Interactomics Protein Arrays and Label-Free Biosensors

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Module 05
Lecture 25

Recombinational cloning and its Application for Protein Microarrays

Welcome to the MOOC NPTEL course on interactomics. Today we will talk about recombinational cloning and it is application in protein microarrays. This will be useful for the next lecture on cell free expression based protein microarrays. The era of high throughput biology began with the inception of the human genome project. Subsequently, high throughput techniques were increasingly used for proteomics research.

In this light, the functional proteomics has immerged as an exciting new approach to study protein functions in high throughput manner. It enables the expression of proteins and subsequent studies to decipher its sub-cellular location, protein partners, biochemical activity, etc. These are regulated at a 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 and various types of protein modifications. All these complexities arise whenever you want to purify the protein and print on the protein microarrays.

To overcome these limitations scientist have tried new methods like cell-free expression based system, but for such microarrays a pre-requisite for achieving high throughput is to access the clone library harbouring numerous clones that is where recombinational cloning comes into the play. Development of high throughput methods remains a challenging a figure, all the high throughput methods start with one common step expression of a protein from a cloned copy of gene cDNA.

The pre-requisite for these approaches including the protein microarray application is the need for large collection of cDNAs in a format, which is conclusive for the high throughput protein expression. Researchers have started creating a large collection of cDNA library using recombinational cloning. The recombinational cloning allows rapid transfer of DNA fragments from one vector to another in a very short time.

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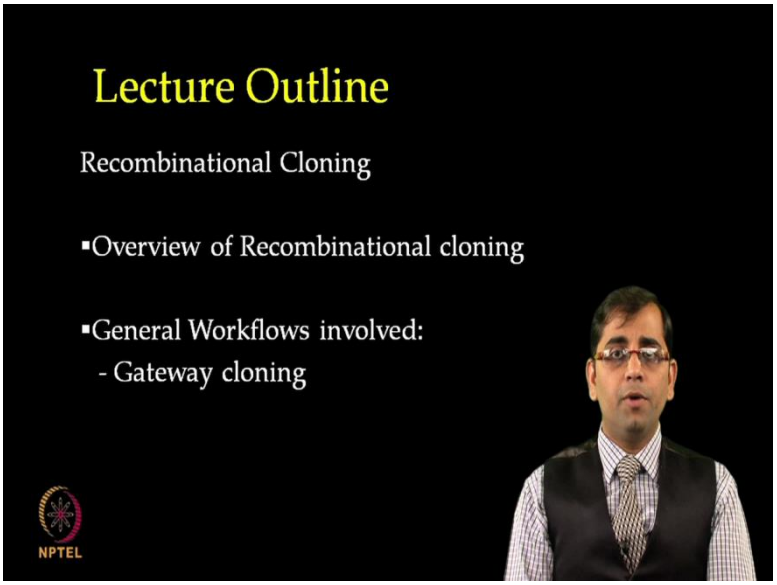

In today's lecture, we will talk about the general workflows involved in performing recombinational cloning. Let us first discuss about recombinational cloning to obtain a large scale production of proteins certain methods are required to transfer the coding region of the gene into an appropriate expression vector for functional proteomics studies site-specific recombination is an efficient genetic recombination technique where DNA strand exchange takes place between the regions possessing resonance sequence (())(4:15), the common size specific recombination strategy include gateway cloning system from (())(4:24) technologies from BD clontech and AB, etc.

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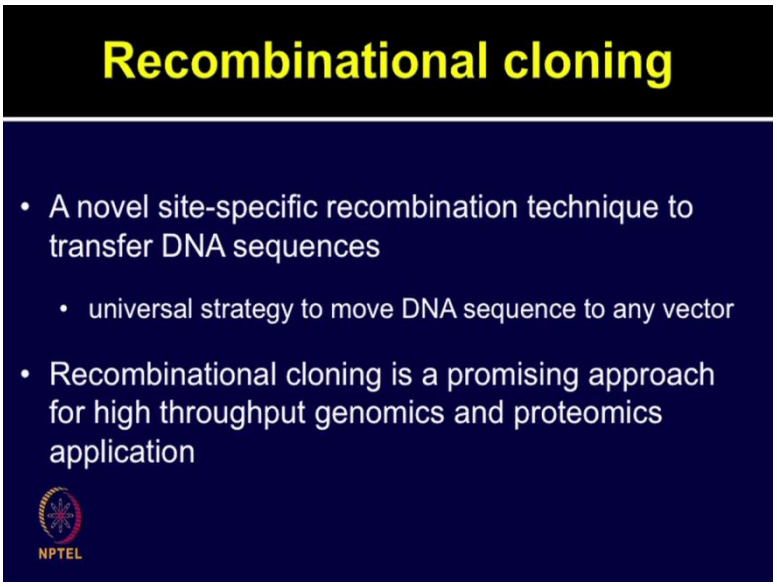

In our discussion on recombinational cloning I will focus more on recombinational cloning system from gateway just more so because of my familiarity with using the system. However, any of these systems can be used for recombinational cloning where the basic principle remains more always the same. As mentioned recombinational cloning is a novel site-specific recombinational technique, which is required for transferring DNA sequence between vectors. These specific recombines enzyme (())(5:14) the DNA backbone and carries out the exchange of DNA between its specific sites on two different molecules.

This is a universal strategy to move DNA sequence to any given vector. This is one of the very promising approach, especially when one looks for high throughput applications in genomics and proteomics. Before I talk about recombinational cloning in more detail, let me introduce you to few terminologies commonly used in recombinational cloning.

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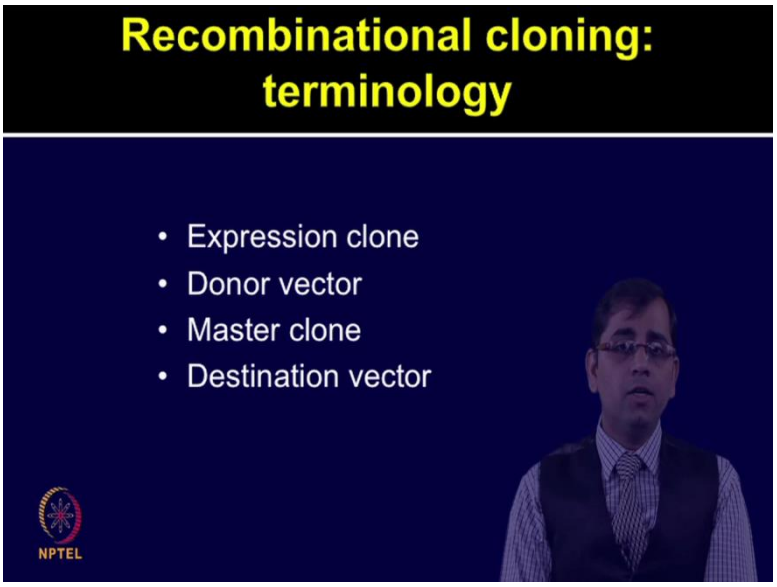

So, what are expression clones this is a clone containing a gene sequence of interest which is flank by at B sites. The orientation of the gene is maintained throughout the process because of its specific interaction between the add sites. So, what are these add sites? The add sites are DNA segments of certain defined length. What is donor vector? The donor vector consists of a counter selectable gene which is flank by at B site which recombines with gene of interest flank by at B site in BP reaction to produce a master or entry clone. The explanation for BP reaction and master clone will be provided in subsequent slides. So, what is a master clone? This is the vector containing gene of interest flank by at L site.

