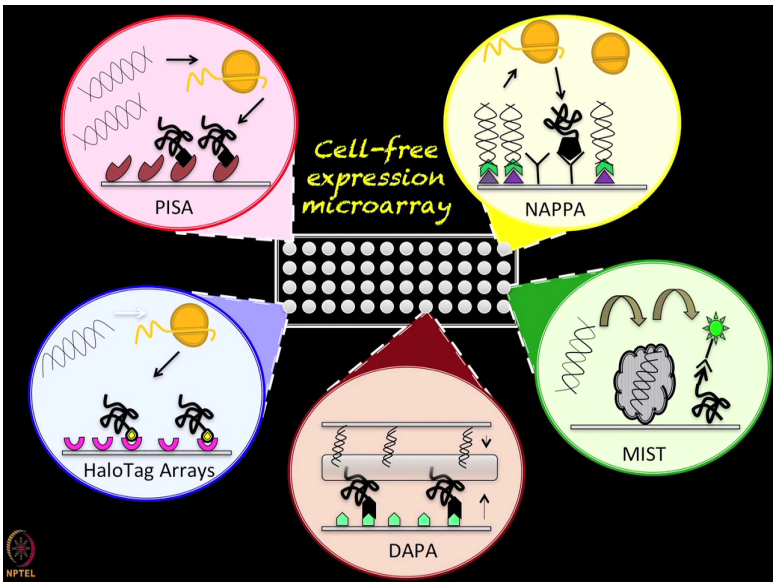

And then using the different type of plasmid or cDNA one could make cell free expression arrays, when you have to express the protein directly on the chip using in vitro transcription translation. And there are different technologies which are available which I have discussed four of them in the last lecture and today the focus will be more on the NAPPA technology.

So, let us talk about NAPPA which is Nucleic Acid Programmable Protein Arrays, this technology has shown the ability to utilize wide variety of DNA templates. Whatever is available in different repositories, all of those DNA could be made use of it to make the proteins on the chip; very simple, quick and cost effective process. And one could do high throughput protein production in a straightforward manner in a simplified and cost effective way.

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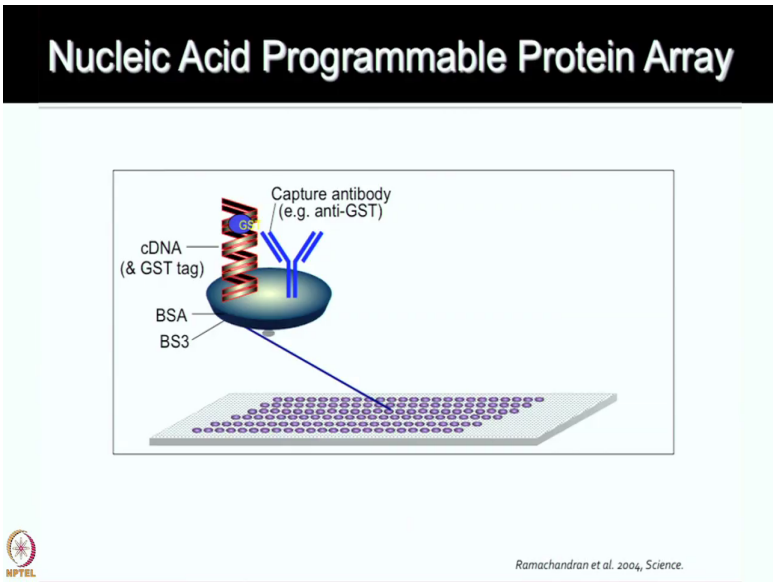

Let us look at how nucleic acid program protein arrays work in this slide. So, if you enlarge one of the spot, you can see there are four features there. One is a cDNA containing a tag which is GST tag or one could also have even any other tag of your interest. But, the caption antibody should be anti tag antibody which is in this case anti GST antibody. If you want to choose make antibody then it will be anti make antibody, then you have the BSA and BS3 which is the cross linker.

Now, the BS3 is going to cross link the antibodies on the chip surface and BSA with some unknown reason and mechanism it helps the complex assembly to get is strongly adhered on the glass surface. After this once you add the in vitro transcription translation mix, then you are hoping that protein is going to be produced and as you can see now the protein is produced which is having GST tag.

So, it is getting adhered with the capture antibody which is anti GST capture antibody. The study was published in Science in 2004 and became one of the very interesting technology to do high throughput protein production on the chip directly using in vitro transcription translation based method. There many merits of using NAPPA arrays, you do not need to express and purify the protein separately, you are simply using a cDNA and, then making the protein on the chip.

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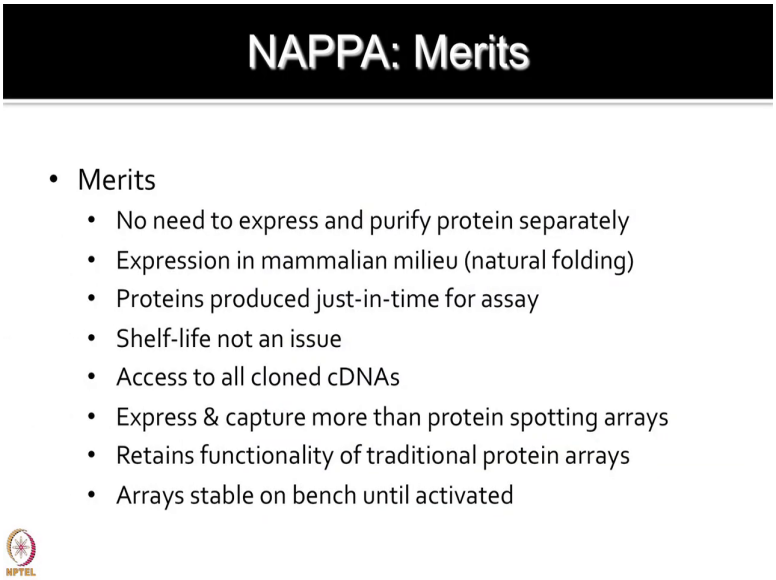

If you are interested in looking at mammalian protein, then if you use in vitro transcription translation mix made from the Rabbit Reticulocyte Lysate or RRL that will be very close for the mammalian proteins to get expressed which will help also for the natural folding of the proteins. Protein production can be just in time for the assays to happen, since you are producing the protein on the same day when you want to do experiment; you are all you know

differently storing the chips which are only having the DNA and those are very stable chips on the room temperature even.

Now, the day when you want to do your assay, your experiment for the biomarker discovery or protein-protein interaction studies, on that day then you are going to express the protein. So, shelf life is not going to be any issue, ideally any cDNA clone which you have access you can print them on the chip and utilize them to make the array for the NAPPA based chemistry.

Expression and capture in this case is much more than if you have to just print the purified protein which will be of course, in very very small volume of nanoliters as compared to the protein produced on the chip itself fresh the day when you are doing the expression. Hopefully the proteins which are expressed they are also maintaining and retaining their functionality.

But, it has been shown from other experiment that these proteins do maintain their functionality and they are very stable on the bench till you are activating them with the IVT mix and incubating at the right temperature. Of course, there are some demerits of using the approach as well.

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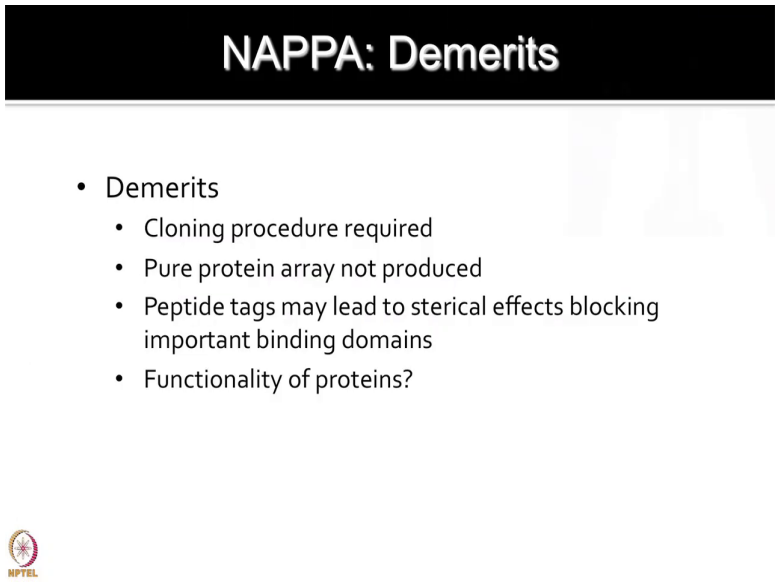

You need to have the clone repository which are having clones with the tag or you have to clone the genes yourself and add those tag which can be utilized for the capture antibody to capture the protein of interest. The chip already having the DNA to start with and the BSA and the BS3 cross linked. So, many things are already on the chip, even a protein is produced it is not going to be pure protein arrays.

Because, if you were to just purify the pure protein and print on the chip what is in this case when you already have a DNA and protein is produced. So, you will have other material which may have some nonspecific signal as well. Although there are experiments which has shown that only the protein is showing the good signal, but you cannot rule out the possibility of some neighboring effect from the other things which are in the part of the master mix.

Peptide tags which is part of the chemistry here may lead to a sterical effect and blocking the important binding domains. And, as I mentioned functionality of the protein is a very challenging question to really address because the proteins are expressed on the chip. But, how well one could ascertain that they are functional you have to do some functional assays to test it out.

And of course, that will be food for thought for you to think about how on the arrays if you have to prove that these proteins which are expressed they are also functional what kind of experiments you can do to prove that hypothesis. So, let me move on and mentioned to you that NAPPA arrays have been used for many applications and subsequently Dr. George Weber is going to talk to you.

And he is going to give you some very interesting applications of using NAPPA arrays, but one application which is very unique in nature is to look at protein-protein interaction study using co expression. Think about you want to study the protein-protein interaction, on the chip you have 10,000 a spots printed, by adding the IVT mix and using NAPPA chemistry you are able to now make 10,000 proteins on the same chip.

And now you want to take one of the protein which is protein of your interest for which you want to identify the protein interactors. Now, this protein when you probe on the chip surface, this is going to bind to the right interactors and then you are going to detect the interaction using different type of antibody (Refer Time: 08:45) tag with the you know either psi 3 or psi 5 or any other fluorophores.

So now, this is the convention experiment right. So, it still while the entire array which is having 10,000 genes of interest they are making the protein of interest. And you are only probing one of the protein of interest, but that protein is actually purified protein. Can we now think about even the protein of interest for which you are looking for the interactors also in the DNA form?.

This was the very elegant experiment which was done to show the co-expression. If you take the DNA of the protein let us say Fos protein and you are measuring the interaction with the jun and other proteins on the chip. So, Fos DNA is added along with the all other printed chip and then you add the IVT mix. So, IVT mix contains your you know the query protein of interest as well in the DNA form.

An idea is if the proteins are expressing then this particular protein the Fos DNA should also make their Fos protein and this should go and bind to the jun protein which is going to express on the chip itself.

