COURSE MSc (BIOTECHNOLOGY) III SEM

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HYBRIDIZATION PROBE

- In molecular biology, a **hybridization probe** is a fragment of DNA or RNA of variable length (usually 100–10000 bases long) which is chemically labelled or tagged.
- This chemical tag could be a fluorescent molecule or it can be an attachment to a colored bead, or photochromic compounds, isotopic labelling, non-isotopic labelling (DIG) etc. It allows us to visualize when a probe attaches to DNA, RNA or other target nucleic acids.
- It can then be used in DNA or RNA samples to detect the presence of nucleotide substances (the RNA target) that are complementary to the sequence in the probe.
- The probe thereby hybridizes to single-stranded nucleic acid (DNA or RNA) whose base sequence allows probe–target base pairing due to complementarity between the probe and target.

DNA probes:

- Conventional DNA probes are isolated by cell-based DNA cloning or by PCR. range from 0.1 kb to a range of ~10kb in length and is often double-stranded.
- These DNA probes are labeled with radioactive nucleotides or with modified nucleotides that can be detected by fluorescence or chemiluminescence, during in vitro synthesis.

RNA probes:

- Detection of specific nucleic acid sequences can be accomplished by hybridization with a labeled RNA probe.
- RNA probes are sequences of a variable length that are used to detect the presence of complementary nucleotide sequences in a sample.
- RNA probes are labeled with radioactive nucleotides or with modified nucleotides that can be detected by fluorescence or chemiluminescence.
- They can be used for Northern blotting, RNase protection assays, Southern blotting, downstream of polymerase chain reaction (PCR), and *in situ* hybridization analysis.

Synthetic probes:

- These probes are chemically synthesized which can generate short single stranded stretches 15–50 bases in length. They are usually highly specific as they are designed particularly for target DNA.
- **Degenerate Probes**: with degenerate sequences can also be synthesized using protein sequences. Degeneracy is introduced when parallel synthesis of oligonucleotides is done with similarity at some sites and difference at others. These probes are usually labeled using ^{32}P isotope or other labeled group at the 5′ end.
- **LABELING OF PROBES:** Nucleotides can be labeled by **isotopic and non-isotopic** methods.
- Probes can be labeled at specific location within the oligonucleotides or internally at multiple sites. Some probes are of defined length and some are heterogeneous populations of labeled molecules.
- Oligonucleotides can be labeled at either the 3' or the 5' end. Using polynucleotide kinase and ATP-gamma-^{32}P, the 5' end is labeled. Using terminal transferase and deoxynucleotide triphosphate labeled on the alpha phosphate, the 3' end is labeled. Traditionally, the isotope of choice has been ^{32}P, however ^{35}S and ^{33}P have been used successfully. Use of ^{35}S and ^{33}P is especially useful when high resolution (as in *in situ* hybridization) or when long probe stability is needed.

Isotopic labeling:

- Isotopes generally used for labeling nucleotides are ^{32}P, ^{33}P, ^{35}S or ^{3}H. They can be detected directly in solution or on X-ray film using autoradiography.
- Properties of radioisotopes used for labeling DNA and RNA probes:

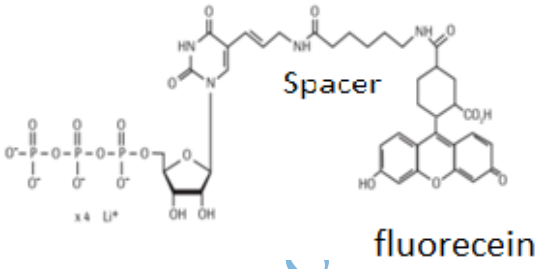
Radioisotope	Half-life	Energy ofemission
3H	12.4 years	0.019 MeV
32P	14.3 years	1.710 MeV
33P	25.5 years	0.248 MeV
35S	87.4 years	0.167 MeV

NON-ISOTOPIC LABELING:

- Non-isotopic labeling systems involve the use of nonradioactive chemicals.
- These methods are developed recently as compared to radioisotope labeling methods, but are finding wide variety of applications in different ways.
- Two types of non-radioactive labeling are conducted: direct and indirect.

Direct non-isotopic labelling

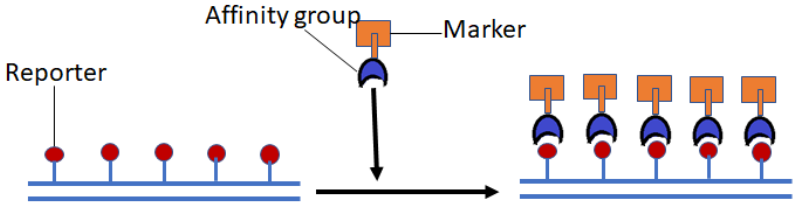
- where a nucleotide containing label such as Fluorescein, Rhodamine that will be detected when incorporated with the help of spacer molecule.
- These modified nucleotides having fluorophore tag, fluoresce when excited by light of certain wavelength.