Entry clones are formed by the BP reaction and further used in LR reaction to produce expression clones of interest. What is a destination vector? This is a vector containing a counter selectable gene flank by at R site which interact with genes form entry clone to produce an expression vector in LR reaction. Broadly recombinational cloning involves two reactions, BP reaction and LR reaction; let us first talk about BP reaction.

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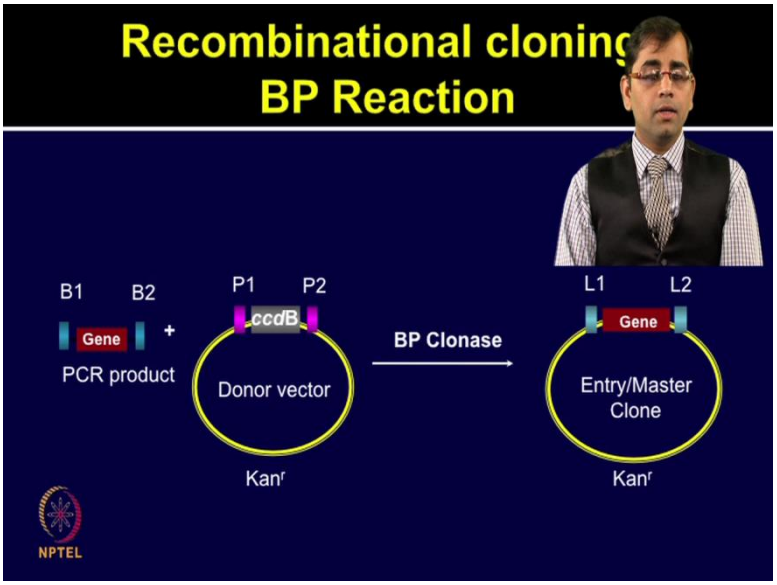

When a reaction is run to mix at B and at P sites such as when making the master clones this step is catalyzed by BP clonase enzyme and it is known as BP reaction because of at B and at P sites involved in it. As you can see in this slide, there is a donor vector or P donor that contains at P sites flanking a ccdB gene or a counter selectable gene that recombines with a gene of interest which is flanked by at B site which you can see on the left hand side.

The gene fragment can be produce from the PCR product and which contains at B site and you have a donor vector, which contains a selectable marker kanamycin resistant marker that contains at P site. The entry or master clone is a vector containing gene of interest flank by the at L site, this happens after the reaction is completed. The recombination between at B and at P sites is catalyzed by enzyme known as BP clonase, this enzyme transfer the gene of interest at B sites to the donor vector, which contains at P sites. So, once the BP reaction is completed, this entry or master clone can be taken for further LR reaction. Let us first talk about how the BP reaction is performed in the laboratory.

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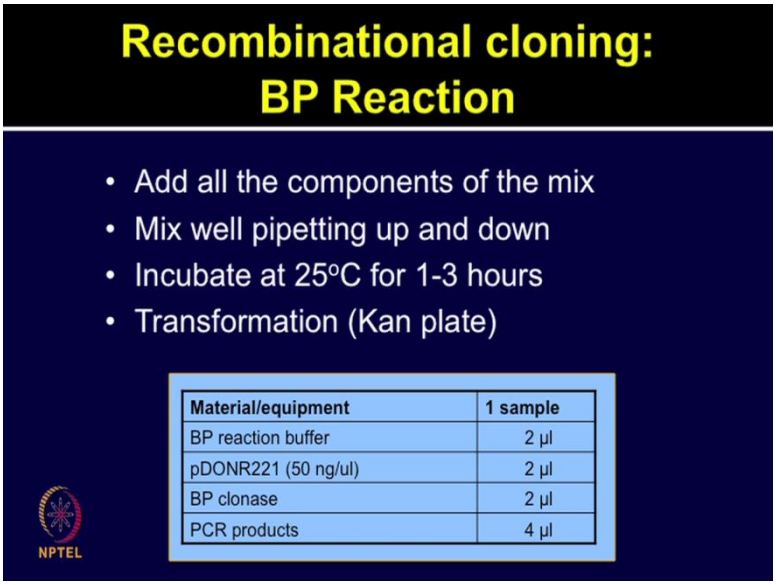

We need to add various components which are shown in the table such as BP reaction buffer, pDONR221 around 50 nanogram per micro drop DNA, BP clonase enzyme and PCR products. These are some of the specifications which can be modified according to the requirements. So, one need to add all these components of BP reaction mix them by doing a pipetting up and down , after this you can incubate them for 2 to 3 hours at 25 degrees and then this reaction mixture can be used for transformation process on kanamycin resistant antibiotics. Since, we have this vector containing kanamycin resistant gene only those clones which will have the entry of your correct insert in the right vector will be able to grow on kanamycin plate.

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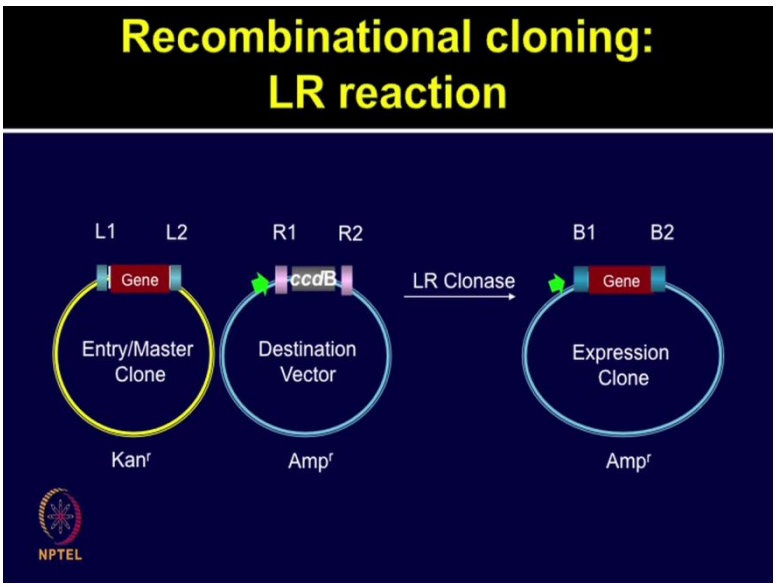

Let us now discuss the next reaction which is LR reaction. In BP reaction you had met entry or master clone, you now that can be further used in the LR reaction. In LR reaction if the interaction between at L and at R site then it can be catalyzed by enzyme LR clonase, reaction is known as LR reaction. LR recombination between entry clone and destination vector as you can see in the slide, you have an entry clone and master clone which was selected on kanamycin plate and now you want to transfer that in destination vector, which contains another antibiotic gene which is ampicillin resistant gene. This destination vector contains R sites R1 and R2.

The gene of interest can move from entry or master clone to the destination vector. Pdest is modified to accept the protein coding sequence in frame from the master or entry clone by using recombinational cloning enzyme known as LR clonase. Once this reaction is completed then the reaction mix can be placed on ampicillin plate and since the vector contains the ampicillin resistant genes, it can be screened on the ampicillin plates, let us now discuss the recipe for LR reaction.

