BIOTECHNOLOGY IN AVIAN MEDICINE

SLIDE STUDY SET #18

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INTRODUCTION

The purpose of this slide study set is to familiarize the individual with various molecular biology techniques so that a higher level of comprehension can be obtained when reading the literature or attending scientific meetings where those techniques are being discussed. This slide study set is not designed to be a methods reference.

A description or definition of each technique will be given along with a schematic representation of the procedure where appropriate. In addition, the applications, advantages, disadvantages, and limitations of each technique will be discussed.

SLIDE #1: BIOTECHNOLOGY OVERVIEW

The composition and structure of DNA and RNA is presented. DNA is composed of nucleotides (bases) A, C, G, and T, the order of which makes up the genetic code. A sugar (deoxyribose) and phosphate backbone make up the structure of DNA. DNA is commonly double stranded, where A pairs with T and C pairs with G by hydrogen bonding. RNA is similar to DNA, except that U replaces T; it is usually single stranded, and a ribose sugar and phosphate backbone make up its structure.

The genetic code of DNA or RNA is directional and is read from left (5' end) to right (3' end).

SLIDE #2: THE FLOW OF GENETIC INFORMATION IN A CELL

DNA contains and stores all of the information for the cell to function. By a process known as transcription, message RNA (mRNA) is synthesized which carries the genetic information for the synthesis of proteins which are produced by a process known as translation.

The basic flow of genetic information in the cell is as follows.

DNA -> mRNA -> Proteins
transcription translation

Replication is the synthesis of a new DNA strand and is necessary when the cell divides.

DNA -> DNA
replication

ENZYMES USED IN BIOTECHNOLOGY

SLIDE #3: RESTRICTION ENZYMES

Restriction enzymes- cut double stranded (ds) DNA at specific sequences called recognition sites. Each RE is named after the bacterium from which it was isolated (i.e., E*co*RI = *Escherichia coli*, H*ind*III = *Haemophilus influenzae*).

SLIDE #4: NUCLEIC ACID MODIFYING ENZYMES

Ligase- brings together two pieces of dsDNA with similar ends.

DNA polymerase- synthesizes a complementary strand of DNA using DNA as the template (Klenow fragment, Taq polymerase).

Reverse transcriptase- synthesizes a complementary strand of DNA using RNA as the template.

RNA dependent RNA polymerase- synthesizes a complementary strand of RNA using RNA as the template (found in many RNA viruses).

NOTE: A definition for Primers and a description of their role in the synthesis of DNA or RNA by polymerase will be presented later in this slide study set.

NUCLEIC ACID CLONING

SLIDE #5: NUCLEIC ACID CLONING

DNA cloning is used to isolate a gene from its natural environment so that it can be obtained in large quantities and molecularly manipulated. Traditionally, cloning is the insertion of a foreign gene into a bacterial plasmid or a phage virus. A plasmid is a small circular DNA molecule that exists in the bacterium separate from the chromosome. A phage virus is a virus that infects bacteria. There are many different approaches to cloning genes. The strategy used will depend on the gene of interest and the cloning vector.

SLIDE #6: NUCLEIC ACID CLONING (RECOMBINANT DNA PRODUCTION)

The basic steps behind the insertion of a foreign gene into bacterial plasmid or phage virus vectors includes the following steps:

1. Purification of the gene of interest (usually mRNA).
2. Production of double stranded copy (c)DNA from the mRNA template (Gene 25:263-269,1983) or the polymerase chain reaction (PCR).
3. Restriction endonuclease digestion of the vector DNA (plasmid or phage).

4. Insertion (ligation) of the cDNA into the vector.
5. Transformation (uptake of DNA into a bacterial cell) of competent (bacteria capable of taking in DNA) *Escherichia coli* cells or transfection (use of a phage virus to insert DNA into the bacterial host).
6. Identification of transformed bacteria containing cDNA inserts by insertional inactivation (color selection on artificial media).
7. Growth of the transformed bacteria in culture medium and purification of the recombinant DNA (mini preps).

