Interactomics Protein Arrays and Label-Free Biosensors
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Module 06
Lecture 29
Digging Deeper into NAPPA: Basic Workflow

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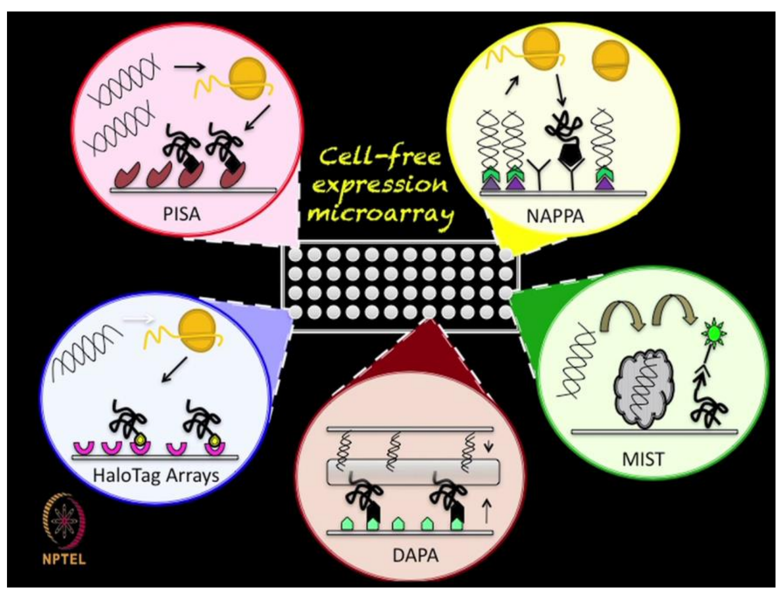

Welcome to the MOOC course on interactomics. In our previous lecture we discussed various types of cell-free expression based protein microarrays. We spoke about protein in-situ arrays, halotag arrays, DNA array to protein arrays, multiple spotting techniques as well as nucleic acid programmable protein arrays or NAPPA. Although each one of those technique offers several unique advantages but NAPPA approach has proof to overcome many practical limitations and let it being applied to generate high density arrays.

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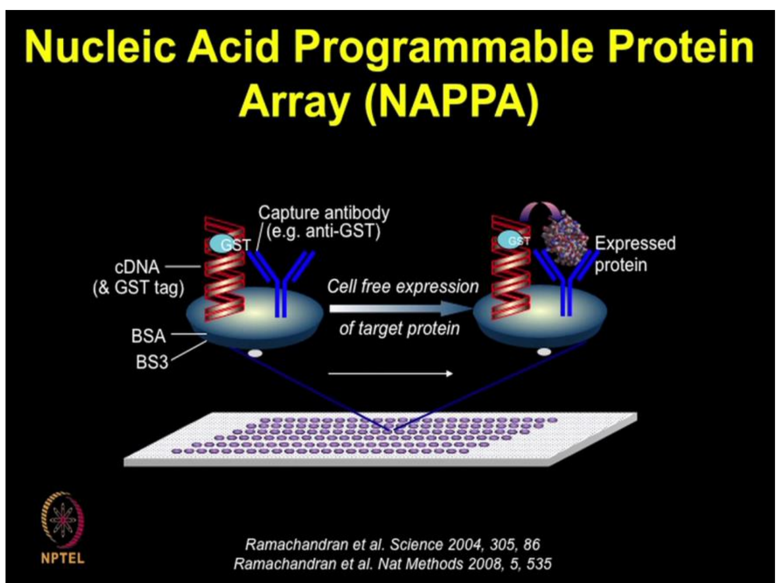

In today's lecture, we will talk about generating protein microarrays using NAPPA chemistry as you can see in the figure, you have a microarray slide and each feature highlighted in the expanded view contain four features. One of this is the c-DNA containing GST tag, then you have bovine serum albumin BSA protein, capture antibody such as anti-GST and BS3 cross linker.

In the NAPPA approach, plasmids encoding the target protein are fused with an affinity tag and those are fixed to the array surface. The array surface is activated by addition of cell-free expression system. The cell-free expression system have yield the path of fabricating arrays and have been widely commercialized from different manufacturers such as (())(2:01), thermo fisher, new England bio labs, etc. The cell extracts of wheat germ cells, rabbit reticulocyte lysate, E. coli cells and insect cells have shown great promise for in-vitro in translating proteins from the template DNA.

Rabbit reticulocyte lysates or HeLa cell lysates are particularly preferred for eukaryotic mammalian protein expression. HeLa cell lysates have shown to less variability from batch to batch as compared to rabbit reticulocyte lysates. This is because the cell lysates comes from an establish cell line which is likely to be more consistent as compare to coming from the animal model and therefore, the HeLa cell lysate protein expression provides very regular protein expression performance and less variability from batches.

The target proteins are expressed an immobilized in-situ and they are detected by universal anti-tag antibody in NAPPA chemistry. In this slide, the GST tag and anti-GST antibody are

shown. So, if you have a GST tag you can use an anti-GST antibody, if you have flag tag you can use anti-flag antibody, if you have mic tag you can use anti-mic antibody. These universal tag strategies can work out depending on the tag you used as well as the corresponding antibody pair.