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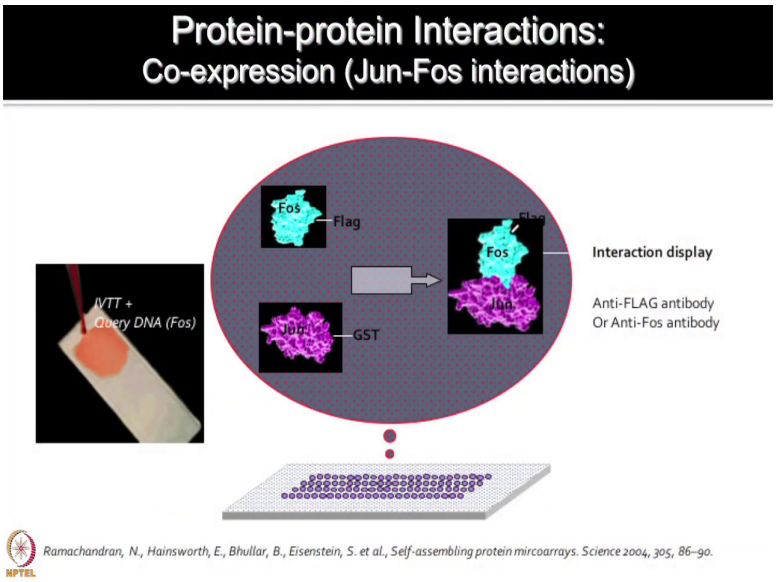

I am showing with the slide now in on the slide and animation here. So, when you are mixing the IVT on the chip to express the protein of interest at that time you are adding the DNA of

the Fos. Only thing you have to keep in mind that if you are looking at the protein-protein interaction using co expression then your query protein should have different type of tag.

So, in this case the protein is having a flag tag with the Fos protein whereas, everything else which is printed on the chip is having the GST tag. If the protein interaction happens between jun and Fos, then Fos contains the flag tag whereas, jun contains GST tag. So, to test out this interaction either you can use anti flag antibody or you can use anti Fos protein specific antibody to test out the interaction.

So, in this manner you do not have to purify the protein even to test out the protein-protein interaction and you can have you know the protein expression in the same environment, even for the query protein of interest. So, different ways of making the arrays which we have talked from the PISA, NAPPA, DAPA and as well as the hello tag base chemistry and the mist, but underlying factor that you want to do the cell free protein synthesis on the chip.

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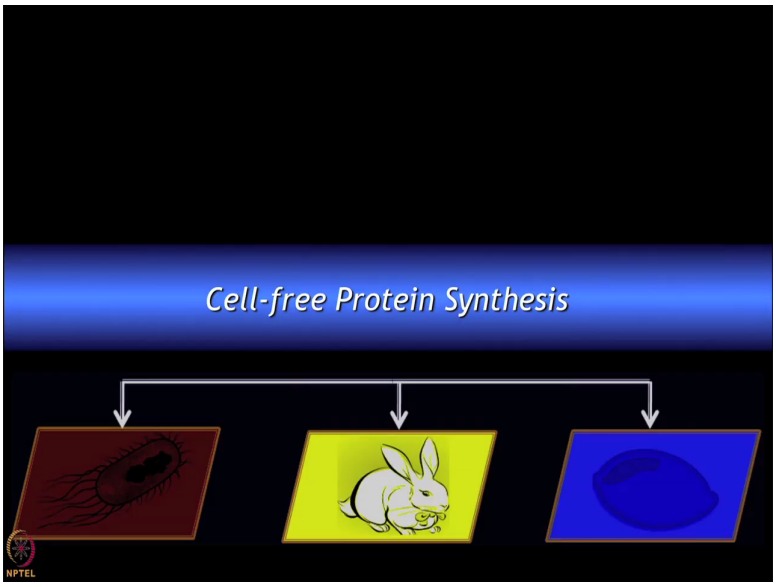

So, let us take a very quick look on how cell free protein synthesis works. One could do cell free protein synthesis either using E-coli based mixture or using wheat germ extracts or using rabbit reticulcyte lysates. By using cell free systems one need not to express and purify the protein which is I think major advantage and how then these kind of arrays have really emerged.

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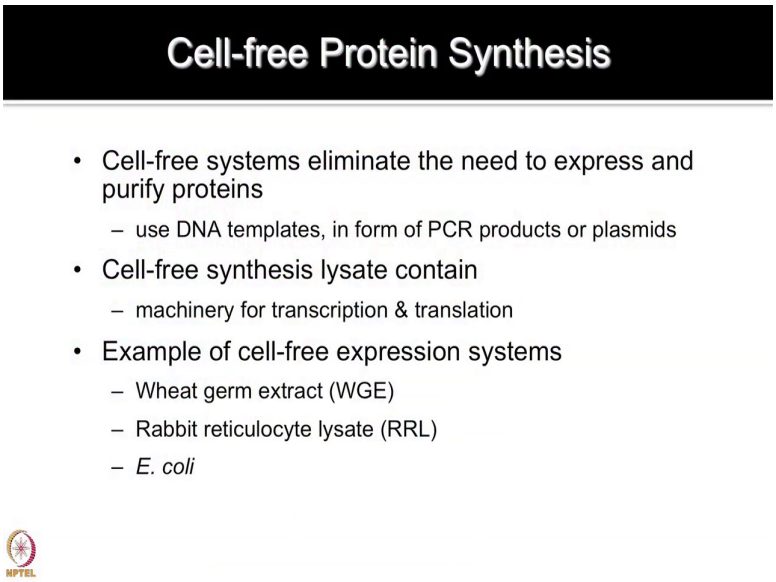

Because, they can utilize the DNA templates in the form of the cDNA a plasmid or PCR products and use that further to make the protein of interest. The cell free synthesis mixture contains the machinery which are required for both transcription and translation, in some way you are trying to do the central dogma of life on the chip directly right. So, there are different ways of doing the cell free expression as I showed in the image last slide, we can use wheat germ extract, a rabbit reticulocyte lysate or RRL and the E-coli based samples to do the cell free protein synthesis.

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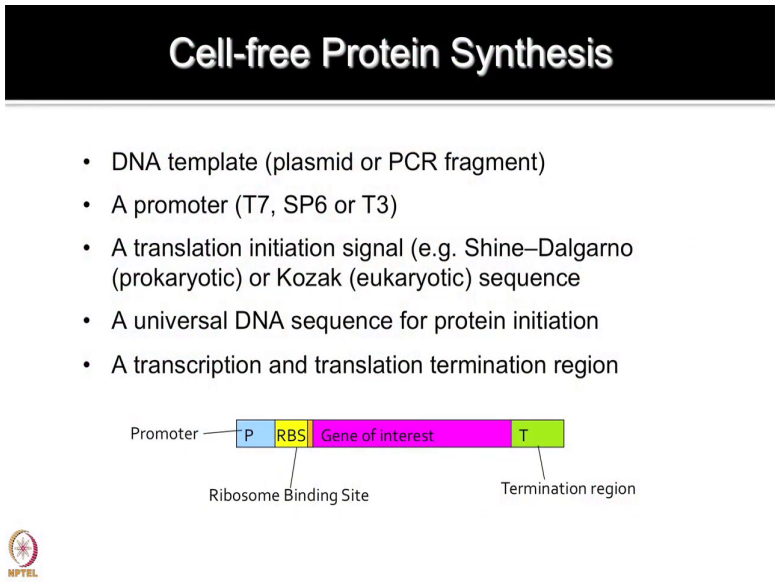

When you want to do cell free protein synthesis you have to have a DNA template the plasmid or the PCR fragment of interest which is linked to a promoter which is having the T 7, SP6 or T3 promoter. A translational initiation signal as you can see the slide I there it will be a Shine-Dalgarno prokaryotic based sequence or it will be Kozak sequence in case of eukaryotes. A unit cell DNA sequence for the protein initiation and transcription and translation termination region.

So, all these are required for doing the cell free protein synthesis, but there are many commercial vendors now who makes the cell free protein synthesis kits from which one could do very efficiently the cell free protein synthesis in the lab. And there have been many biochemist and a structural biologists who have been using this for expressing the difficult to purify proteins using the IVT mix, in vitro transcription transition mix.

I must say that protein microarray community has been you know very sharp to pick up those resources and reagents and utilize it here in this case for the doing the cell free expression based protein micro arrays. Let me now take you another concept briefly which is recombinational cloning.

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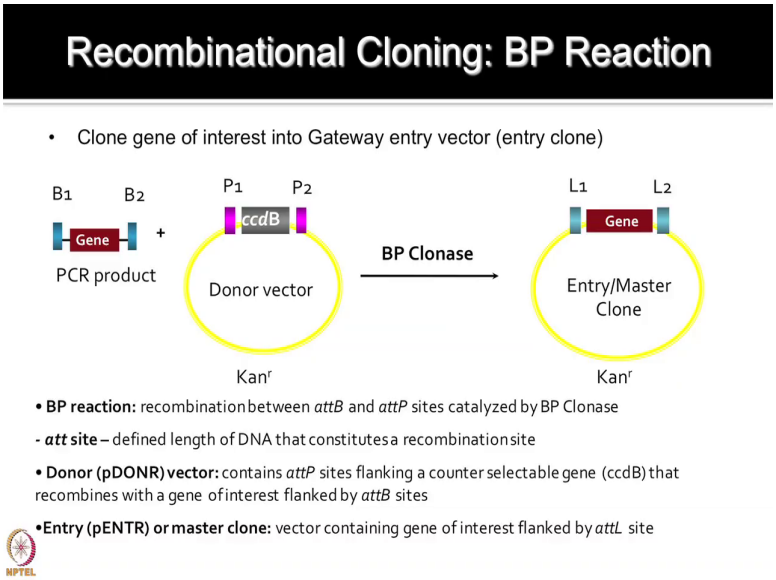

Many times when we are talking about this kind of you know high density arrays and large number of clones to be printed on the chip, it just looks like its so, difficult to do right. But, there are many repositories who are working very systematically to just try to clone the genes in the same format with the same vector backbone; so, that the entire community can utilize them very easily and very efficiently. So, the gateway based recombinational cloning, this involves two reactions BP reaction and LR reaction, I will just define what this means.

So, you have a gene of interest which is let us say PCR product and flanking regions of that is having a defined length of DNA with the ad site that constitutes a recombination site. So, the left side of the image shows you that there is a gene which is flanked with the attB site, attB 1 and attB 1 B 2 sites. And, then you have a donor vector in which you want to move your gene of interest which contains attP site, attP 1 and attP 2 sites.

So, this donor vector that contains a counter selectable gene or ccdB that recombines with the gene of interest which is flanked by the attB sites. Donor vector having the kanamycin resistance gene and by adding now the enzyme BP clone is you are initiating the reaction. And, now the gene of interest will move in the donor vector and that is not termed as the master clone or the entry clone.

This is now, we are terming as the L 1, so after the reaction of B and P happened that has now become the L. So, this is now become the entry clone which we are going to use for our second reaction with the LR.

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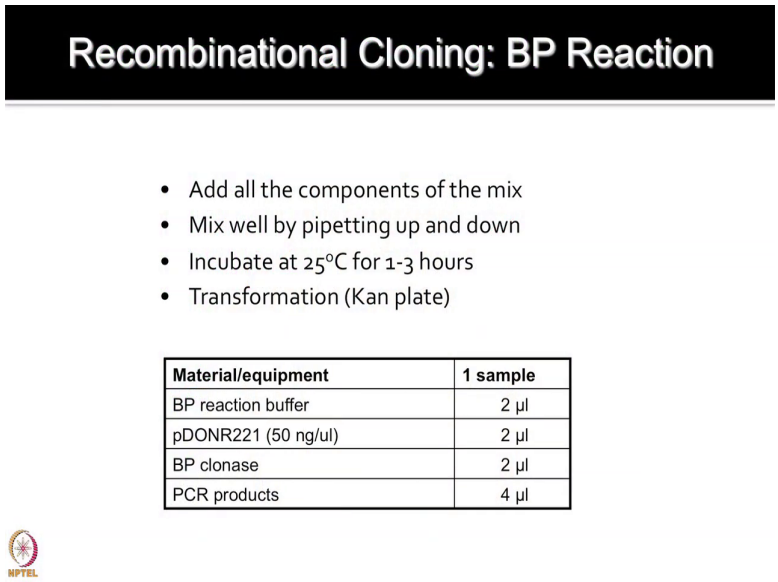

So, this whole thing looks a little complex, but happens in a very simple to you with a very simple reaction mix which consists of the BP reaction buffer, the p donor vector with the which in the DNA 50 nano gram per micro litre, BP clone is enzyme and the PCR product. You mix them in the you know as a recipe shown on the slide, incubate it at 25 degrees for an hour and then do the transformation on the kanamycin plate. Next day you will have the colonies grown on the kanamycin plate.