SLIDE #7: CLONING

A schematic representation of the primary steps in cloning is presented. Starting with mRNA (top center of the slide), cDNA is synthesized by PCR or the Gubler and Hoffman method. The cDNA is either inserted into a plasmid (left side of the slide) or into a phage virus (right side of the slide) and transformed into *E. coli.*

POLYMERASE CHAIN REACTION (PCR)

SLIDE #8: POLYMERASE CHAIN REACTION (PCR)

The PCR amplifies very small quantities of DNA to detectable levels.

SLIDE #9: TWO INNOVATIONS LEAD TO THE AUTOMATION OF PCR

The discovery of a DNA polymerase (Taq polymerase) that is stable at very high temperatures and the development of computerized temperature blocks have automated PCR and made it practical. A stable DNA polymerase is needed because one of the steps in the PCR that is repeated over and over is heating to 95C. An automated temperature block eliminates the need to manually switch to different reaction temperatures during the PCR cycle.

SLIDE #10: PCR MIXTURE

T*aq* polymerase- the thermostable enzyme that synthesizes a new strand of DNA from a DNA template.

Nucleotides A, C, G, and T- the building blocks of DNA.

Primers- short single-stranded pieces of DNA that specifically anneal (hydrogen bond) to the template DNA and flank the region to be amplified. Primers are used by the T*aq* polymerase to initiate the synthesis of a new strand of DNA.

Reaction buffer (containing $MgCl_2$)- the buffer contains a tris salt and Mg^{++} which is needed for the activity of the T*aq* polymerase enzyme.

Template DNA- this is the DNA containing the region to be amplified.

SLIDE #11: THERMAL CYCLER

This thermocycler is a Perkin-Elmer Cetus 9600 (Norwalk, CT) and is based on a 96 well format.

SLIDE #12: THE PCR CYCLE

There are 3 steps in the PCR which are repeated 30 to 40 times. Those steps are:

1. Denaturation (95C)- separation of the two strands of the DNA template.
2. Primer annealing (30C to 65C)- primers specifically attach to the template flanking the region to be amplified.
3. Polymerization (72C)- polymerization occurs at the optimal temperature for the T*aq* polymerase enzyme, begins at each of the primers, and proceeds in a 5′ to 3′ direction.

SLIDE #13: PCR TECHNIQUE

A schematic representation of the PCR technique is presented. Generally, the DNA to be amplified is flanked by a pair of synthetic primers. The target DNA is denatured (the strands of DNA are separated) by heating, and the primers are allowed to anneal (hybridize) to the DNA by lowering the temperature of the sample. When the polymerization step is conducted, the sequence between the primers is copied, producing twice as much DNA as was originally present in the sample. By repeating the denaturation, hybridization, and polymerization steps many times, the target DNA is effectively amplified.

The utility of the PCR is still being discovered, but it has been used for cloning experiments, DNA probe production, DNA sequencing, and many other genetic engineering techniques requiring large quantities of DNA.

SLIDE #14: AGAROSE GEL OF PCR PRODUCTS

Agarose gel electrophoresis is used to separate DNA according to size (large pieces at the top and small pieces at the bottom). Following electrophoresis, the DNA is stained with ethidium bromide, which attaches to the DNA and fluoresces when exposed to UV light. In this agarose gel, PCR product is visualized in lanes 1 through

6. Molecular weight size markers are in the lane to the far right (lane 7). The hazy bands at the bottom of lanes 1-6 are excess primers always present at the end of the PCR.

NUCLEIC ACID PROBES

Nucleic acid probes are single-stranded segments of DNA or RNA which are labeled. The sequence or source of a probe is known so it can be used to identify unknown nucleic acids by complementary base pairing. The characteristic which makes probes different from any nucleic acid molecule is the presence of a label. Labels make probes easy to detect. Many types of labels and systems to detect them are available commercially.