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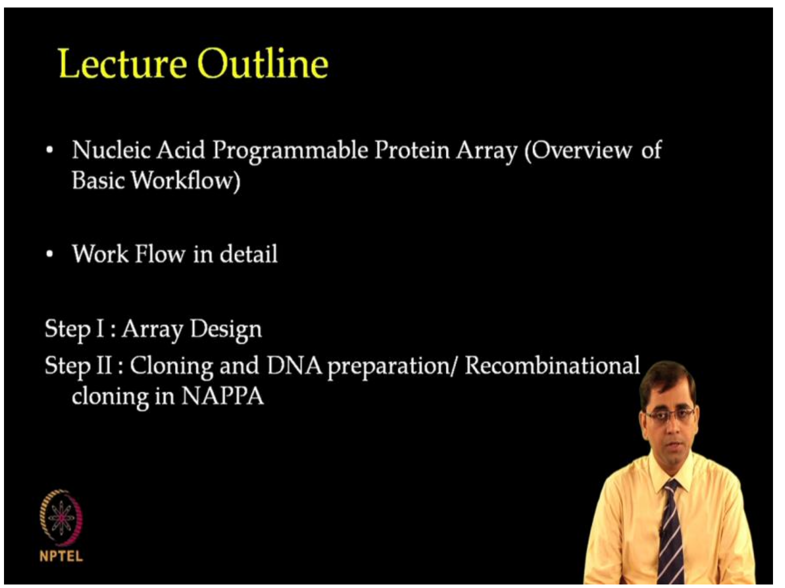

Based on the overview of different types of protein microarray platforms and cell-free expression based systems from our previous lecture, we will now focus on how to make protein microarrays by using NAPPA approach, we will go through with a stepwise workflow of monitoring various parameters. This includes a step like cloning and plasmid preparation, which will be discussed in this lecture as well as amino acyl silent coating of the glass slide printing parameters, which will be discussed in next lecture.

In the NAPPA approach, one need to ensure that DNA printing is of good quality as well as protein expression is good so that all the quality control checks are well established. So, what were the requirements to assemble protein arrays? In today’s lecture, we will discuss about making protein arrays which is similar for most of the cell-free expression based protein microarrays. This workflow can be largely applied for all types of protein microarrays platforms, but it will be essentially in the context to the NAPPA.

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Assembling protein array: requirements

- A repository of expression-ready clones in flexible cloning system that enables easy sub-cloning between various expression constructs
- A pipeline for quickly purifying DNA constructs and arraying them
- Robust printing chemistry
- HT capability at every step

First of all, we need repository of the expression ready clones, which should be in flexible cloning system. In the previous lecture, we have talked about recombinational cloning which provides very high quality master clones. Now, those master clones can be easily transferred by sub-cloning them in a different type of expression system. One must there after establish a pipeline for very quick verification of DNA constructs and it is printing on the chip surface. So, you need a robust printing chemistry since you are aiming for high throughput experiments with thousands of features to be printed on these arrays, high throughput capability is thus required so is the automation at every step. We will therefore first talk about the array design and the experimental planning required for performing these arrays.

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NAPPA protein array: work-flow

- Array design and experimental planning
- Selection of surface and printing chemistries
- Culture expression construct clones
- Purification and concentration of DNA
- Re-suspension of expression construct DNA in array spotting buffer
- Array printing
- Quality control check

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So unlike other proteomic techniques, where one starts with unknown things to identify the protein by using mass spectrometry. In protein microarray, scientists are already aware what type of contents are printed on the chip surface to start with and those contents have to be very well planned along with control features. If you have a specific objective in mind, for example, you want to study protein-protein interactions; you need to have some good positive controls for the protein interactors as well as some negative controls to rule out the false positive.

Even though to identify some biomarkers, for example, you are looking for serum screening you need to have some good control spots such as human IgG or mouse IgG. Thus, lot of thought process goes in designing the experiments for the microarrays as opposed to what one does in other type of proteomic technology. What type of surface one need to select? What printing chemistry one need to use are different questions which are always in mind of microarray surface? Since, we are talking about, the cell-free expression based system you need DNA to start with. So how to express those and construct those clones are other considerations.

Once you have done the culturing of clones, now you need to concentrate on DNA preparation. Almost all the molecular biology laboratories perform these experiments on routine basis and it is very straight forward to do the DNA extraction. However, when we talk about doing the experiments in high throughput, then it possess several challenges because quality of all your features have to be good.

So, various type of quality controls and various observations one need to keep in mind for doing the even simple experiments like DNA purification and concentration. Once your DNA is prepared then you need to mix it with proper printing mix. In this case, we are talking about NAPPA arrays, so we need to have those components which are required to make the protein by c-DNA to ensure the printing quality is good and various type of quality control checks are required.

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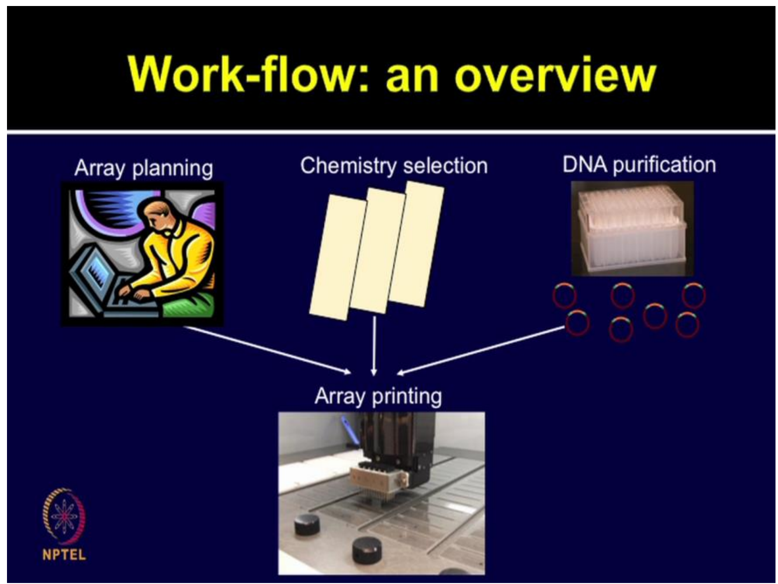

So, to overview the experimental planning, one must design the arrays; establish the features to be printed on the arrays surface. One must design these experiments thoughtfully; establish the features which are going to be printed on these arrays. What type of chemistry needs to be selected? How to purify the DNA and once you have the slide functionally ready along with a DNA in the printing mix then all you need to do print those in large number of slides for various applications.

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Points to Ponder:

- NAPPA is one of the most sought after cell-free expression based array platform available
- It involves printing and immobilization of purified cDNA/ plasmid DNA containing clones of desired genes on a chip
- These genes are expressed using cell-free expression systems which are captured using a capture antibody/ ligand
- NAPPA requires a robust printing capability, availability of a library of clones which allows high-throughput performance at every stage.