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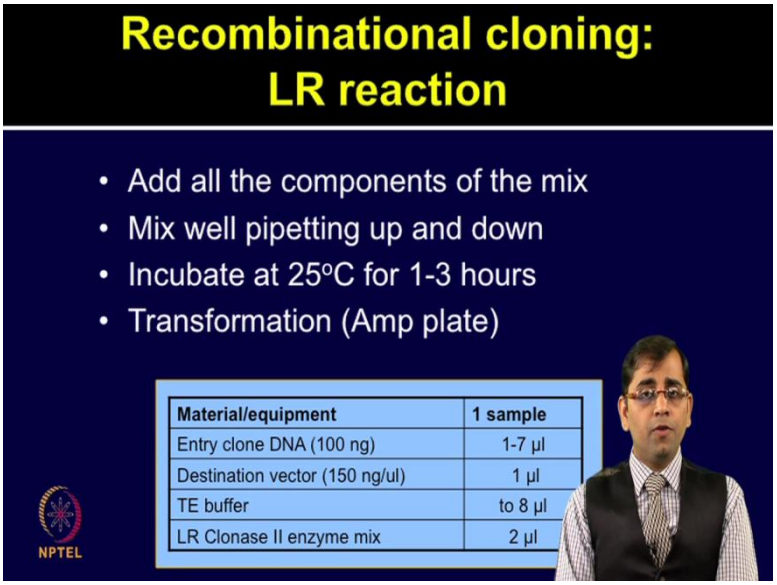

To perform the LR experiment one need to take the entry clone DNA obtained from the BP reaction around 100 nanogram is ideally required; there you need a destination vector which is additive of your choice, 150 nanogram per microliter concentration, you also need TE buffer and LR clonase 2 enzyme mix. Again these volumes are just to give an estimate about how to do the reactions. Add all the components of this mix by doing the pipetting up and down, incubate them at around 25 degrees for 1 to 3 hours and then this mixture can be plated

on ampicillin plate, antibiotic will be able to screen only those clones which have the gene of interest in the correct vector.

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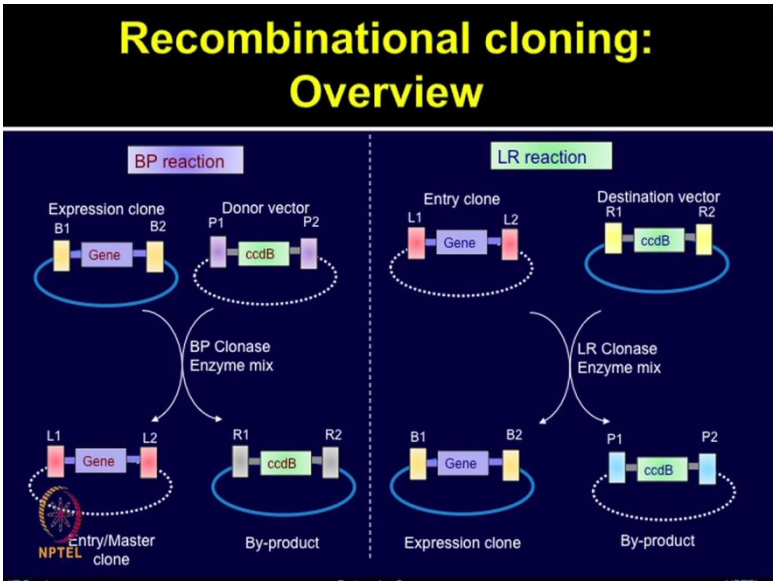

So, broadly there are two steps involved in recombinational cloning; BP reaction and LR reaction. The recombination technology facilitates protein expression and cloning of PCR products by using site-specific recombination enzymes rather than restriction endonucleases and ligase is which are commonly used in conventional cloning. The recombinational cloning makes use of a master clone as you can see in this slide having a particular gene that is rapidly transferred to the desired destination vectors and thereby, it can provide significant benefit over the conventional cloning methods in BP reaction, which is catalyzed by BP clonase enzyme facilitates recombination of at B site and at B substrate. The at B obtained from PCR product or at B containing the expression clones and this reaction with the at P substrate which requires the master vector can create at L containing master clones.

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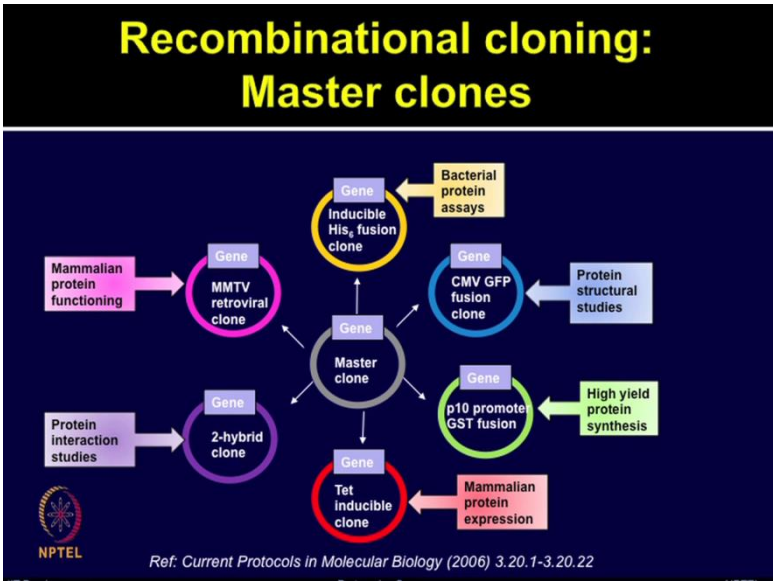

So the two steps are very quick, for the BP reaction you can generate entry or master clones which can further be used for transferring gene of interest to the destination vector of your choice. Once, these master clones are generated, these are immensely useful, you can transfer the gene of your interest from these master clones to different types of destination vectors based on applications. In this slide, few vectors are shown and few applications have been mentioned, for example, one can use inducible histidine 6 fusion clone. This can be used for 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 a protein is located in the cell then P10 promoter GST fusion clone results in a good protein yield if protein synthesis is your requirement you would like to transfer your gene of interest into this vector. Now, teracycle inducible clones, mammalian protein expression system that can turn the protein on and off in the mammalian cells. 2-hybrid clones, if your objective is to study the protein interactions then test the protein functions in the mammalian cell by using two hybrid clones MMTV retroviral clones if you want to perform mammalian protein functioning you might want to use this vector to know what other proteins does the protein interact with.

So, there are various types of vectors which one can make use of it. One can transfer, the gene of interest from these master clones to variety of vectors depending upon their applications. The recombination cloning provides you the feasibility and opportunity to perform multiple experiments once these clones are available. I will now provide you a

glimpse of high throughput cloning, it is important because automation is the key to achieving accuracy in microarray or other high throughput experiments.

So the error rate for the robots during repetitive manipulations is extremely low and all the operations can be verified by examining log files for the robotic run. Errors are likely to occur, which is laborious manual processes like gel loading or (())(18:12), this is why many of the these steps have been now automated and the labs which are using a high throughput platforms have modified the systems accordingly. I will show you few images from a previous tenure (())(18:31) working a Dr. Joshua Labaer lab at Harvard institute of proteomics. These images will provide you a glimpse of various steps involved in high throughput cloning and how one can perform these experiments in a high throughput manner.

