LECTURE-28

Microarray related concepts: Recombinational cloning cell-free expression

TRANSCRIPT

Welcome to the proteomics course. Today we will talk about concepts related with microarray. We will talk about recombinational cloning and cell-free expression. Both recombinational cloning and cell-free expression systems are being used for microarrays. This will be useful when you hear the next lecture on cell-free expression based protein microarrays. The high throughput biology began in earnest with human genome project and increasingly these high throughput techniques are used for proteomics research. In this light, the functional proteomics is an exciting new approach to study protein functions in high throughput manner. It will enable the expression of proteins and subsequent assays of various properties such as sub-cellular location, interacting protein partners, biochemical activity. These are regulated at the scale to achieve the high throughput. There are large numbers 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 types of proteins modifications. All these complexities arise when one wants to purify the protein and apply that to protein microarrays. So to overcome these limitations people have tried various new methods and cell-free expression systems are one among them. But for the microarray when the requirements are high throughput you need large number of clones to be studied and that's where recombinational cloning comes into play.

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

researchers 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's start our discussion with 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-specific recombination is a genetic recombination technique where DNA strand exchange takes place between the regions possessing resonance sequence homology. The common site-specific recombination technologies include gateway cloning system from Invitrogen and created technology from BD Clonetech. In our discussion on recombinational cloning I will focus more on recombinational cloning system from Gateway. It is more because of my own familiarity using in my own research but both the systems can be used for recombinational cloning.

As I mentioned, recombinational cloning is a novel site-specific recombinational technique, which is required to transfer the DNA sequences. The specific recombinase enzymes cleave DNA backbone and carryout interchange of DNA helices between specific sites on two different molecules. The recombinational cloning is a universal strategy to move DNA sequence to any given vector. This is a very promising approach, especially when one looks for high throughput applications such as genomics and proteomics.

Before I talk about what recombinational cloning is and how it works, let me introduce to the terminology, which is used in recombinational cloning.

Expression clone: a clone containing a gene sequence of interest which is flanked by att B sites. The orientation of the gene is maintained through out the process because of specific interactions between the att sites. So what are the att sites? att sites are DNA segments of certain defined lengths.

Donor vector: donor vector consists of a counter selectable gene which flanked by attP site which recombines with gene of interest flanked by attB site in BP reaction to produce a master or entry clone. The explanation for BP reaction and master clone will be given in the subsequent slides.

Master clone: the vector containing gene of interest flanked by attL site. Entry clones are formed by the BP reaction and further used in LR reaction to produce expression clones of interest.

Destination vector: this is a vector containing a counter selectable gene flanked by attR sites which interact with genes from entry clone to produce an expression vector in the LR reaction.

Now slowly this terms will be easier for you to follow up when we explain this in the subsequent slides where I have shown you some pictorial representation of these reactions.

Broadly recombinational cloning involves two reactions, BP reaction and LR reaction. Let's talk about BP reaction. When a reaction is run to mix att B and att P sites such as when making master clones, this step is catalyzed by BP clonase enzyme and it is known as BP reaction because of attB and att P sites involvement. As you can see in this slide there is a donor vector or pDONR that contains attP sites flanking a ccdB gene or a counter selectable gene that recombines with a gene of interest which is flanked by att B site which you can see on the left hand side. So the gene fragment can be produced from the PCR product and which contains attB site and you have a donor vector which contains a selectable marker, kanamycin resistance marker that contains attP sites. So entry or master clone is a vector containing gene of interest flanked by the attL site. This happens after the reaction is completed. The recombination between attB and attP sites is catalyzed by an enzyme known as BP clonase. This enzyme transfers the gene of interest at B sites to the donor vectors which contains attP sites. Again what is att site? This can be defined as length of DNA that serves as the binding site for

recombinant protein. So once the BP reaction is completed this entry or master clone can be taken for further LR reaction.

Before I move to LR reaction, let me give you a brief explanation on how BP reaction can be performed in the laboratory. You need to add various components, which are shown in table such as BP reaction buffer, pDONR221 around 50 ng/µL 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 components of BP reaction mix and then mix them well by pipetting them up and down. After that you can incubate them for 1- 3 hours at 25°C and then this reaction mix can be used for transformation process on kanamycin resistant antibiotics. Since we have this vector containing kanamycin resistance gene, only those clones which will have the entry of your correct insert in the right vector will able to grow on the kanamycin plate.

