Preparation of Labeled Nucleotides:

Nucleotides can be labeled by **isotopic and non-isotopic** methods.

Isotopic labeling:

Isotopes generally used for labeling nucleotides are 32P, 33P, 35S or 3H. 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 of emission
^{3}H	12.4 years	0.019 MeV
^{32}P	14.3 years	1.710 MeV
^{33}P	25.5 years	0.248 MeV
^{35}S	87.4 years	0.167 MeV

The strength of autoradiography signal depends on intensity of radiation emitted by radioisotope and duration of exposure.

32P emits high energy β-particles which offer high detection sensitivity. Thus, it is widely used in Southern blot hybridization, dot-blot hybridization, colony hybridization. But it is relatively unstable and when fine resolution is required to interpret results, the image is unambiguous due to its high energy β-particle emission.

Due to this,35S-labeled, 33P-labeled (moderate half-lives) and3H-labeled nucleotides are used which emit less energetic β- radiation. They are used in DNA sequencing and in-situ hybridization.3H requires long exposure time due to low energy β-particle emission.

Non-isotopic labeling:

Non-isotopic labeling systems involve the use of **nonradioactive probes**. 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 labeling, where a nucleotide containing label such as Fluorescein, Texas Red, 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. The fluorescein group is linked to the 5' carbon atom of the uridine by a spacer group. Similarly, Rhodamine can also be used in place of fluorescein.

Indirect non-isotopic labeling 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.

Two widely used non-isotopic labeling methods are:

Biotin-Streptavidin Method: This method uses two ligands which has high affinity towards each other: Biotin works as the reporter and the bacterial protein streptavidin is used as the affinity molecule. Biotinylated nucleotides like bio-11-dUTP are used as labeling agents with a spacer of 4-16 C atoms long between biotin and dNTP. However, Biotin is a ubiquitous constituent of mammalian tissues and tends to stick easily to certain type of nylon membranes which leads to high levels of background during in situ, northern and southern hybridization. To overcome this background problem, digoxigenin is used.

Digoxigenin, a plant steroid obtained from *Digitalis* plant and is used as a reporter and an affinity molecule. Digoxigenin is thus an all-purpose immuno-tag, and in particular a standard immunohistochemical marker for **in situ hybridization.**

Enzymatic methods are mostly used to label DNA probes with biotinylated nucleotides. Photochemical labeling of biotin to nucleic acids can also be used. The label is linked to nitrophenylazido group that is converted by UV irradiation to highly reactive nitrene that form stable covalent linkages to DNA or RNA.

End labeled DNA can be used as:

1. Molecular-weight standards in Southern blotting
2. Probes in gel-retardation experiments
3. Tracers for small quantities of DNAs on gels
4. Probes for screening bacterial colonies or plaques

5. Substrates for Maxam-Gilbert sequencing

6. Probes for RNA mapping with S1 Nuclease or Mung bean nuclease

7. Primers in primer-extension reactions.

Detection of non-radioactively labeled probes after hybridization:

Affinity molecules (streptavidin or digoxigenin-specific antibody) are conjugated with a variety of marker groups or molecules. They include various fluorophores or enzymes such as alkaline phosphatase and peroxidase which can permit detection via colorimetric assays, chemical luminescence assays or fluorescent assay.

In **colorimetric assays**, alkaline phosphatase catalyzes removal of phosphate group from BCIP (5-bromo-4-chloro-3-indolyl phosphate), generating a product that dimerizes to di-bromo-di-chloro indigo, which reduces NBT (Nitrobluetetrazolium) to insoluble purple dye, diformazan that becomes visible at sites where probe has hybridized.

Fluorescent assays make use of HNPP (2-hydroxy-3-naphthoic acid 2'-phenylanilide phosphate). After de-phosphorylation by alkaline phosphatase, HNPP generates fluorescent precipitate on membranes that can be excited by irradiation at 290nm. The response signal emitted at 509nm are captured by CCD cameras.

Chemiluminescence is the fastest and most sensitive assay using HRP (Horseradish peroxidase) – luminal detection system. HRP catalyzes the oxidation of luminal in the presence of H2O2, generating reactive peroxide that emits light at 425nm during decomposition to its ground state.

Applications: This technique is used in physical mapping, karyotyping and phylogenetic analysis, gene expression profiling in developmental biology, pathogen profiling, and abnormal gene expression in pathology and for morphology and population structure of microorganisms.

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