Unit 8

PROBE DESIGN AND SYNTHESIS

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8.1 INTRODUCTION

In Unit 8, we shall learn about probe, an important tool of recombinant DNA technology. As the name suggests it is related to searching for the answers. Probe is used to detect the presence of specific sequences of nucleotides in a sample. In this Unit, we shall discuss about two types of probes; oligonucleotide and gene probe. On what basis these are designed and used for detection will be described. Methods used for their synthesis and purification will be elaborated. These probes have vast number of applications in the area of recombinant DNA technology which will also be discussed.

Expected Learning Outcomes

After studying this unit, you should be able to:

- explain what probe is;
- differentiate between oligonucleotide and gene probe;
- understand different criteria to design a good probe;
- write about methods used for probe synthesis; and
- discuss applications of probes in cloning, library screening and mutagenesis.

In genetic engineering, a nucleic acid probe is a specially designed segment of single stranded nucleic acid (DNA or RNA) that is used to detect the presence of specific sequences of nucleotides in a sample (referred as target). The sequences of probe and target are complementary and therefore, have high affinity for each other. The probe-target complex is revealed by different labelling and detection techniques. Depending on the conditions, sometimes they may not be exactly complementary to each other. Probes are synthetic and can be of two types: oligonucleotide probe and gene probe.

8.2 SYNTHETIC OLIGONUCLEOTIDES

Synthetic oligonucleotide probes are complementary to specific region in the gene. Let us look at some of the important features which must be kept in mind while designing these probes. You may find these features to be quite similar to those required for designing a primers for PCR.

Probe Length: Oligonucleotide probes should be between 18 and 50 bases long. With current synthesizers, it is possible to make oligonucleotides as long as 100bp, however, longer probes may require extended hybridization times and yield lower synthesis results. Shorter probes may lack specificity.

Base Composition: The ideal G-C content should be between 40% and 60%. Lower GC content may result in increased nonspecific hybridization.

Complementary Regions: Care should be taken so that there are no complementary regions within the probe to prevent the formation of "hairpin" structures, which can hinder hybridization to the target.

Repetitive Bases: Long stretches (more than four) of a single nucleotide in an oligonucletide must be avoided to enhance specificity.

After identifying a suitable sequence, computerized sequence analysis should be conducted. The probe should be compared against the target region as well as its reverse complement. Probes that have more than 70% homology or eight or more consecutive bases matching with non-target regions should not be used

It is important that optimal hybridization conditions for the synthesized probe should be tested against specific and nonspecific target nucleic acids under varying conditions.

The phosphoramidite method of oligonucleotide synthesis was introduced by McBride and Caruthers in1983.

8.2.1 Synthesis of Oligonucleotides

Solid support method has been the mainstay method for the oligonucleotide synthesis for last more than 40 years. It is a chemical process by which nucleotides are specifically linked on a solid support to form a desired sequence product. The phosphoramidite method is the standard synthetic method used in most automated synthesizers today. The advantage of this method is its high efficiency for the synthesis of long oligonucleotides with less failure sequences.

Activated DNA nucleosides (phosphoramidites) act as the building blocks and are commonly referred to as “monomers”. The dimethoxytrityl (DMT) group is used to protect the 5’-end of the nucleoside, while a β-cyanoethyl group protects the 3’-phosphate moiety. Many additional groups may be included

that serve to protect reactive primary amines in the heterocyclic nucleoside bases. The protective groups are selected to prevent branching or other undesirable side reactions during synthesis (Fig. 8.1). Oligonucleotides are synthesized on solid supports. Typically, the support is a small column filled with control pore glass (CPG), polystyrene or a membrane. More recently polystyrene with a UnyLinker molecule is attached. The synthesis is computer-controlled and fully automated, and the reagents are flowed through the column sequentially to perform the reactions on the solid support. The oligonucleotide is usually synthesized from the 3' to the 5'