Let's now discuss the next reaction which is LR reaction. In the BP reaction you have made entry or master clone and now that can be further used in the LR reaction. In the LR reaction if the interaction is between the att L and att R site then it can be catalyzed by the enzyme LR clonase and the 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 the master clone which was selected on kanamycin plate and now you want to transfer that in a destination vector which contains another antibiotic gene which is amplicillin resistant gene. Now this destination vector contains R sites, R1 and R2. The gene of interest can move from entry or master clone to the destination vector. The destination vector a.k.a DEST is modified to accept the protein coding sequence in-frame from the master or entry clone by using the recombinational cloning enzyme known as LR clonase. Once this reaction is completed then the reaction mix can be plated on the ampicillin plate and since the vector contains the ampicillin resistance gene it can be screened on the ampicillin plates.

Now let's 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 BP reaction; around 100 ng will be optimal. Then you need a destination vector, vector of your choice in which you want to insert the gene of your choice; 150 ng/µL. you also need TE buffer and LR clonase II enzyme mix. Again these volumes here are just to begin with and can be modified according to the reaction requirements. So add all the components of this mix of LR reaction. Mix them well by pipetting up and down. Incubate the reaction at 25°C for 1-3 hours and then this mix can be put on the plate for the transformation process on the ampicillin plate. This antibiotic will be able to screen only those clones, which have the gene of interest in the right vector.

So now as an overoview of this reaction of recombinational cloning there are two steps involved, BP reaction and LR reaction. So the recombinational technology facilitates protein expression and cloning of PCR products by using site-specific recombination enzymes rather than the restriction endonucleases and ligases which are used in conventional cloning. Recombinational cloning makes use of a master clone, as you can see in the slide, having a particular gene that 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 BP clonase enzyme, facilitates recombination of attB site, attB substrate. It could the attB obtained from PCR product or attB contiaing the expression clone. And this reaction with the attP substrate, which requires the master vector, can create attL containing master clones. So the two steps are very quick. From the BP reaction you can generate entry or master clones which can be further used for the transfer of the gene of interest to the destination vector of your requirements.

Once these master clones are generated these are almost like 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 types of destination vectors. In the slide few vectors are shown and few applications are mentioned here. For example, one can use inducible histidine6 fusion clone. Now 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 my protein is located in the cell.

Then p10 promoter GST fusion clone which produces lots of proteins for the experiments. So protein synthesis if that is your requirement you would like to transfer your gene of interest into this vector. Now, tetracycline inducible clones: Mammalian protein expression system that can turn the protein on and off in the mammalian cells. 2-hybrid clone: if your objective is to study the protein interactions then test the protein functions in the mammalian cells by using 2-hybrid clones. MMTV retroviral clones: if you want to perform mammalian protein functioning you may want to use this vector to know what other proteins does this protein interact with. So there are various types of vectors which one can make use of. One can transfer the gene of interest from these master clones to a variety of vectors depending upon their applications. 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 a 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 manipulations is exceedingly low and all operations can be verified by examining the log files for the robotic run. So it is especially evident when you are doing the gel loading or colony picking which are very much error prone processes during the cloning process, then if you are performing the manual steps, thousands or even more large numbers, there is a good probability that there will be errors. That's why lot of these steps have been automated and the labs, which are using this high throughput platforms, have modified the systems according requirements and system has been automated based on the robotic platforms. I'll show few images obtained during my Postdoc at Dr. Joshua LaBaer's 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.

So this image shows you the high throughput platform for loading DNA gels. A robotic system is used here where 192 lanes can be loaded in only 20 mins. If you are doing this manually it will require large amount of time as well as there will be sample spilling

on the gel and different types of problems will come especially when you are talking about 192 lanes. By the time you start from one sample and reach to the last one many samples would have diffused. So automation is very important as well as speed.

Now let's look at this image of the high throughput agarose gel-loading. So once the reaction done as we loaded in the last slide, you can visualize the gel with ethidium bromide staining. This reaction set-up 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 pattern for loading the gels, which makes the bands size identification easier and avoids the contamination of the samples from the neighbouring lanes. By doing the alternate way you are avoiding the contamination, which can go to the neighbouring wells due to the overflow of the sample and also you can easily identify your sample in the molecular weight.

Now high throughput requirements also include the plating for the transformation by robot. So the bacterial transformation can be plated on to the robot on to the custom design bio-assay dishes which contain 48 compartments, as you can see in this slide. And these colonies can be further picked automatically by another robotic platform which is a colony-picking robot.