SLIDE #15: TYPES OF NUCLEIC ACID PROBE LABELS

Types of labels:

Labels can be attached directly to the nucleic acid molecule. The methods used to attach labels are discussed in Slide #21.

Biotin, horseradish peroxidase, digoxigenin, and sulphonated cytosine have been used to label probes. Each of these labels, except for horseradish peroxidase, is detected using another molecule (usually a monoclonal antibody). The biotin molecule is attached to nucleic acid and a streptavidin-label conjugate is used to identify the biotin. This combination suffers from high background if endogenous biotin is present in the sample being tested. Digoxigenin and sulphonated cytosine are detected using a monoclonal antibody conjugated to alkaline phosphatase or fluorescein. Horseradish peroxidase is detected directly using chemiluminescence.

Types of detection strategies:

Radioactivity is detected using autoradiography: exposure to x-ray film. Radioactive labels which are most commonly used are ^{32}P, ^{33}P, and ^{35}S. Isotopes are usually included as part of a nucleotide triphosphate, for example 32P-dCTP, which is incorporated into the probe during nucleic acid synthesis. Although these labels are not practical for most diagnostic laboratories, they are still popular in research laboratories because they are easy to detect and rarely produce high background or false positive reactions.

Colorimetric detection requires enzymes (alkaline phosphatase) which cause the production of a color in the presence of the appropriate substrate, similar to an enzyme-linked immunosorbent assay (ELISA).

Fluorescence can be used to detect nucleic acids; however, detection of these molecules requires an ultraviolet light source.

Chemiluminescence is one of the newest non-radioactive detection strategies. When compounds such as horseradish peroxidase come in contact with their substrate, they produce a photon of light which can be detected using autoradiography or Polaroid film.

PROBE SYNTHESIS

Synthesis of a probe constitutes the incorporation of a label into a nucleic acid. The actual synthesis of a new nucleic acid molecule is necessary when direct conjugation of the label and nucleic acid is not possible or is impractical. There are several methods used to synthesize probes. Each method relies on the incorporation of a label directly into the nucleotide backbone of the DNA or RNA molecule.

SLIDE #16: PROBE SYNTHESIS: METHODS OF LABELING NUCLEIC ACID PROBES

The 4 most commonly used methods to produce nucleic acid probes are nick translation, oligonucleotide primed synthesis, kinase end labeling, and RNA polymerase transcription. Each of these methods is discussed in the next four slides.

SLIDE #17: PROBE SYNTHESIS: NICK TRANSLATION

The enzyme DNase I introduces random single-stranded nicks into the double-stranded DNA. DNA polymerase I provides 2 functions to synthesize the new probe. First, it finds the nicks and removes nucleotides using its 5′ to 3′ exonuclease activity. The new piece of DNA (probe) is then synthesized, beginning at the site of each nick, by DNA polymerase I. One of the deoxynucleotides (dNTP) used to synthesize the new DNA strand is labeled with either a radioactive or non-radioactive molecule.

SLIDE #18: PROBE SYNTHESIS: OLIGONUCLEOTIDE PRIMED SYNTHESIS

Double-stranded DNA must first be denatured to form a single-stranded template. Small single-stranded DNA fragments called oligonucleotides are added to the reaction and allowed to anneal to the single-stranded DNA template. This creates double-stranded regions randomly along the template which are recognized by the Klenow fragment of DNA polymerase I.

When DNA polymerase I is proteolytically cleaved, a fragment containing the 5′ to 3′ exonuclease activity is removed and the resulting Klenow enzyme only contains the 3′ to 5′ exonuclease activity and the polymerase activity. Removal of the 5′ to 3′ exonuclease activity prevents degradation of the oligonucleotides and insures that newly incorporated nucleotides are not removed.