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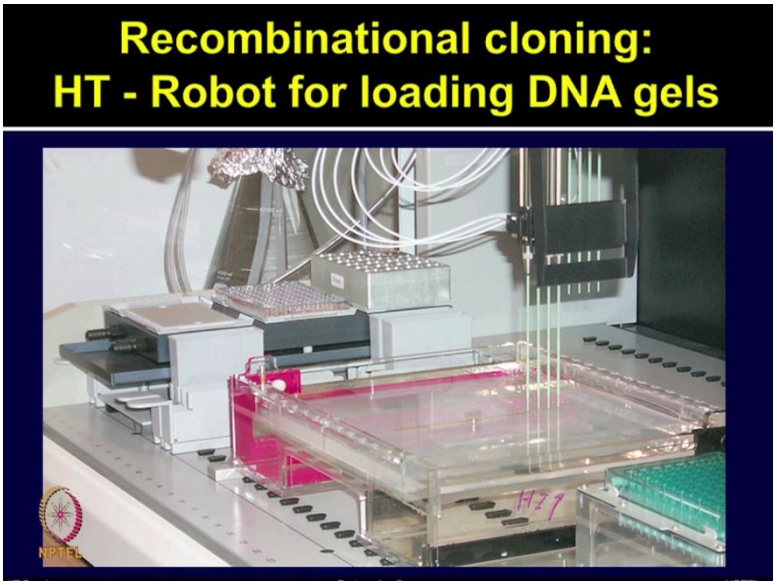

This image shows you the high throughput platform for loading DNA gels. A robotic system is used here where 192 lenses can be loaded in only 20 minutes. If you are doing this manually, it will require large amount of time as well as there will be sample is spinning on the gel along with various other manual errors in loading including the gel assembly and gel loading. Also, by the time you start from the first sample and reach to the last one, many samples are likely to be defused, so automation as well as speed are very important.

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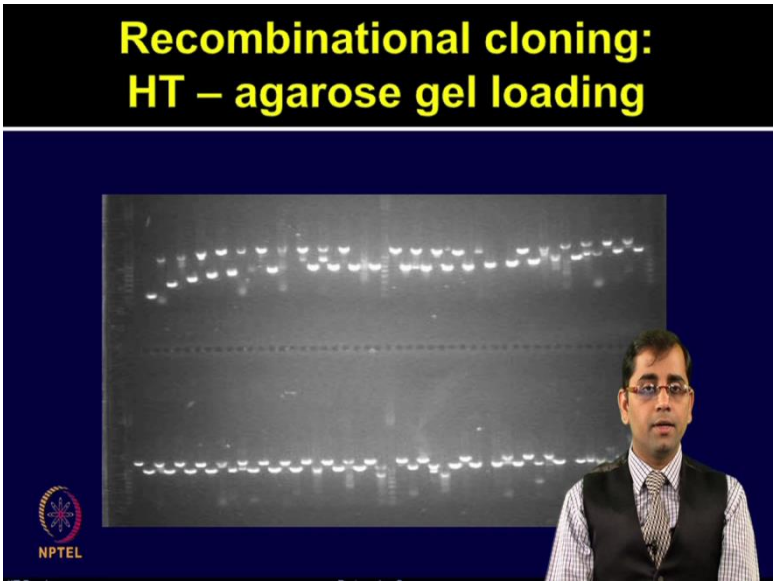

Let us look at this image of the high throughput agarose gel loading. Once, the run is done you can visualize the gel by a ethidium bromide staining. This reaction setup can be performed by liquid handling robots and agarose gels can be loaded by the robotic system. In the gel here, you can see this is saw tooth pattern for loading gels which makes the band size identification easier and avoids the contamination of the samples from the neighboring wells. Manual loading could lead to contamination into neighboring wells and makes it difficult to identify the accurate molecular weight of your sample.

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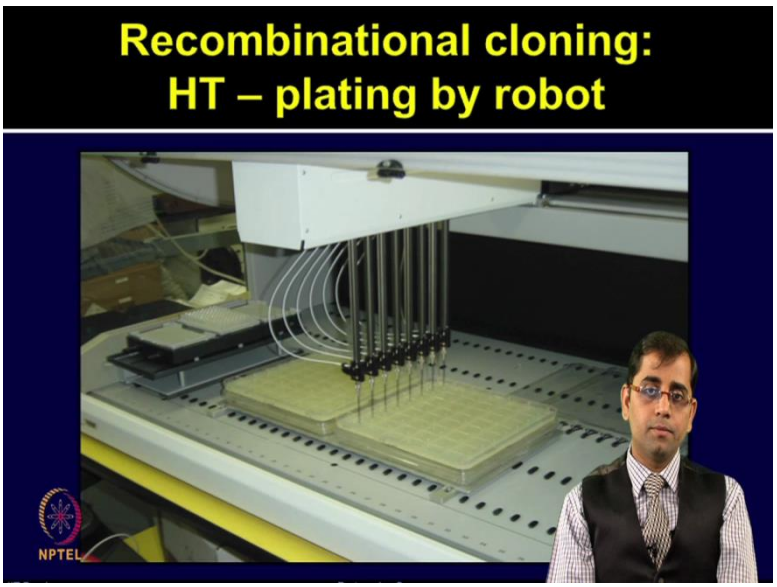

Now high throughput requirements also include the plating for the transformation by robot. The bacterial transformation can be plated by the robot on to the custom design bio-assay

dishes which contain 48 compartments as you can see in this slide and these (())(20:43) can be further picked automatically by another robotic platform which is a colony picking robot.

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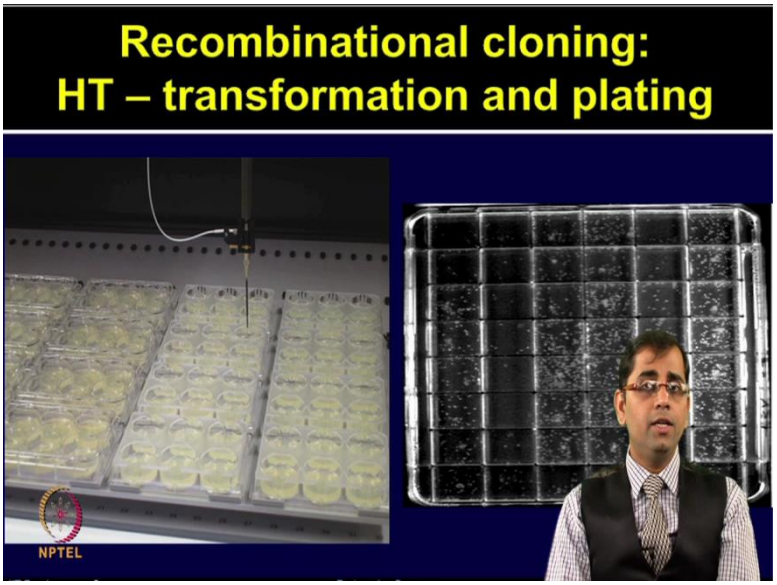

In recombination cloning, a high throughput method of doing transformation and plating is very crucial. Therefore, automation during the bacteria plating and colony picking reduces the error rates. The development of 48 sector plate suppose plating of 96 transformations on two plates instead of using 96 plated dishes. The low throughput way in conventionally labs utilize single plate for transformation.

So, if you have 96 clones and you want to do this transformation in 96 plates handling all these plates labeling, storing and identifying the right clones becomes very laborious. Now, if you have these 48 well plates your only requirement is to handle the two plates at a time by using robotic system, one can easily pick the bacterial colonies without touching the neighboring clones. Therefore, single colonies can be picked with much more accuracy by using this automation system.

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The bacterial colonies can be picked by such robotic systems, which increases the efficiency of the process and due to this decontamination.

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At the end if you want to extract DNA for subsequent applications, high throughput platforms can be used to perform DNA prepping in 96 well plate formats. We will discuss these when we talk about protein microarrays in more detail.