So in the recombinational cloning the high throughput way of doing transformation and plating is very crucial. It provides automation during the bacterial plating and colony picking reduces the error rates. The development of 48 sector plate supports the plating of 96 transformation on 2 plates instead of 96 petridishes. The low throughput way is conventionally labs use single plate for doing these transformation. So if you have 96 clones and you want to do this transformation in 96 plates, handling all those plates, labeling, storing and identifying the right clones becomes very tedious. Now if you have these 48 well plates, so your only requirement is to handle two plates. And again by using this robotic system one can easily pick the colonies with out touching the neighbouring colonies. So single colonies can be picked up very accurately by using this system.

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 automation can be modified to do the DNA prep in the 96 well plate format.

I will touch upon these more when we talk about how to make the protein microarrays in the lab, how can one do those experiments. I will elaborate on that more but this just gives a glimpse on how various types of steps have been modified to do the automated requirements of the experiments.

Now here a cartoon is shown to explain you the cDNA clone repositories. Sorting these clones is very tedious because in the scale thousands in the high throughput experiments. Now once these clones are stored and you want to take a clone of your interest from a large number of plates it is very tough to identify the right clone. Therefore some of the very automated clone storage systems have been generated and these clones are stored properly at -80°C in these types of clone storage systems. These genes, which are generated from recombinational cloning, which we discussed just few minutes ago, can be transformed into any vector. These genes are broadly available, there are no restrictions, there's 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 this system with out effecting the other clones.

So the Gateway recombination cloning strategy allows the DNA fragments flanked by the homologous recombination sites to move from one vector to another in single step procedure, in-frame and with out any mutations. These reactions are very straightforward. As you have seen, BP reaction can be done in 1 hour, so is the LR reaction. It allows the high throughput automation. So virtually these are almost 100% efficient, if all the pipetting and all the reaction mix are added accurately and with proper

pipetting. So let me explain you the basics of cloning as well as recombinational cloning in the following animation.

Animation - 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 versus recombinational cloning.

The conventional cloning protocol makes use of a restriction enzyme that fragments the selected plasmid vector as well the DNA sequence containing the gene of interest at the same recognition sites. The complementary sequence overhangs that are produced in the plasmid and gene insert during restriction digestion are useful for proper orientation of the fragment during insertion. The insert is then ligated by means of the DNA ligase enzyme. Insertion of the fragment within an antibiotic resistance gene leads to inactivation of this gene. After this the transformation process occurs.

Transformation

Once the insert has been introduced into the plasmid vector in the desired orientation, they are transformed into suitable bacterial host cells. This can be done by techniques such as electroporation, chemical sensitization etc. which make the cell membrane relatively permeable thereby allowing the plasmids to enter the cell.

Screening and selection:

After transformation you need to select the right clone. The cells are 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 grow on medium containing both tetracycline and ampicillin. Those cells that have taken up the plasmid and contain the gene insert will l grow on a tetracycline containing medium but will not grow in presence of ampicillin. Those cells that do not grow in presence of antibiotics have not taken up any plasmid and therefore do not have resistance to the antibiotics. Comparison of colonies grown in the presence of both

antibiotics and in presence of only tetracycline will reveal those that have taken up the gene insert by a technique known as replica plating.

Recombinational cloning:

Let's now talk about recombinational cloning. The recombinational cloning involves two BP reaction and LR reaction.

BP Reaction - The BP reaction of GATEWAY cloning is a site-specific recombination reaction between the attB site of an expression clone or a PCR product and attP site of a donor vector in the presence of BP Clonase enzyme master mix. The reaction is incubated for just an hour at 25°C to obtain the entry or master clones containing the gene of interest. Once this master clone, flanked by attL sites is produced, it can then be transferred into any destination vector to produce expression clones for a specific desired application. The reaction yields more than 90% or almost 100% correct clones. The colony selection is performed on kanamycin resistance plates. Once BP reaction is successfully performed then one needs to take this clone, extract DNA and then further move on to LR reaction.

LR Reaction - The LR reaction is essentially the reverse of the BP reactions where the master clone, flanked by attL sites, recombines with a destination vector with attR sites. This reaction which takes place in the presence of the LR Clonase enzyme mix results in transfer of the gene from the master clone to the destination vector to produce an expression clone for a specific purpose. This reaction enables generation of several expression clones for various applications in very short time. The transformation results in to colonies which can be selected on ampicillin resistant plates. Once the right colonies have been selected, DNA can be extracted and these clones can be stored for various applications. Therefore this recombinational cloning provides significant advantages over conventional cloning techniques. These master clones can be used for several applications. Once these recombinational clones are produced these master clones can be used for various applications.