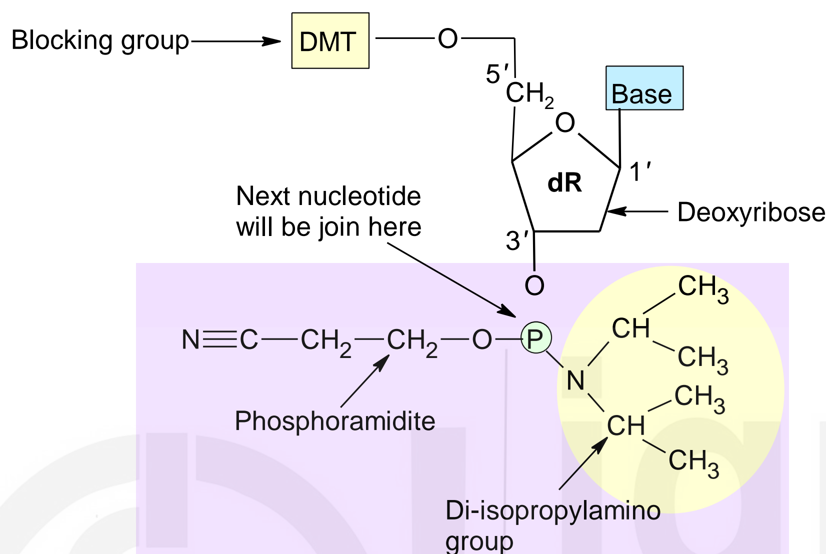

Fig. 8.1: Use of DMT in oligonulceotide synthesis.

Synthesis of oligonucleotide takes place in cycles of four steps with each step followed by a solvent wash (Fig. 8.2). Each cycle adds one nucleotide. As mentioned earlier, the synthesis begins with a solid support resin, on which a nucleoside with its 5′-hydroxyl group protected is preloaded

(1) Detritylation: The dimethoxytrityl (DMT) protecting group on the 5′-hydroxyl group is removed with a solution of an organic acid, typically dichloroacetic acid (DCA) in toluene. This step makes the hydroxyl group available for the next nucleotide coupling.

(2) Coupling: The next nucleoside phosphoramidite in the sequence is activated with an acidic activator and coupled with the 5′ hydroxyl group of the growing oligonucleotide chain.

(3) Oxidation/Sulfurization: Oxygen or sulfur is added to the phosphorus atom using iodine or a sulfurizing reagent to give either a phosphodiester or a phosphorothioate group.

(4) Capping: Unreacted 5′ hydroxyl groups are capped with acetic anhydride to prevent further chain extension of coupling failures and minimize N-1 impurities.

The synthesis cycle is carried out for each nucleotide in the sequence until the desired number of bases is added. Oligonucleotides from length 20 to 120 bases can be synthesized. Each reaction in the cycle is quick, with a single cycle taking around 45 minutes and the full synthesis of a 20-mer completed in under 24 hours. The final step involves removing the cyanoethyl protecting

groups from the phosphodiester/phosphorothioate backbone by rinsing with an amine solution.