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Array design

- Design experiment type & appropriate target protein test set
- Fit NAPPA chemistry utilized to assay being executed
- Decide array density & mapping
- Select robotic parameters for array execution

So, let us start with the array design first, as I mentioned lot of thought process goes into designing these experiments, one need to perform the thorough literature review, one need to look at the experimental objective carefully even before moving forward for any type of microarray printing. So, design the experiment type and designate an appropriate protein test set. Once, your test set works one can actually expand it for 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 define the robotic parameters for these experiments, all these steps have to be a well-planned in advance.

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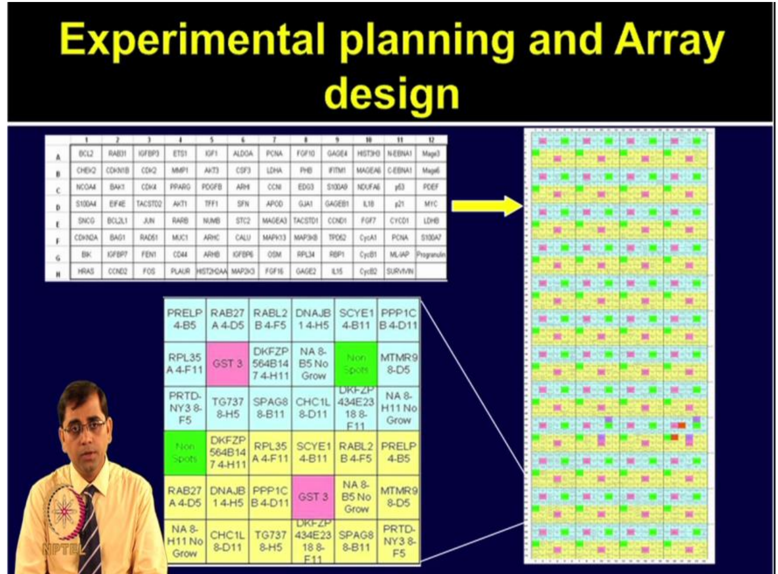

For example, I have shown you few insets here, the very top panel in these slide show different genes, which you would like to make on the 96 well plates while doing DNA prepping. First of all, we have clones and now you want to do the DNA prepping you know in each well, for example, in A1 you know what gene it would contain, it has B cell 2G, then H12 has been left blank, G12 has a define gene. Similarly, all the wells have a define gene, on the right hand side I have shown you the array map which shows the very high density map. How different types of features can be printed on the chip all these things have to be designed prior to the experiment.

Now, each spot needs to be printed on duplicate, also you need to ensure that control features are spread throughout the array surface. For example, if you see the bottom panel, once which are highlighted in the red color are showing GST protein, green are the known spots. So these spots are printed in duplicates and they are spread on the array surface not adjacent to each other so that you can look up the duplicate results and ensure that there is no (())(12:28) issues or spreading on the neighboring spots.

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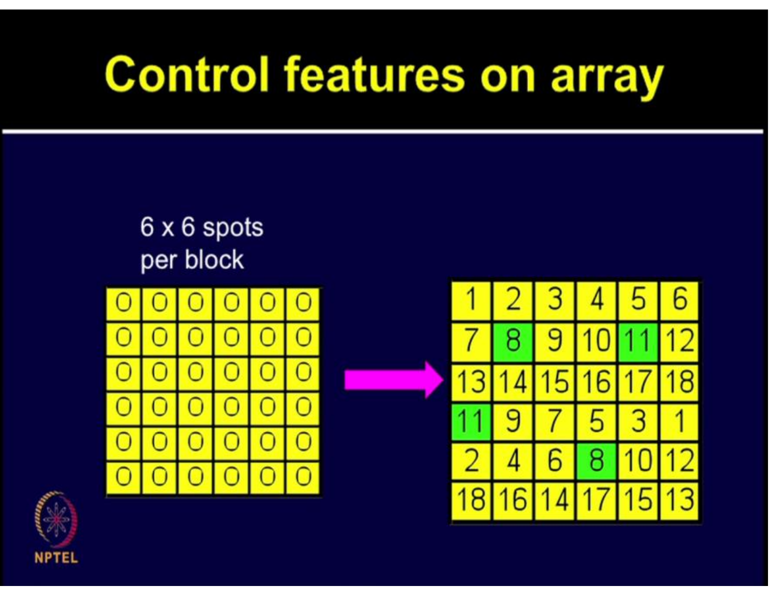

This is expanded view here of a 6 by 6 spots for block. These are small blocks and obviously on the (())(12:39) you have several features. The yellow ones show the genes and green one shows the control features. So for example, here one can use 4 different types of control features, no spot, no DNA control, a mouse IgG, a known gene, GST protein, human IgG, etc. It all depends on the objective of your experiment and then suitable controls can be incorporated in the array map.

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Points to Ponder:

- Array designing is one of the crucial primary steps in NAPPA based experiments
- Since chip printing involves several robotic parameters, the array design and desired density must be established before hand.
- The scheme and order of assay must be fed to the arrayer where it would follow the input scheme from multiple blocks of 96 well plates onto chips
- The array design must incorporate several positive, negative and in-built quality control features for undertaking experiments optimally

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Cloning and DNA extraction

- Bioinformatics selection of target cDNAs
- Primer design and PCR amplification
- Gel purification of PCR amplicons
- Cloning into entry construct
- Selection and culturing of isolated transformants

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First of all, one need to do a bioinformatics selection of all the target c-DNAs. If you are focusing on a particular cancer or a particular disease obviously, you have to think about all those genes which are relevant for that disease as well as different type of control features which could be a marker for that given disease, so now you need genes of interest to print. So, let us talk about cloning and plasmid preparation. Earlier we talked about recombinational cloning.