The reaction variation is very specific and very efficient, from these colonies now you can make the DNA further and you can proceed for the LR reaction. So, now let us talk about the recombination cloning second step which is the LR reaction which is the recombination between the entry clone and the destination vector.

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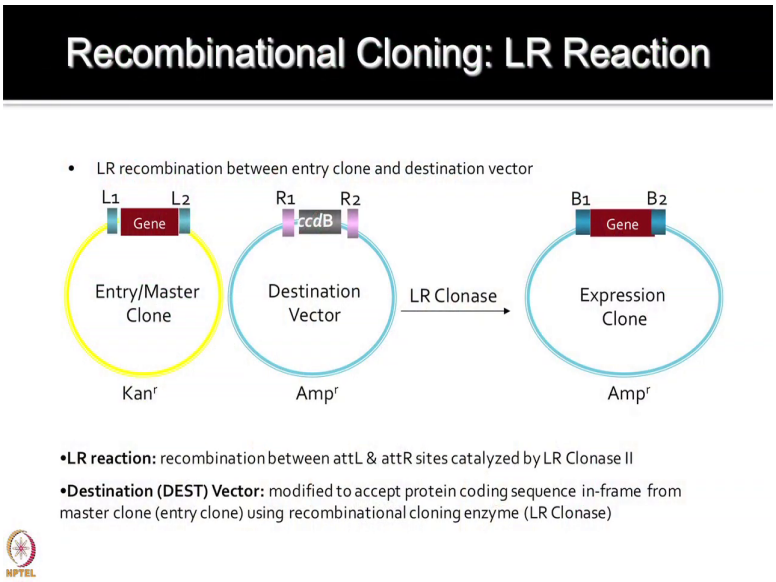

So, here we are bringing the entry or the master clone from our first step which is having the flanking at sight of attL 1 and attL 2. And, then we have destination vector which is having the R 1 and R 2 sights, keep a note that destination vector is having ampicillin resistance gene. Now, in the presence of enzyme LR clonase when the reaction happens then you your gene is going to move inside the destination vector and this destination vector could be any vector of your interest.

So, now, you can screen this on the ampicillin plate and then you can find the right colony which is having your gene of interest. So, the destination vector is modified to accept the coding sequence in frame from the master clone which will the entry clone from the BP reaction, by using the recombinational enzyme LR clonase. And then you can move your gene of interest to any suitable vector in this manner.

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Recombinational Cloning: LR Reaction

- Add all the components of the mix
- Mix well by pipetting up and down
- Incubate at 25°C for 1-3 hours
- Transformation (Amp plate)

Material/equipment	1 sample
Entry clone DNA (100 ng)	1-7 µl
Destination vector (150 ng/ul)	1 µl
TE buffer	to 8 µl
LR Clonase II enzyme mix	2 µl

Again this reaction is also very simple and as you can see on the slide you are taking the entry cloned DNA, destination vector, the TE buffer and LR clonase enzyme. Mixing them together incubate at 25 degrees and then performing transformation and selecting the colonies on the ampicillin plate.

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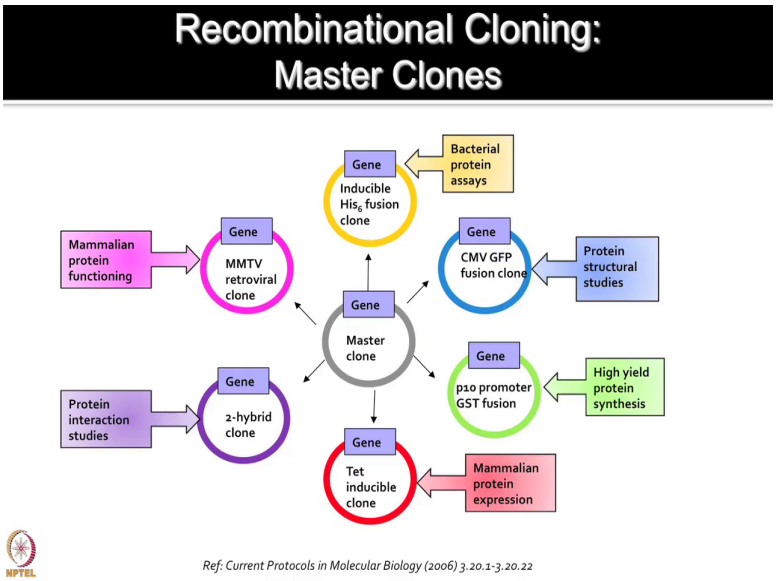

So, now if your lab has access to the master clones or you can purchase these master clones or obtain them from the various repositories, you can transfer these genes of interest to any other vector backbone. So, what you see here that you know your master clones, the gene of interest could now be moved to the you know another expression vector which is used for the bacterial protein assays.

You can also move them to the CMV GFP fusion clones for the protein structural studies or you can move them for the high yield protein synthesis work, mammalian protein expression work, protein interaction studies or mammalian protein functioning. You can use different type of vector backbones and you can move your gene of interest to any of them in the single reaction.

So, the beauty of this part is your reaction is very efficient and gene is going to just replace in the cassette. So, even you need not to do the sequencing to verify the proper insertion of the gene of interest. Although its good idea to recommend, if you are doing 100 genes in the recombination cloning at least few you should pick up and do sequencing, but the reaction is usually very specific. So, thing should be in frame and in the right sequence.

So, I hope you got some idea that you know how cell free protein synthesis works, how recombinational cloning works and the brief concept of NAPPA; nucleic acid program protein arrays. So, these arrays make use of the clones which is obtained from recombination cloning, make use of the reagents which is able to do these cell free protein synthesis.

And now one could think about how to do the NAPPA arrays, I will now walk you through with the workflow of how to do these experiments using NAPPA protein microarrays. And same concept will be applicable for any type of protein microarrays or any type or cell free expression arrays; I am just giving you an illustration by using and walking you through with the protocol of NAPPA arrays.

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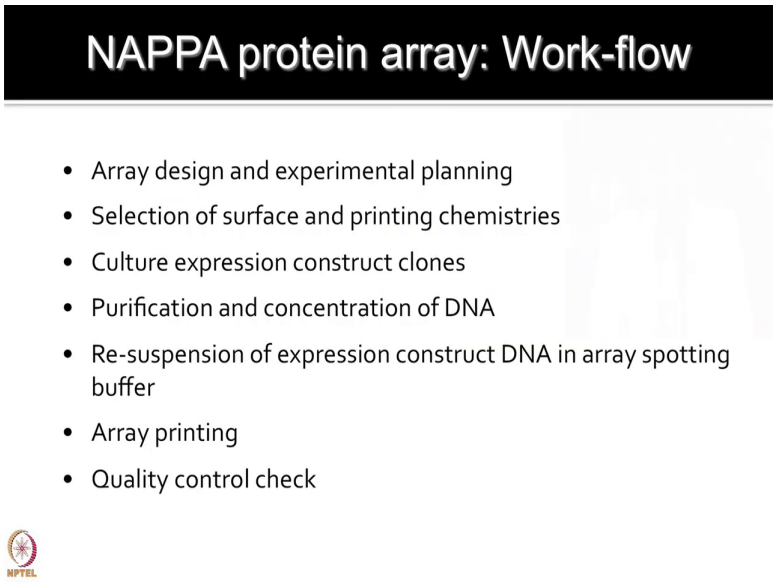

So, very first thing is that you want to decide what is the layout of your array which are all genes you want to print, in which form you want to make all your blocks and where you want to place your control spots. You have to also identify which are your best control spots, what should be the positive control, what should be negative control and where they should be placed on the array layout.

Then of course, the selection of the surface and the printing chemistry, how these biomolecules are going to adhere and printed on the chip. Then you have to use the ways to do the culturing for your clones to make the DNA and then re suspend them in the printing buffer. Once you are ready now you can start doing the high throughput printing for the large number of slides with all the genes of interest. And then the next step involves the quality control checks to make sure that you know you are going to work on the right type of slides.

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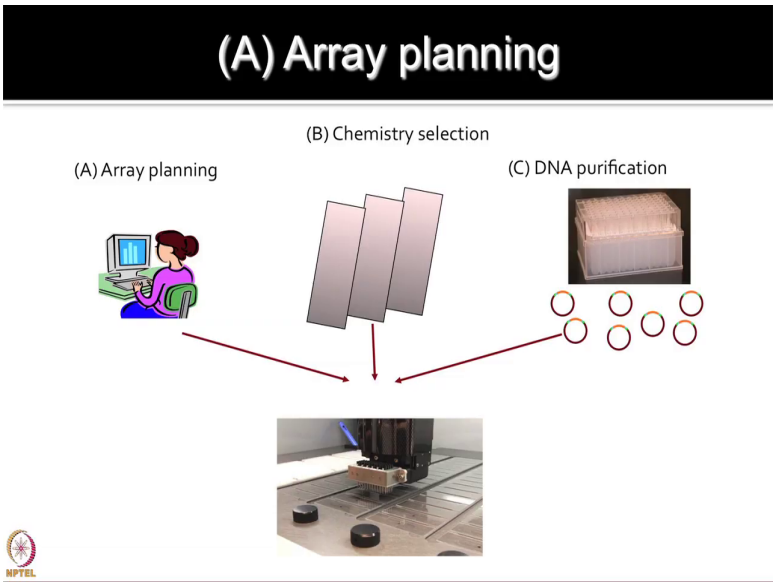

So, let me take you some of these steps briefly. So, this slide illustrates you the major step which is involved before you come for the printing arrays, you have to do lot of bioinformatics work to decide the gene list and decide which block contains which kind of gene and where the controls will be placed. All of these information you decide and you do lot a literature review to identify the positive control, negative control.

And then you make the very thoughtful way your array layout. Once you do that then you are also looking at the chemistry selection which type of slide you are going to use nitrocellulose, glass, gold which type of array you are going to make. And then of course, the strategy to do the DNA purification, all these contents are mixed into the master mix the printing buffer and then you are now ready to do the printing.

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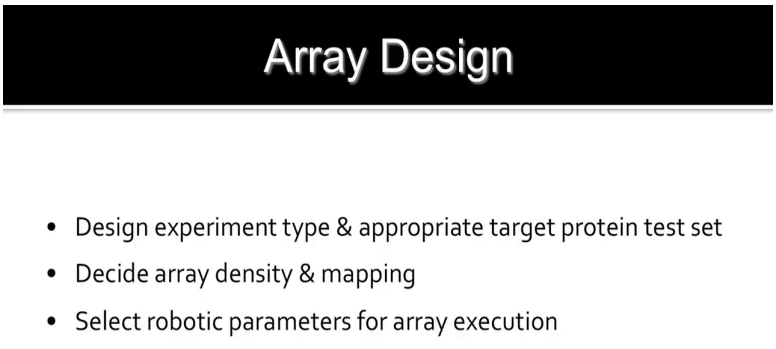

So, array design as I mention is the most crucial part and that is where, it takes most of the time before any experiment actually starts; you will have to spend your months and months of time to think about what all you want to print on the chip. Many times you do not want to just do the skinning on all 20,000 spots, you want to look at a careful design array which can give you the very meaningful answer.