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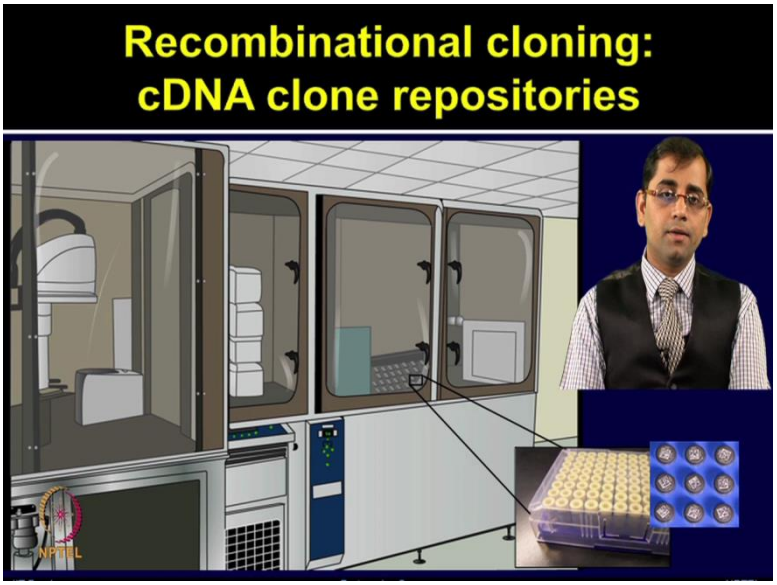

Here is an image shown to explain the functioning of cDNA clone repositories. The throughput of microarray experiments makes sorting these clones tedious. A thorough documentation or automation is very essential to identify a clone of interest within such a large repository therefore; these storage systems are highly automated where temperatures are strictly maintained at minus 80 degrees. These genes which are generated from recombinational cloning can then be located and clone into any vector. These genes are available in flexible formats; they are expression ready sequence verified and affordable.

All of these clones are stored in these automated clone repositories so that each tube contains a full length coding region that can be easily located and utilized without disturbing the other clones. The gateway recombinational cloning strategy allows, the DNA fragments flank by the homologous recombinational site to move from one vector to another in a single step procedure in frame and without any mutations. These reactions are very straight forward as you have seen BP reaction can be performed in one hour so as the LR reaction, it allows high throughput and automation. So, virtually these are almost 100% efficient if all the pipetting and reaction mix are added accurately. Let me explain you the basics of cloning as well as recombinational cloning in following animation.

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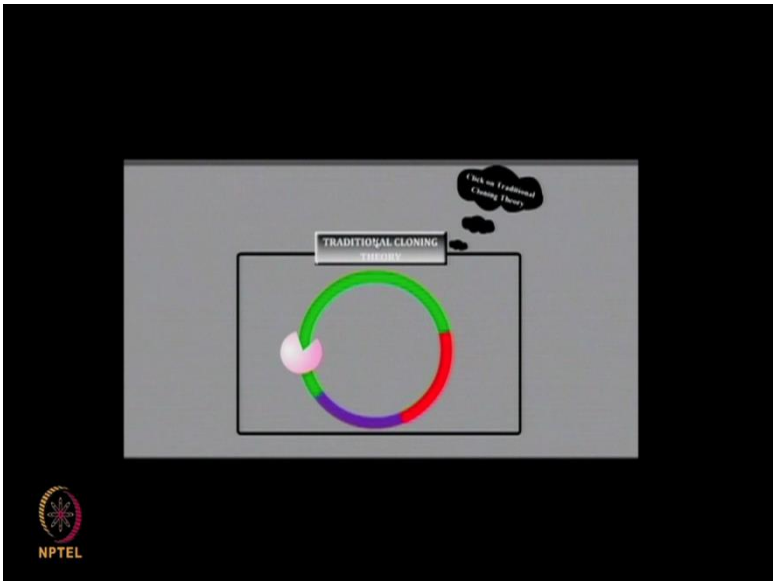

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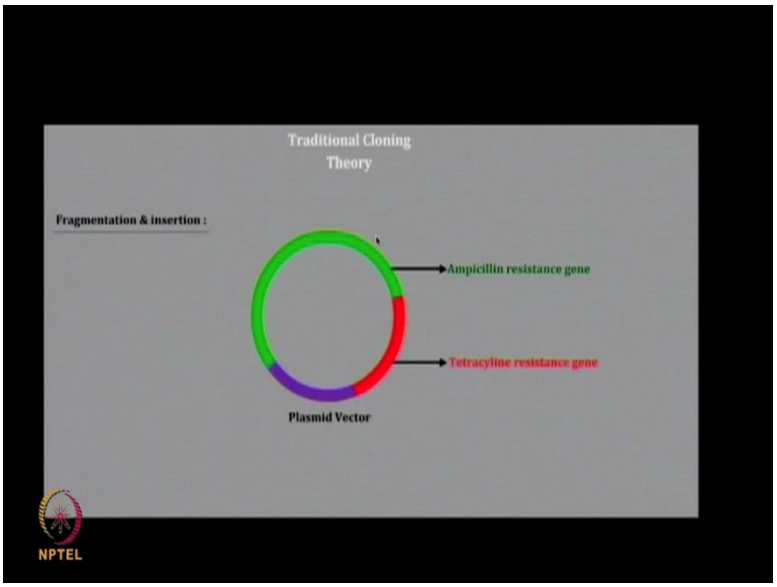