An example of fluorescein conjugated dUTP

Indirect non-isotopic labeling

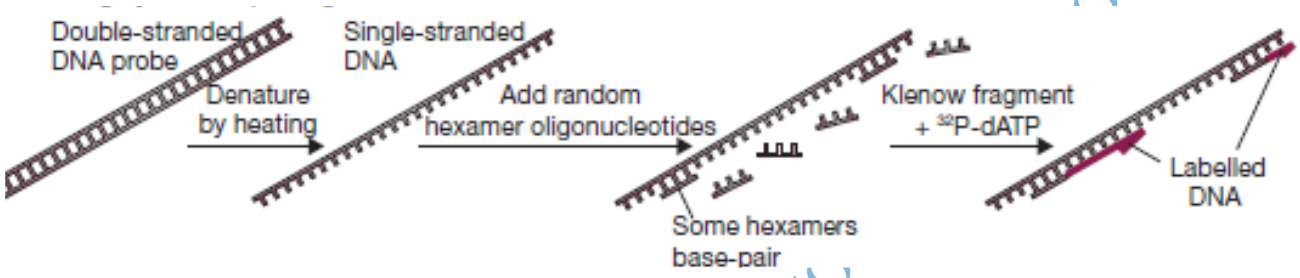
- It involves chemical linkage of reporter molecule to a nucleotide.
- When this modified nucleotide is incorporated into DNA, then it is specifically bound to a protein or other ligand which has high affinity against the reporter group.
- Long spacer is introduced between nucleotide and reporter so as to reduce steric hindrances for binding of affinity molecule.
- Example: Biotin-Streptavidin Method



Random Primer method

- The basic technique for primer extension labelling was first introduced by Feinberg and Vogelstein, and used a mixture of hexanucleotides to prime DNA synthesis randomly on single stranded DNA.
- DNA synthesis can be accomplished by either DNA polymerase I, or the Klenow fragment
- use of Klenow fragment in primer extension avoids the loss of incorporated label.

Labelling by random priming

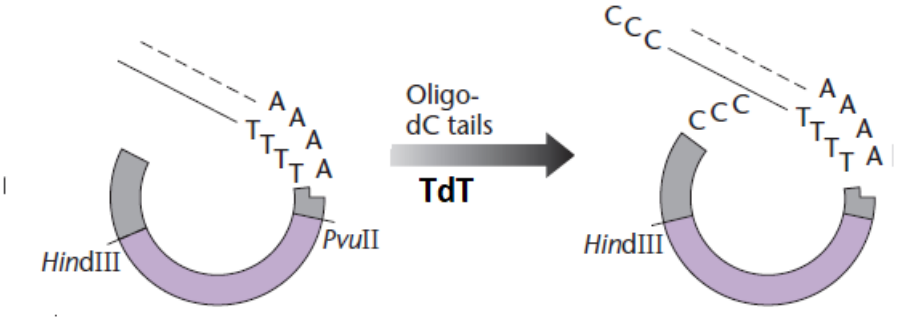


Nick Translation

- In nick translation, the DNA is treated with DNase to produce single-stranded "nicks." DNA polymerase I is then used to replace the nicked sites, elongating the 3' hydroxyl terminus, removing nucleotides by 5'-3' exonuclease activity, and replacing with dNTPs.
- To radioactively label a DNA fragment for use as a probe, one of the incorporated nucleotides provided in the reaction should be radiolabeled on the *alpha* phosphate position.
- The translated nick can be sealed by DNA ligase.
- **Klenow fragment** lacks the 5' → 3' exonuclease activity of DNA polymerase I, which can be advantageous in preparing radiolabeled probes.
- Klenow fragment can be used to label the termini of DNA fragments by using radiolabeled dNTPs to fill recessed 3' termini, or to end-label DNA molecules with protruding 3' tails.
- **(a) Labelling by Nick Translation** **(b) Labelling by end - filling**

minal deoxynucleotidyl transferase (TdT)

- Terminal deoxynucleotidyl transferase (TdT) is a DNA polymerase enzyme expressed in certain populations of lymphoid cells.
- TdT typically adds numerous deoxynucleotides to the 3′ terminus of a DNA strand, TdT is template independent and not significantly affected by DNA sequence, but DNA structure



