Proteomics : Principles And Techniques

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Lecture No. # 28
Microarray related concepts: Recombinational cloning cell-free expression

Welcome to the proteomics course in today's lecture, we will talk about few concepts related with the microarrays, we will talk about recombinational cloning, and we will talk about cell free expression both recombinational cloning and cell free expression systems are being used for microarrays. And this will be useful when you hear the next lecture, on the cell free expression based protein microarrays. The high throughput biology begin in Ernest with human genome project and increasingly, these high throughput techniques are used for proteomics research. In this light the function of proteomics is an exciting new approach to study the protein function in high throughput manner, and it will enable the expression of proteins and subsequent assay of various properties, such as sub cellular location interacting protein partners bio chemical activity these are regulated at the scale to achieve the high throughput.

There are large number of tools to study the activity of individual proteins, including methods to measure sub cellular localization of proteins, identifying interacting partners, as well as various type of protein modifications. All these complexities arise when one wants to verify the protein and apply that for the protein microarrays. So, to overcome these limitations people have tried various new methods, and self reexpression systems are one among them, but for the microarrays the new requirements are high throughput you need large number of clones to be studied, and that is where recombinational cloning comes into the plate.

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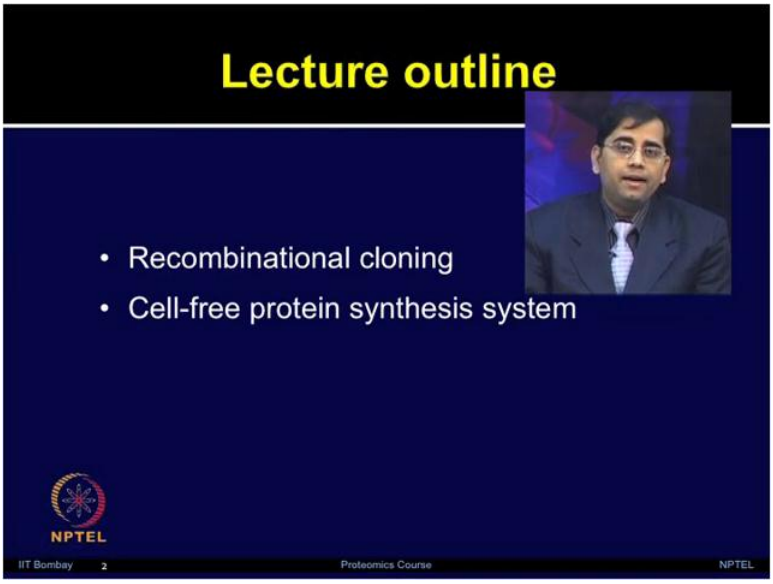

In today's lecture, we will talk about recombinational cloning and cell free protein synthesis system. So, the development of high throughput methods that still remains very challenging, all the high throughput methods they start with the one common step, which is expression of a protein from cloned copy of the gene Cdna. A prerequisite for these approaches including the protein microarray applications is need for large collection of cDNA's in a format which is conducive for the high throughput protein expression.

So, researcher's have started to create such large collection of cDNA by using recombinational cloning, which allows very rapid transfer of DNA fragment from one vector to another in a very short time. So, let us start our discussion with the recombinational cloning, to examine the collection of proteins in mass methods are required to transfer the coding region collectively into the appropriate expression vectors, for functional proteomic studies. The site is specific recombination is a genetic recombination technique, where DNA strand exchange takes place between the regions possessing resonance sequence homology.

The common site is specific recombination technologies include gateway coding system from invitrogen and create a technology from b d clontech. In our discussion on recombination cloning I will focus more on recombination cloning system from the gateway. It is more so because of my own familiarity using in my own research, but both the systems can be used for recombinational cloning. So, as I mentioned recombinational

cloning is a novel site specific recombination technique, which is required to transfer the DNA sequences. The specific recombinase enzymes they cleave DNA backbone and carryout interchange of DNA helices between the specific sides on two different molecules.

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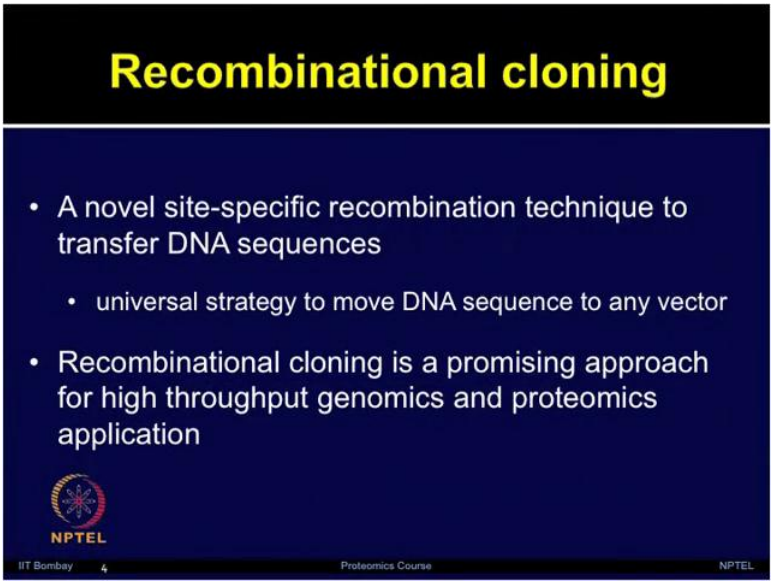

The recombinational cloning is the universal strategy to move the DNA sequence to any given vector. This is a very promising approach especially, when one looks for the high throughput applications, such as genomics and proteomics. Before I talk about what recombinational cloning is and how it works. Let me introduce you to first terminology which is used in recombinational cloning.

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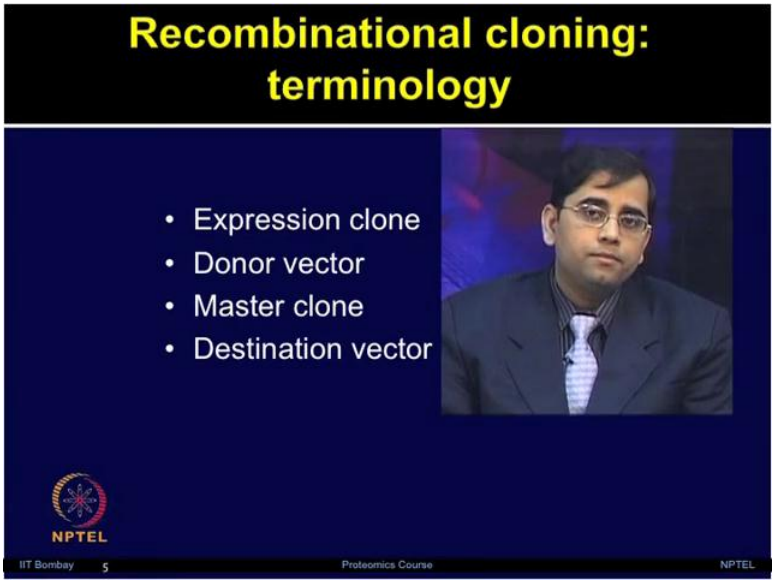

So, what is expression clones. A clone containing gene sequence of interest which is flange by the B sides. The orientation of gene is maintained throughout the cloning process, because of specific interactions between the add sites. So, what are the add sites these are the DNA segments of a certain defined length. Now let us talk about donor vector. Donor Vector consist of counter selectable gene which is flange by the at P side, which recombines with gene of interest flange by the at B side in BP reaction to produce a master or entryclone. What is BP reaction what is master entry clone these things will be slowly cleared to you in the subsequent slides. So, let us define what is master clone or infriclone the vector containing gene of interest flange by at L side, entry clones are formed by the BP reaction and further used in alar reaction to produce expression clones of interest.

What is destination vector? This is vector containing a counter selectable gene flange by at R sides, which interacts with genes from entryclone to produce an expression vector in the LR reaction. Now slowly these terms will become easier for you to follow up when we will explain this in subsequent slides where I've shown you some pictorial representation of these reactions. So, broadly recombinational cloning involves two reactions BP reaction and LR reaction.

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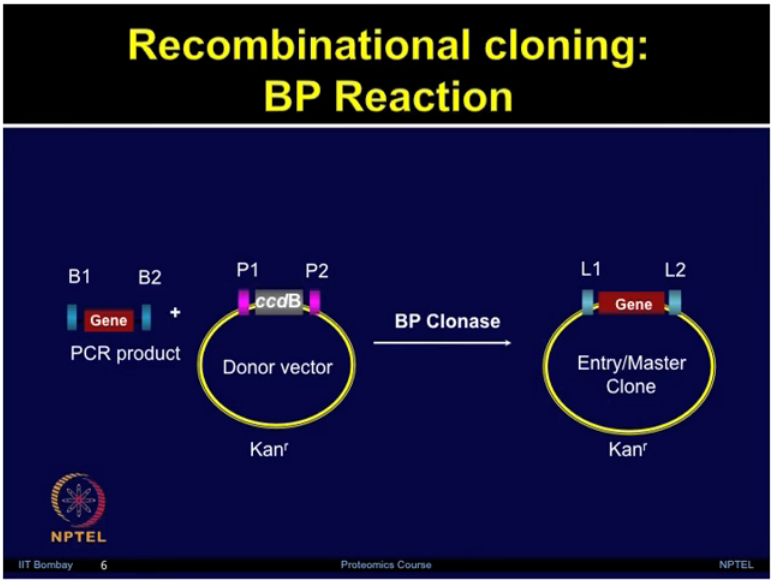

So, let us talk about BP reaction, when reaction is run to mix at B and at P sides, such as when making master clones this step is catalysed by BP clone is enzyme and it is known as BP reaction, because of at B and at P side involvement. So, as you can see in the slide there is a donor vector or Pdonr, that contains at P slides flanking a ccdB gene or a counter selectable gene that recombines with gene of interest which is flange by the at B side which you can see on the left hand side. So, this gene fragment can be produced from the PCR product and it contains at B side and you have a donor vector which contains a selectable marker kanamycin marker that contains P sides. So, entry or master clone is a vector containing gene of interest flange by the at L side, this happens after this reaction is completed.

Now the recombination between at B and at P side is catalysed by an enzyme known as BP clonase. Now this enzyme transfers the gene of interest the at B sides to the donor vector which contains at P sides. Again what is ad side? So, these can be defined as length of DNA that serves as the binding site for recombination protein. So, once the BP reaction is completed this entry or master clone can be taken for further alar reaction.