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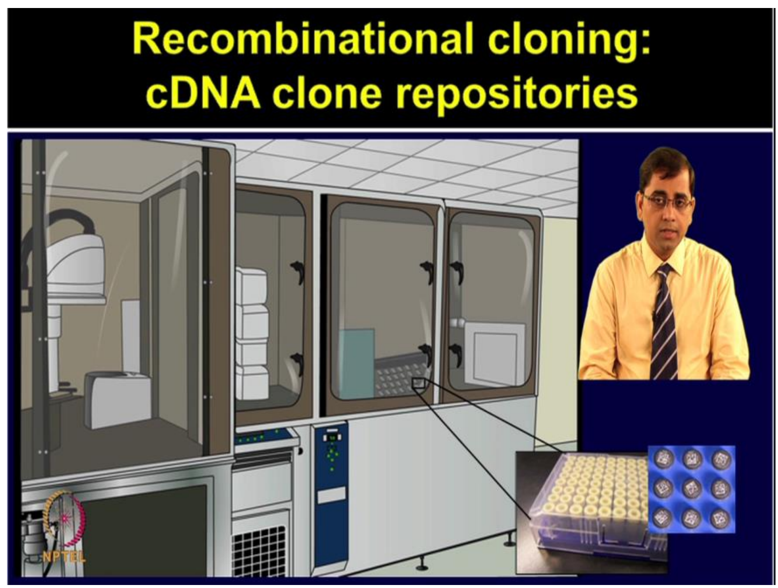

Now, researchers have started creating c-DNA repositories using recombinational cloning, which allows for the rapid transfer of DNA fragments from one vector to another in frame and without any mutation. These clones are collected in repositories such as one at a (())(14:42), MCG clones and there are several other commercial repositories as well.

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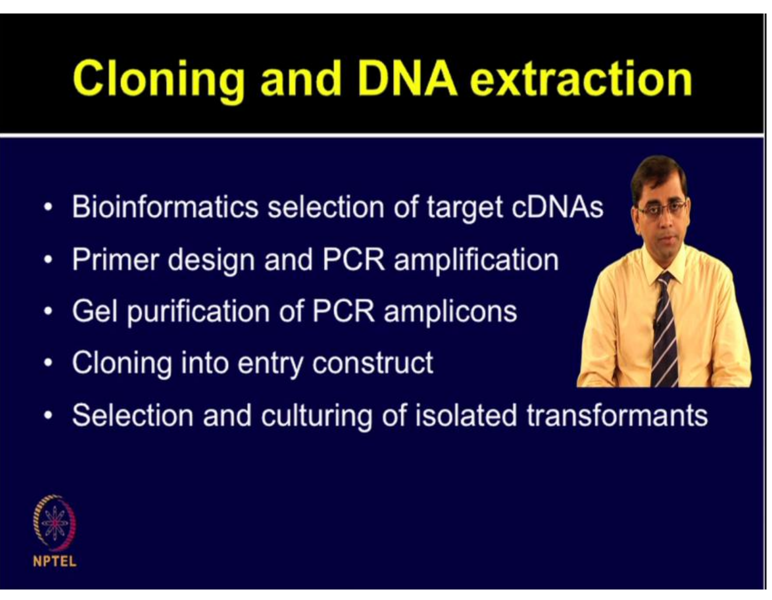

Ones you have selected the gene of interest. Now, you need to design the primers and use PCR to amplifying those clones. Gel purify the right size band and then move them in the entry construct, You need to then transform them in E. coli, select the right transformant colony, then you would like to use those colonies in the long term by making a stock in this all stock and use it for the future reference.

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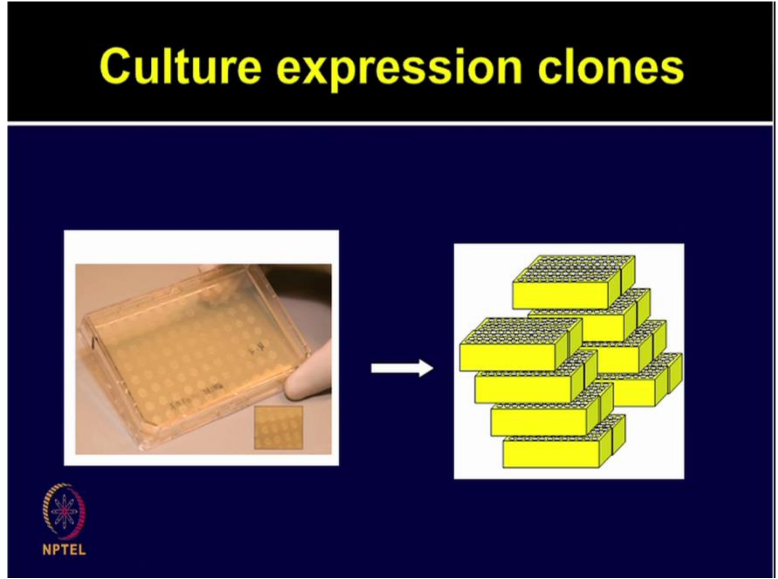

DNA preparation process is very similar to what everybody performs in a very routine molecular biology lab, only difference here is that things have to be much more automated this steps has to be much faster and in high throughput manner. If you are performing these plasmid (())(15:43) in tubes, then you have to really do it many times which will increase the irreproducibility and manual artifacts. So, these steps are preferably performed by using automated liquid handling system. It is difficult to achieve high density, so all the steps need to be automated.

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An image here shows the type of arrayers and platforms which can be used for performing these experiments. In this manner, you can do the prepping in 96 well plate format and lysate

can be loaded directly on to the DNA binding plates by using these types of robots. Let me explain you the cloning as well as plasmid preparation in high throughput manner by showing this animation Automated clone storage system.