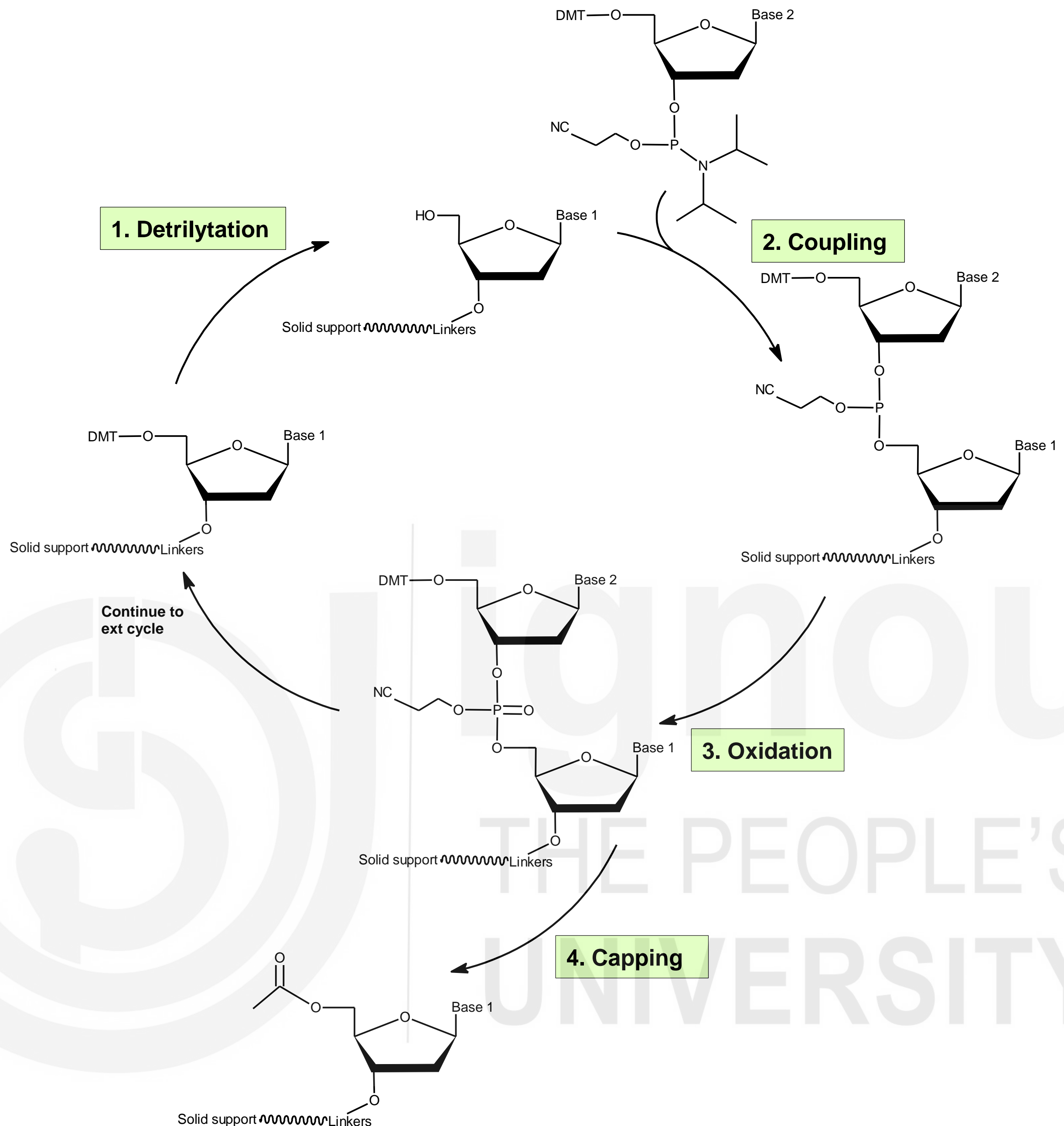

Fig. 8.2: Steps involved in Oligonucleotide synthesis.

Cleavage from solid support: Complete oligonucleotide is cleaved from the solid support, often using a cleavage reagent (aqueous ammonium hydroxide) that does not damage the oligonucleotide. For RNA-containing oligonucleotides, the 2′-O-silyl protecting groups are cleaved using fluoride. Cleavage is necessary so that the free 3'-OH may take part in biochemical reactions.Ester hydrolysis of the linker (and, simultaneous removal of the solid support) is carried out by treatment with concentrated aqueous ammonium hydroxide. The product is the oligonucleotide with a terminal, free 3'-OH (Fig. 8.3).