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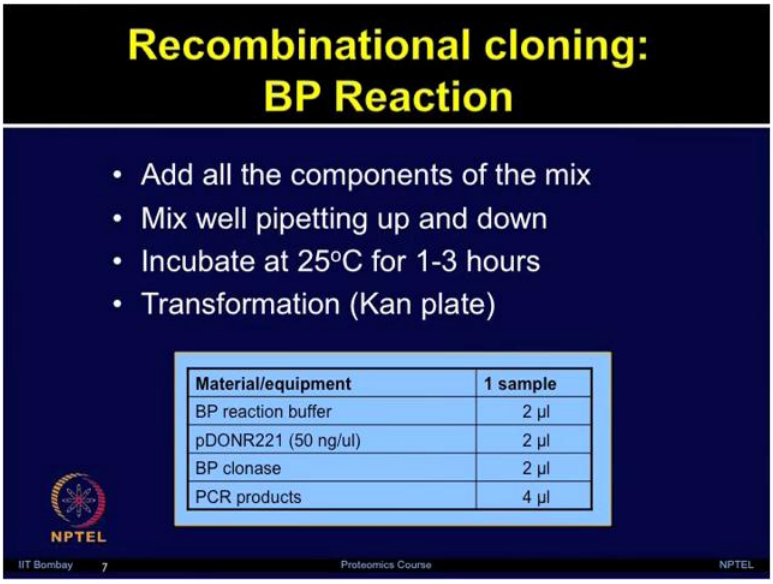

So, let before I move to the alar reaction, let me give you a brief overview of how BP reaction can be performed in the laboratories. So, you need to add various components which are shown in the table such as the BP reaction buffer P donor around 50 nano gram of DNA, BP clonase enzyme and PCR products these are some of the specifications which can be modified according to the requirements. So, add all these component of BP reaction mix and then mix them well by pipetting them up and down. After that you can incubate for 1 hour or 1 to 3 hours at 25 degree centigrade, and then this reaction mix can be used for transformation process on kanamycin resistant antibiotics. Since we have this vector containing kanamycin resistant gene, now only those clones which will have the entry of your correct insert in the right vector will only able to grow on the kanamycin plate.

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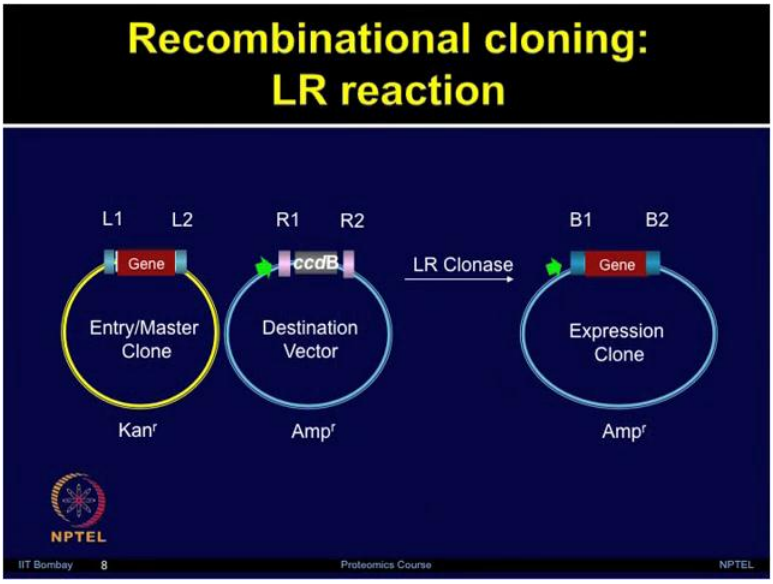

Let us now, discuss the next reaction which is LR reaction. So, in the BP reaction you have made one entry or master clone and now that can be further used in the LR reaction. So, in the LR reaction if the interaction is in between the at L and at R side, that is how the name LR came then it can be catalysed by the enzyme LR clonase, and the reaction is known as LR. So, LR recombination between entry clone and the destination vector. As you can see in the slide you have the entry clone or the master clone which was selected on the kanamycin plate. Now next you want to transfer that in the destination vector which contains another antibiotic gene which is ampicilin resistant gene, now this one the destination vector contains R sides at R 1 and R 2.

So, the gene of interest can move from the entry or the master clone to the destination vector. The destination vector also known as d e s t dest it is modified to accept the protein coding sequence in frame from the master clone or the entry clone, by using the recombinational cloning enzyme known as LR clonings. Once this reaction is completed then reaction mix can be plated on the amphicyon plate, and since the vector contains amphicyon resistant gene it can be screen on the amphicyon plates.

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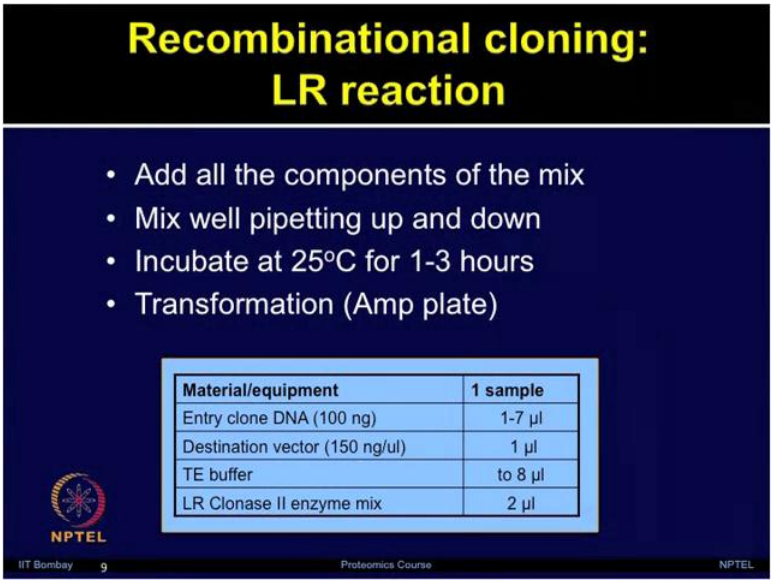

Now let us discuss, the recipe for LR reaction what are the components required. Again to perform the experiment in the laboratory one need to take the entry clone DNA obtained from the BP reaction around 100 nano grams will be optimal. Then you need a destination vector of your choice in which you want to insert this gene of interest, around 150 nano gram per micro litre, you need TE buffer and you need LR cloning enzyme mix. Again these volumes are just to begin with analytically modified according to the reaction requirements. So, add all the components of this mix the LR mix them well by doing the pipetting again do proper up and down, incubate the reaction at 25 degrees for an hour or 1 to 3 hours, and then this mix can be put on the plate for the transformation process on the amphicyon resistant plate. Now this antibiotic will be able to screen only those clones which have the gene of interest in the right vector.

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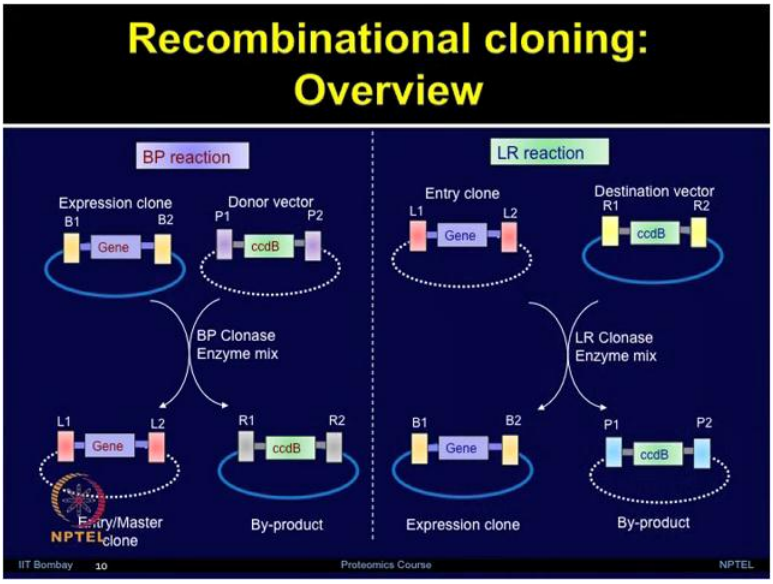

So, now as the overview of this reaction of recombinational cloning. There are two steps involved BP reaction and LR reaction. So, the recombinational cloning technology facilitates protein expression and cloning of PCR products by using side specific recombination enzymes rather than the restriction endonucleases and ligases which are used in the conventional cloning. The recombinational cloning makes use of a master clone as you can see in the slide having a particular gene that can be rapidly transferred to the desired destination vectors, and thereby it provides significant benefit over the conventional cloning methods. In the BP reaction which is catalyzed by the BP clone is enzyme it facilitates recombination of at B site at B substrate, it could be the at B obtained from the PCR product or att B containing the expression clone and this reaction with the att P substrate which is requiring the master vector can create the att L containing master clone.

So, the two steps are very quick from the BP reaction you can generate the entry or master clones, which can be further used for transferring the gene of interest to the destination vector of your requirements.

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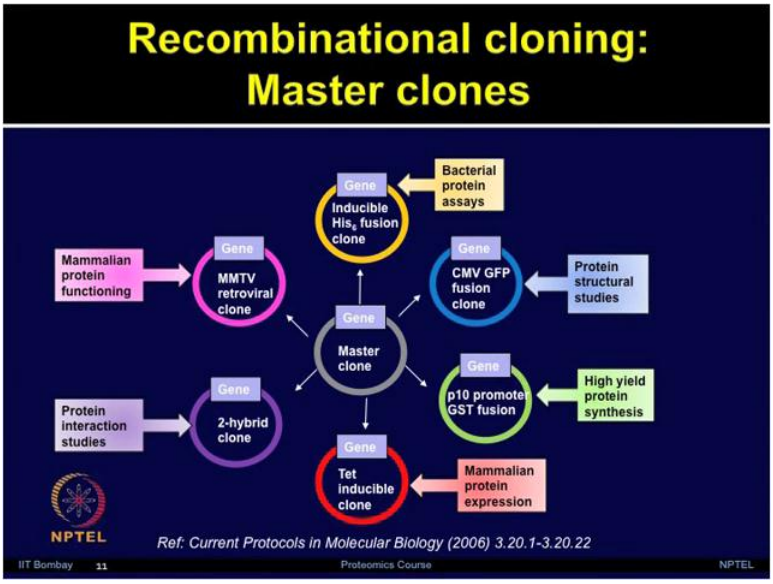

Now, once these master clones are generated, these are almost like a gold clones this repository can be very useful for various applications. So, you can transfer your gene of interest from these master clones to different type of destination vectors. In the slide there are few vectors are shown and few applications are informed here for example, one can use inducible historine 6 fusion clone. Now this can be used further performing assays using protein made in the bacterial system. One can use CMV GFP fusion clones for the protein structural studies if you want to know where my protein is located in the cell. Then P 10 promoter GST fusion clone, which produce lots of proteins for the experiments. So, high yield protein synthesis if that is your requirement you would like to transfer your gene of interest into this vector. Now tetracycline inducible clones by mammalian protein expression system that can turn the protein on and off in the mammalian cells two hybrid clone. If your objective is to study the protein interactions then, test the protein function in the mammalian cells by using two hybrid clones. MMTV retroviral clones if you want to perform mammalian protein functioning, you may want to use this vector to know what other protein this protein interacts with.