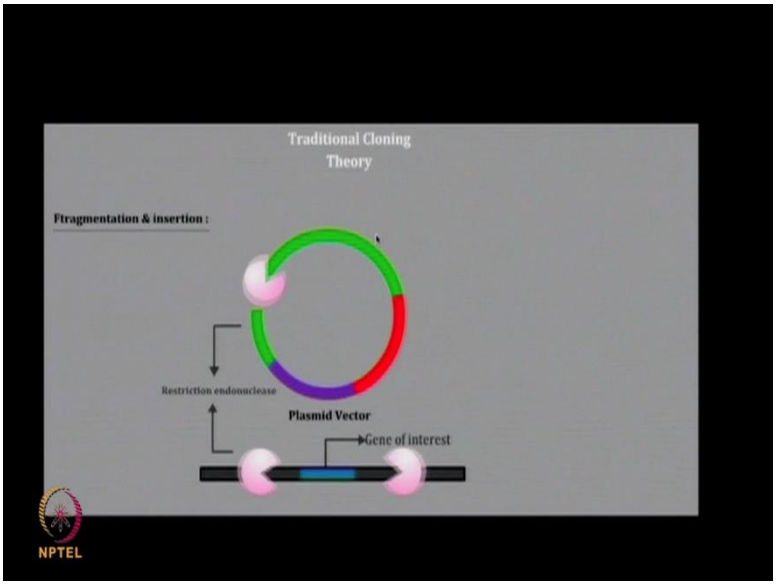

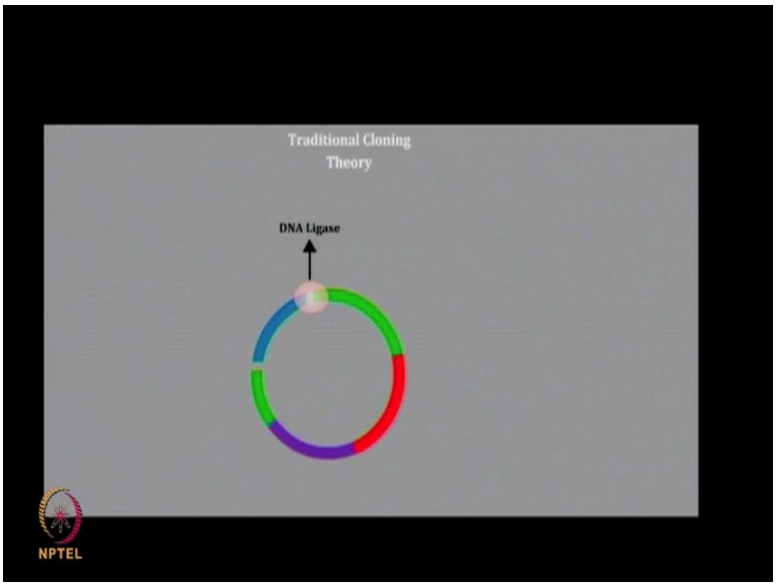

Since we were discussing about recombinational cloning, let us also briefly touch upon traditional cloning so, that it will be easier to compare traditional cloning verses recombinational cloning. The conventional cloning protocol makes use of a restriction enzyme that fragments, these selected plasmid vector as well as the DNA sequence containing gene of interest at the same recognition site. The complimentary sequence overhands that are produced in the plasmid and gene insert during restriction digestion are useful for proper orientation of these fragments during the insertion.

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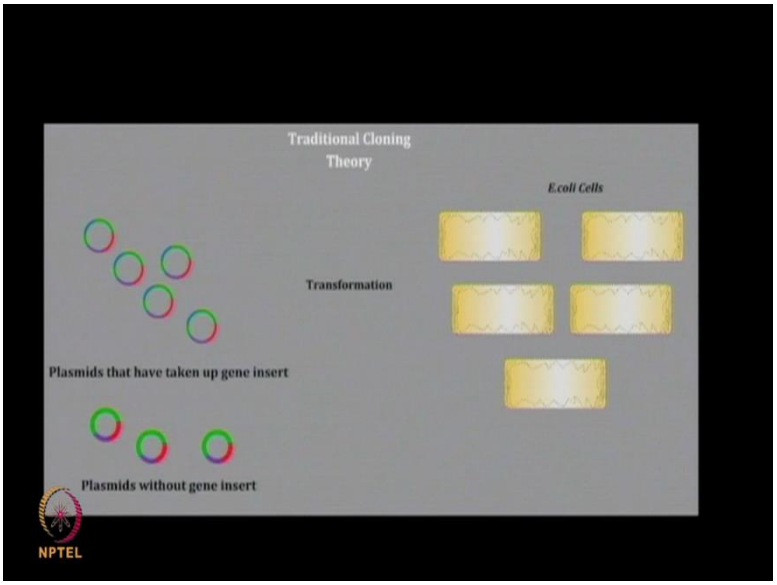

The insert is ligated by means of DNA ligase enzymes. The insertion of fragments within an antibiotic resistant gene leads to the inactivation of this gene, after this the transformation process occurs. So once the gene the insert has been introduced into the plasmid vector in desired orientation, they are transformed into suitable bacterial hole cells.

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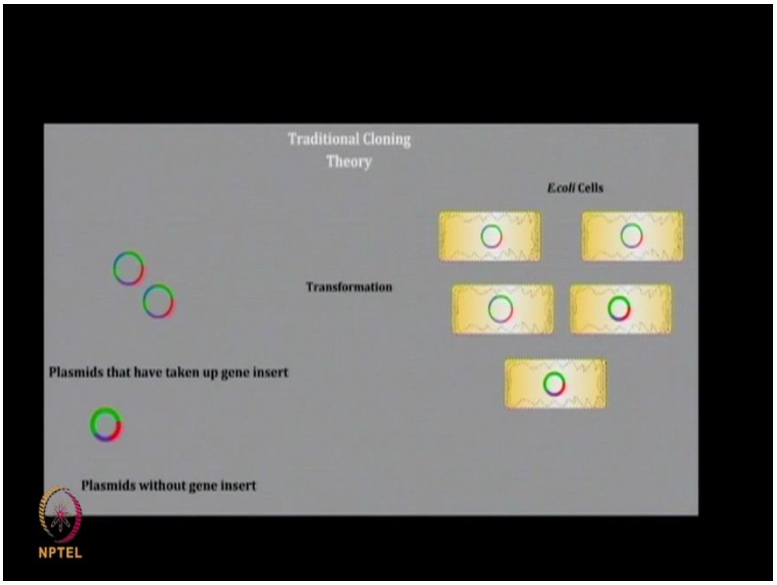

This can be done by technique such as electroporation, chemical sensitization, etc, which makes a cell membrane relatively permeable thereby allowing the plasmids to enter the cell.

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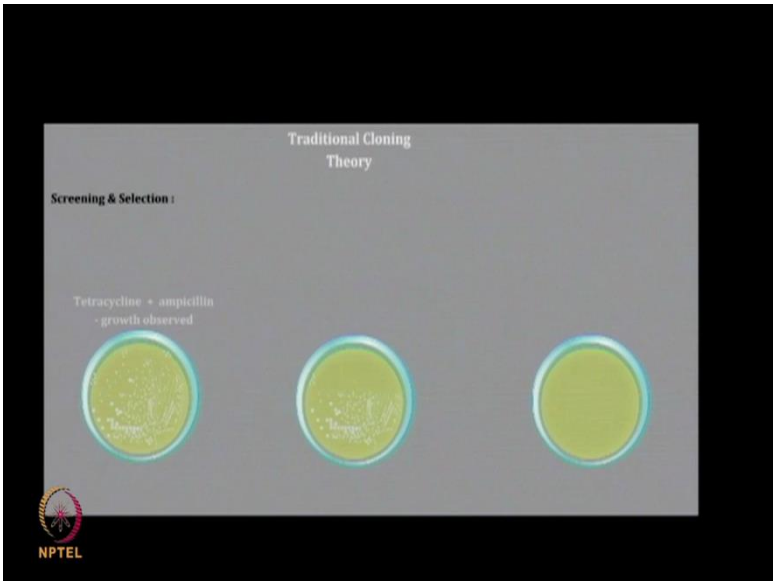

After transformation you need to select the right clone. The cells are grown on a suitable medium which contains the specific antibiotics that allow only certain bacterial cells to grow. Cells that have been transform with the plasmid but do not contain the gene of interest; they grow on the medium containing 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 resistant 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 of the gene insert by technique known as replica plating.