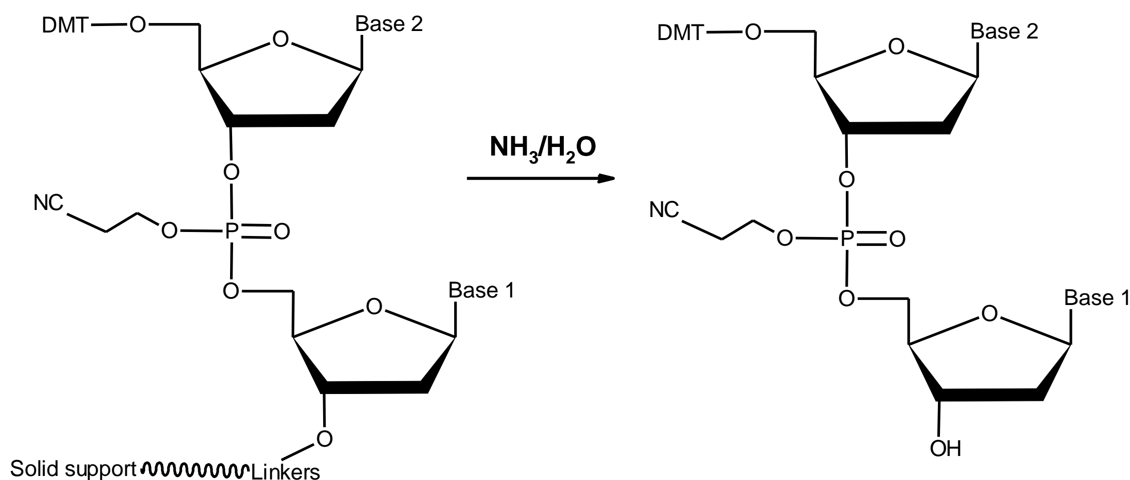

Fig. 8.3: Cleavage from solid support.

DEPROTECTION: After cleavage, the solution of oligonucleotide in concentrated aqueous ammonia is heated to remove protecting groups from the bases and phosphates. The protecting groups must be removed so that proper hydrogen bonds between the oligonucleotide and the target nucleic acid may form. While thymine does not require a protecting group, adenine, cytosine, and guanine do, since they contain exocyclic primary amino groups.

Once cleaved from the solid support, the crude oligonucleotide can be analyzed for concentration, identity, and purity. The synthesized oligonucleotide is purified and characterized.

8.2.2 Purification of Synthetic Oligonucleotides

It is essential to purify synthetic oligonucleotides to ensure their high quality and specificity for downstream applications. Many methods are used which are briefly discussed here.

1. Polyacrylamide Gel Electrophoresis (PAGE): Oligonucleotides are separated based on size using a polyacrylamide gel. After electrophoresis, the desired band is excised and the oligonucleotide is extracted. High resolution and effective separation of oligonucleotides of similar lengths are the advantages of this method.

2. High-Performance Liquid Chromatography (HPLC): Oligonucleotides are separated based on their chemical properties (size, charge) using a liquid chromatography system. Common types include reverse-phase and anion-exchange HPLC. The method is preferred due to its high efficiency and large-scale purification.

3. Reverse-Phase High-Performance Liquid Chromatography (RP-HPLC): This technique separates oligonucleotides based on hydrophobic interactions. Oligonucleotides are eluted from a hydrophobic column using a gradient of solvents. The method is very effective for removing contaminants and achieving high purity.

4. Anion-Exchange Chromatography: Oligonucleotides are separated based on their negative charge. This method exploits differences in charge to

separate oligonucleotides from impurities. It is particularly useful for separating phosphorylated and non-phosphorylated oligonucleotides.

5. Precipitation: Ethanol or isopropanol can be used to precipitate oligonucleotides from solution. After precipitation, the oligonucleotide is collected by centrifugation and washed. It is a very simple and cost-effective method, though may not achieve the same level of purity as other methods.

6. Affinity Purification: This technique uses specific interactions (like biotin-streptavidin) to selectively isolate oligonucleotides from a mixture. The method is highly specific and effective for particular sequences.

With availability of so many methods, the choice of purification method often depends on the intended application, with higher purity needed for sensitive applications like PCR or sequencing. Some methods may result in lower yields than others, so it’s essential to balance purity and yield based on experimental needs.

Purified oligonucleotides are then characterized using methods like mass spectrometry or capillary electrophoresis to confirm their identity and purity.

8.2.3 Detection of the Probe

The synthetic oligonucleotides are labeled to ease their detection. Labels may by radioactive or non radioactive such as enzymatic or fluorescent markers.

Radioactive Labels are added using radioactive isotopes (e.g., 32P, 35S, 125I, 3H) to the oligonucletide probes which are then detected by autoradiography or Geiger–Muller counters. Radiolabeled probes used to be the most common type because of their highest degree of sensitivity as well as resolution in the currently used hybridization assays discussed in the following section. However, concern over the safety considerations, cost and disposal of radioactive waste products resulted in decline in their use. We shall now briefly discuss about the features, advantages and disadvantages various radiolabels commonly used.