So, there are various type of vectors which one can make use of one can transfer the gene of interest from these master clones to variety of vectors depending upon their application. So, recombinational cloning in that way gives you flexibility and opportunity to perform multiple experiments once these clones are available. So, I will give you glimpse of high throughput cloning it is important, because the automation step requires increasingly for the

accuracy in microarray or other high throughput experiments. So, the error rate for the robots during repetitive manipulation is exceedingly low and all operations can be verified by examining the lock files for the robotery Cruz.

So, it is especially evident that when you are doing the gel loading or colony picking which are very error prone processes during the cloning process, then if you are performing the manual steps in the high throughput 1000s or even more large numbers there is a good probability that there will be errors. That is why lot of these steps have been automated and laboratories which are using these high throughput systems high throughput platforms they have modifies the systems according to the requirements and system has been automated based on the robotic platforms. I will show you few images which are obtained from during my post talk in George Shellay lab at Harward institute of proteomics. So, these images will provide you a glimpse of various steps involved in the high through put cloning and how one can perform these experiments in the high throughput manner.

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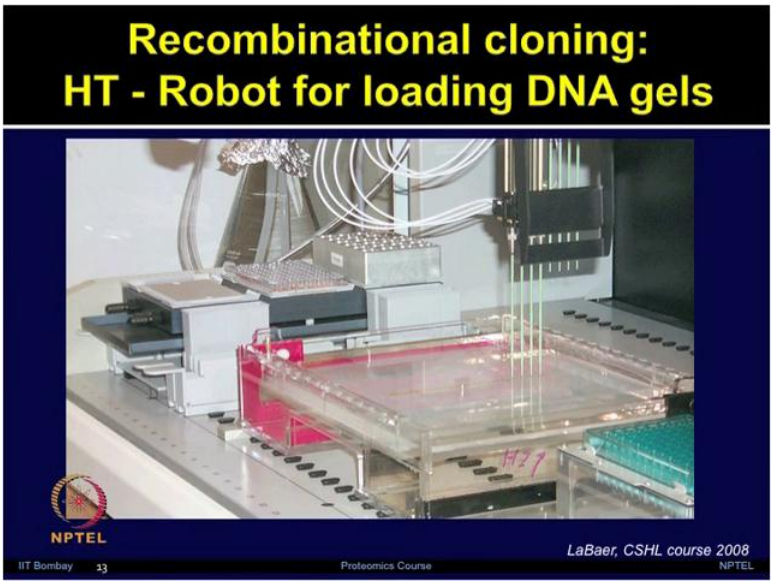

So, this image shows you the high through put platform for the loading DNA gels a robotic system is used here, where 192 leases can be loaded in only 20 minutes. If you are doing these manually it will require large amount of time as well as there will be sample spilling on the gel and different types of problem will come, especially when you are talking about 192 lease by the time you start from your one sample and reach towards the last one actually you have already many samples are diffused. So, automation is very important as

well as speed. Now let us look at this image of the high throughput agarose gel loading. So, once the reaction is done as we loaded in the last slide after that you can visualize the gel in the from the ethidium bromide staining.

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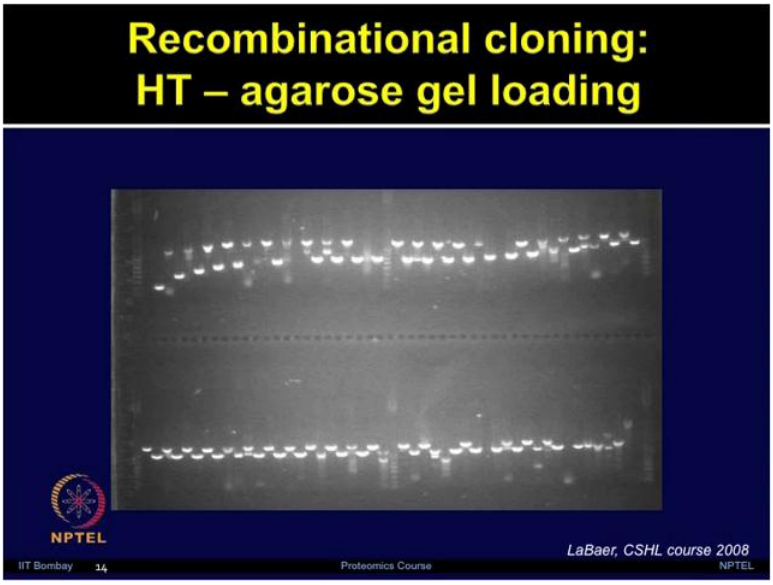

So, this reaction set ups can be performed by liquid handling robots and agarose gels can be loaded by the robotic system. In the gel here, you can see the saw tooth with pattern for loading the gels, which makes the band size identification easier and avoids the contamination from the neighbouring lanes by doing the alternate way you are avoiding the contamination which can go to the neighbouring wells due to the over flow of the sample and also you can easily identify the your sample and the molecular weight. Now high through put requirements also include the plating for the transformation by robot.

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So, the bacterial transformation can be plated by robot onto the custom design which contains 48 compartments. As you can see in this slide and these colonies can further be picked automatically by another robotic platform which is colony picking robot. So, in the recombinational cloning the high throughput way of doing transformation and plating is very much crucial, it provides the automation during the bacterial plating and colony picking reduces the error rates.

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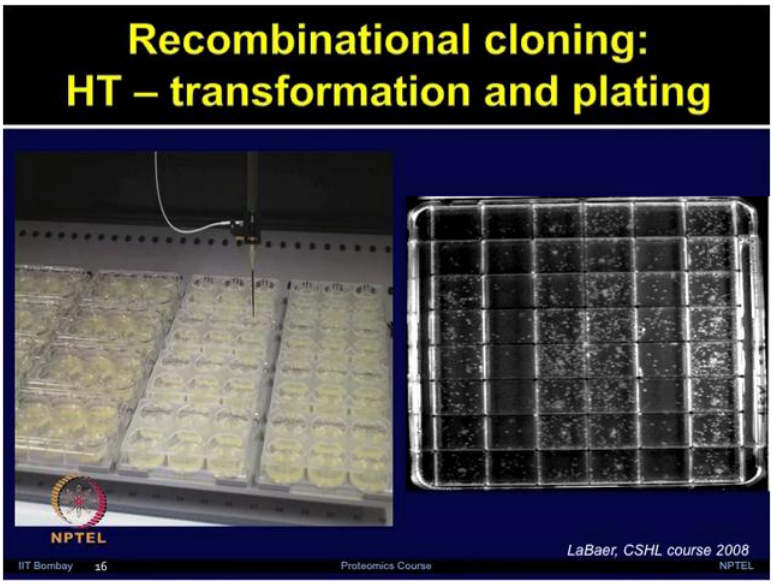

The developments of a 48 sector plate actually, suppose the plating of 96 transformations on two plates instead of 96 Petri dishes. So, in the low throughput way or conventionally lapse use single plate for doing these transformation. So, if you have 96 clones and you want to do this transformation in 96 plates handing all those plates labelling those and storing identifying the right clones all those becomes very tedious. Now if you have these 48 well plates and so only your requirements is to handle two plates, and again by using these robotic system one can easily pick up the colonies without touching the neighbouring colonies so single colonies can be picked up very accurately by using these system.

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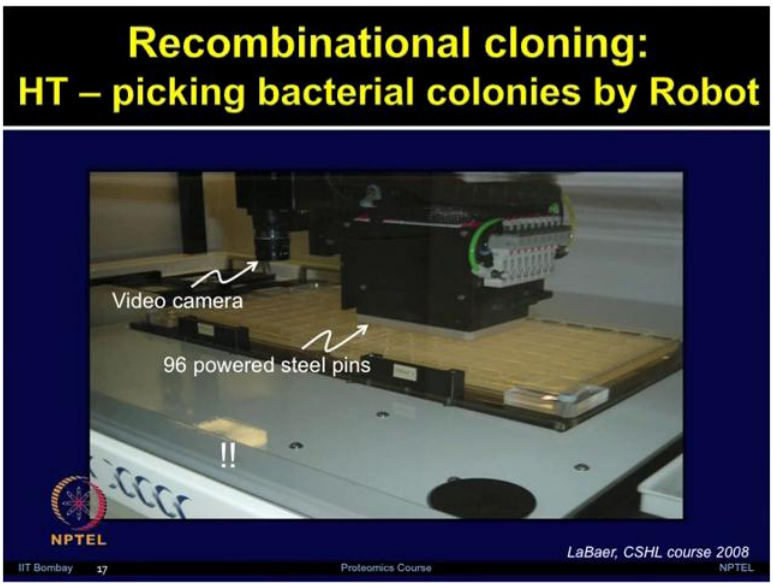

So, the bacterial colonies can be picked by bacterial colony picker robotic system, and that increases the efficiency of the process, and reduces the contamination. At the end when you want to make the DNA for subsequent applications even high throughput platform and automation can be modified to do the DNA preparing in the 96 well plate forming. I will touch upon these more when we talk about how to make the protein microarrays in the laboratory.

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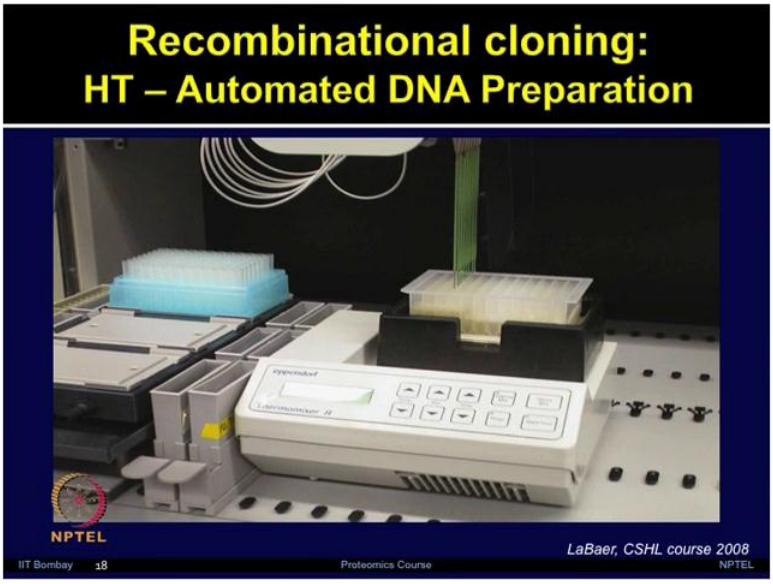

How one can do those experiments I will elaborate on that more, but this just gives you a glimpse of how various type of steps have been modified to do the automated requirements of the experiments.