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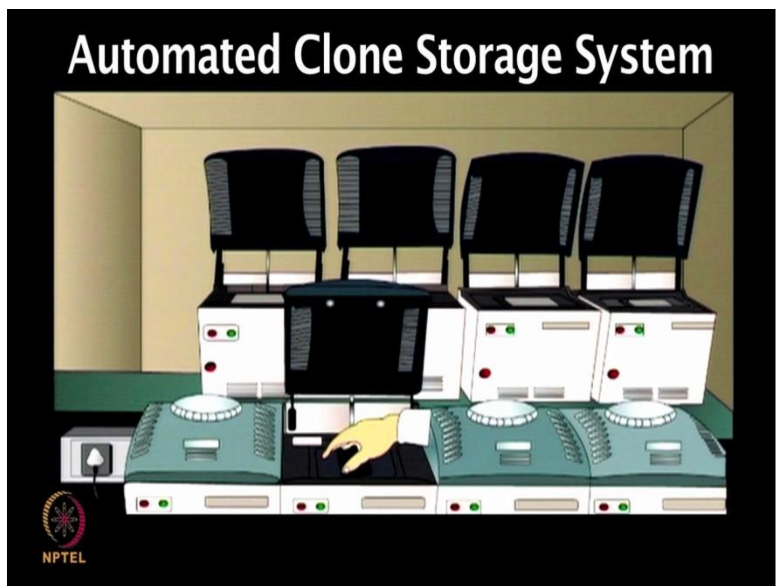

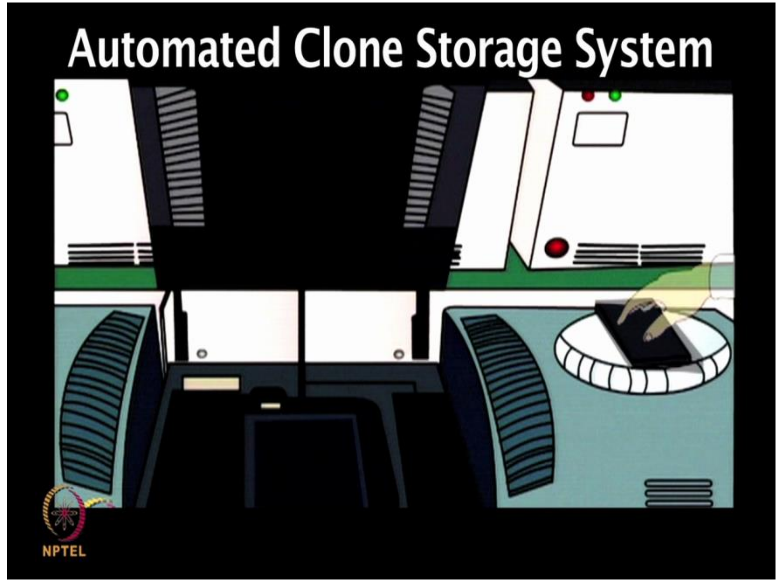

In order to program the NAPPA arrays, first of all we will require access to the clone c-DNA representing the target proteins. The c-DBNA clones are stored in repositories in -80 degrees; all the genes should be a fully sequenced. The full length open reading frames captured into a recombination based cloning system. Genes of interest can be easily transferred into any expression vector by simply adding the appropriate recombinational 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 recombinational

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 on the LB agar plate.

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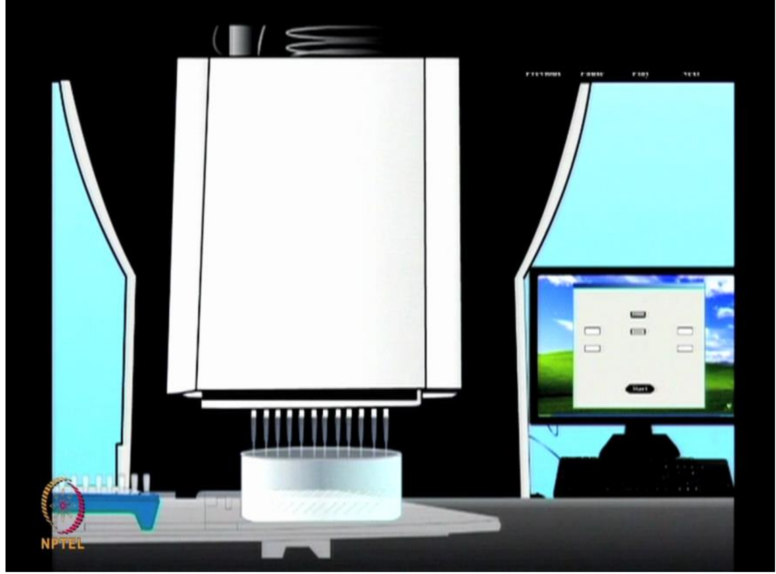

So transfer the bacteria from the glycerol stocks to an LB agar plate that contains appropriate antibiotic, you can use that either using multichannel pipetters or using liquid handling systems. It is prefer to do this in 96 well plate format so that you can avoid any miss-labeling of the samples.

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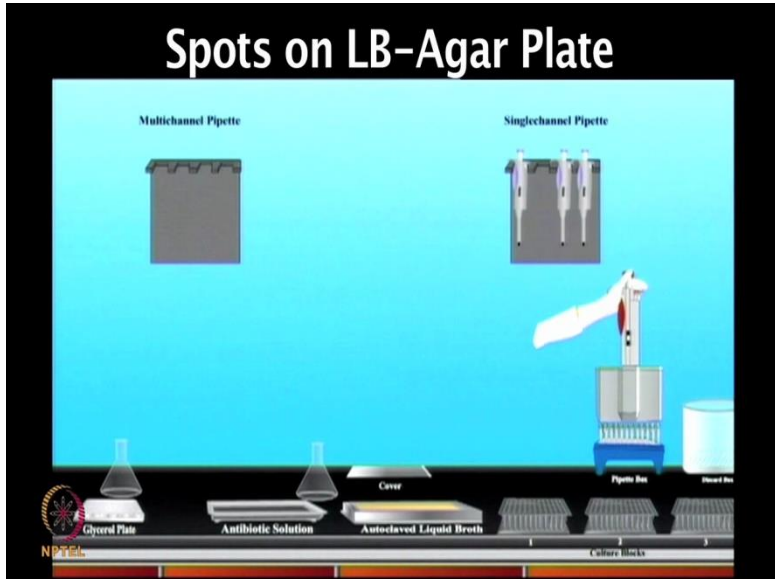