Radiolabel	Advantages	Disadvantages
P^{32}	1. Highest sensitivity: It can detect single-copy genes in only 0.5 µg of DNA because of highest specific activity and emission of high energy β-particles. 2. Its structure is essentially similar to that of the nonradioactive counterpart, therefore, its labelling does not inhibit the activity of DNA-modifying enzymes	Due to high penetration and their relatively short half-life (14.3 d), P^{32} cannot be used for high-resolution imaging and is dangerous to the user.

S^{35}	1. The lower energy and longer half-life (87.4 d) of S^{35} make this radioisotope more useful than 32P for the preparation of more stable, less specific probes. 2. S^{35}-labeled probes, although less sensitive, provide higher resolution in autoradiography and are especially suit able for in situ hybridization procedures. 3. Another advantage of is that S^{35} presents little external hazard to the user as the low-energy β-particles barely penetrate the upper dead layer of skin and are easily contained by laboratory tubes and vials	
H^3	1. H^3-labeled probes have traditionally been used for in situ hybridization because the low-energy β-particle emissions result in maximum resolution with low background. 2. It has the longest half-life (12.3 yr)	

Non radioactive labels: Radioactive labels pose concern about the laboratory safety and the economic and environmental aspects of radioactive waste disposal resulting in decline of their use and development of non radioactive alternatives. Non-radioactive labeling of oligonucleotides is a versatile and safer alternative to radiolabeling, enabling researchers to detect and quantify nucleic acids in various applications without the hazards associated with radioactive materials.

Their advantages include safety, higher stability of probe, and higher efficiency of the labeling reaction, ability to be detected *in situ and* less time taken to detect the signal. Some of the frequently used methods are discussed here.

Biotin Labeling:The method takes advantage of the high affinity of avidin or streptavidin for biotin. The probe is biotin linked which interacts with reporter enzyme (like alkaline phosphatase or horseradish peroxidase) is linked through avidin (Fig. 8.7). It is an indirect method of detection since the reporter enzyme is linked to probe through streptavidin–biotin bridge, however, it works well in most cases.

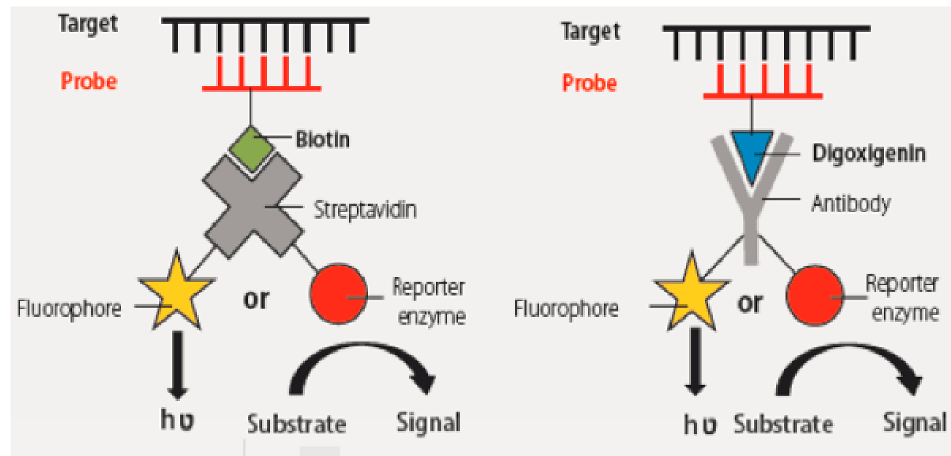

Fig. 8.7: Biotin labeling of the probe and its detection.

A limitation of this method is occurance of high level of background noise due to the ubiquitous presence of biotin (Vitamin H) in mammalian tissues and tendency of biotinylated probe to stick to nylon membranes. This can be addressed by using inucleotide derivatives such as digoxigenen-11-UTP, -11-dUTP, and -11-ddUTP, and biotin-11-dUTP or biotin-14-dATP.

After hybridization, these are detected by an antibody or avidin, respectively, followed by a color or chemiluminescent reaction catalysed by alkaline phosphatase or peroxidase linked to the antibody or avidin. The enzyme reacts with substrates, leading to a color change (colorimetric) or light emission (chemiluminescence) (Fig. 8.7). For HRP, chloronaphthol produces a purple insoluble product.