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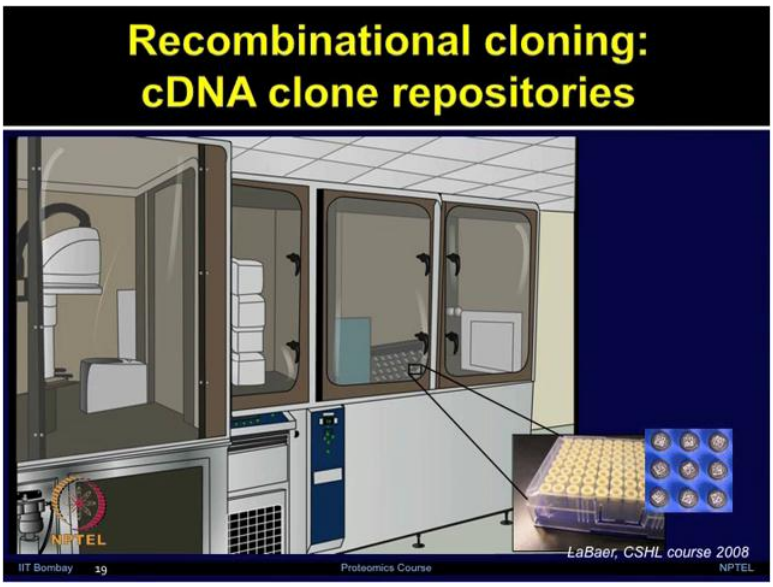

Now here a cartoon is shown to explain you the cDNA clone repositories storing these clones is very tedious, because we are talking about 1000s scale in the high throughput experiments. Now once these clones are stored and you want to take out a clone of your interest a gene of your interest from the large number of plates it is very tough to identify

the right clone. Therefore, some of the much automated system the clone storage system have been generated and the these clones are stored properly in the minus 80 degrees in these type of clone store system. Now these genes which are the generated from the recombinational cloning which we discussed just few minutes ago. Now these are having no restriction further they can be transferred into any format into any vectors.

They are these genes are broadly available there is no restrictions there is a flexible format they are expression ready sequence verified and affordable. So, all these clones are stored in these automated clone repositories. So, that each tube which contains the full length coding region can be taken out from the system without affecting the other clones. So, the gateway recombinational cloning strategy allows, the DNA fragments flange by the homologous recombination site, to move from one vector to another in single step procedure, in frame and without introducing any rotations. These reactions are very straight forward as you have seen BP reaction can be done in one hour. So, as the LR reaction and it allows for the high throughput automation. So, virtually these are almost 100 percent efficient if all the pipetting all the reaction mix are added accurately and with the proper pipetting. So, let me explain you the basics of cloning, as well as recombinational cloning in following animation.

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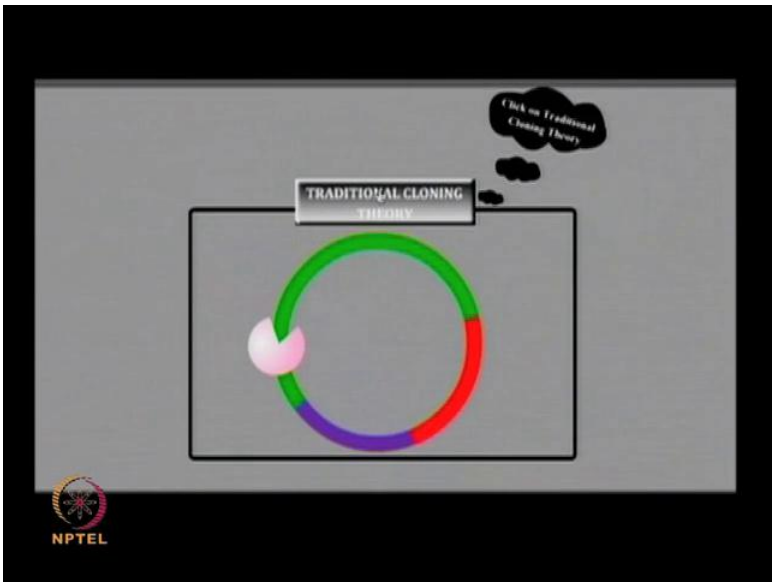

So, since we are discussing about recombinational cloning. Let's also briefly touch upon traditional cloning. So, that it will be easier to compare traditional cloning verses

recombinational cloning. The conventional cloning protocol makes use of a restriction enzyme that fragments the selected plasmid vector as well as, the DNA sequence containing gene of interest at the same recognition site. The complimentary sequence overhands that are produced with the plasmid.

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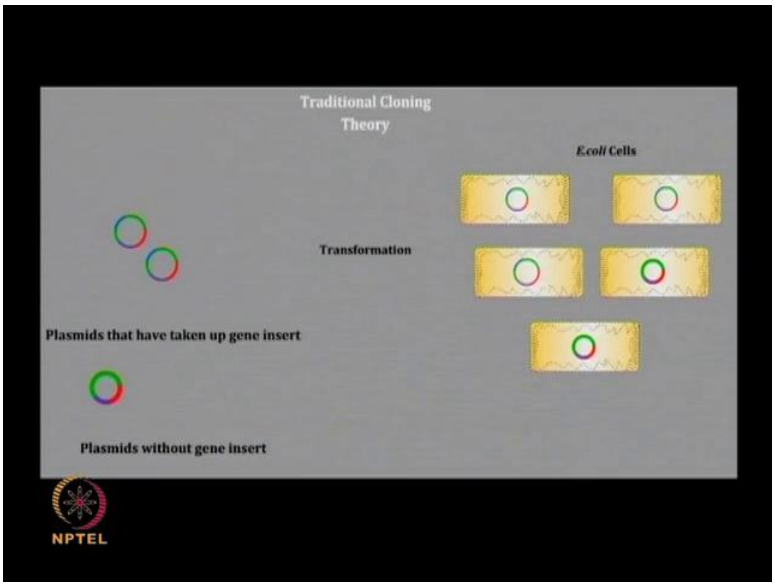

And gene insert during restriction digestion are useful for proper orientation of these fragments during the insertion the insert is ligated by means, of DNA ligase enzymes. The insertion of fragments within an antibiotic resistant gene leads to the inactivation of this gene. After this the transformation process occurs. So, once the gene the insert has been introduced in to the plasmid vector in desired orientation, they are transformed into suitable bacterial host cells. This can be done by technique such as electro oration chemical sensor digestion etcetera, which makes the cell membrane relatively permeable there by allowing the plasmids to enter the cell.

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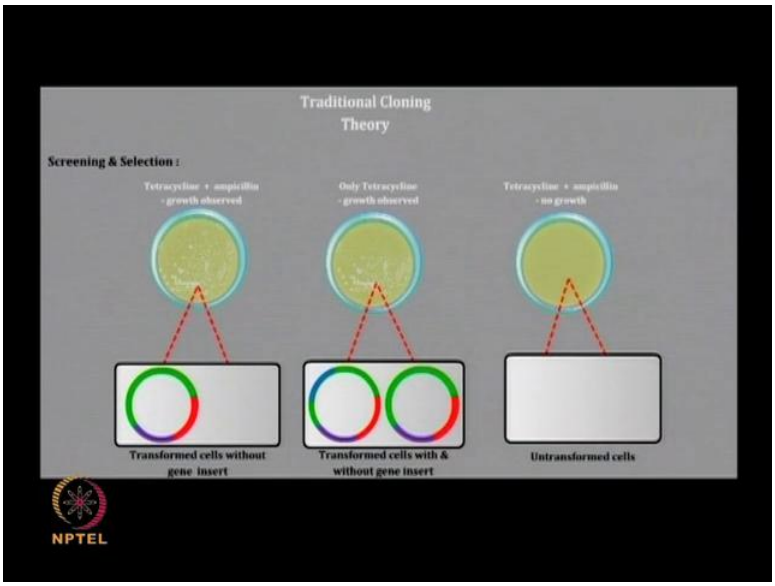

After transformation you need to select the right clone. The cell is grown on a suitable medium which contains specific antibiotics that allow only certain bacterial cells to grow. Cells that have been transformed with the plasmid, but do not contain the gene of interest they grow the medium containing both tetracycline and ampicillin. Those cells that have taken up the plasmid and contain the gene insert will grow on a tetracycline containing medium, but will not grow in the presence of ampicillin. Those cells that do not grow in the presence of antibiotics have not taken up any plasmid and therefore, do not have resistance to the antibiotics. The comparison of colonies grown in the presence of both antibiotics and in the presence of polytetracycline will reveal those that have taken up the gene insert by a technique known as replica plating.

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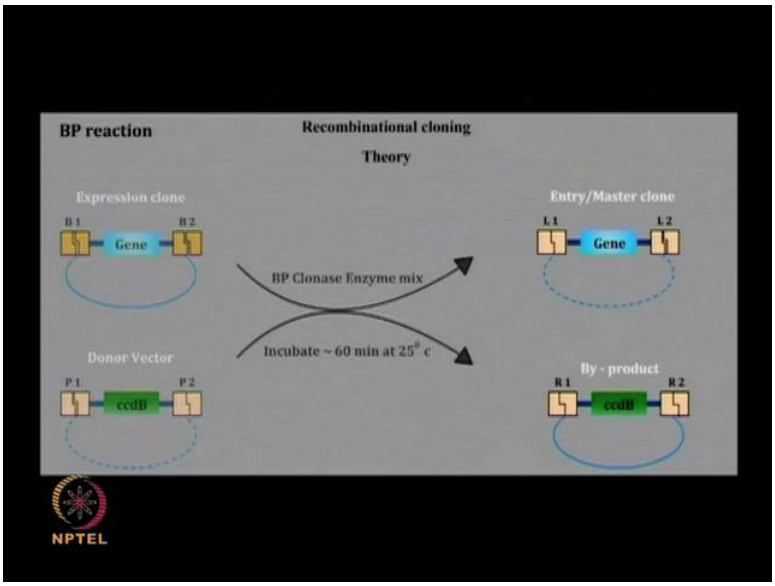

Let us now, talk about recombinational cloning. The recombinational cloning involves two reactions BP reaction and LR reaction. The BP reaction of gateway cloning is a side specific recombination reaction between the att B site of an expression clone or a PCR product and att P site of a donor vector in the presence BP clonase enzyme master mix. The reaction is incubated for just an hour at 25 degree centigrade to obtain the entry or master clones containing genes of interest.

