MSc III Sem – Biotechnology

Course – Principles of Genetic Engineering

Nucleic Acid Labelling techniques

- Nucleic acids may be modified with tags that enable detection or purification. The resulting nucleic acid probes can be used to identify or recover other interacting molecules.
- Common labels used to generate nucleic acid probes include **radioactive phosphates, biotin, fluorophores and enzymes**.
- Numerous reagents are available for quick and efficient benchtop oligonucleotide labeling, and they are most useful for making small amounts of probe or when many different probes with the same label are required (i.e., for mutational analysis).
- For small-scale probe generation needs, enzymatic methods are an economical method for labeling probes. There are enzymatic and chemical methods for creating probes labeled at either the 5′ or 3′ ends of the oligonucleotide as well as randomly incorporated throughout the sequence.
- Nucleic acids hybridization reactions (i.e., northern blotting) benefit from the high specific activity gained through random incorporation of label into a probe.
- However, assays requiring protein interactions (i.e., gel shift and pull-down assays) require end-labeling to allow protein binding.

Chemical methods for nucleic acid labeling

- **Periodates** are anions that are formed from oxygen and iodine, and are commonly found as salts of sodium or potassium. Aldehyde groups that are created in solutions with periodates are spontaneously reactive towards amine-containing surfaces or molecules. Thus, periodate oxidation of RNA is a common chemical method for nucleic acid labeling.
- **EDC or 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide** is a water-soluble compound that is preferably used in aqueous reactions when the pH is between 4.0 and 6.0. EDC-mediated conjugation is an economic approach for coupling both DNA and RNA to any primary amine-containing surface or molecule.
- Random chemical labeling of DNA or RNA along the length of nucleic acids is also a labeling method carried out that enables a higher degree of labeling when compared to end-labeling techniques.
- However, a disadvantage of random chemical labeling is direct modification of nucleotide bases, which reduces or prevents the pairing of bases between complementary strands while hybridization experiments are underway. Hence, balancing the degree of labeling with the probe hybridization efficiency in some specific experiments is definitely warranted.

Enzymatic methods for nucleic acid labeling

- DNA polymerase is an enzyme used to create DNA polymers by DNA elongation or primer extension processes. These enzymes are used to generate nucleic acid probes by randomly incorporating modified nucleotides during DNA replication, most notably by simple primer extension procedures or by polymerase chain reactions. Such probes exhibit high specificity enabling detection of even minute quantities of the target.
- **Terminal deoxynucleotidyl transferase** (usually abbreviated as TdT) is a DNA polymerase enzyme that is expressed in certain lymphoid cells. Usual sources of DNA templates that can be modified with TdT include single-stranded and unlabeled polymerase chain reaction (PCR) primers, as well as double-stranded endonuclease restriction fragments with 3' overhangs.
- **T4 RNA ligase** (an ATP-dependent ligase) from the T4 bacteriophage catalyzes the bonding between a terminal 5′-phosphate and a terminal 3′-hydroxyl group on the RNA molecule. Despite the primary substrate for this specific ligase being RNA, the reaction conditions can be optimized for DNA molecules (more specifically, single-stranded DNA molecules) as well, with somewhat lower efficiency.
- **T4 polynucleotide kinase** (abbreviated as T4 PNK), also found in T4 bacteriophage, helps in the transfer an organic phosphate from the ATP molecule to the 5′-hydroxyl group of a nucleic acid. This enzyme is template independent and can efficiently modify 5′ overhangs and single-stranded polynucleotides.

Summary of nucleic acid labeling methods

Method		Effective for	Labelling site	Recommended for
Enzyme	TdT	ssDNA	3′	modified nucleotide incorporation
	T4 RNA ligase	ssDNA, RNA	3′	modified nucleotide incorporation (including isotopes)
	T4 PNK	ssDNA, RNA	5′	phosphate isotopes
	DNA polymerase	DNA, RNA	5′,3′, random	modified nucleotide incorporation (including isotopes)
	RNA polymerase	RNA	random	modified nucleotide incorporation (including isotopes)
Chemical	Periodate	RNA	3′	amine- or hydrazide-modified tag addition
	EDC	DNA, RNA	5′	amine- or hydrazide-modified tag addition
	Nonspecific crosslinkers	DNA, RNA	random	psoralen-, phenyl azide-, or ULS-modified tag addition

DNA and RNA labeling techniques

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 has been used successfully. Use of ^{35}S is especially useful when high resolution (as in in situ hybridization) or when long probe stability is needed.