Enzymes: In this method, an enzyme alkaline phosphatase and horseradish peroxidase (HRP) is directly attached to the probe and its presence is usually detected by reaction with a substrate that changes color. That is why enzyme is sometimes referred to as a “reporter group,” HRP in the presence of peroxide and peroxidase converts chloronaphtol to a purple insoluble product.

Chemiluminescence: In this method, chemiluminescent chemicals attached to the probe are detected by their light emission using a luminometer. Chemiluminescent probes (including the above enzyme labels) can be easily stripped from membranes, allowing the membranes to be reprobed many times without significant loss of resolution.

Fluorescence chemicals: These chemicals when attached to probe fluoresce under ultraviolet (UV) light. This type of label is especially useful for the direct examination of microbiological or cytological specimens under the microscope—a technique known as fluorescent in situ hybridization (FISH).

Antibodies: An antigenic group is coupled to the probe and its presence is detected using specific antibodies. Also, monoclonal antibodies have been developed that will recognize DNA–RNA hybrids. The antibodies themselves need to be labeled, using an enzyme.

DIG system: It is the most comprehensive, convenient, and effective system for labeling and detection of DNA, RNA, and oligonucleotides. Digoxigenin (DIG), like biotin, can be chemically coupled to linkers, and nucleotides and DIG-substituted nucleotides can be incorporated into nucleic acid probes by any of the standard enzymatic methods. These probes generally yield significantly lower backgrounds than those labeled with biotin. An anti-digoxigenin antibody–alkaline phosphatase conjugate is allowed to bind to the hybridized DIG-labeled probe. The signal is then detected with colorimetric or chemiluminescent alkaline phosphatase substrates. If a colorimetric substrate is used, the signal develops directly on the membrane. The signal is detected on an X-ray film (as with ^{32}P- or ^{35}S-labeled probes) when a chemiluminescent substrate is used.

SAQ 1

a) Name the methods used for chemical synthesis of oligonucleotide probe.

b) Which four steps are repeated in the automated synthesis of oligonucleotide?

c) Which method of purification separates oligonucleotides based on;

i) specific affinity; ii) negative charge and iii) hydrophobic interactions

d) Which radioactive label has the highest sensitivity and specificity?

e) Name two non radioactive labels used for oligonucleotide probes.

8.3 APPLICATIONS OF OLIGONUCLEOTIDE PROBES

Probes play a crucial role in cloning by allowing researchers to identify and isolate specific DNA sequences. Key to their success is hybridization of probe to the target sequence which is like finding needle in the haystack. The properties which contribute to the versatile applications of nucleic acid probes include their complementarity, sequence specificity as well as relative chemical stability, ease of manipulation and labeling. Here are some applications of probes. You have already learnt about some of these while others will be discussed in the next upcoming units.

1. **Gene Identification**: Probes can be designed to bind specific genes of interest, helping to locate them within a genomic library.

2. **Screening Recombinant Clones**: After cloning, probes are used to screen libraries for clones that contain the desired DNA insert by hybridization techniques.

3. **Verification of Inserts**: Probes can confirm the presence and integrity of inserted DNA in plasmids or other vectors.

4. **Expression Analysis**: In expression cloning, probes help assess the expression levels of cloned genes in various conditions or tissues.

5. **Mapping Genomes**: Probes are utilized in physical mapping of genomes, facilitating the identification of the locations of genes and genetic markers.

6. **Detecting Mutations**: Changes in certain gene sequences can cause inherited diseases such as cystic fibrosis, muscular dystrophies, phenylketonuria, apolipoprotein variants, and sickle cell anemia. . Nucleic acid probes have successfully been used to detect those mutations.

7. **Site directed mutagenesis**: Synthetic oligonucleotides find use in introduction of one or more mutations at specific sites in DNA. The oligonucleotide with more than one mismatched base or desired mutation is annealed to the target DNA resulting in hybridization. This oligonucleotide acts as a primer for the synthesis of DNA strands. By this method multiple insertions and deletions, substitutions can be brought about in the DNA strand. Such site directed mutations provide invaluable information about protein structure and functions.