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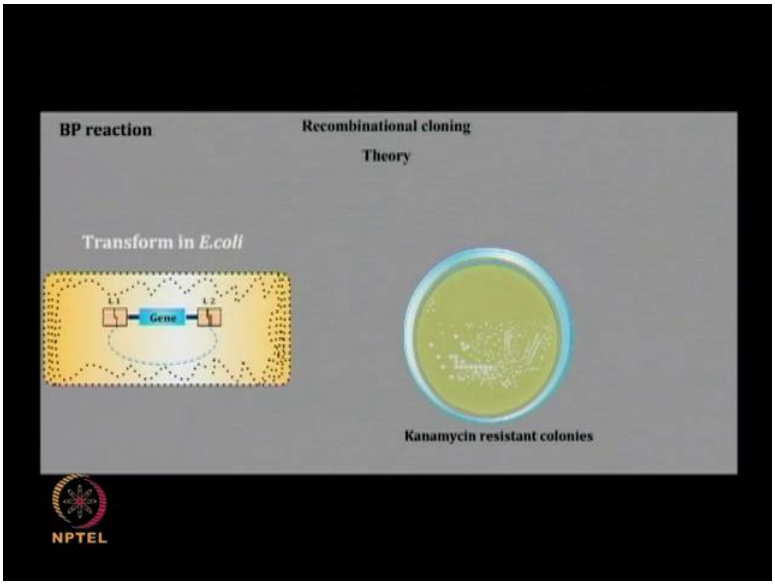

Once this master clone flange by the att L site is produced it can then be transferred into any destination vector to produce expression clone for specific desired application. The reaction yields more than 90 percent or almost 100 percent correct colonies.

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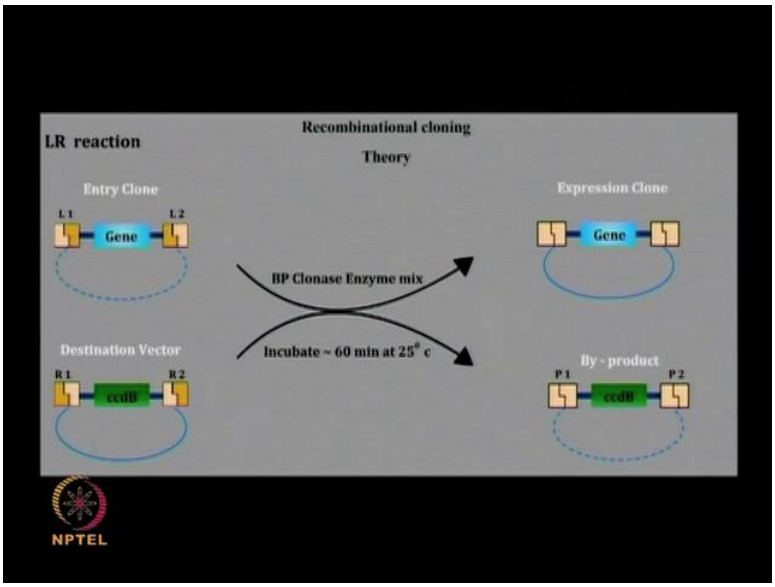

The colony selection can be performed on kanamycin resistant plates. Once BP reaction is successfully performed then one need to take this clone extract DNS and then further move on to LR reaction. The LR reaction is essentially the reverse of the BP reaction, were the master clone flange by the att L site recombines with a destination vector with att R sites. This reaction which takes place in the presence of LR clonase enzyme mix results in transfer of gene from master clone to the destination vector to produce an expression clone for specific purpose.

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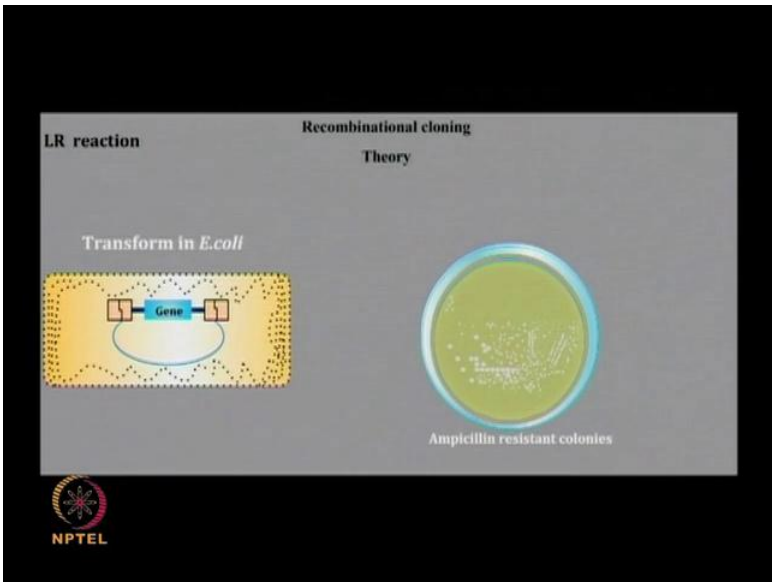

The LR reaction enables generation of several expression clones for various expression various applications in very short time. The transformation results into the colonies which can be selected on ampicillin resistant plate. Now once the right colonies have been selected the DNA can be extracted and these clones can be stored for various applications.

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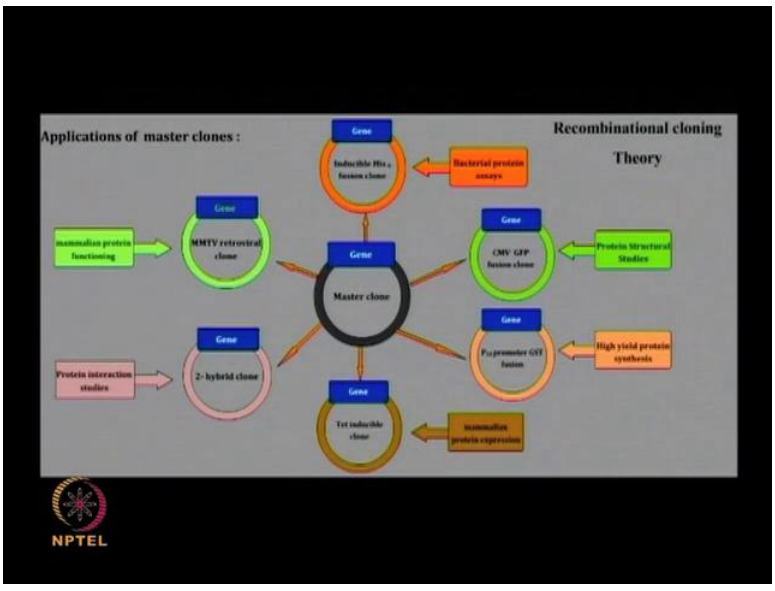

Therefore, this recombinational cloning process provides significant advantages over conventional cloning techniques, these master clones can be used for several applications. So, once these recombinational clones are produced these master clones can be used for

various applications. So, the gene in the master clone can be transferred to various destination vectors by means, of LR reaction to produce expression clones for several applications. Proteins can be efficiently expressed in bacterial yeast and mammalian systems, an used for a variety of applications such as structural and functional studies protein interaction studies, protein assays producing high yield of protein for experimentation etcetera. The rapid recombination between clones that is possible with gateway system cannot be done by using conventional cloning techniques, due to which this recombinational cloning protocol is now widely used. After watching this animation. Now, let us talk about what are the advantages of using recombinational cloning.

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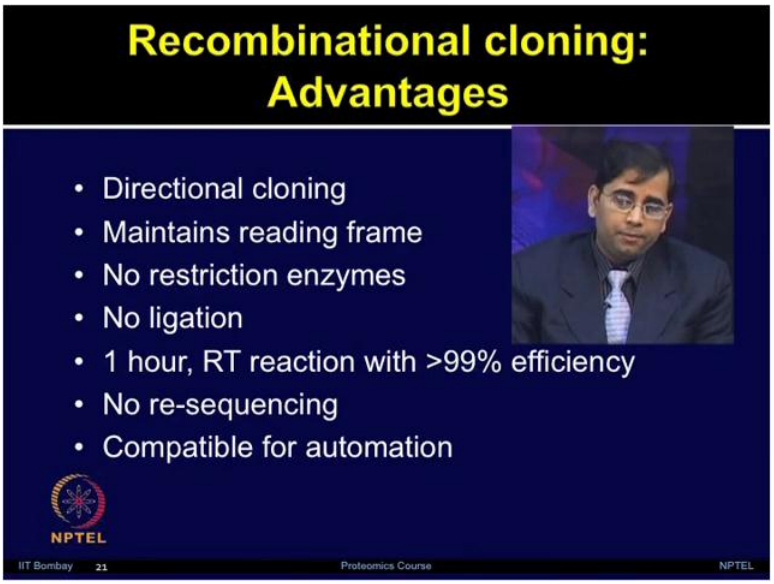

So, as you have seen this is a directional cloning, it maintains the reading frame. There is no need for adding restriction enzymes which is a case for the conventional cloning, no need for doing the ligation steps the room temperature 1 hour reaction which increases efficiency close to the 99 percent. Once the reactions are performed successfully there is no need to do the re-sequencing it is almost 100 percent correct sequence inserted in the right vector and the system is compatible for doing the automation for high throughput requirements.