Application of master clones

The gene in the master clone can be transferred to various destination vectors by means of the 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 structural & functional studies, protein interaction studies, protein assays, producing high yields of proteins for experimentation, etc. The rapid recombination between clones that is possible with the GATEWAY system cannot be done with conventional cloning techniques due to which the GATEWAY protocol is now being extensively adopted.

After watching this animation, now let's talk about using recombinational cloning. So as you have seen this is directional cloning. It maintains the reading frame. There's no need for adding restriction enzymes which is the case for conventional cloning. No need for doing the ligation steps. One hour reaction at room temperature which increases the efficiency close to 99%. Once the reactions are done successfully there's no need to do the resequencing. It is almost 100% correct sequnce inserted in the right vector and the sytem is compatible for automation for doing the high throughput requirements.

Let's talk about the requirements for cell-free microarrays. Once you have done the recombinational cloning and obtained several master clones the repository of expression ready clones in a flexible cloning system enables the easy sub-cloning between various expression constructs. So a pipeline for quickly purifying DNA constructs and arraying them for the microarray applications can be generated after the recombinational cloning is performed. So in the subsequent lecture we will talk about cell-free expression systems and we today we will touch upon the concepts of cell-free expression technology. But all this requires large number of genes of interest, the cDNA clones and in the right expression vectors. So by doing the recombinational cloning one can actually have the flexibility to perform various types of applications including microarray applications.

So now you have generated lot of master clones which are gold clones. Now one can use these cDNA clones for various applications including protein microarrays. One can transfer these clones into the right expression vectors and purify the proteins or one can use these vectors for producing proteins by using cell-free protein synthesis system. So these clones are important and are required regardless of whatever way you want to make the protein, you want to make cell-based or cell-free method. But again since the requirements for making the microarrays in a high throughput manner, it is very difficult to purify thousands and thousands proteins and especially when you want to preserve their activity you want to do the functional activity testing. So eliminate all these problems people have started using the cell-free protein synthesis method. Cell-free synthesis system makes use of template DNA in the form of plasmids or PCR products for direct *in vitro* protein synthesis in the presence of a crude cell lysate which contains all the necessary machinery required for transcription and translation with essential amino acids, nucleotides, salts and other energy generating factors which are added exogenously.

So the cell-free synthesis system eliminates the need for protein expression and purification in cell-based system. Various types of DNA templates can be used such as PCR product or plasmids. The cell-free synthesis lysate contains machinery for transcription and translation. The commonly used cell-free expression systems include wheat germ extracts (WGE), rabbit reticulocyte lysate (RRL) and *E. coli* based systems. So the cell-free expression based 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 rabbit reticulocyte lysate (RRL). Others include those obtained from *Xenopus* oocyte, hybridomas, insect and mammalian cells.

One can use any of these cell-free protein system but it depends on their application requirements. So let's do a comparison of these 3 widely used cell-free protein synthesis system, *E. coli* extract, rabbit reticulocyte lysate and wheat germ extracts (WGE). Post-translational modification is not possible using *E. coli* extracts whereas RRL and WGE that are eukaryotic systems can provide the post-translational

modifications. The synthesized proteins majorly are incomplete polypeptides in case of *E. coli* extracts whereas in RRL and WGE it is full-length protein. The template source in *E. coli* is mainly from bacteria, in RRL it is mainly from animals especially rabbits and in WGE mainly from the plants.

Since we are talking about proteomics applications for the eukaryotic systems, let's talk about eukaryotic cell-free expression systems, WGE or RRL, their advantages and disadvantages. Advantages of this system include higher stability; so the longer lifetime of the cell-free systems, better compatibility with eukaryotic mRNAs and the synthesis of eukaryotic proteins. However, there are certain disadvantages such as lower translational 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 system provides various benefits such as an adjustable environment to allow the proper protein folding, disulfide bond formation and addition of labeling agents during the translational process which enables easy detection of synthesized proteins. So let's discuss the commonly used cell-free expression systems.

Animation 2: - The commonly used cell-free expression systems include *E. coli* s30, rabbit reticulocyte lysate (RRL) and wheat germ extracts (WGE).

E. coli S30: This is a commonly used bacterial expression system that is capable of producing protein yields of ~6 mg/mL (Jackson et al., 2004). This system however is not capable of carrying out post-translational modifications (PTMs) of proteins due to the absence of required machinery for this process and very often produces incomplete protein chains. DNA templates obtained from bacterial sources are commonly used with this cell-free lysate.