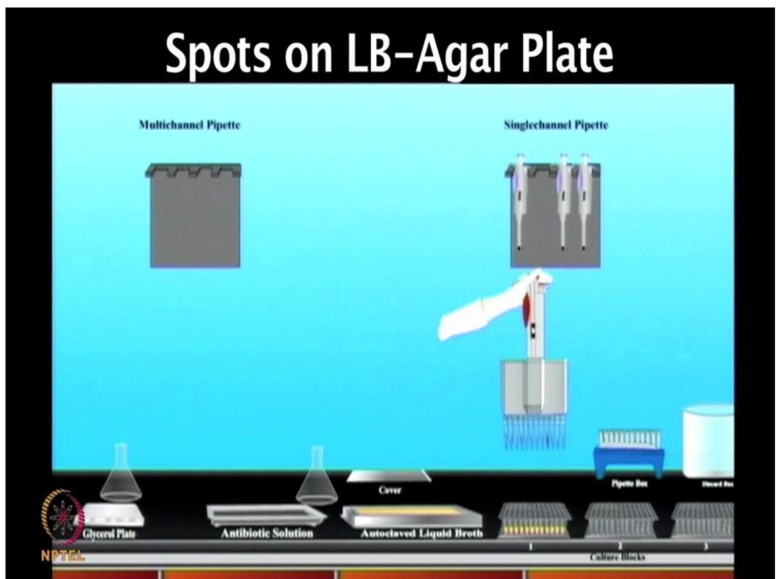

So, allow the bacteria to grow overnight at 37 degrees before inoculating that into the liquid medium. So, as shown here you can use a multichannel pipetter or you can also use the liquid handling system. So, first of all you need to prepare the culture blocks and add the liquid block in 96 well plate formats, in order to ensure that you have added the antibiotic solution in each well. Once culture blocks are ready, and then inoculate the liquid culture from the bacteria grown on the agar plates.

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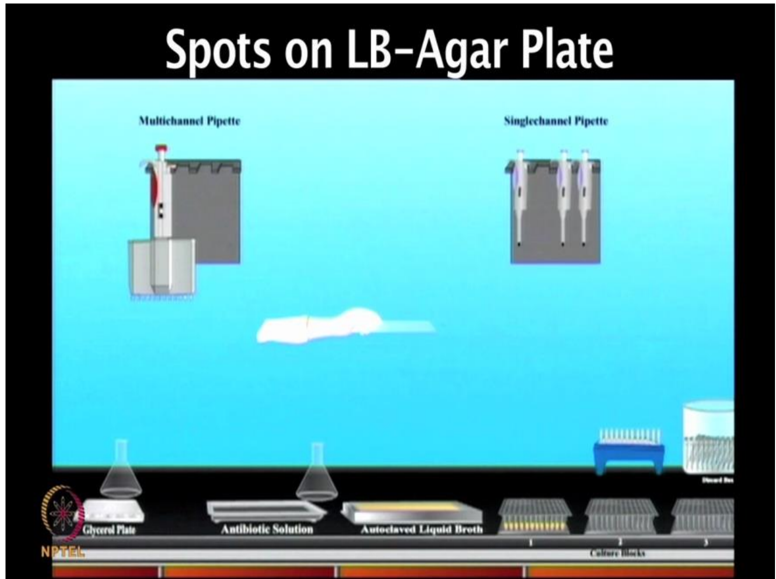

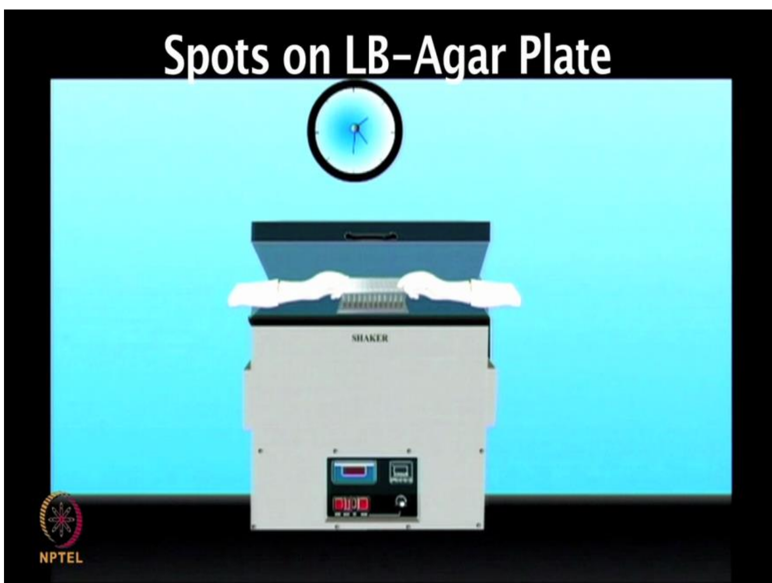

Similarly, you can fill the all the wells or then cover the deeper block with a permeable seal, ensure that sealing is proper and then incubates the cultures on a shaker you can glow a culture at 37 degrees at 300 RPM, ensure that shaking is uniform, after these blocks have completely grow on the bacteria for the overnight or 10 to 12 hours of incubation, then we need to pellet down these blocks.