So, then you have to do lot of intense work on knowing the literature, understanding which builds the question you want to address. And, accordingly you can now decide what should be my array density, the array map and then the robotic parameters which are going to execute this kind of array printing.

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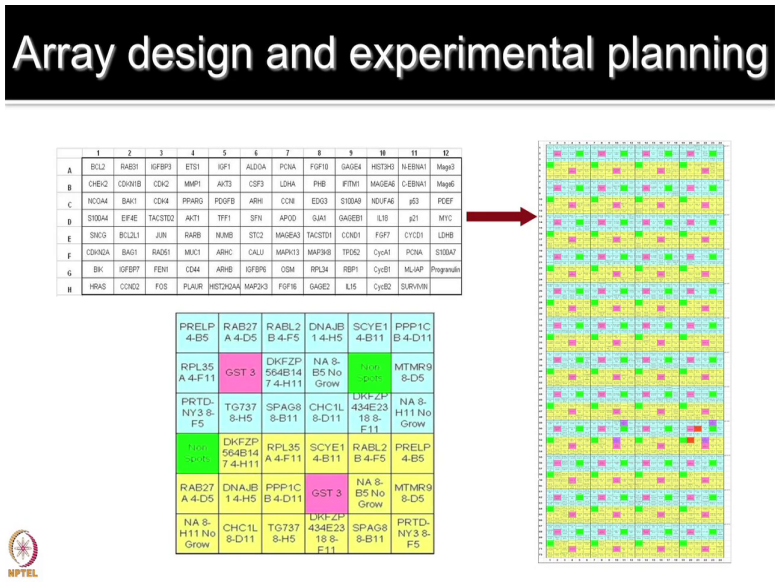

Here shown in the slide one of the you know actual screenshot from the printing which we are trying to do, its shown that you know how you are making your 96 well plate where the DNA will be placed. And eventually after printing how the slide will look like on the right hand side and then each block, one of the block I have highlighted in the below panel here.

What you can pay attention here that you know the same spot like GST 3 shown in the purple color that actually your you know same spot which is duplicate, you are trying to avoid to keep them together; you want them to be slightly far apart. So, that there is no neighboring effect, you know if there is something is spilling over then both the spots should not get contaminated.

And likewise there is some non spot which sometimes you want to keep an empty area or just the buffered where no DNA is there or only vector control. So, many of these controls you

want to keep them for a part on the full block; so, that you can ascertain the quality of the whole array.

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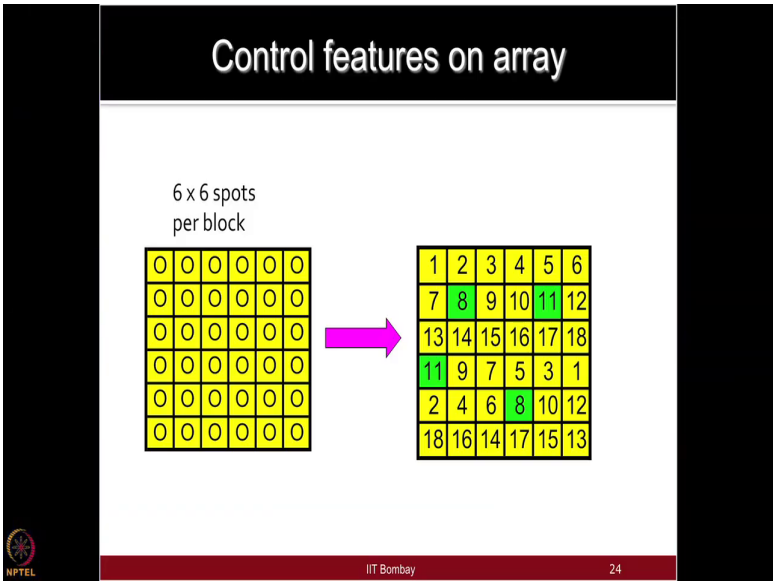

This is the more close look of one of the block of this whole high density array chip and you can see the number 11 gene is shown far apart, number 8 gene again diagonal and they are actually quite far apart from the to being on the neighboring side. So, that one could look at a signal of both in a certain how good and reproducible these signals are. So, many of these you know the work what we are talking for the high density arrays, they require the cDNA clone repositories.

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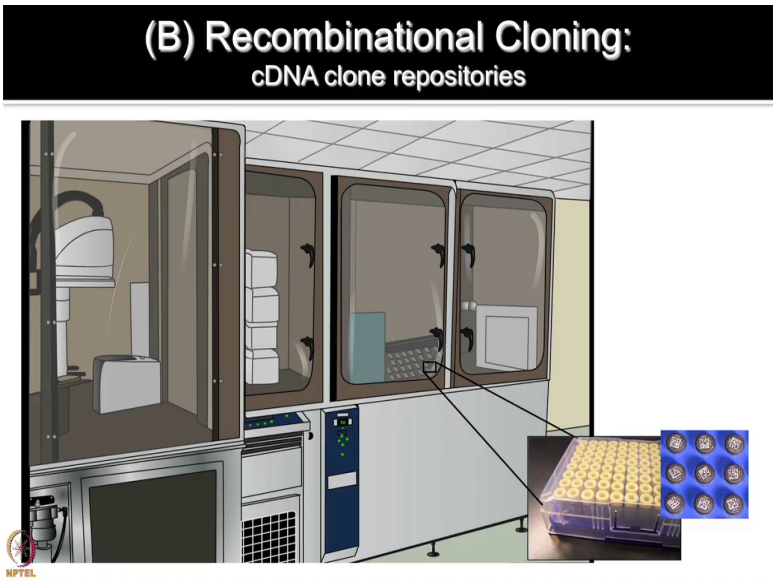

And, one such clone repository is shown here, where in the automated manner you can actually obtain the clones outside and then now these are all bar coded. So, that you are not influencing the other genes you know in the glycerol plates with the freezing thawing. And, then you can make your you know the glycerol stroke of genes of interest and then do the DNA prepping and followed by the DNA printing. So now, next couple of slide I will just you know walk you through very fast, just keep looking at the images.

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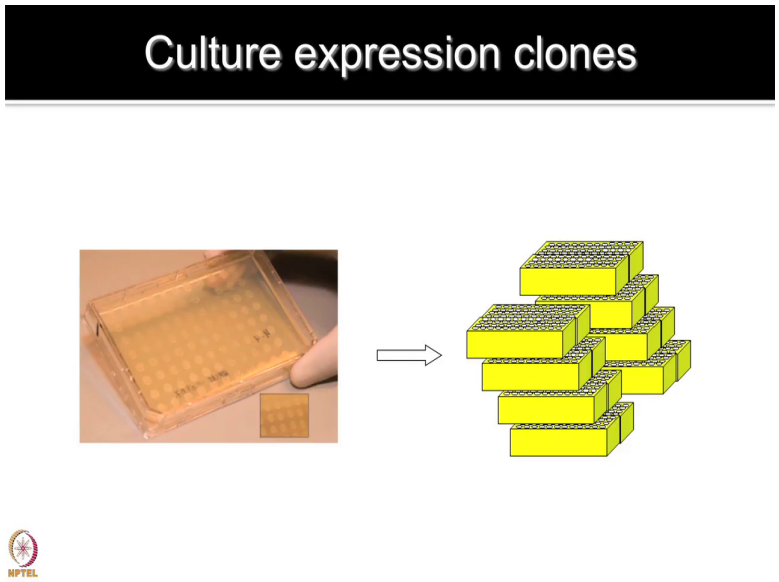

These are just some illustration about how you are I am sure you have been able to do already DNA you know the purification work yourself. But, in this case we are just showing you in more high throughput manner how you are doing everything in 96 (Refer Time: 23:54) format or 384 will plate format because, you are talking about 10,000 to 20,000 genes of interest to be printed on the chip.

So, you cannot do in the you know in the tubes; so, the whole setup is essentially meant for doing with high throughput ways of bio molecular sample preparation part. So, in this case you know you are even recombination cloning when you have done you are selecting the clones of interest, you are putting those in this plates which is having all the 96 genes you are putting at the same time.

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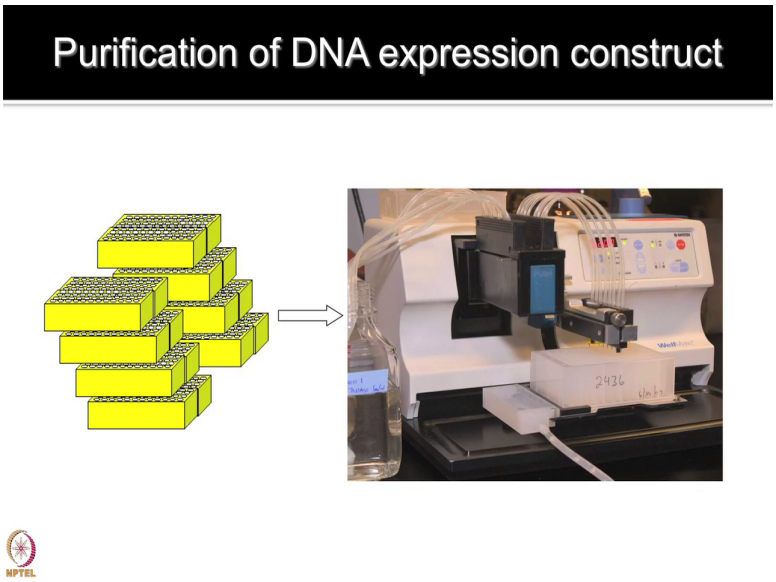

Then you are culturing those and again you are using different type of you know various type of liquid handlers which can facilitate and more make the process very fast.

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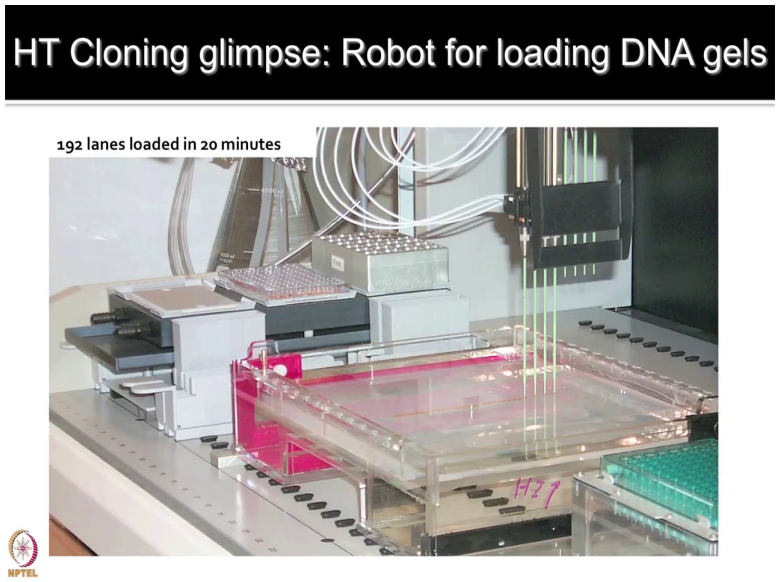

This particular unit for the agarose running unit for looking at the DNA quality is again you are loading many samples together, you are looking at almost 48 samples and 96 samples to be loaded on the same gel.