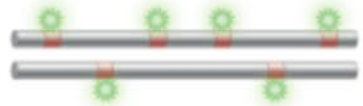
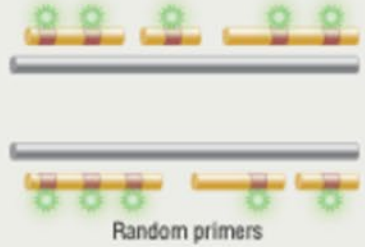
Description of some common DNA and RNA labeling methods and terms:

Application	Description
Random Primer	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, which requires a primer and a template, can be accomplished by either the holoenzyme DNA polymerase I, or the Klenow fragment of DNA polymerase I. Because Klenow fragment lacks the 5’ → 3’ exonuclease activity, the use of Klenow fragment in primer extension avoids the loss of incorporated label. This allows routine incorporation of radiolabeled nucleotide at greater than 60%, in less than 30-60 minutes, providing DNA probe specific activities of greater than 109 dpm/µg DNA.

Application	Description
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 is radiolabeled on the alpha phosphate position. The translated nick can be sealed by DNA ligase.
Klenow	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.
Reverse Transcriptase	In this reaction, RNA is reverse transcribed to cDNA using reverse transcriptase.
Thermophilic DNA Polymerase	Like other DNA polymerases, thermophilic DNA polymerases catalyze synthesis of DNA from nucleotide triphosphates. These thermostable enzymes are used frequently in PCR. A free 3' hydroxyl is required to initiate synthesis and magnesium ion is necessary. In general, optimal activity is achieved at 75 degrees C to 80 degrees C. At lower temperatures, the activity is reduced. Some common thermophilic DNA polymerases are Taq, Pfu and Vent.
DNA 3' End and DNA 5' End labeling	The techniques for end labeling oligonucleotides with radioisotopes have driven nucleic acid probe technology. Oligonucleotide probes can be custom made based on sequence information of the target DNA or RNA in several hours on a DNA synthesizer. Use of a DNA synthesizer eliminates the usual cumbersome and time consuming steps involved in cloning and isolation of restriction fragments to be used as probes. Oligonucleotide probes are highly specific and can be designed to detect single base changes in a gene. 3' end labeling of DNA is usually carried out using terminal transferase. 5' end labeling of DNA (or RNA) is usually carried out using T4 PNK.

Application	Description
SP6	SP6 is a DNA-dependent RNA polymerase. It uses a DNA template that has a specific SP6 phage promotor. SP6 is used in the synthesis of labeled RNA probes for use in hybridization. SP6 DNA-dependent RNA polymerase, like T7 RNA polymerase, can be used to synthesize RNA sequences from short DNA templates which contain the appopriate 18 base pair promoter region. Use of SP6 polymerase extends the range of possible 5' sequences of RNA products since the preferred SP6 start site (of the RNA product) is 5'GAAGA, while T7 polymerase prefers 5'GGGAG. The SP6 start site can be advantageous in large-scale syntheses, where high concentrations of RNA can lead to aggregation. SP6 works best with UTP, CTP, and GTP but not well with ATP.
T7 RNA polymerases	T7 RNA polymerase catalyzes the formation of RNA from a DNA template containing the appropriate promoter. T7 polymerase is extremely promoter-specific and only transcribes bacteriophage T7 DNA or DNA cloned downstream of a T7 promoter. This reaction requires a DNA template and Mg2+ ion as cofactor for the synthesis of RNA. T7 RNA polymerase activity is stimulated by BSA or spermidine. In contrast to bacterial RNA polymerases, T7 polymerase is not inhibited by the antibiotic rifampicin. T7 works well with UTP, CTP, and ATP but not with GTP.
RNA 3' End labeling	T4 RNA ligase can be used to 3'-end label RNA molecules. The enzyme catalyzes the ligation of the 5' phosphate terminus of a radiolabeled nucleotide to the 3'-hydroxyl terminus of a single-stranded DNA or RNA oligo in an ATP-dependent manner.

Products for DNA Labelling:

Application	Label	Reaction	Recommended Enzyme
DNA 5´ End Labeling	γ-^{32}P rATP		T4 Polynucleotide Kinase
Labeling by PCR	α-^{32}P dNTP, Biotin-dNTP, Fl-dNTP		*Taq* DNA Polymerase
DNA 3´ End Labeling	α-^{32}P dNTP, Biotin-dNTP, Fl-dNTP		Terminal™ Transferase Klenow Fragment (3´ → 5´ exo-)
Single Nucleotide Terminator Labeling	Fl terminator nucleotide		Therminator DNA Polymerase
Random Priming	α-^{32}P dNTP, Biotin-dNTP, Fl-dNTP		Klenow Fragment (3´ → 5´ exo-)
Nick Translation	α-^{32}P dNTP, Biotin-dNTP, Fl-dNTP		DNA Polymerase I (*E. coli*)

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