8. **Detection of pathogenic microorganisms**: Nucleic acid probes have been increasingly used for isolation and detection of pathogenic or unculturable microorganismin the environment. Through the development of DNA–DNA and RNA–DNA hybridization procedures and recombinant DNA methodology, the isolation of species-specific gene sequences is readily achieved Oligonucleotide hybridization probes complementing either small ribosomal subunits, large ribo somal subunits, or internal transcribed spacer regions have now been developed for a wide variety of microorganisms Detection of a nucleic acid sequence unique to a particular microorganism would demonstrate its presence in a specimen and, perhaps, confirm an infectious disease.

9. **DNA fingerprinting**: Tandem repeat sequences are usually 30–50 bp in length. Their size and distribution are distinctive for an individual. They can be detected using nucleic acid probes and PCR. They are the basis of so-called "DNA fingerprinting," which can be used in forensic science to confirm the identity of a suspect from specimens (any body fluid, skin, and hair) left at the scene of a crime. This technique can also be used for paternity tests, sibling confirmation, and tissue typing.

10. **Novel uses of oligonucleotides:** Recently, oligonucleotides are being used as aptamers and antisense oligonucleotides. **Aptamers** are the single-stranded oligonucleotides (DNA or RNA) that can bind to specific target molecules with high affinity and specificity. They are often referred to as "chemical antibodies" because they can perform similar functions to antibodies, such as recognizing and binding to proteins, small molecules, or even cells.

These molecules are easy to synthesize and modify chemically, cost effective with high specificity and reproducibility as well as show lower immunogenicity than antibodies. Due to these advantages of aptamers over antibodies, they are being widely used as drugs and drug delivery systems, for identifying molecular markers of disease, and as molecular switches for various purposes, including in some biosensors

Antisense oligonucleotides (ASOs) are short, synthetic strands of nucleic acids designed to bind to specific messenger RNA (mRNA) molecules. By hybridizing with their target mRNA, ASOs can modulate gene expression, providing a valuable tool for therapeutic and research applications. ASO are invaluable as they are easier to synthesize and modify chemically than traditional antibodies or small drug molecules. Moreover, their high specificity to bind and modulate the expression of desired gene reduces side effects. However, they are still being explored and improved because of challenges of effective delivery to target tissues and cells and possibility of inducing immune responses limiting their therapeutic applications.

8.4 SYNTHETIC GENE ASSEMBLY

Gene probes are single stranded strands which are generally longer than 500 bases and comprise all or most of a target gene. They can be generated in two ways.

1. Cloning: You have already read about this process in the previous blocks. This method is used when a specific clone is available or when the DNA sequence is unknown and it must be cloned first in order to be mapped and sequenced. Cloned gene probe is then cut with restriction enzymes and excised from an agarose gel. This step is not needed if the if the vector used for cloning has no homology.

2. Polymerase chain reaction (PCR) is a powerful method for making gene probes. It allows the amplification and labeling of the gene at the same time.

Complete sequence of a gene can easily be obtained from databases (GenBank, EMBL, DDBJ) and primers can be designed to amplify the whole gene or gene fragments (see Unit 7). Amplification of a gene by PCR is highly economical as it saves time. Lots of steps such as restriction enzyme digestion, electrophoresis, and elution of DNA fragments from vectors can ce avoided. Gene probes generally provide greater specificity than oligonucleotides because of their longer sequence and because more detectable groups per probe molecule can be incorporated into them than into oligonucleotide probes.

SAQ 2

a) What is the difference between oligonucleotide and gene probe?

b) What are aptamers and their use?

c) Write full form of ASO and its application.

8.5 SUMMARY

- A nucleic acid probe is a specially designed segment of single stranded nucleic acid (DNA or RNA) that is used to detect the presence of specific sequences of nucleotides in a sample. It can be an oligonucleotide probe or gene probe.

- Essential feature of a good oligonucletide probe are length between 18-50 bp, 40% and 60%. G-C content, absence of internal complementary regions and repetitive bases.

- Synthesis of oligonucleotides is done using automated synthesizers which is based on the phosphoramidite method. The advantage of this method is its high efficiency for the synthesis of long oligonucleotides with less failure sequences.