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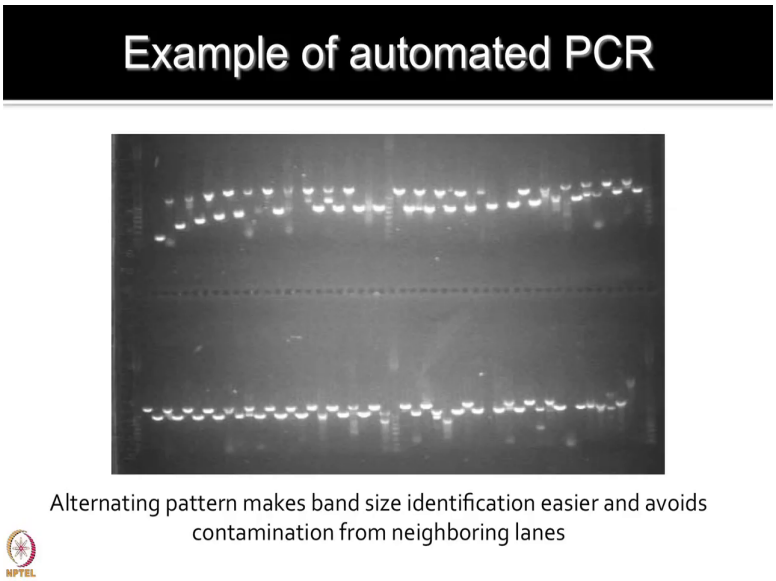

And like you know now you can see the pattern of these images like how these PCR look like, you are putting alternate wells. So, there is no contamination in the two neighboring wells. So, one is up, one is low and although the you know loading is being then more automated manner.

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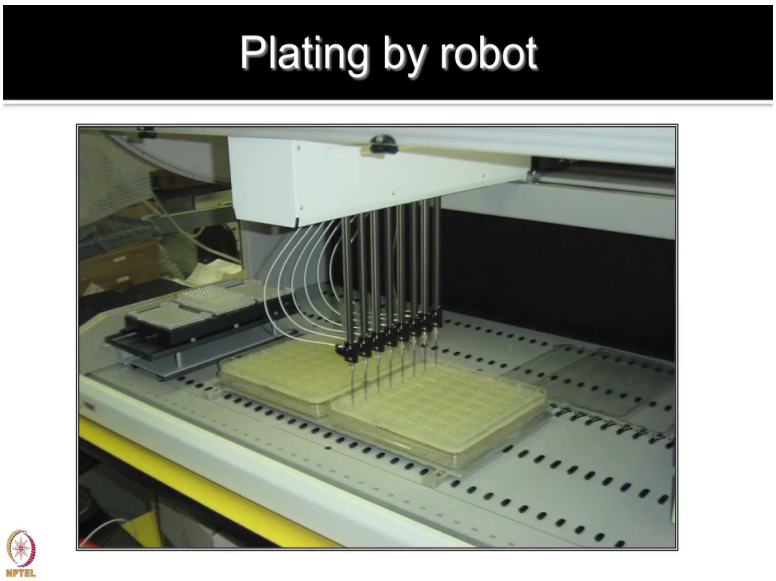

Now, even the plating of the you know once you are doing BP reaction, LR reaction and want to do the you know for the transformation those plating could also be done by the robot.

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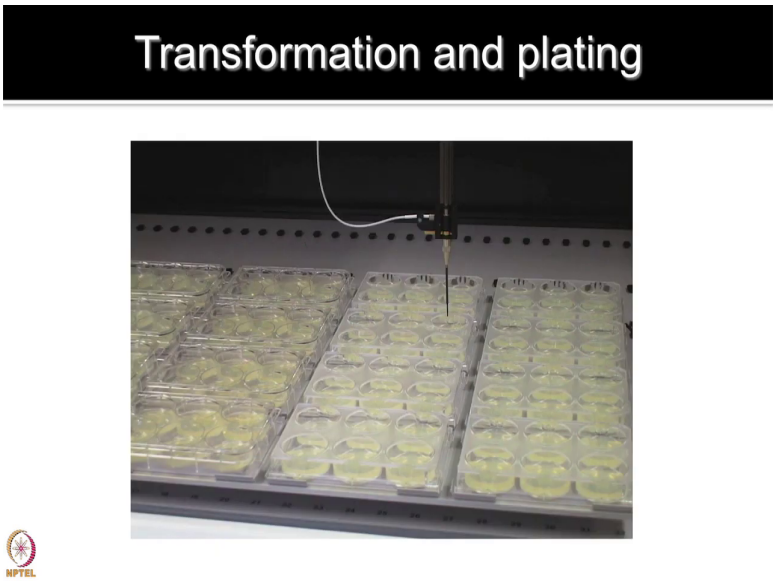

Transformational plating again are shown here.

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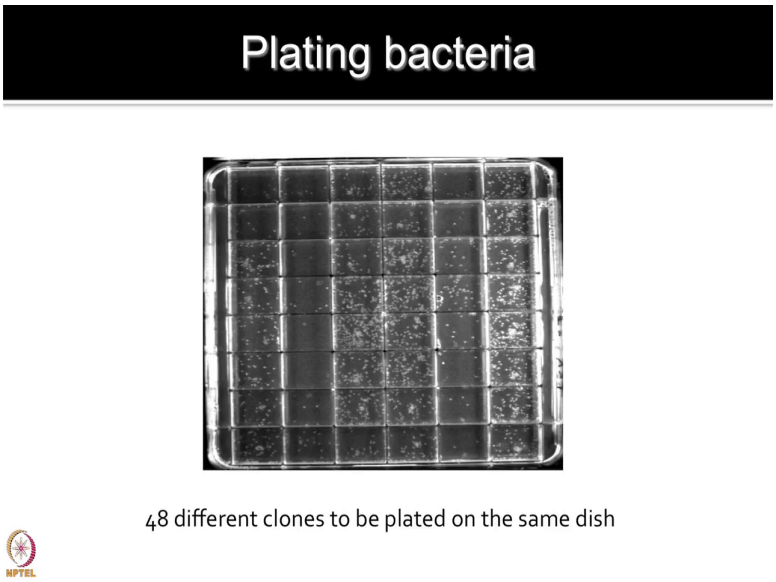

These colonies are now made on the this particular plate.

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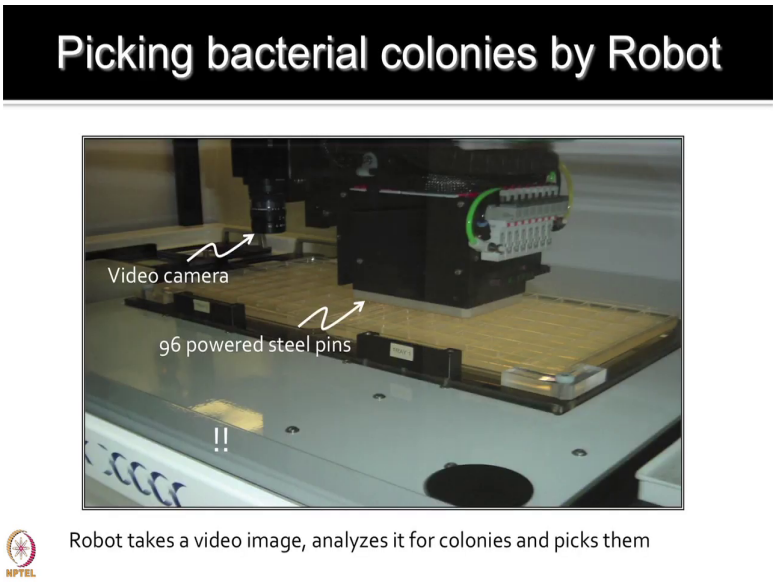

You can also monitor these things with the robot which can look at the bacterial colonies pick up, a single bacterial colony and being also video recorded. So, that you can see that is a single colony or not.

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Again the set up for showing the automated DNA preparation.

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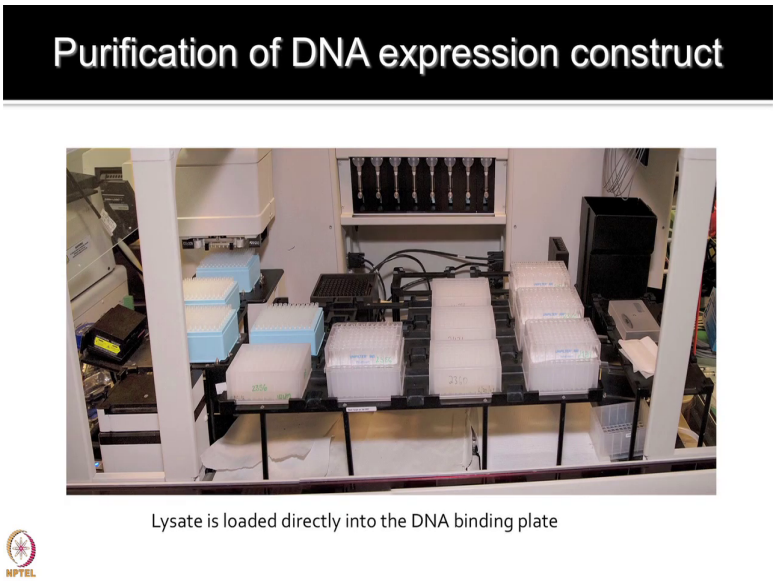

And, now this particular liquid handler which is very advanced used for the purification of DNA expression constructs.

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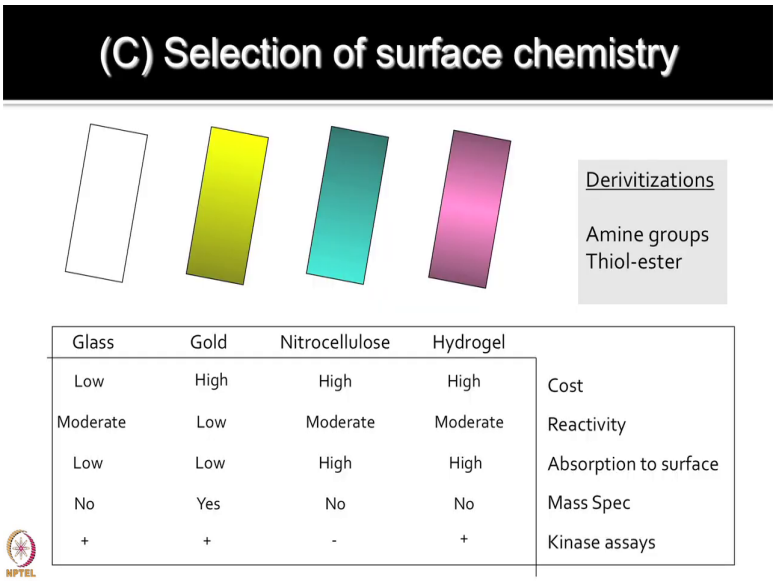

Now, finally, comes the important aspect which is looking at the surface chemistry which a substrate you are going to use for printing your you know the material of interest, in this case you are looking at the NAPPA chemistry. So, you can choose from variety of options glass, gold, nitrocellulose or hydrogels. Of course, glasses the most you know commonly used because, it is very cheap on one hand; also most of the commonly used instruments they are meant to use this kind of substrates.

So, glass slides are popularly used. Nevertheless, for a specific application people also use gold, nitrocellulose and hydrogel. In this slide you can look at the different you know pros and cons of using different substrates. Of course, when it comes a cost gold will very high, but if you are looking at low reactivity and also the compatibility of your further measurement using mass spectrometer.

If you want to look at the protein interactions and then want to strip off the interactor and read on the mass spec, then gold will be much better way of doing your protein interactions and then further analyze them on the mass spec. Adsorption to surface both in case of glass and gold, it is low and moderate in case of nitrocellulose, in hydrogels.

The kinase based assays have been performed on both glass and gold, not on the nitrocellulose and also on the hydrogel membrane. So, you can look at you know different pros and cons and then decide which is the right substrate and surface chemistry which you are going to use for printing your biomolecules of interest, many a times you are going to derivatize these using amino groups thiol ester based chemistry.