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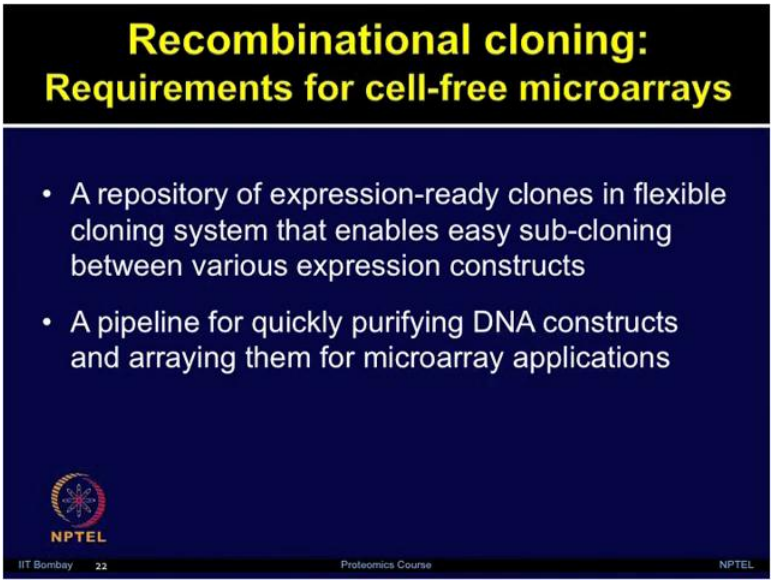

The recombinational cloning let us talk about the requirements for cell free microarrays. So, once you have done the recombinational cloning and obtained several master clones the repository of expression ready clones in the flexible cloning system, enables the easy sub-cloning between various expression constructs. So, a pipeline for quickly purifying the DNA constructs and arraying them for the microarray application can be generated after the recombinational cloning is performed. So, in the subsequent lecture, we will talk about cell free expression systems and even today we will touch upon the concepts of cell free expression technology.

But, all this requires large number of gene of interest the cDNA clones and in the right expression vectors. So, by doing the expression by doing the recombinational cloning one can actually have the flexibility to perform various type of applications including microarray applications. Now you have generated lot of master clones which are gold clones, these repositories and now one can use these c DNA clones for various applications including protein microarrays.

Now one can transfer these clone into the right expression vectors and purify the proteins or one can use these vector for producing protein by using cell free protein synthesis system. So, this clones are important and required regardless of whatever way you want to make the protein you want to make cell based or cell free method, but again like will the requirements for doing the microarrays in high throughput manner it is very difficult if you have to purify

1000 and 1000s of protein and especially, when you want to preserve their activity you want to do the functional assays functional activity tested. So, to eliminate all those problems people have started using the cell free protein synthesis methods. So, cell free synthesis system make use of template DNA in the form of plasmids or PCR products for direct invitro protein synthesis in presence of a crude cell lysate which contains all the necessary machinery which is required for transcription, and translation with essential amino acids nucleotides sars and other energy generating factors which are added exogenously.

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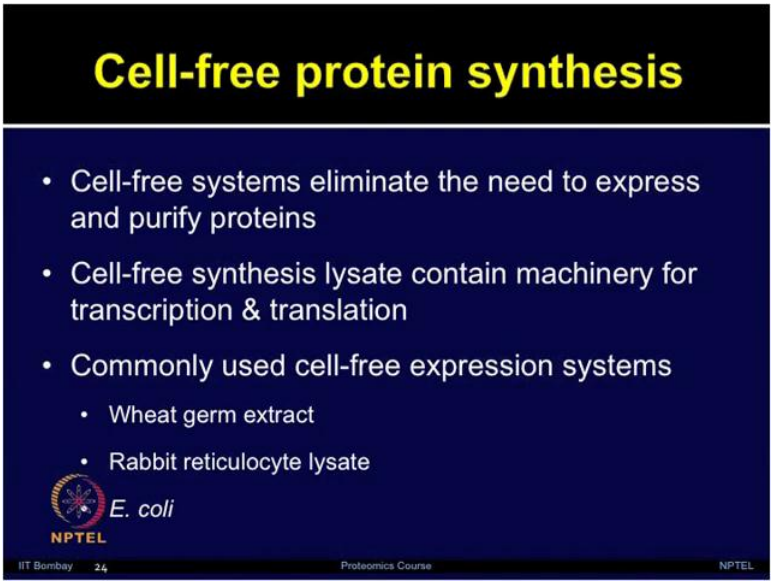

So, the cell free synthesis system the eliminate the need for protein expression and purification in the cell based system. The various type DNA templates can be used such as PCR products or plasmids. The cell free synthesis lysate contain machinery for transcription as well as translation. The commonly used cell free expression systems include wheat germ extracts or WGE rabbit retculocyte lysate or RRL and Escherichia coli or E coli base systems.

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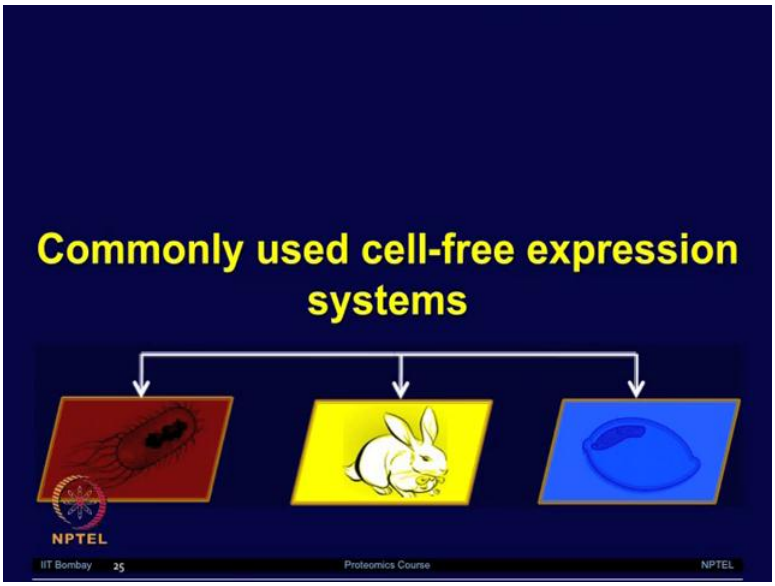

So, the cell free expression systems have been extracted from several different species and cells. The commonly used cell free expression systems are E coli, wheat germ extracts, WGE and the rabbit retculocyte lysate or RRL. Others include those obtains from xenopus oocyte, hybridomas insect and mammalian cells. One can use any of these cell free protein synthesis system, but it depends on their application requirements.

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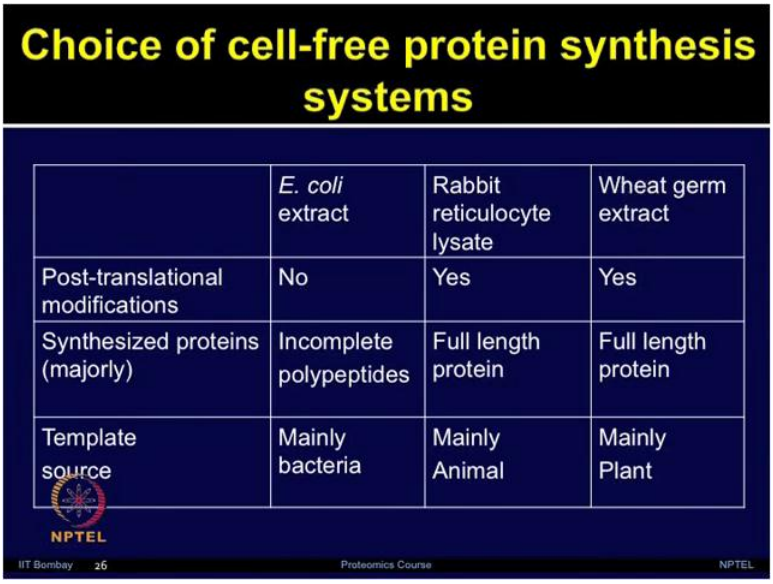

	E. coli extract	Rabbit reticulocyte lysate	Wheat germ extract
Post-translational modifications	No	Yes	Yes
Synthesized proteins (majorly)	Incomplete polypeptides	Full length protein	Full length protein
Template source	Mainly bacteria	Mainly Animal	Mainly Plant

So, let us do a comparison of these three most widely used cell free protein synthesis system. E coli extract rabbit retculocyte lysate and wheat germ extract. So, post translational

modification that is not possible in the using E coli extracts, whereas rabbit reticulocyte lysate and wheat germ extract eukaryotic systems can provide the post translation modifications. The synthesize proteins majorly are incomplete poly peptides in case of E coli extracts where as in RRL and WGE it is full length protein. The template source in E coli it is mainly from the bacteria, rabbit reticulocyte lysate mainly animals especially rabbits and in the wheat germ extracts mainly the plants. Since, we are talking about applications proteomic applications more for the eukaryotic system. Let us talk about eukaryotic cell free expression systems the wheat germ or the rabbit reticulocyte lysate what are their advantages and disadvantages.

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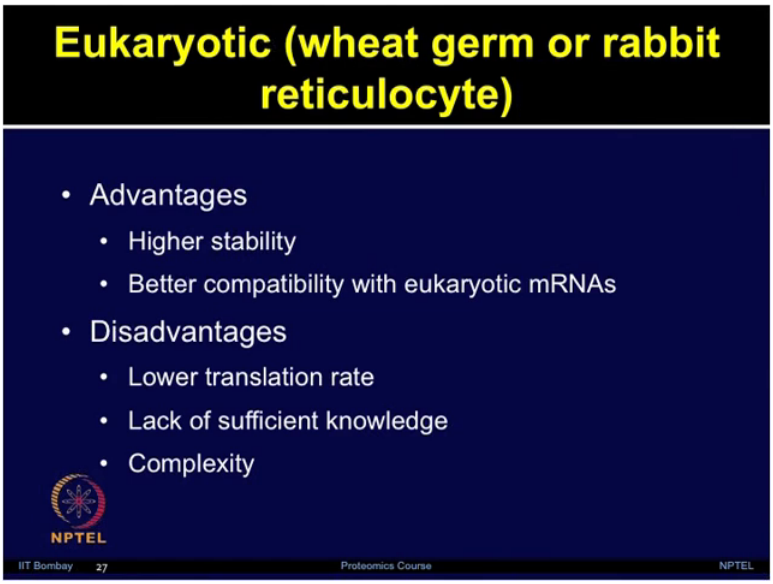

So, advantages of this system include higher stability. So, the longer life time of the cell free systems. Better compatibility with the eukaryotic mRNA's and synthesis of eukaryotic proteins. How about their certain disadvantages such as lower translation rate, lack of sufficient knowledge to construct effective genetic vectors and the complexity of genetic constructs for the effective protein expression. So, we will discuss the commonly used cell free expression systems in the following animation. The open nature of the cell free expression systems provides various benefits, such as an adjustable environment to allow the proper protein folding disulphide bond formation and addition of labeling agents during the translation process which enables easy detection of synthesize protein.