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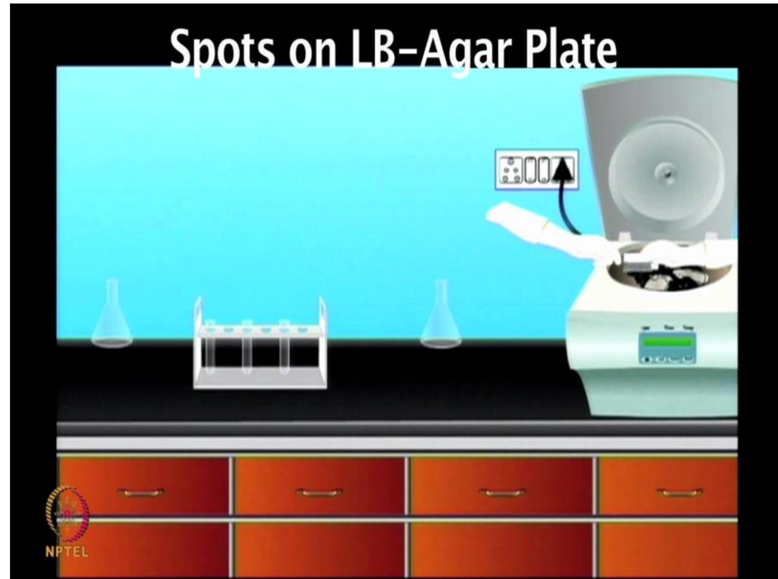

So, you can centrifuge these blocks for 15 minutes at 2000 to 2500 RPM, while doing centrifugation ensures that centrifuge is balanced. So, once centrifugation is complete then the bacterial pellet will be in the well and then you need to remove the supernatant. So, remove the supernatant from these bacterial cultures, discard the tips and once we have completed that for all the wells then you are ready to perform the DNA plasmid isolation.

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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 micro liter of solution 1, which is resuspension buffer that contains 50 milimolar of (())(22:42) at pH 8, 10 milimolar of EDTA and 0.5 mg per ml of riboneucleus. So, you need to add the resuspension buffer around 200 microliter to each well. So, remember in the last step we had made the bacterial pellet from those culture blocks and now you can add this 200 microliter of resuspension buffer in each well.

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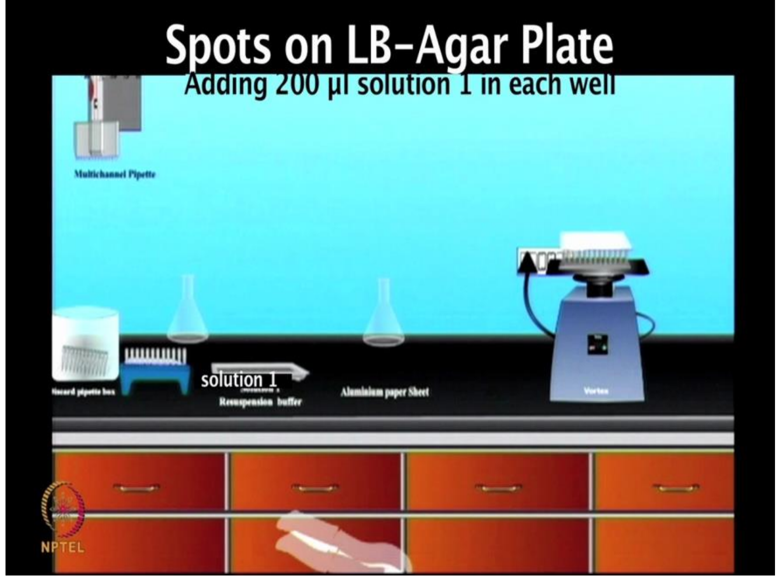

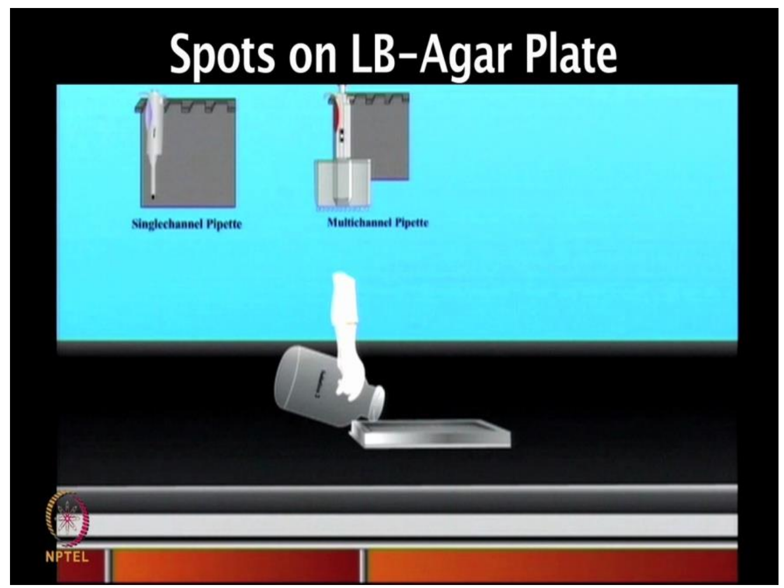

After adding the solution then you can close the plate by adding a cover seal. Once the seal is placed there you need to vertex it, one can also do it by pipetting up and down or doing the (())(23:49) few times. In a similar way, add 200 microliter of solution 2 to each well. Solution 2 contains 0.2 normal NaOH and one percent of sodium dodecyl sulphate. This is a lysate solution, after adding this solution a need you repeat the same process of adding 200 microliter of solution two to each well, fill the plate and then inward the plate few times.