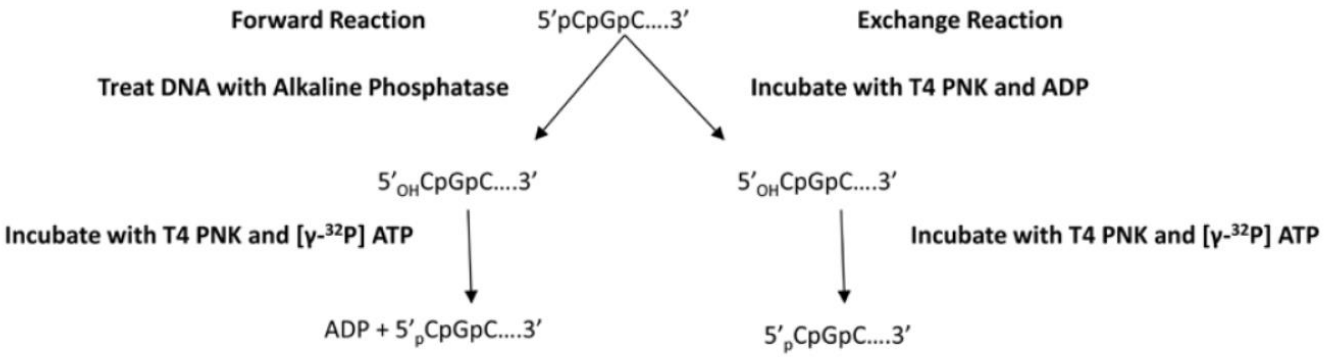
- TdT has the highest activity towards the 3′ end of single-stranded DNA but can also modify the 3′ overhang of double-stranded DNA with lower efficiency.
- TdT has poor activity towards double-stranded DNA with blunt ends or 5′ overhangs. Common sources of DNA templates modified with TdT include unlabeled, single-stranded PCR primers and double-stranded restriction endonuclease fragments with 3′ overhangs (“sticky ends”, 5′ recessed ends).
- TdT is often used to label DNA probes for **RACE** (Rapid Amplification of cDNA Ends), **TUNEL** (Terminal deoxynucleotidyl transferase dUTP Nick-End Labeling) assays and as a method for adding 3′ overhangs to DNA fragments to facilitate cloning.
- TdT can also be used to label the 3′ end of DNA probes with radioactive and nonradioactive tags for a variety of detection and affinity applications. For example, TdT addition of biotin-11-UTP to the 3′ end of complementary DNA probes is an effective way of creating probes for use in nonradioactive **electrophoretic mobility shift assays** (EMSA) and **DNA pull-down assays**.

T4 POLYNUCLEOTIDE KINASE (PNK)

- T4 polynucleotide kinase (T4 PNK) is an enzyme coded for in the genome of the T4 bacteriophage.

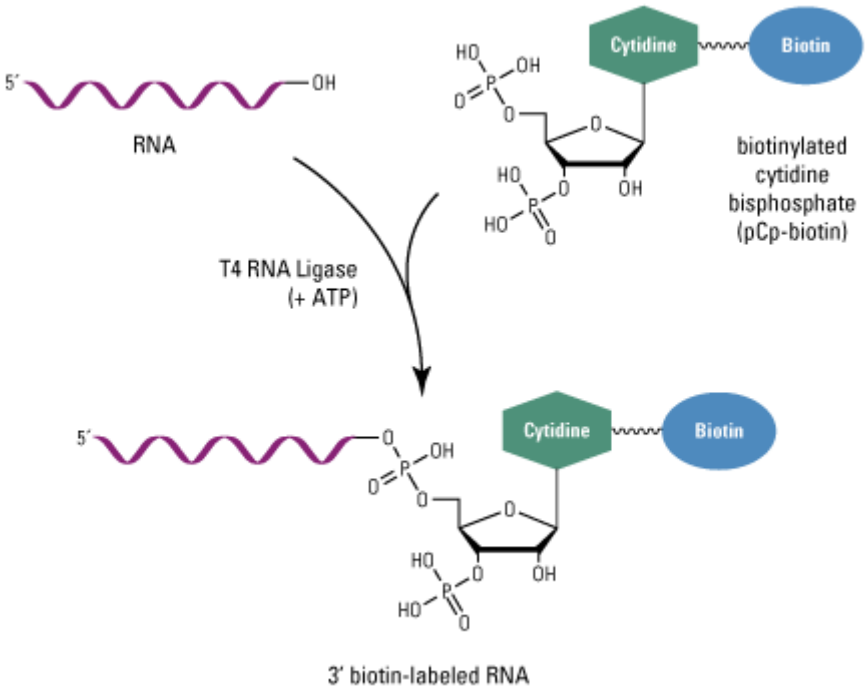
- T4 PNK transfers an organic phosphate from the gamma position on ATP to the 5′-hydroxyl group of DNA and RNA.
- The wild-type enzyme also has 3′-phosphatase activity. T4 PNK ligase is template independent and modifies single-stranded polynucleotides and 5′ overhangs efficiently.
- Blunt-ended and 5′ recessed ends can be modified with reduced efficiency.
- T4 PNK is used primarily for labeling the 5′ ends of polynucleotides with radioactive phosphate from isotope-modified ATP.
- PNK also perform phosphate-exchange reactions, PNK labeling is most efficient when the 5′ end of the target molecule has been dephosphorylated.