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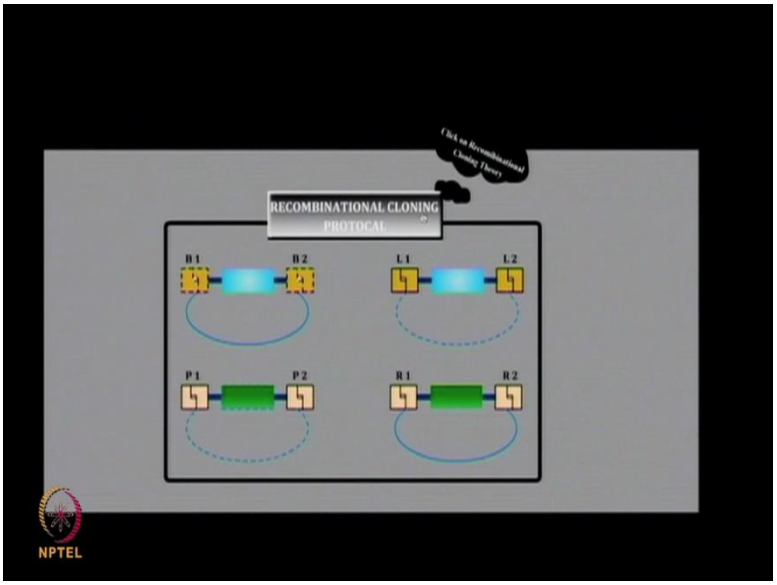

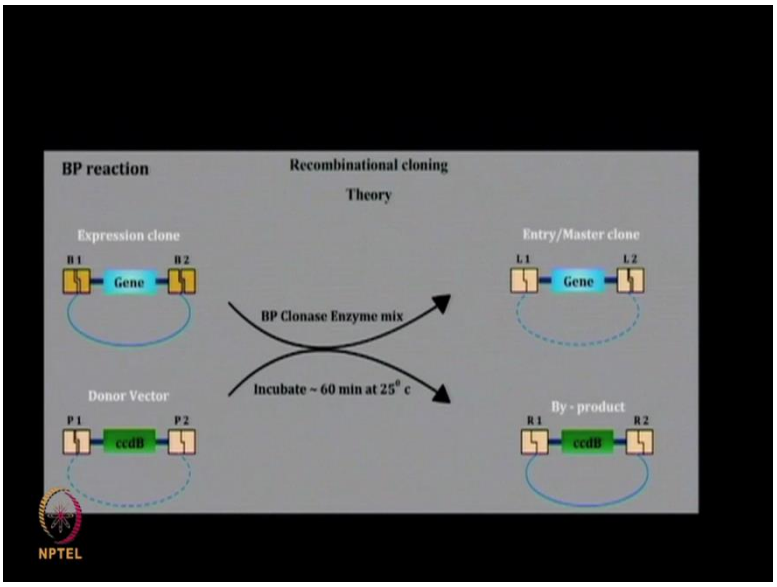

Let us now talk about recombinational cloning; the recombinational cloning involves two reactions BP reaction and LR reaction. The BP reaction of gateway cloning is a site-specific recombination reaction between the at B site of an expression clone or a PCR product and at P site of a donor vector in the presence of BP clonase enzyme master mix.

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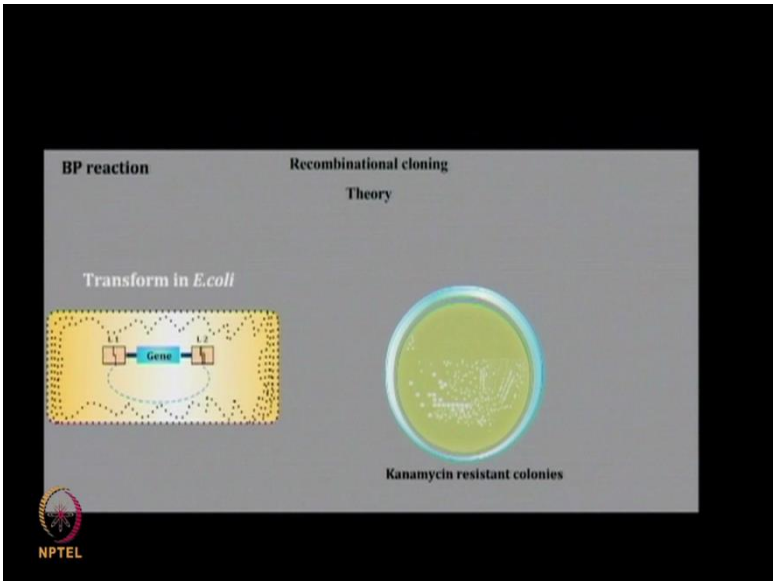

The reaction is incubated for just an hour at 25 degree centigrade to obtain the entry or master clones containing genes of interest. Once, this master clone flank by the at L site is produced, it can then be transferred into any destination vector to produce expression clone for a specific desired application. The reaction yields more than 90 percent or almost 100 percent correct colonies. The colony selection can be performed on kanamycin resistant plates.

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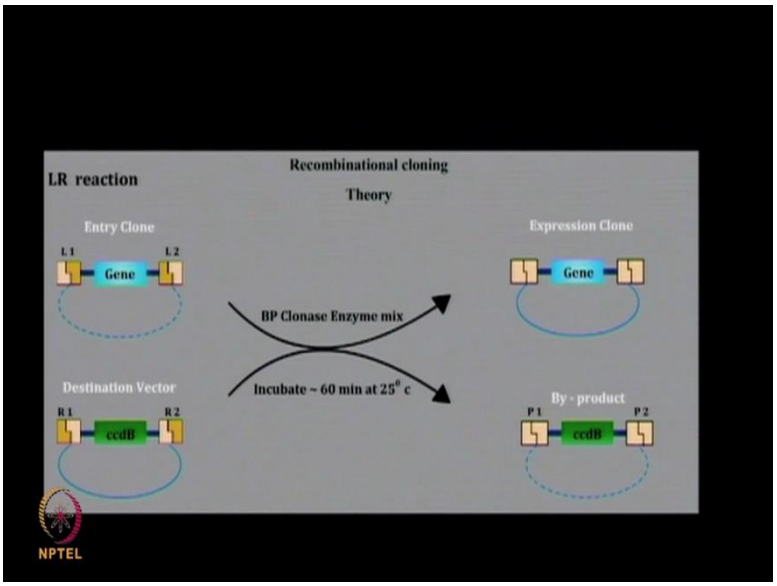

First BP reaction is successfully performed then one need to take this clone a (())(29:24) DNA and then further move on to LR reaction. The LR reaction is essentially the reverse of the BP reaction, where the master clone flank by the at L site recombines with a destination vector with at R sites. This 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 a specific purpose.

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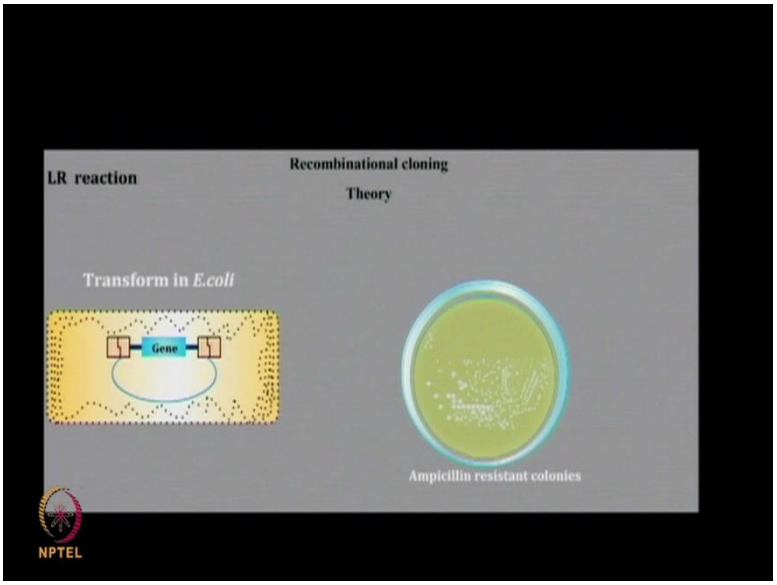

The LR reaction enables generation of several expression clones for various expression various applications in very short time. The transformation results into the colonies which can be selected on ampicillin resistant plate. Now, once the right colonies have been selected, the DNA can be extracted and these clones can be stored for various applications therefore, this recombinational cloning process provides significant advantages over conventional cloning techniques.