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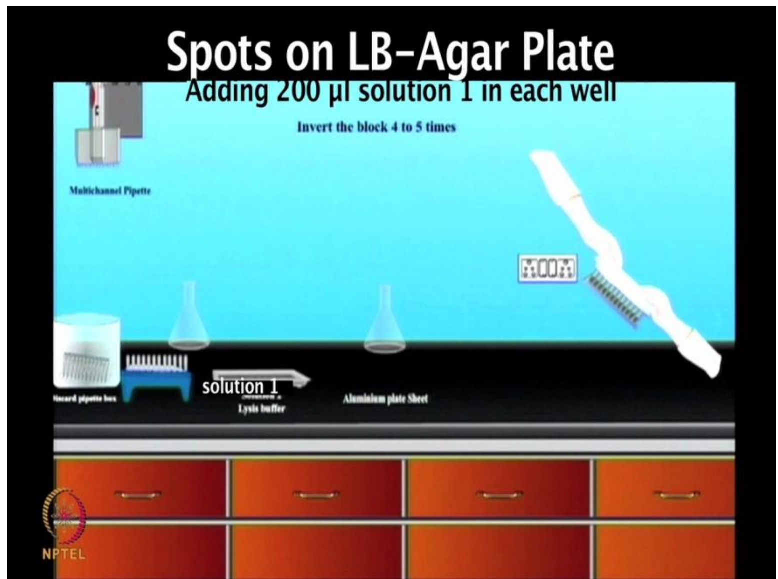

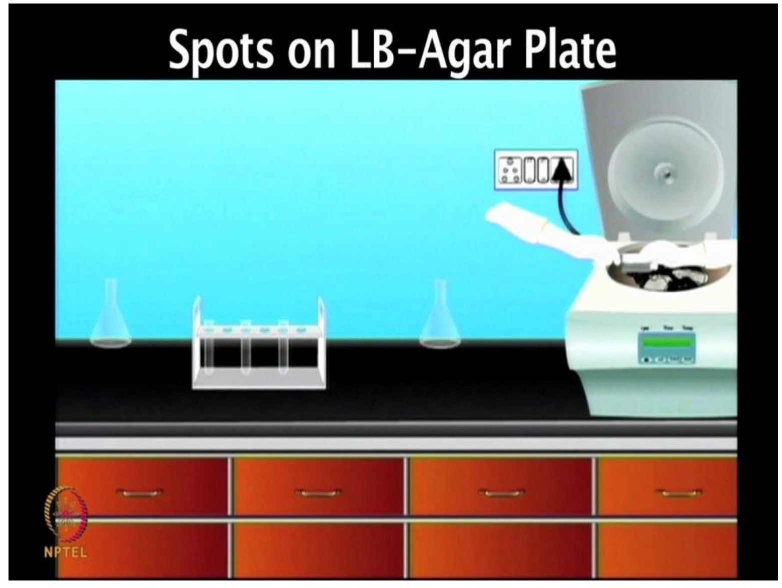

Once solution two is added make sure that you inward the plate few times. Now, add 200 microliter of solution 3 to each well, similar to what we have seen earlier. The solution 3 is then utilization solution which contains 3 molar of potassium acetate at pH 5.5. Now, centrifuge a block at maximum speed for 20 minutes at the room temperature. Once centrifugation is complete, then you need to discard these supernatant by dumping the block over waste container.

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Points to Ponder:

- The cDNA/ plasmid DNA which requires to be printed onto the slide needs to be available in expression ready clones
- One must amplify the desired genes from an available clone library and sub-clone it onto a desired vector using recombinational cloning, or must procure clones from a clone repository in expression vectors compatible with the cell-free lysate being used
- These steps can be performed in a high-throughput format using liquid handling robots

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So, with this we have now learned how to perform plasmid preparation for printing on NAPPA arrays. This would serve as the primary template for protein production. In the next lecture, we will learn further as how to fabricate the surface of these chips, print arrays and express pure proteins for the functional protein assays Thank you.

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Summary

- NAPPA is a new-age protein array platform
- Array designing and procuring the right clones in a compatible expression vector are the two primary steps involved in a NAPPA experiment
- While designing the array, one must ensure that the broad goals of the experiment and inherent quality control checks are maintained. The chip must be designed such that it aids in future trouble shooting of the experiment, meeting the goals of the experiment

References

- Atak, A., Mukherjee, S., Jain, R., Gupta, S., Singh, V., Gahoi, N., Kp, M., and Srivastava, S. (2016). Protein microarray applications: Autoantibody detection & Post-translational modification. PROTEOMICS.
- Chandra, H., and Srivastava, S. (2010). Cell-free synthesis-based protein microarrays and their applications. Proteomics 10, 717–730.
- Chandra, H., Reddy, P.J., and Srivastava, S. (2011). Protein microarrays and novel detection platforms. Expert Rev. Proteomics 8, 61–79.

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References

- Gupta, S., Manubhai, K.P., Kulkarni, V., and Srivastava, S. (2016). An overview of innovations and industrial solutions in Protein Microarray Technology. Proteomics 16, 1297–1308.
- Hu, C., Huang, W., Chen, H., Song, G., Li, P., Shan, Q., Zhang, X., Zhang, F., Zhu, H., Wu, L., et al. (2015). Autoantibody profiling on human proteome microarray for biomarker discovery in cerebrospinal fluid and sera of neuropsychiatric lupus. PloS One 10, e0126643.
- Liotta, L.A., Espina, V., Mehta, A.I., Calvert, V., Rosenblatt, K., Geho, D., Munson, P.J., Young, L., Wulfkuhle, J., and Petricoin, E.F. (2003). Protein microarrays: meeting analytical challenges for clinical applications. Cancer Cell 3, 317–325.

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References

- Ramachandran, N., Hainsworth, E., Bhullar, B., Eisenstein, S., Rosen, B., Lau, A.Y., Walter, J.C., and LaBaer, J. (2004). Self-Assembling Protein Microarrays. Science 305, 86–90.
- Ramachandran, N., Raphael, J.V., Hainsworth, E., Demirkan, G., Fuentes, M.G., Rolfs, A., Hu, Y., and LaBaer, J. (2008a). Next-generation high-density self-assembling functional protein arrays. Nat. Methods 5, 535–538.
- Ramachandran, N., Srivastava, S., and Labaer, J. (2008b). Applications of protein microarrays for biomarker discovery. Proteomics Clin. Appl. 2, 1444–1459.

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References

- Ray, S., Mehta, G., and Srivastava, S. (2010). Label-free detection techniques for protein microarrays: prospects, merits and challenges. Proteomics 10, 731–748.
- Yan, H., Park, S.H., Finkelstein, G., Reif, J.H., and LaBean, T.H. (2003). DNA-Templated Self-Assembly of Protein Arrays and Highly Conductive Nanowires. Science 301, 1882–1884.