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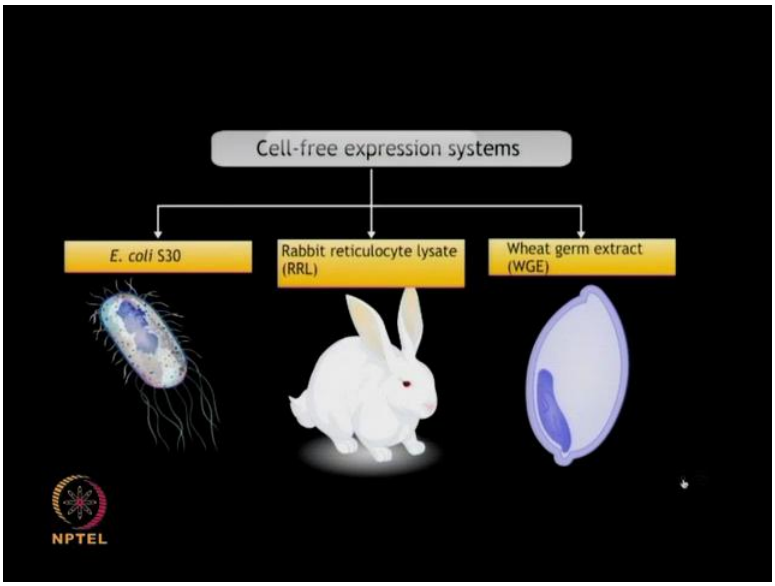

So, let us discuss the commonly used cell free expression systems. The commonly used cell free expression systems include e coli as 30 rabbit reticulocyte lysate or RRL and wheat germ extracts WGE E coli s 30. This is a commonly used bacterial expression system that is capable of producing protein yield of around 6 mg per m l the system. However is not capable of carrying out the post translation modifications or PTM's of proteins due to the absence of require machinery for this process. And very often produces incomplete protein chains. DNA templates obtained from bacterial sources are commonly used for this cell free lysate. Next we will talk about rabbit reticulocyte lysate or RRL a mammalian cell free expression system, that also gives protein yield of around 6 mg per m l. This system is more suitable for expression of full length eukaryotic proteins from plant and animal sources that require proper protein folding and post translation modification. Wheat germ extracts or WGE this is a cell free expression system that is capable of producing full length proteins with correct folding and PTM's form plant sources yields obtain in this system.

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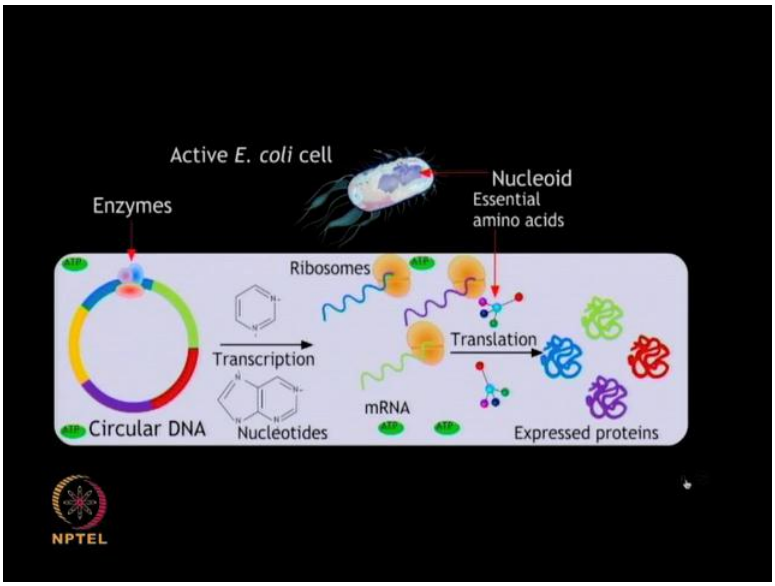

However slightly lower than the E coli and RRL based cell free expression system. E coli s 30 extract they actively growing and replicating E coli cells can be used for extracting cell free lysates. These cells that can in the process of growth and division are constantly producing proteins and other factors required for various cellular process. The co factors and enzymes such as RNA polymerises, peptidyl transferase are available in significant quantities due to the cellular process of transcription and translation taking place in the cell. As you can see in the animation the two steps have occurred here the transcription and translation and the required material have been provided exogenously.

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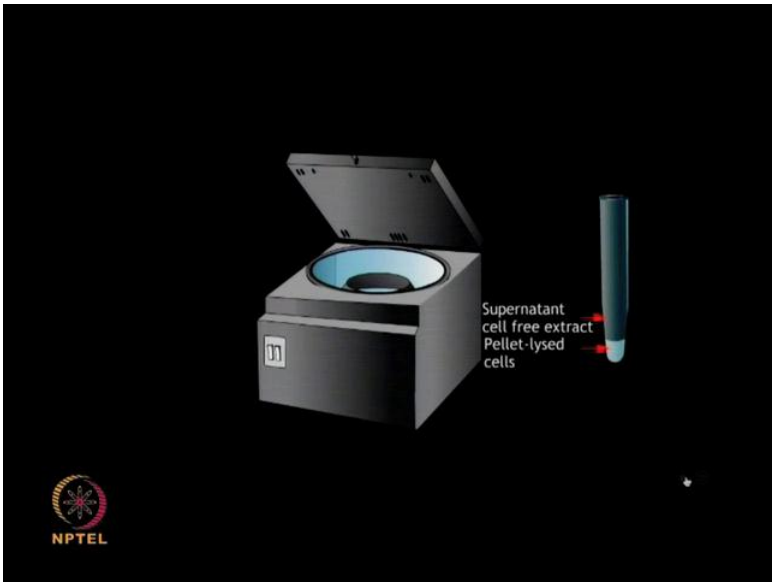

These cells are lysed in a suitable buffer and after that they are centrifuged at 30000 g to collect the supernatant containing the extract. Now this lysate which is present in the supernatant after the centrifugation step, that contains the cell free extract. So, lysate that will extracted from such actively growing and dividing cells will contain all the required cellular machinery to carry out in vitro protein synthesis, and requires addition of essential amino acids nucleotides salts and other energy generating factors.

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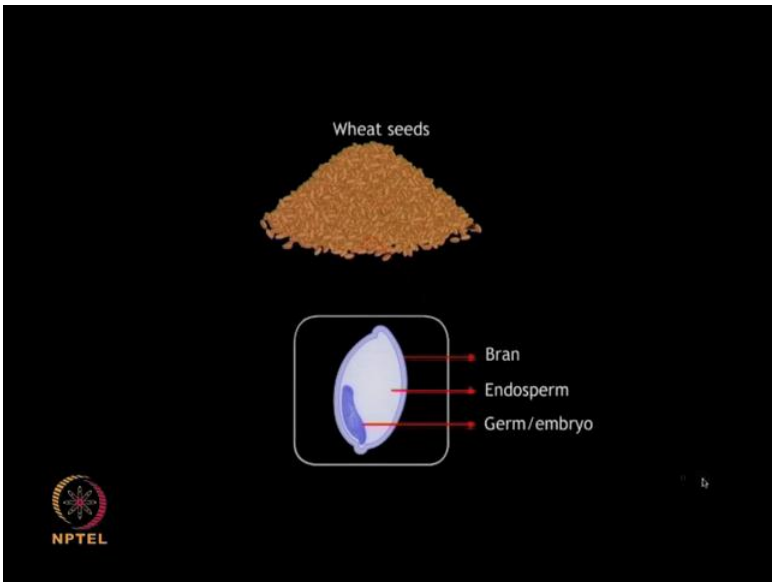

Let us now talk about wheat germ extract or WGE. This is one of the most commonly used eukaryotic cell free expression system, which is obtained from the embryo of wheat seed. The seeds are grinded and then sieve to remove their outer coating fragments. Once the grinding is finish the embryos and other small particles are floated in organic solvents, such as cyclohexane after these embryos are floated in the solution.

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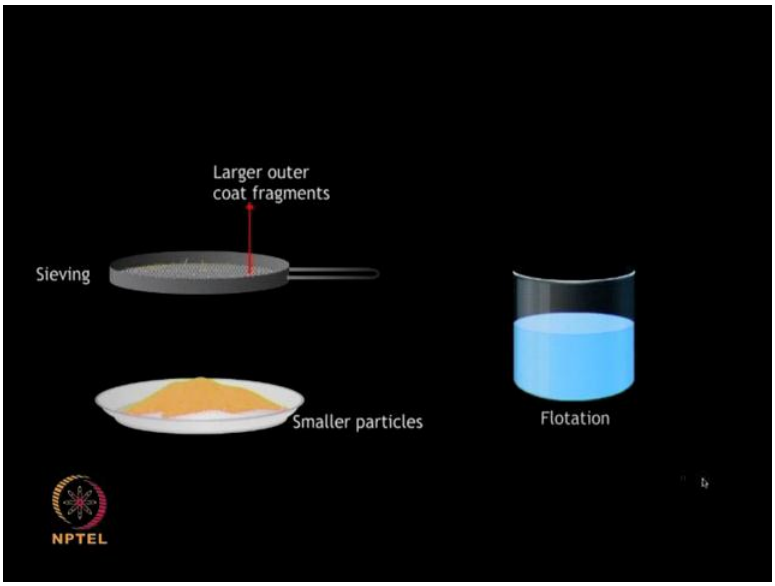

The floating embryos are quickly removed and dried avoid any damage from the organic solvent. The dried embryos are carefully sorted such that only good embryos without any endosperm coating are selected. One needs to repeat this washing step few times, because the endosperm contains certain inhibitors of protein synthesis which must be removed. The selected embryos are washed thoroughly with cold water after which they are mixed with extraction buffer and again grind it.

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Now, this solution must be centrifuged at 30000 g at 4 degree centigrade which results in the wheat germ extract forming a layer between the top fatty layer fraction and the pellet at the bottom. This you can see in the tube here. So, this fraction can then be separated and purified by chromatographic methods to remove any components of the extraction buffer. This cell free lysate is capable of synthesizing full length eukaryotic proteins which provides yields of around 4 microgram 4 mg per m l. So, how cell free synthesis occurs, for that you need certain elements, you need a DNA template plasmate or PCR fragment a promoter which could be T 7 SP 6 or T 3 a translation initiation signal.