T4 RNA ligase



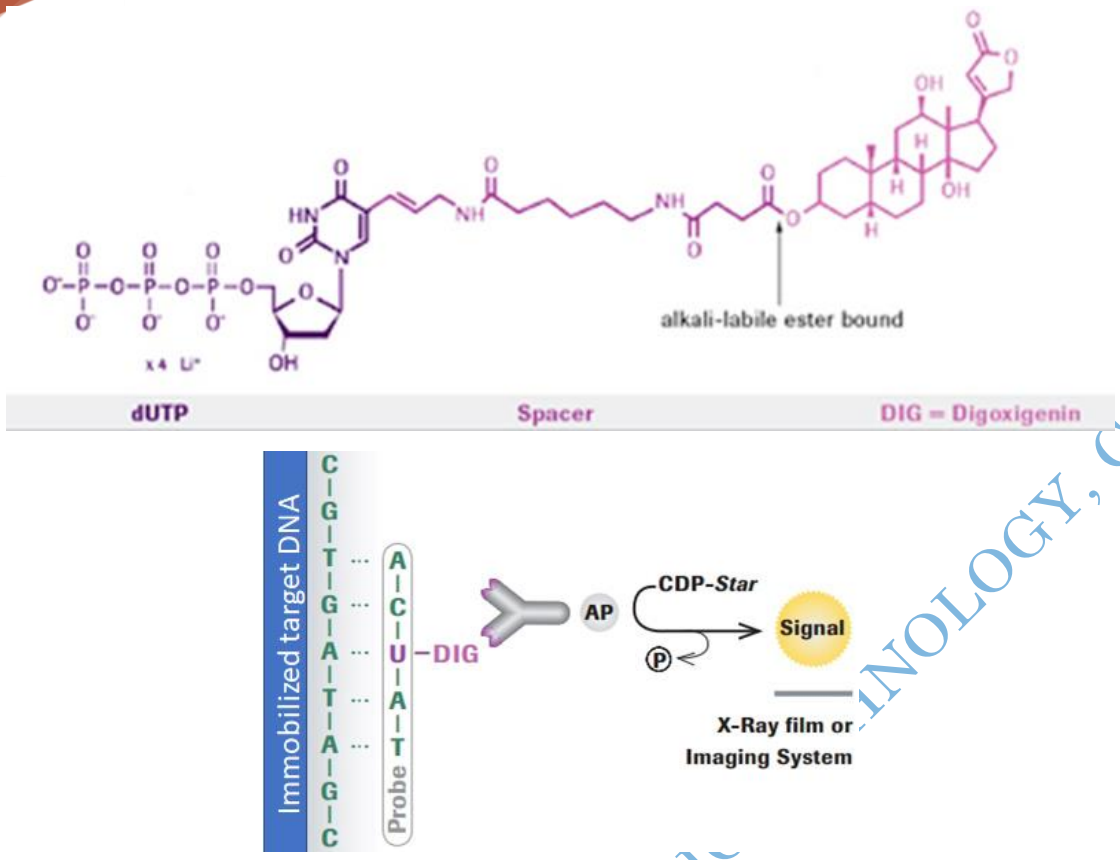
T4 RNA LIGASE

- an enzyme coded for in the genome of the T4 bacteriophage. T4 RNA ligase catalyzes the attachment of a terminal 5′-phosphate to a terminal 3′-hydroxyl group on RNA.
- T4 RNA ligase is template independent but requires single-stranded RNA and ATP.
- T4 RNA ligase is used for labeling the 3′ end of RNA with [5′ ^{32}P]pCp (cytidine-3',5'-bis-phosphate), modifying mRNA for cDNA library generation and performing 5′-RACE.
- T4 RNA ligase can also be used to 3′ end-label RNA with nonradioactive tags using an appropriately modified nucleoside 3',5'-bisphosphate.



Digoxigenin (DIG) Labeling and Anti-DIG Antibody

- The DIG System is the nonradioactive technology of choice to label and detect nucleic acids for multiple applications. The system is based on a steroid isolated from digitalis plants (Digitalis purpurea and Digitalis lanata).
- These plants are the only natural source of digoxigenin, so the anti-DIG antibody does not bind to other biological material, ensuring specific labeling.
- Due to this high specificity, less material is needed compared to radioactive labeling making the DIG system ideal for nucleic hybridization analysis.
- Immobilized nucleic acids are hybridized with a DIG-labeled probe and subsequent detection is performed using high affinity **Anti-Digoxigenin antibodies**, coupled either to alkaline phosphatase (AP), horseradish peroxidase (HRP), fluorescein or rhodamine for colorimetric, and chemiluminescent or fluorescent detection.



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