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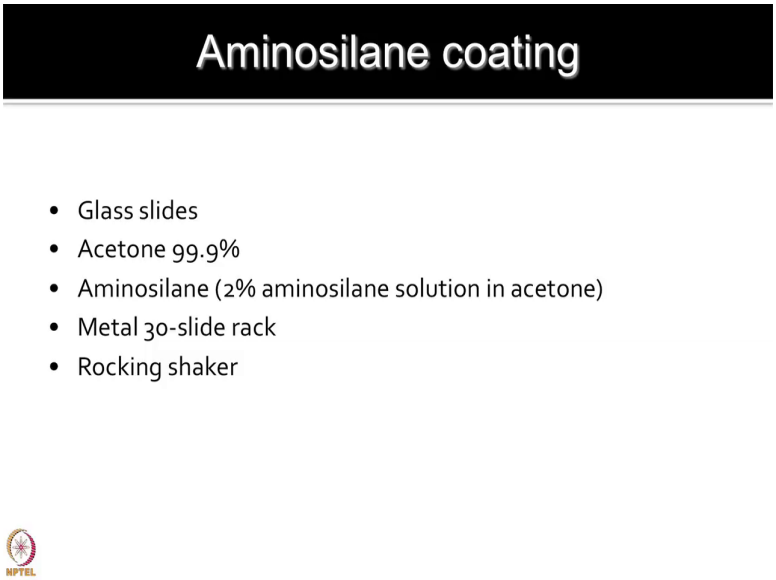

So, for the NAPPA printing first you are going to functionalize your glass slide with aminosilane which is you can take the aminosilane in the acetone, take your slides in the

metal rag and simply dip them in the aminosilane solution. And after that then you are you can again rinse it in acetone, then you can dry them or centrifuge them and our slides are ready to be printed.

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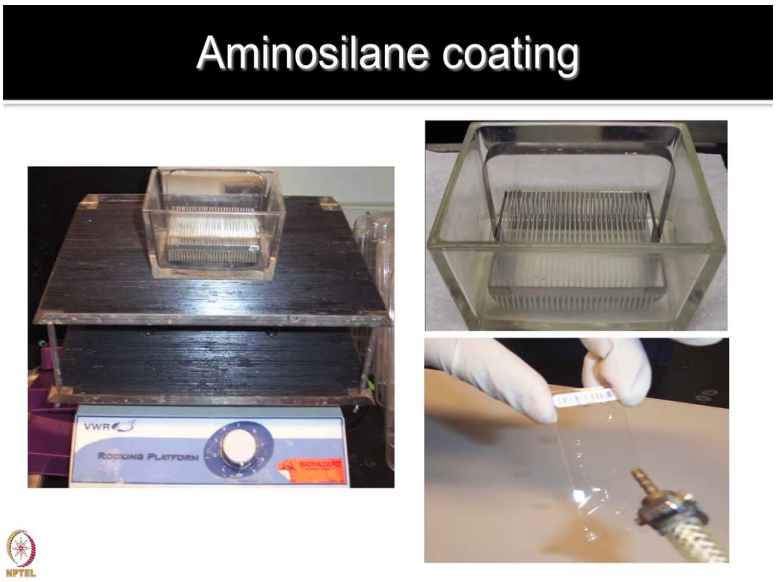

Here in the slide shown is the this setup, you can simply use your you know routine lab setup for doing this aminosilane coating. And now dry the glass slides after the washing steps.

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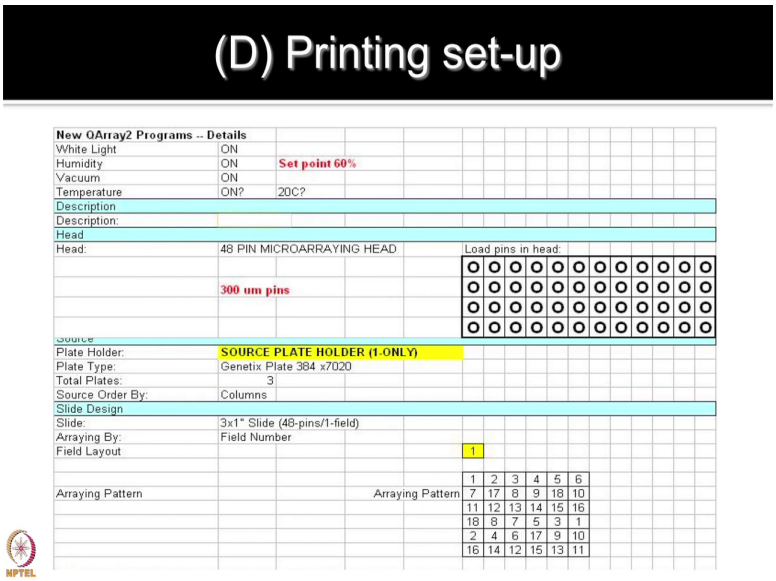

And then now you are ready, you know you got the DNA made, you have the layout prepared already, you have the slides which are functionalized now and then you can start doing the printing. So, you can set up the robotic parameter at how many pins you want for the printing, what is the depth you want it to go and you want to see like how much time you want for printing, the washing time. And, all of these parameters has to be defined including the humidity and the temperature what you want to maintain during the printing hours.

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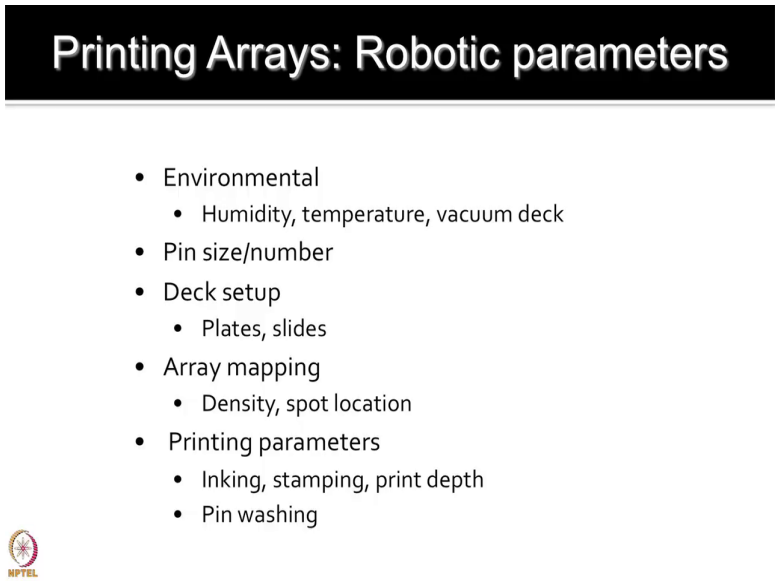

Then once you have done these optimization; so, as I mentioned that you know you have to look for many parameters. For example, environmental conditions for the printing which is humidity, temperature, the vacuum deck; how many pins you want to use for printing, you know the deck setup how many slides you can have on that deck or how many plates you can keep for doing the printing.

Array mapping is very crucial which is how many density you want, how many spots you want to print, which is spot on which location and then different parameters for the printing. Finally, comes you know if you have printed in arrays then you want to do the protein expression, one could use different ways of doing this cell free expression work.

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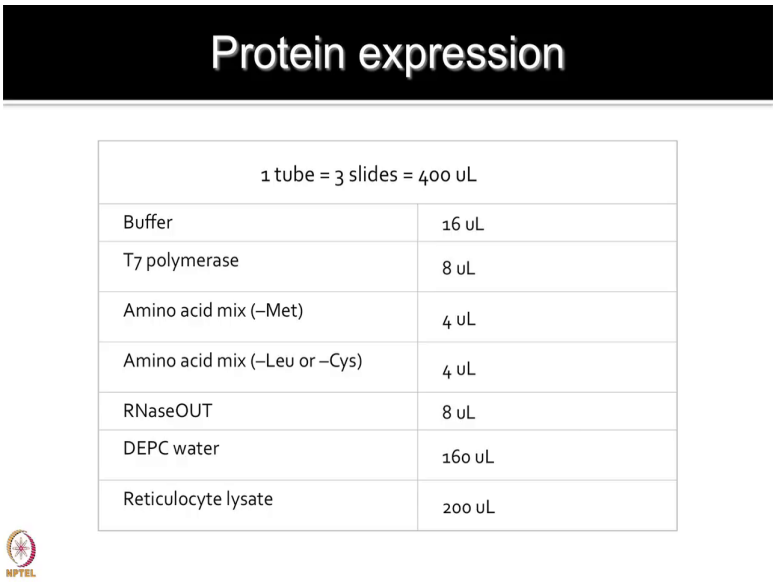

One example is shown here when you can use the IVT mix which contains T 7 polymerase, amino acid mixture, ribonuclease OUT, DEPC water and rabbit reticuloocyte lysate. All of this comes in a tube and then you are using this to expel the protein of interest which is shown in next slide here.

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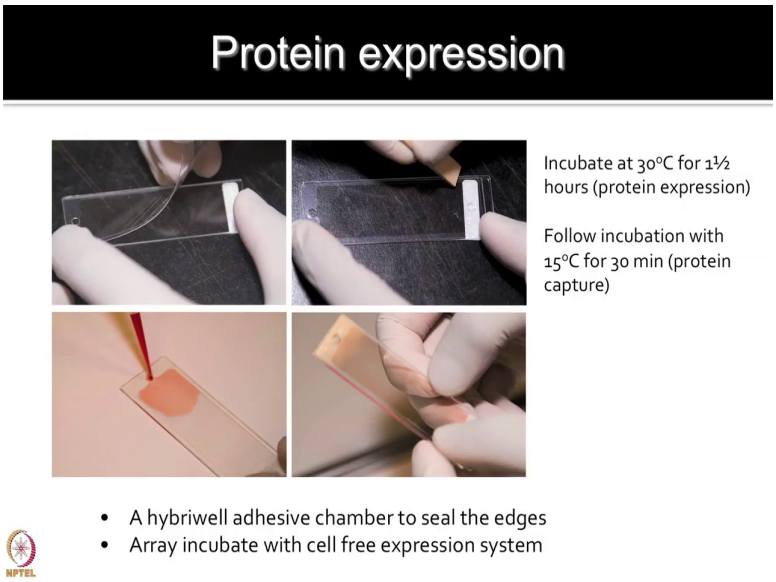

So, on the glass side once you have printed your genes of interest, then you have to create some boundary; so, that you know your IVT mix should not wash off. So, its good idea to keep some sort of hybriwell or the plastic which can adhere on the edges. This is what is shown on the slide here and once you do that, then you are able to add your IVT mix from the hole which is made on one side and other hole is there on the other side. So, that the capillary action can be used for moving this IVT mix on the whole slide.

An idea is that you know every spot which is DNA now has to make the protein. So, you to be very careful in this process of moving the IVT mix on the slide. So, that you are not losing out with any bubbles or anything and otherwise those spots will not have any protein expression. So, once you are ready with this part then you can do incubation at 30 degrees for one and half hours.

And then you can also lower the temperature for the capture a strip at 15 degrees for 30 minutes and then now your you are ready to starting from the DNA on that day and now you got the proteins made on the same chip.