Klenow DNA polymerase I synthesizes a new strand of DNA (probe) using labeled deoxynucleotides (dNTP). Each new strand will be extended in the 5′ direction from one oligonucleotide primer to the next. A polymerase which can be used to

replace Klenow is the modified T7 polymerase obtained from the bacteriophage T7. The modified T7 polymerase also lacks the 5′ to 3′ exonuclease activity and is marketed under the name Sequenase™.

SLIDE #19: PROBE SYNTHESIS: KINASE END LABELING

This method is used to label existing molecules of DNA or RNA. The enzyme alkaline phosphatase is used to remove the phosphate (P) from the 5′ end of a nucleic acid. This is called dephosphorylation, and the resulting molecule will have hydroxyl groups (OH) at the 5′ end. Polynucleotide kinase from the bacteriophage T4 can attach a radioactive phosphate to these hydroxyl groups. The molecule which donates the radioactive phosphate is usually adenosine triphosphate (ATP), with a radioactive phosphate in the gamma position. The resulting probe is radioactive and, although radioactivity is hazardous and impractical for most diagnostic laboratories, this is a very efficient reaction and results in a probe which is easy to identify.

SLIDE #20: PROBE SYNTHESIS: RNA POLYMERASE TRANSCRIPTION

Transcription is the process of making RNA from a DNA template. The enzyme responsible for this RNA synthesis is RNA polymerase. There are many different RNA polymerases. Each recognizes a different promoter (specific sequence of DNA). For example, the T7 RNA polymerase would recognize the T7 bacteriophage promoter but not the SP6 bacteriophage promoter. Only the SP6 polymerase will recognize the SP6 promoter. Insertion of a nucleic acid between the SP6 and T7 promoters allows transcription of either strand of DNA (remember: RNA transcription proceeds in a 5′ to 3′ direction only). The 2 strands are often referred to as (+) and (-).

The circular plasmid vector containing a cloned foreign DNA insert is cut with restriction enzymes to make it a linear molecule. The appropriate RNA polymerase is chosen and transcription is allowed to proceed until the polymerase falls off the end of the linear cloned DNA template. This is called run-off transcription and insures that only the cloned DNA sequence and no vector sequences are transcribed. Transcription of vector sequences into an RNA probe could cause high background or non-specific positive reactions in the subsequent hybridization assay.

During synthesis of the new RNA molecule, RNA polymerase uses ribonucleotides (rATP, rGTP, rCTP, rUTP) to build the RNA. One of these molecules (usually rUTP) is labeled with a radioactive or non-radioactive molecule.

SLIDE #21: PROBE SYNTHESIS: METHODS OF LABELING NUCLEIC ACID PROBES

Non-radioactive labels can be incorporated into probes during synthesis of a new molecule of nucleic acid. Alternatively, they can be attached directly to nucleic acids by a process called direct conjugation. The advantage of direct conjugation is that cloning and synthesis of a new nucleic acid molecule is not necessary. Existing

molecules can be labeled and used as probes. An example of this is kinase end labeling to produce a radioactive probe (Slide #19). Examples of direct conjugation using non-radioactive labels is described in this slide.

Photobiotin is an aryl azide derivative of biotin which can photochemically couple the biotin molecule to a nucleic acid in the presence of visible light. The covalently bound biotin can then be detected using a strepavidin-label conjugate (horseradish peroxidase is most often used as the label in this conjugate).

Photodigoxigenin is an aryl azide derivative of digoxigenin and can be used to directly conjugate the hapten digoxigenin to nucleic acid in the presence of visible light.

Horseradish peroxidase/polyethyleneimine can be used to directly label double- or single-stranded DNA molecules. Polyethyleneimine is a positively charged polymer which is covalently cross linked to horseradish peroxidase. The positively charged conjugate reacts with the negatively charged nucleic acid to create an electrostatic bond. This bond is converted to a covalent bond by adding glutaraldehyde. The horse-radish peroxidase/polyethyleneimine label is usually combined with a chemiluminescent detection system.