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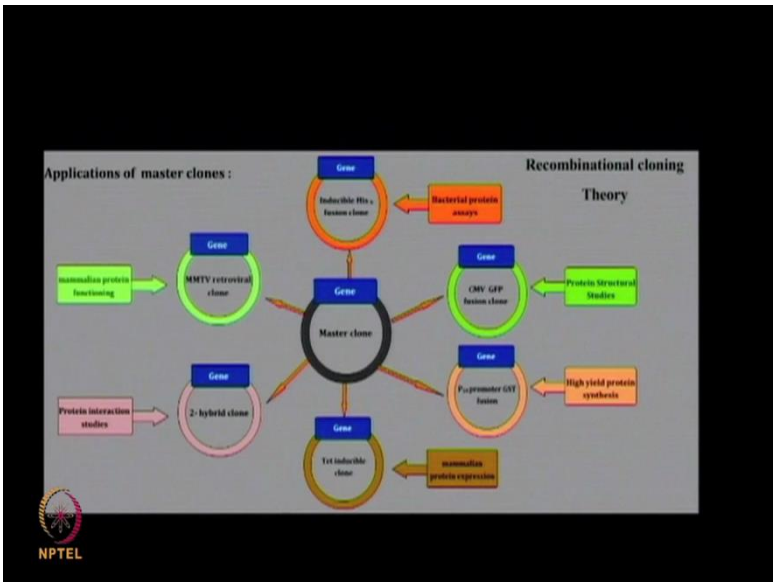

These master clones can be used for several applications. So, once these uhh recombinational clones are produced, these master clones can be used for various applications. So, the gene in the master clone can be transfer to various destination vectors by means of LR reaction to produce expression clones for several applications. Proteins can be efficiently expressed in bacterial, yeast and mammalian systems and used for a variety of applications such as a structural and functional studies, protein interaction studies, protein assays producing high yield of protein for experimentation, etc. 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.

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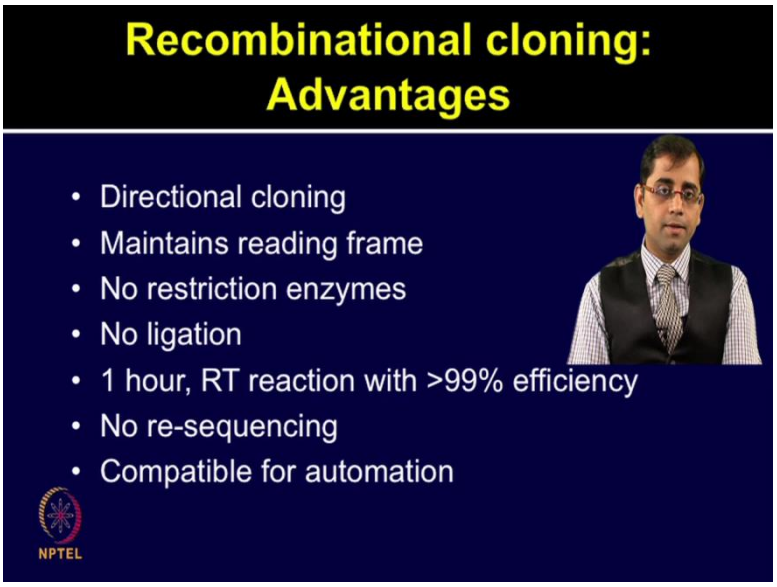
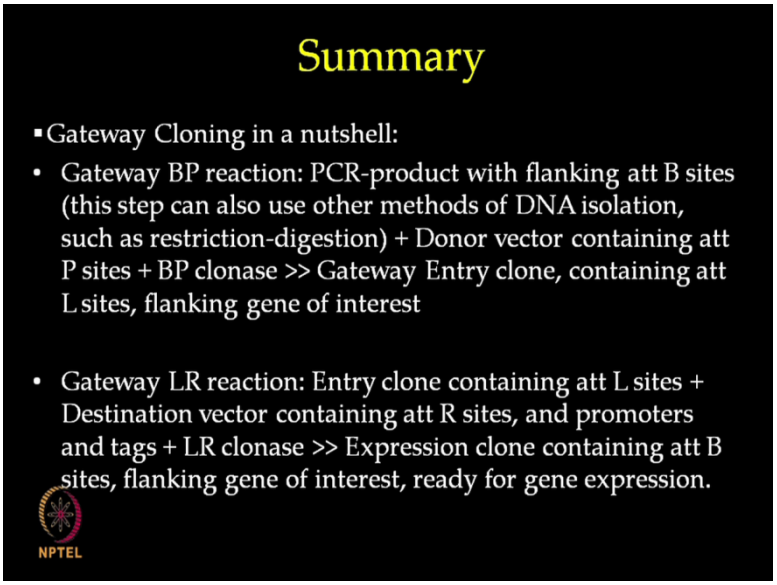


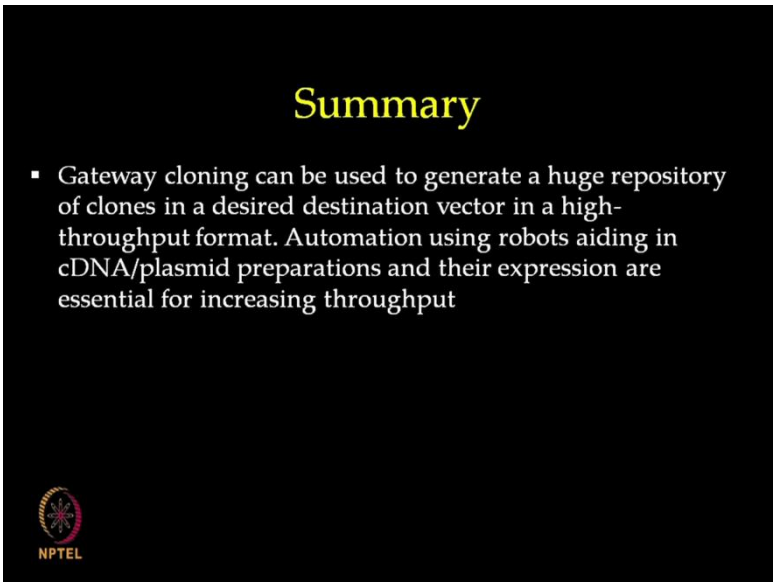
After watching this animation, now let talk about use of recombinational cloning for protein microarray applications. You have seen that recombinational cloning is directional cloning. It maintains the reading frame, there is number need for adding restriction enzymes or ligation procedures which is performed in the case of conventional cloning. The one hour reaction at room temperature increases the efficiency close to 99 percent.

Once the reactions are done successfully, there is no need to do re-sequencing, it is very accurate and the system is compatible for automation for the high throughput experiments. In summary, today we talked about few concepts which are required for the protein microarray generation and high throughput applications in genomics and proteomics namely recombinational cloning. The concept of protein microarrays has still a great deal (())(33:35) excitement in the proteomics community.

Once, the technology is fully realized, it promises to enable the study of broad variety of protein features at an unprecedented pace and scale however, generating protein contents in high throughput manner remains a challenge therefore, recombinational cloning is being widely used to establish clone repositories. In the next lecture we will discuss how we could use these techniques along with cell-free expression systems to produce proteins easily by circumventing the tedious process of purifying proteins from the conventional methods, Thank you.

(Refer Slide Time: 34:41)





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