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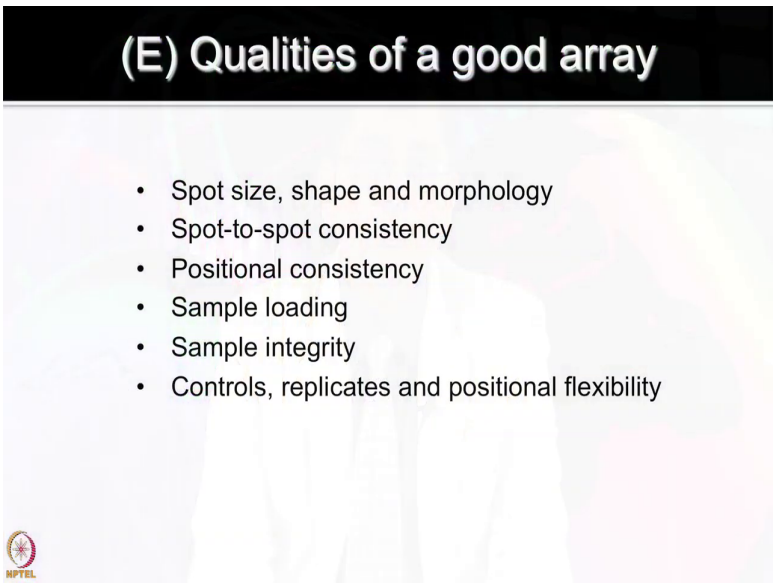

So, let us also talk about what are the qualities of a good array because, once you are you know in the process of doing the whole work of protein microarrays you want to make sure that is spot size, shape, morphology how consistent they are. And you know until unless your controls, your replicates these things are not in place; you cannot use the arrays for the actual experiments.

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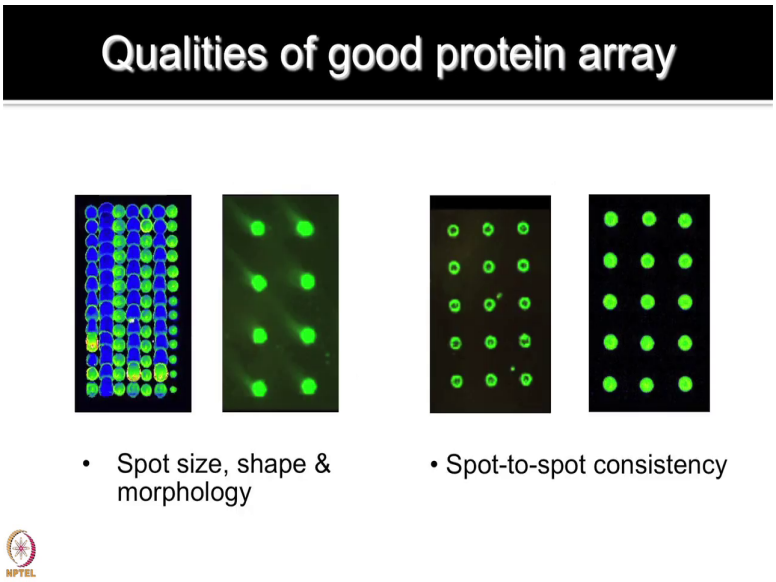

So, I have shown here some a couple of examples, on the left side it was some example of bad slides, that slides were some hallo effect. In the middle you have you see a slide with the do not affect the third slide from the left. And the last one is more you know the consistent slide with the proper spot morphology. And, you want to use these kind of a spots for the printing, while the rest three printing will be actually and not used.

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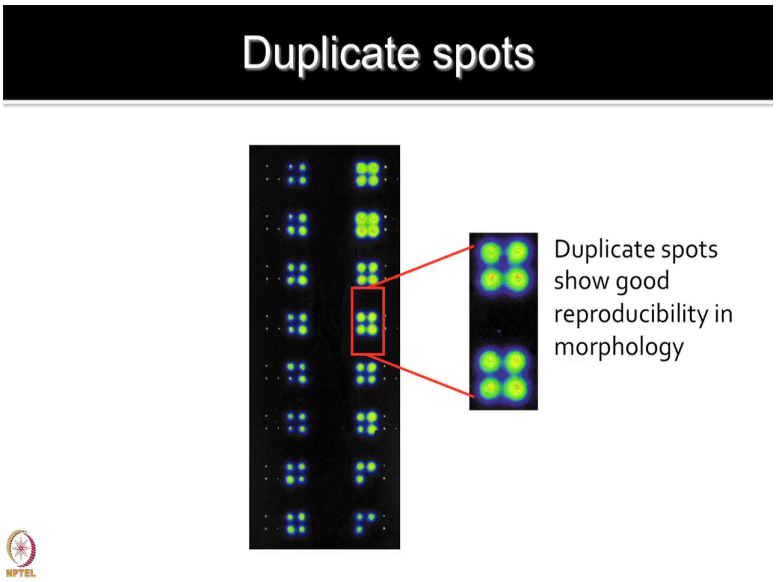

We also want to see how good your duplicated spots look like, how reproducible they are and this is shown in this slide which looks pretty good.

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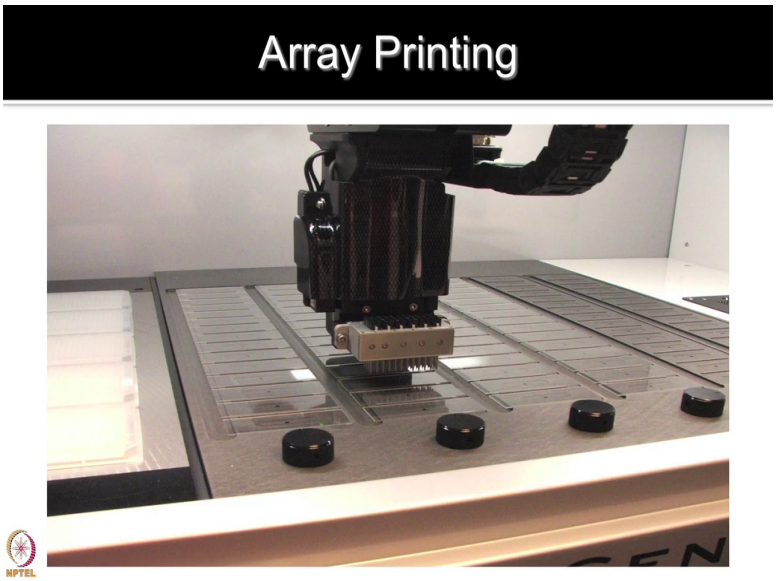

So, array printing we which we have talked this just shows your setup of doing array printing.

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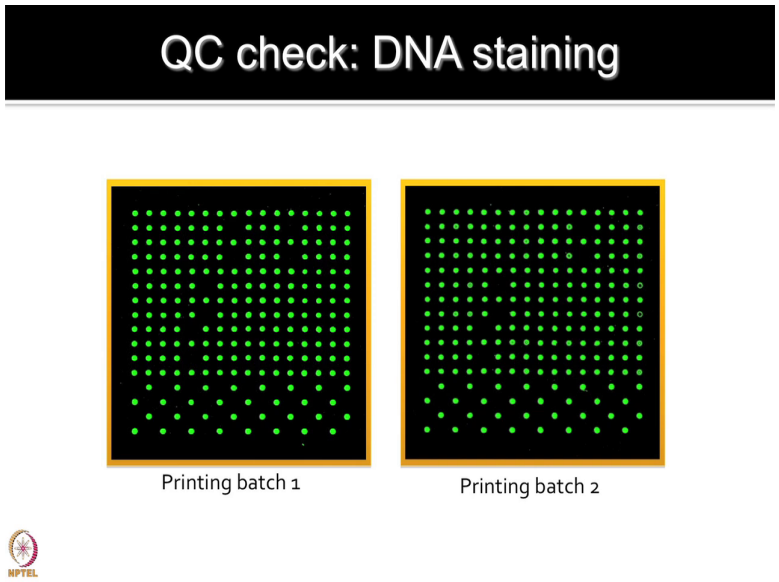

And then you are looking at the quality control checks especially the DNA staining because you are printing DNA. So, you want to first make sure their DNA is printed properly and you are looking at the batch to batch, how good the printing has come. If there are spot which are missing so, it means printing batch 1 and printing batch 2 are not very good. What I meant to the with the batch for example, you are making the DNA and the master mix, keeping it 384 well plate and keeping on the deck for printing.

Now, from the same material you can first print let us say 90 slides or 100 slides in one batch and then on the deck then you will remove these slides. Now, you keep the next set of the fresh slides and with the same DNA now the printer will go and start printing on all these two you know 100 slide, now you got 200 slides. Now, from the same DNA you are going third one more round and let us see you are doing the third round printing that is the third batch.

So, then you are going to eventually compare how good my batch 1 versus batch 2 and batch 3, how reproducible and consistent they are. Because, then only you can use these 300 slides for the same big experiment which is required for biomarker discovery based work.

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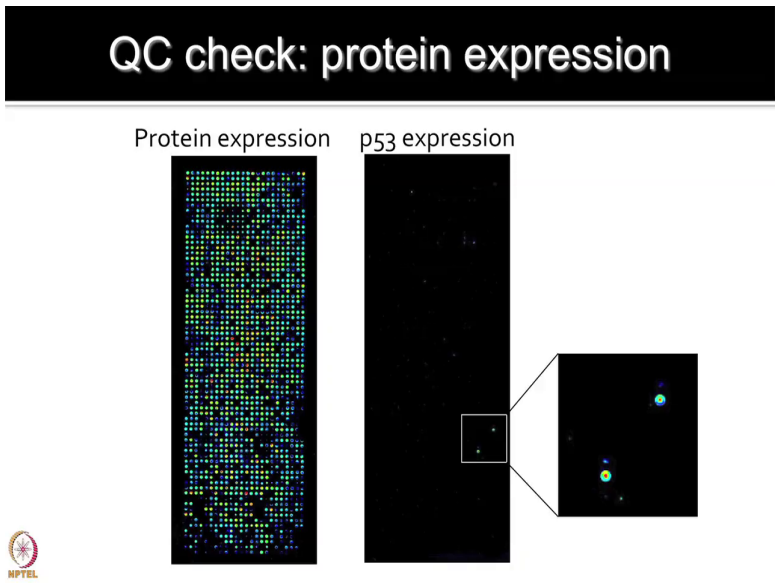

Now, the quality control check for the protein expression, once you have expressed your protein of interest you would like to see first the anti GST that you know the all the spots which are having the GST tag whether they are showing the spot you know intensity. And also to check the specific protein expression, in this case we had used anti 53 antibody which shows that you know 53 antibody is binding to the right feature of 53 protein.

And, that shows the you know that in the entire array while every protein is expressed you are able to pick up the right protein which is p 53 using this antibody of interest. So, again you

know in this entire workflow of doing arrays important considerations are keep testing the reproducibility.

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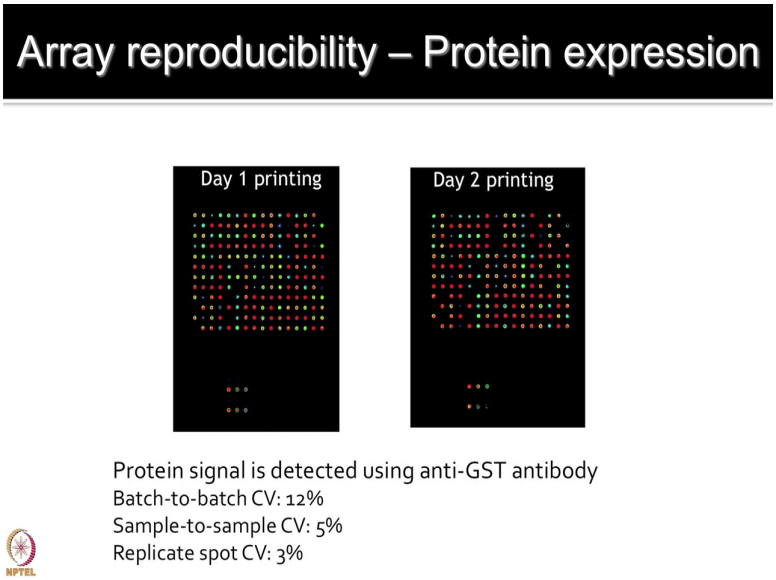

How good the quality of your assays are, how good the arrays are, how much variability you have from sample to sample, day to day and different individuals and different batches. All of these things are important and you have to keep monitoring them, what is the coefficient of variation by doing these assays.