HYBRIDIZATION

SLIDE #22: HYBRIDIZATION ANALYSIS

Hybridization is a process during which a probe binds to a target nucleic acid molecule. In this case the target molecule is DNA, but it could be RNA. The sequence of bases in the probe will only hybridize to a complementary sequence in the target molecule. This is due to specific base pairing in which adenosine (A) always binds with thymidine (T) and cytosine (C) always binds with guanosine (G). Since each species has unique nucleotide sequences, hybridization can be used to distinguish them from all other species. In many cases, individual organisms can be distinguished using hybridization.

The double-stranded DNA molecule in this example must first be denatured into a single-stranded molecule. This is usually accomplished using heat. The bases in the single-stranded molecule are now available for the probe. Random collisions between the probe and DNA target eventually result in nucleation, which is the hydrogen bonding between a small number of bases. If nucleation is in a region where the probe and target DNA are complementary, the remaining bases can hydrogen bond to each other in a zipper-like effect which occurs very rapidly.

Hybridization can be conducted in solution or after immobilization of the target or probe to a solid support. Hybridization using immobilized nucleic acids is usually easier and more practical for diagnostic assays. The most common types of hybridization

assays using immobilized nucleic acids will be described in the slides which follow. Hybridization of nucleic acids in situ is a type of immobilized nucleic acid hybridization assay conducted on tissue sections but will not be described in this slide set.

SLIDE #23: HYBRIDIZATION ANALYSIS: IMMOBILIZED NUCLEIC ACIDS

The target single-stranded nucleic acid is attached to a solid support. This solid support is usually some type of nylon membrane. Nitrocellulose membranes have been used successfully, but they have been replaced by nylon for many applications because of the strength, resiliency, and versatility of nylon membranes. Immobilization of nucleic acids to nitrocellulose requires baking at 80C in a vacuum oven. Nucleic acids can be immobilized on nylon membranes using a similar procedure or by cross linking the nucleic acids to the filter using UV irradiation. Some nylon membranes can be given a positive or negative charge to their surface. Baking or UV irradiation is not necessary when using these types of membranes.

The probe can be added to the immobilized nucleic acids. If a complementary sequence is present, the probe will hybridize. Unbound probe can be washed away and the filter examined for the label carried on the probe.

Alternate procedures call for binding a capture nucleic acid (this is a probe without a label) to the solid support followed by hybridization of the target nucleic acid. The target nucleic acid is then detected using a probe complementary to a sequence which is different than that recognized by the capture nucleic acid.

SLIDE #24: HYBRIDIZATION ANALYSIS: IMMOBILIZED NUCLEIC ACIDS DOT OR SLOT BLOT, SOUTHERN BLOT, NORTHERN BLOT

Several types of hybridization assays have been developed. Three of the most popular are represented in this slide.

Dot blot or Slot blot hybridization employs a vacuum manifold to attach the target DNA or RNA to the solid support. The name of this hybridization comes from the shape of the wells in the manifold. Following transfer to a solid support, the target nucleic acid is detected by hybridization.

Southern blot hybridization was named after the scientist who first described this procedure. In this assay, DNA is cut with restriction enzymes to create many smaller fragments. The fragments are separated by electrophoresis, denatured to single strands while they are in the electrophoresis gel, and then transferred to a solid support before hybridization.

Northern blot hybridization is similar to Southern blot hybridization, except that RNA is the target molecule. The RNA molecules do not usually need to be cut into smaller fragments because commonly mRNA is used. Each mRNA is relatively small compared to genomic DNA used in the Southern blot assay, which can be quite large.

An RNA molecule usually represents only a single gene or, in the case of some viruses, a small number of genes. In the Northern blot assay, RNA molecules are separated by electrophoresis and transferred to a solid support. These molecules are usually single-stranded, but since they contain many double-stranded regions (due to secondary structure), they must be separated in a denaturing electrophoresis gel. Following electrophoresis, the RNA is transferred to a solid support and hybridized with a probe.