- Synthesis of oligonucleotide takes place in cycles of four steps with each step followed by a solvent wash. Each cycle adds one nucleotide. Tour steps are detritylation, coupling, oxidation/Sulfurizationand capping.The synthesis cycle is carried out for each nucleotide in the sequence until the desired number of bases is added.

- Following synthesis, the oligonucleotide is cleaved from the solid support, often using a cleavage reagent (aqueous ammonium hydroxide) that does not damage the oligonucleotide and is heated to remove protecting groups from the bases and phosphates.

- Synthesis is followed by purification using chromatographic and electrophoresis methods.Choice of the method depends on the intended application, with higher purity needed for sensitive applications.

- The synthetic oligonucleotides are labeled to ease their detection. Labels may be radioactive or non radioactive such as enzymatic or fluorescent markers.

- The nucleic acid probes have immense applications. These are Gene Identification, Screening Recombinant Clones, Verification of Inserts, Expression Analysis, Mapping Genomes, Detecting Mutations, Site directed mutagenesis, Detection of pathogenic microorganisms and DNA fingerprinting.

- Recently these have been used as aptamers and antisense oligonucleotides. While aptamers are being widely used as drugs and drug delivery systems, for identifying molecular markers of disease, and as molecular switches for various purposes, including in some biosensors. Antisense oligonucleotides (ASOs) can modulate gene expression, providing a valuable tool for therapeutic and research applications.

8.6 TERMINAL QUESTIONS

1. What are the desired features of a good nucleic acid probe?

2. Explain the applications of nucleic acid probes in detection of inherited diseases and pathogenic microorganism.

3. Describe the method of oligonucleotide synthesis.

4. Discuss about the radioactive labels used for detection of nucleic acid probes.

5. What are the advantages of non radioactive labels over the radioactive labels? Name different types of non radioactive labels used to detect nucleic acid probes.

8.7 ANSWERS

Self-Assessment Questions

1. a) Solid support phosphoramidite method

   b) Detritylation, coupling, oxidation/Sulfurizationand capping

   c) i) Affinity chromatography; ii) Anion exchange chromatography
   iii) Reverse-Phase High-Performance Liquid Chromatography

   d) P^{32}

   e) Biotin and Antobodies

2. a) Oligonucleotide is shorter than gene probe. Oligonucleotide probe is complementary to a part of gene with length between ranging 18-50 bp while gene probe is generally longer than 500 bases and comprise all or most of a target gene. Specificity of gene probe is more than the oligonucleotide probe.

   b) Aptamers are the chemically synthesized single-stranded oligonucleotides (DNA or RNA) that can bind to specific target molecules with high affinity and specificity. They are often referred to as "chemical antibodies" because they can perform similar functions to antibodies, such as recognizing and binding to proteins, small molecules, or even cells. They are being widely used as drugs and drug delivery systems, for identifying molecular markers of disease, and as molecular switches for various purposes, including in some biosensors

   c) Antisense oligonucleotides (ASOs) are short, synthetic strands of nucleic acids designed to bind to specific messenger RNA (mRNA) molecules. By hybridizing with their target mRNA, ASOs can modulate gene expression, providing a valuable tool for therapeutic and research applications. ASO are invaluable as they

are easier to synthesize and modify chemically than traditional antibodies or small drug molecules. Moreover, their high specificity to bind and modulate the expression of desired gene reduces side effects.

Terminal Questions

1. Please refer to the section 8.2.
2. Oligonucleotide hybridization probes complementing either small and large ribosomal subunits, or internal transcribed spacer regions have been developed for a wide variety of microorganisms. Detection of a nucleic acid sequence unique to a particular microorganism would demonstrate its presence in a specimen and, perhaps, confirm an infectious disease.
3. Synthesis of oligonucleotides is done using automated synthesizers which is based on the phosphoramidite method. Please refer to section 8.2.1 for more details.
4. Radioactive labels such as 32P, 35S, 125I, 3H are added to the oligonucleotide probes which are then detected by autoradiography or Geiger–Muller counters. For more detaqils, please refer to the section 8.2.3.
5. Non radioactive labels are are hazardous to human health and safe to use. Biotin, enzymes, chemiluminescence, DIG are some of the non-radioactive labels used to detect nucleic acid probes.