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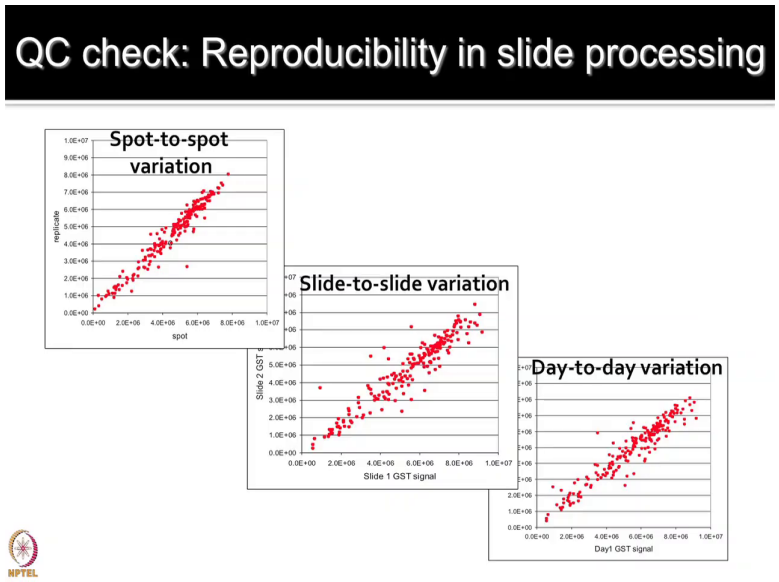

So, next slide you can see that you know how much rigor goes on before the actual experiments are being performed to look at a spot to spot variation, slide to side variation and day to day variation. Once you are very happy with these parameters, now you are good to go for screening large number of samples for the actual biomarker discovery program. So, I hope you got the glimpse of how to do the NAPPA based cell free expression protein microarrays. In general any microarray platform will utilize this kind of workflows and applications are unlimited, infinite.

You can think about any biochemistry, just at the miniaturized scale; think about arrays just you know that you have reduced the volume of all the regions of interest. And, now you want to test out the clinical samples of interest or the drugs of interest or the other protein of interest in a very very small volume which is now you can test out on the entire chip; with the

2 microlitre, 5 microlitre of the volume of the serum sample which you are diluting further in the buffer.

Now, you can probe on the entire chip which contains 20,000 proteins. So, with very very small volume with very precious samples now, you can do these assays which was otherwise not possible. Also good idea for you to keep thinking and comparing different technologies for example, you must have heard about mass spectrometry is making lot of revolution in the whole field of proteomics.

And whether microarray is to be comparable to that, each technology is unique in its own nature. But, in this context if we are talking about patient sample and especially serum sample, think about you know for doing mass spectrometry you have to first process your serum sample and remove all the abundant proteins. Using either you know some of the anti albumin or anti igG columns.

Once you remove those then only your serum sample contains the less abundant protein which could be further processed and digested to make the peptides which could be analyzed in the mass spectrometer. In this case on the arrays you are simply taking the serum, diluting it in the buffer and putting on the chip, directly screening. So, the dynamic range for the different you know order of magnitude of the protein is much larger here for measurement as compared to the mass spectrometry which will be only 4 to 5 order of magnitudes.

So, in many ways you know microarray is much more robust to provide with some unique applications which is if you compare with the mass spectrometry, it may not be possible. And you know many proteins which are actually bound with the abundant protein which are the carrier with the albumin and IgGs, those will also be removed when you are doing the abundant protein removal which will not be case in this case here.

If you are doing biomarker discovery program that is where lot of you know the array based approaches are still very powerful and they have shown lot of promises. If you are thinking about doing protein-protein interaction studies, you can know different ways; many ways are there even precipitation followed by mass spec or protein arrays. But, think about that if you

got an unknown protein of interest for which you do not have an antibody or you have is some protein of interest purified.

So, this unknown protein if you now probe on the chip, this is going to bind to some other proteins on the chip if there is interaction happen. And, based on this information you can get the clues that these proteins are going to be potentially involved the same network. So, they will have probably the similar role, similar function and this is what is known as functional proteomics. You can determine the function of the protein by utilizing these kind of high throughput approaches.

So, again microarrays can be powerful, way of getting understanding for the unknown function of the proteins. I hope you are convinced that protein microarrays are very rapid, efficient, high throughput platform to provide various type of applications both cell free and cell based arrays have been used. Cell free arrays have overcome some limitations which was otherwise not possible by using cell based arrays.

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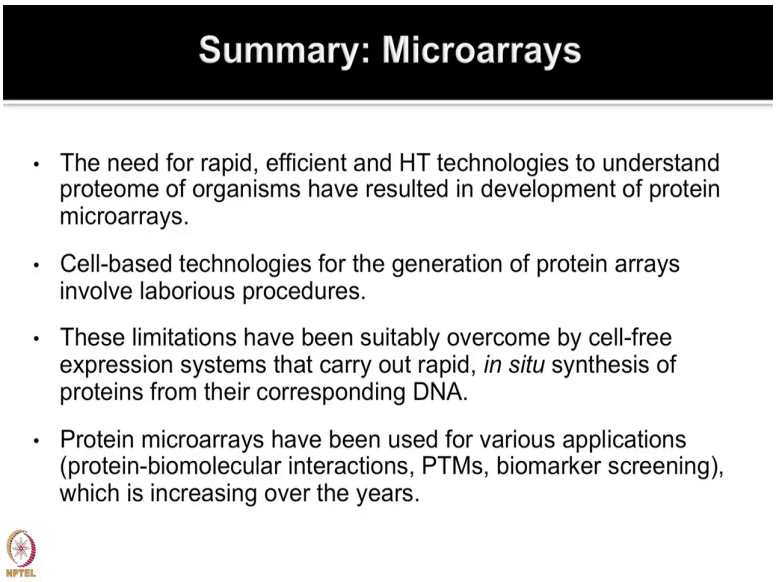

You can go to the (Refer Time: 37:50) and look for literature, you will find n number of applications whether its biomarker discovery looking at post translation modification, protein interaction, kinase substrate and assays; variety of thing you will see using arrays for every organism of interest. Of course, more on the human, but you will see n number of applications for all the life sciences.

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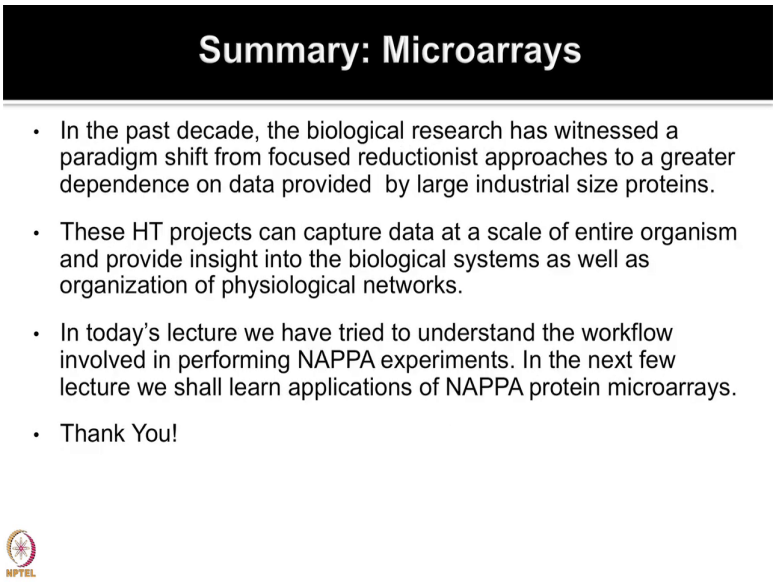

So, in the past decade the biological research has witnessed a paradigm shift from the focused reductionist approach to a greater dependence on the data provided by the large industrial sized proteins. These high throughput projects can capture data at a scale of entire organism and provide insight into the biological system as well as organization of physiological networks. I hope in today's lecture I was able to explain you how best you can do the protein microarrays.

While, the whole procedure looks a little complicated, but if you think about it is very straightforward just you have to change your mindset from the single tube and move out to the 96 well plate format or 384 well plate format; in everything you can achieve in the high density format. Now, this array platform once is ready, now can we use for many applications.

In the next lectures Dr. Joshua Labaer is going to talk to you about NAPPA technology, as well as the next generation high density NAPPA technique which is now able you know to much higher reproducible way of printing arrays and much more high density of the NAPPA arrays for variety of applications both for human and other pathogens.

So, I hope these lectures are going to be very exciting for you and they will add the perspective to some of the lectures which I have been delivering to give you an idea that how these arrays could be utilized for the real clinical problems and real biological problems and applications of interest.

Thank you.

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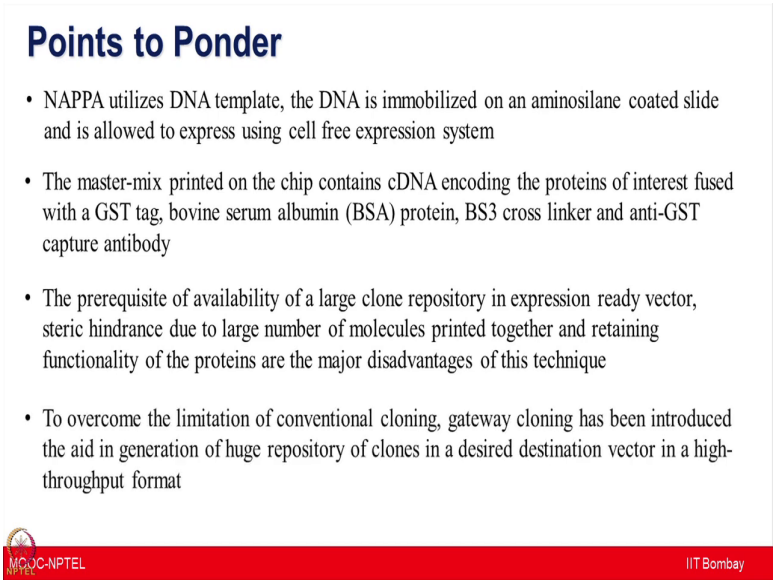

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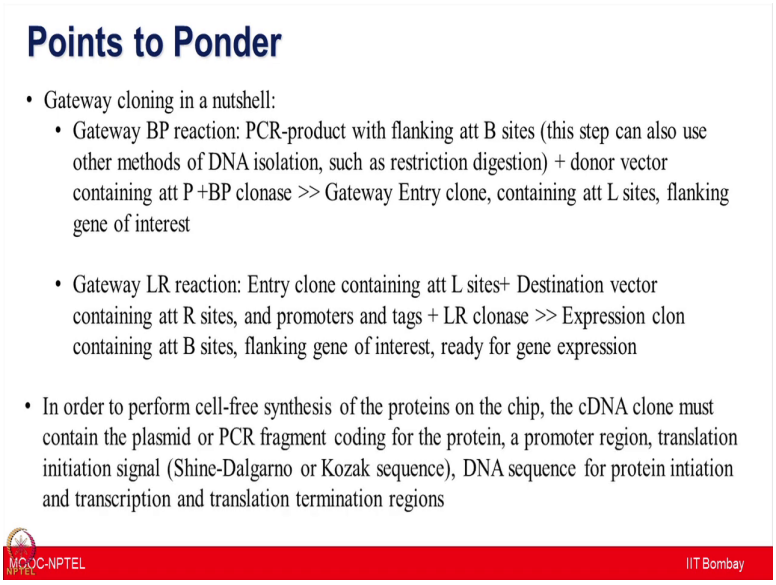

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