Rabbit reticulocyte lysate (RRL): A mammalian cell-free system that also gives protein yields of ~6 mg/mL (Jackson et al., 2004). This system is more suitable for expression

of full-length eukaryotic proteins from plant and animal sources that require proper folding and post-translational modifications (PTMs).

Wheat germ extract (WGE): This is a cell-free expression system that is capable of producing full length proteins with correct folding and PTMs from bacterial, plant or animal sources. Yields obtained in this system are however slightly lower than *the E. coli* and RRL based cell-free expression systems.

Actively growing and replicating *E. coli* cells can be used for extracting cell-free lysates. These cells that are in the process of growth and division are constantly producing proteins and other factors required for the various cellular processes. Co-factors and enzymes such as RNA Polymerase, peptidyl transferase etc are available in significant quantities due to cellular processes of transcription and translation taking place in the cell. As you can see in the animation, the two steps have occurred here, transcription and translation and the required material has been provided exogenously

The cells are lysed with a suitable buffer and then centrifuged at 30,000g to collect the supernatant containing the extract. Lysate that is extracted from such actively growing and dividing cells will contain all required cellular machinery to carry out *in vitro* protein synthesis and requires addition of essential amino acids, nucleotides, salts and other energy generating factors.

Wheat germ extract (WGE):

One of the most commonly used eukaryotic cell-free expression systems is obtained from the embryo of the wheat seeds. The seeds are grinded and then sieved to remove their outer coating fragments. The embryos and other small particles are floated in an organic solvent such as cyclohexane. The floating embryos are quickly removed and dried to avoid any damage from the organic solvent.

The dried embryos are then carefully sorted such that only the good embryos without any endosperm coating are selected. One needs to repeat this washing step few times. The endosperm contains certain inhibitors of protein synthesis that must be removed.

The selected embryos are washed thoroughly with cold water after which they are mixed with the extraction buffer and grinded.

This solution must be centrifuged at 30,000g at 4°C which results in the wheat germ extract forming a layer in between the top fatty layer fraction and the pellet at the bottom. This fraction can be separated and then purified by chromatographic methods to remove any components of the extraction buffer. This cell-free lysate is capable of synthesizing full length eukaryotic proteins with yields of around 4 mg/mL.

So how cell-free synthesis occurs? For that you need certain elements. You need a DNA template, plasmid or PCR fragment, a promoter which could be T7, SP6 or T3, a translation initiation signal, for eg., Shine dalgarno in case of prokaryotes or Kozak in eukaryote, a universal DNA sequence for protein initiation and a transcription and translation termination region. All these are required for cell-free protein synthesis.

The cell-free expression systems allow rapid conversion of genetic information directly to the functional protein. It facilitates synthesis of several proteins in a single reaction. So let me show you an animation for *in vitro* protein synthesis which will explain you these concepts very easily.

Let's talk about *in vitro* protein synthesis. The DNA template is thawed 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 promoter sequence which can initiate the transcription process, a translation initiation sequence for binding of the ribosome as well as suitable termination sequences to correctly synthesize only the proteins of interest.

The thawed cell-free lysate containing the essential cellular machinery for protein synthesis is added to the DNA template followed by the other exogenous factors that are required for the process such as essential amino acids, nucleotides, ATP, etc. 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^{o}C. Enzymes for transcription bind to the promoter sequences and in the presence of other factors like ATP and nucleotides, they carry out synthesis of the mRNA transcript. This mRNA is then translated into the corresponding proteins with the help of ribosomes, tRNA, enzymes and other factors required for the process.

So the cell-free expression systems are required for microarrays. But why it can be used? Because it has the ability to utilize wide variety of DNA templates. One can make use of all the master clone obtained from the recombinational cloning. Cell-free expression system is a simple, quick and cost-effective process. It provides a high throughput production in a very short time and in a single reaction. So all these requirements are very useful for high throughput protein production and for microarray generation.

In summary, today we talked about few concepts, which are required for the protein micrarray generation as well as high throughput applications in genomics and proteomics. The concept of protein microarrays has stirred a great deal of 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 challenging. Therefore the recombinational cloning to produce clone-repository and cell-free expression systems to produce proteins becomes very important. In today's lecture we have discussed about recombinational cloning, various steps involved such as BP and LR reactions, we talked about how high throughput cloning can be performed. I walked you through some glimpses of the pictures for cloning process. I also showed you some animations on comparison of cloning, conventional and recombinational. We then discussed about cell-free protein synthesis system. We talked about wheat germ extracts, rabbit reticulocyte lysate and *E. coli* based systems. These concepts will be very important when we discuss further in the following lectures about protein microarray and especially the cell-free based protein microarrays.