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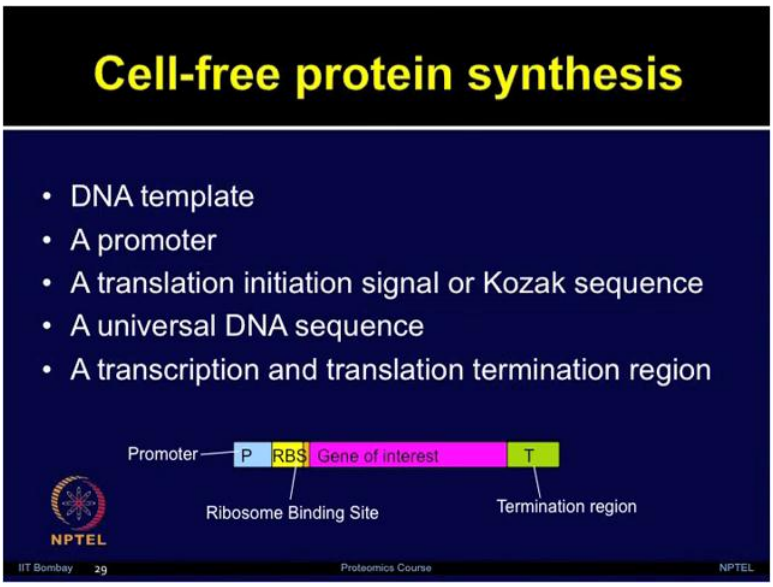

For example, shine-dalgarno in case of prokaryotic or kozak in eukaryotic these sequences a universal DNA sequence for protein initiation and a transcription and translation termination region, all these are required for the cell free protein synthesis. The cell free expression systems allow rapid conversion of genetic information directly to the fictional proteins; it facilitates synthesis of several proteins in single reaction. So, let me show you this animation for in vitro protein synthesis which will explain you these concepts very easily.

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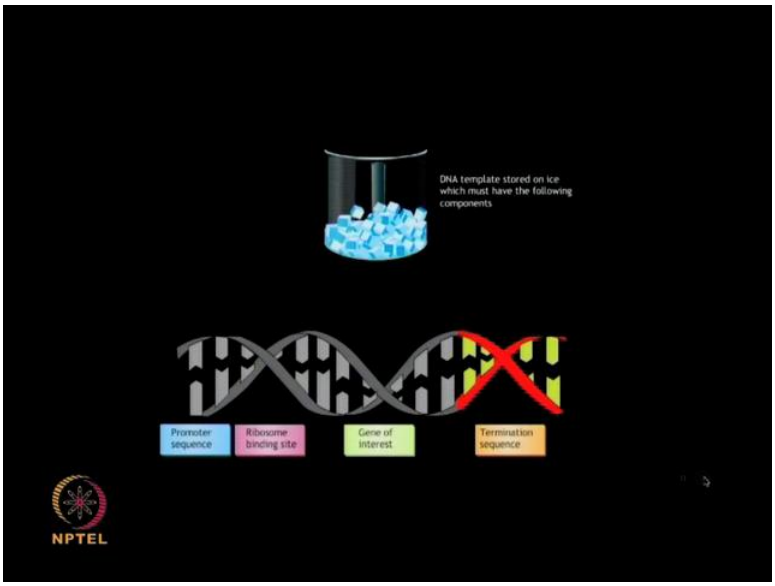

Let us talk about in vitro protein synthesis. First the DNA template is thought and then placed on ice during the preparatory process. For in vitro protein synthesis to take place the DNA template must contain the gene coding for the protein of interest, in addition to this there must be a promoted sequence, which can initiate the transcription process a translation initiation sequence for binding of ribosome, as well as a suitable termination sequence to correctly synthesize only the proteins of interest.

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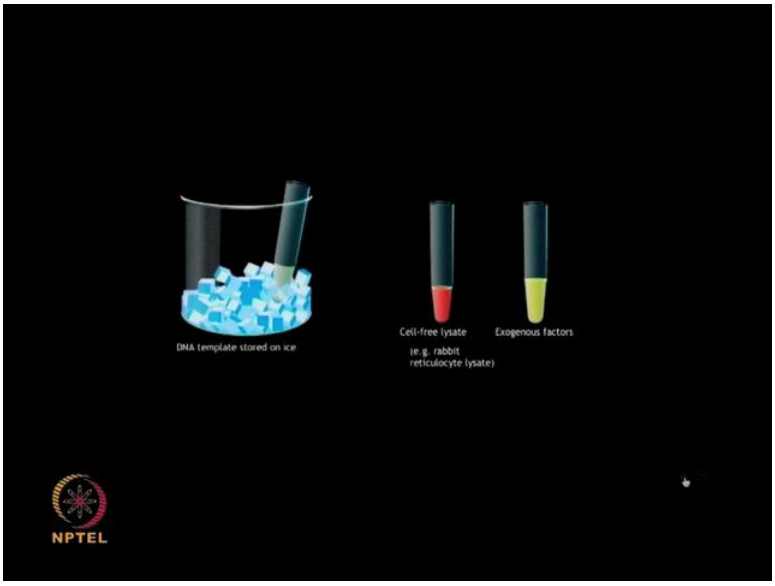

The though cell free lysate containing the essential cellular machinery for protein synthesis is added to the DNA template followed by other exogenous factors that are required for the process. Such as essential amino acids, nucleotides ATP etcetera. All these are done while storing the template on ice to ensure that there is no loss of activity. The tube containing all the required components is then incubated at 30 degree centigrade.

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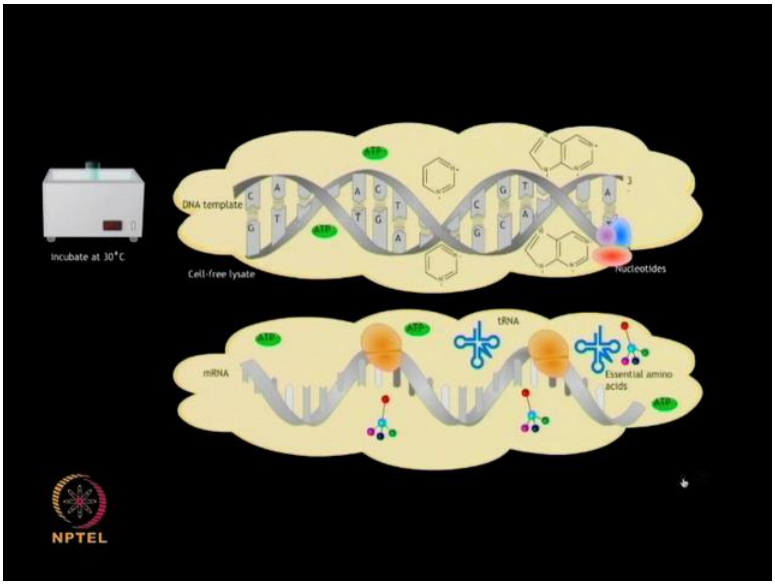

The enzymes for transcription bind to the promoter sequences and in the presence of other factors. Such as ATP and nucleotides they carry out synthesis of mRNA transcript. This mRNA is then translated into the corresponding proteins due to the help of ribosome's tRNA enzymes and other factors which are required for the process. So, cell free expression systems are required for the microarrays, but why it can be used, because it has ability to utilize wide variety of DNA templates one can make use of all that gold DNA repositories the master clones obtained from the recombination cloning.

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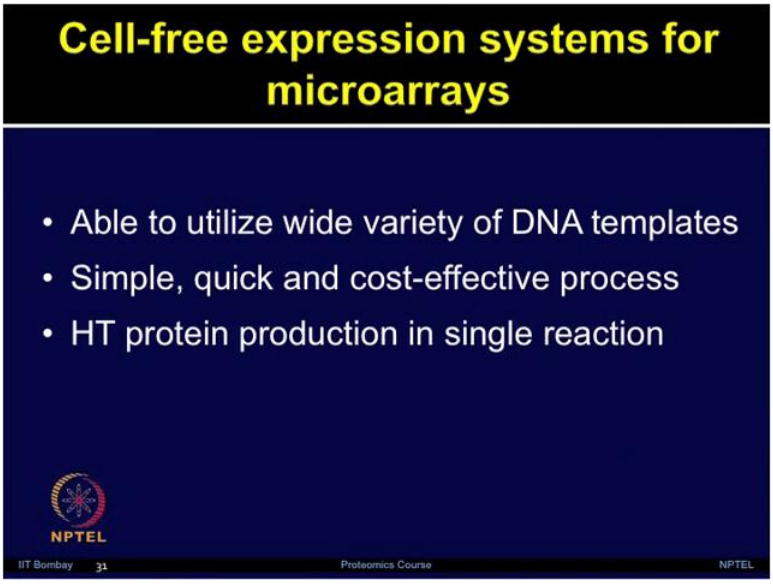

This cell free expression system is very simple quick and cost effective process. It provides the high throughput protein production in very short time and in single reaction. So, all these requirements are very useful for high throughout protein production and for protein micro origin ratio.

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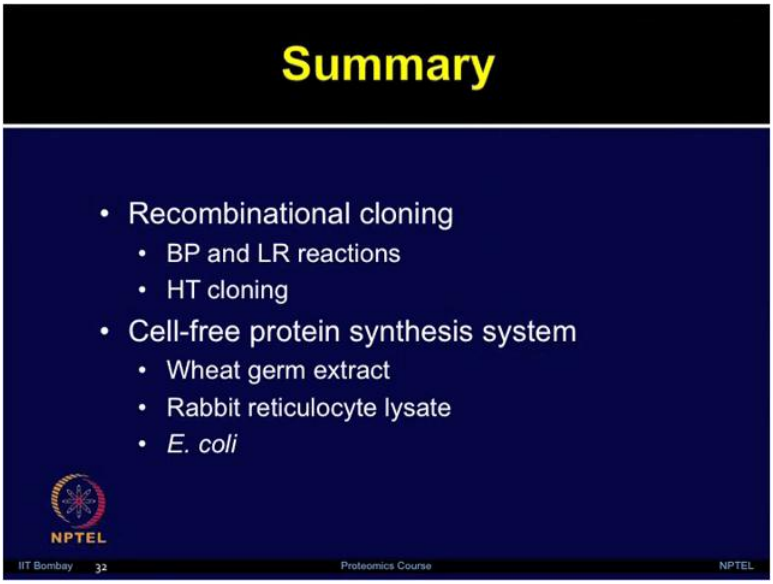

So, in summary today we talked about few concepts which are required for the protein microarray generation. As well as for various high throughput applications in genomics and proteomics. The concept of protein microarrays has estered a great deal of excitement in the

proteomics community. Once the technology is fully realized it promises to enable the study of a broad variety of protein features at an unprecedented pace and scale. However generating protein contents in high throughput manner remains challenging. Therefore, the recombinational cloning to produce clone depository and cell free expression systems to produce proteins becomes very important.

In today's lecture, we have discussed about recombinational cloning we discussed about various steps involved such as BP and LR reaction we talked about how high throughput cloning can be performed, I walked you to the process of some glimpses or the pictures for cloning process, also showed you some animation for comparison of cloning conventional as well as recombinational cloning. We then discussed about cell free protein synthesis system. We talked about wheat germ extracts rabbit reticulocyte lysate as well as Escherichia coli base systems. Now these concepts will be very important when we discuss further in following lectures, about protein microarrays and specially the cell free expression based protein microarrays. Thank you.