SLIDE #25: SAMPLE PROCESSING

The first step in conducting any of these hybridization assays is sample processing. All samples must be processed to expose the DNA or RNA for hybridization. In the case of bacteria and viruses, the cell walls and capsids, respectively, must be removed. This can be accomplished using a variety of proteolytic enzymes and organic chemicals (phenol and chloroform). Protein and lipid molecules, which contaminate many samples, can cause background and may need to be removed prior to conducting a hybridization assay. Various products designed to "clean up" a sample of nucleic acid are available commercially.

SLIDE #26: DOT BLOT VACUUM MANIFOLD

This is an example of a dot blot vacuum manifold used to transfer RNA or DNA to a solid support. Notice that the holes in this unit are round (dot shaped) and in a 96-well format. Sandwiched between the layers of plexiglas is a support membrane. A vacuum is pulled from the bottom, which pulls the aqueous sample through the membrane. Large molecules (DNA and RNA) cannot pass through and collect on top of the membrane. Once all the samples are added to the manifold, the unit is disassembled and the membrane is removed. Following attachment of the nucleic acids to the membrane using heat or UV irradiation, hybridization can be conducted.

SLIDE #27: DOT BLOT HYBRIDIZATION OF IBDV EXTRACTED FROM TISSUES

This is an example of the results obtained following a dot blot hybridization assay. In panel A, samples 1 through 6 are positive; samples 7 and 8 are negative. In panel B, samples 1 through 5 are positive and samples 7 and 8 are negative. Three 10-fold dilutions of each sample were prepared and applied to the membrane (the highest concentration is on the left). The dot shape results from using the dot blot vacuum manifold to apply the samples to this membrane.

SLIDE #28: AGAROSE GEL ELECTROPHORESIS OF NUCLEIC ACID

Prior to the transfer of nucleic acid to a solid support, electrophoresis must be used to separate the molecules in the Southern and Northern blot assays. This is an example of DNA separated on a 1% agarose gel following restriction endonuclease digestion. The DNA bands are visualized using ethidium bromide, which binds DNA and fluoresces under a UV transilluminator.

SLIDE #29: TRANSFER OF SEPARATED NUCLEIC ACIDS TO A SOLID SUPPORT

Following electrophoresis, the separated fragments of nucleic acid must be transferred to a solid support. This slide demonstrates one method for accomplishing this transfer. A membrane is cut to the exact size of the electrophoresis gel and applied so it is in direct contact with the gel surface. A buffer chamber on the bottom of the unit contains a high salt (20X SSC) buffer which is wicked to the bottom of the gel via filter paper. The buffer travels vertically up through the gel and membrane because it is being pulled by capillary action into the sponges on top of the unit. This movement of buffer through the gel and across the membrane causes the nucleic acid molecules in the gel to be transferred to the membrane surface which is in contact with the gel. The entire capillary blot transfer process can take from 1 to 48 hours, depending on the concentration of the gel and the size of the nucleic acid fragments. Since this method can be a slow--albeit inexpensive and easy--process, other blotting methods have been developed which employ an electrical current to transfer the nucleic acid.

There are several products on the market which employ an electrical current to transfer the fragments horizontally out of the gel and onto a membrane. The membrane is cut to the shape of the gel and applied so it is in direct contact with the gel surface. An electrical field is applied across the gel which drives the nucleic acid against the membrane. This procedure is relatively fast, compared with capillary blotting, and is more suitable for some Southern and Northern blot assays.

SLIDE #30: NORTHERN BLOT HYBRIDIZATION

This slide contains the results of a Northern blot hybridization. The RNA bands (labeled "A" and "B") can be observed in the lanes marked BB in panel 1; lane C is the control. In panel 2, only RNA "B" is observed because the probe used in this panel was specific for RNA "B" and did not contain complementary sequences for RNA "A".

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