

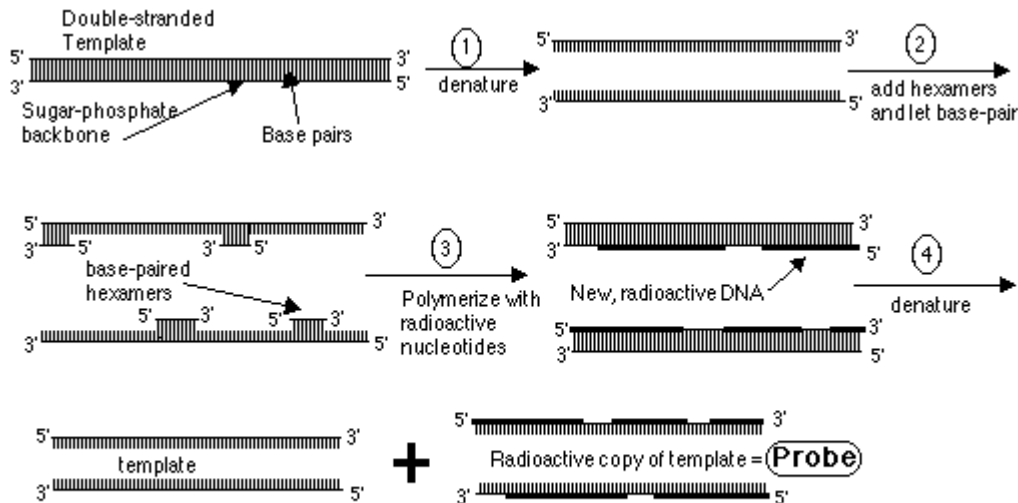
## Southern blotting: Probe labeling & Detection

Southern blotting (and other molecular techniques) takes advantage of the specific hybridization of complementary single stranded nucleic acids with each other to form double stranded molecules. One strand is the “target” sequence being tested, and the other is the **probe** whose **sequence is known**. Typically, the **probe is labeled** to allow detection of where it has hybridized.

### Probe labeling:

In the following labeling methods, the label is attached to one of the 4 nucleotide triphosphates and then incorporated into the probe.

- **PCR labeling.** A straightforward and now very popular method of incorporating labeled nucleotides into a probe. Design primers that will amplify a sequence you wish to use as a probe, and simply include a labeled dNTP during PCR.
- **Nick translation** involves the action of 2 enzymes on the dsDNA you wish to label: DNaseI and DNA polymerase I. Under conditions designed to limit its activity, DNaseI will randomly introduce a few nicks (i.e. single strand breaks) in the DNA backbone. These nicks are repaired by DNA polymerase I. As you know, DNA polymerase synthesizes DNA in a 5'-3' direction. It ALSO has exonuclease activity. These two activities act together to remove and then replace a few nucleotides down from each nick. The newly formed sequence bits are now labeled.
- **Random oligo primed synthesis** in which DNA is denatured into single strands and annealed to random hexamer oligonucleotides. These random primers can then be extended using DNA polymerase (Klenow), incorporating labeled nucleotides (as above).



- **End labeling** in which the end of a DNA (or RNA) molecule is specifically labeled. The **5' end** can be labeled with the enzyme polynucleotide **kinase**, which donates the terminal (gamma) phosphate group (usually radioactive  $^{32}\text{P}$ ) from a dNTP to the 5' OH. Note that only one marked residue per probe molecule is incorporated by this method, so the specific activity of

the label (radioactive counts per minute per microgram of DNA) is lower than in the other methods. {3' ends can also be labeled, with terminal transferase; this enzyme can add a small chain of identical labeled nucleotides, so it gives higher specific activity than the 5' method, but also creates potentially undesirable new sequence in your probe.}

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## **Probe Detection**

Classically, probes were radioactively labeled, often with  $^{32}\text{P}$  because of its high energy and ease of incorporation into the phosphate groups of dNTPs. Radioactively labeled probes are detected using X-ray film.

Disadvantages of  $^{32}\text{P}$ :

- Short half life (about 2 weeks) means probes must be used immediately, and the labeling reagent cannot be stored for long.
- Contamination problems: all materials and equipment must be dedicated to radioactive work only. Regular lab-wide testing for contamination is required. Expense of disposal of radioactive waste.
- Must have access to a dark room to set up and develop films.

Now, MANY different detection systems are available, depending on the label used. Broadly, the categories of **nonradioactive** detection are colorimetric, fluorescent, and chemiluminescent.

**Colorimetric** detection generally involves the production of a colored precipitate which can be seen with the naked eye. In a typical system, the DNA probe itself is labeled with an antigen such as *digoxigenin*; following hybridization to its target it would be exposed to an anti-digoxigenin antibody conjugated to an enzyme capable of catalyzing a colorimetric reaction (one commonly used example is **alkaline phosphatase** which will act on substrates NBT & BCIP to produce a dark purple product).

**Fluorescent** detection involves probes which are directly labeled with fluorophores, or more likely, probes which are coupled to fluorescent molecules indirectly. For example, if probe is labeled with biotin, it would be exposed to avidin conjugated to a fluorescent tag. (Biotin and avidin strongly and specifically bind together, like an antibody and its antigen.) Fluorophores emit light when excited by light of an appropriate wavelength.

**Chemiluminescence** is sort of a combination of these two: an enzymatic reaction that triggers the release of ordinary visible light (think: firefly luciferase).

### **Direct vs. Indirect**

Each detection system will have certain advantages and disadvantages in a given setting. Some will not be sensitive enough (false negatives), some may too sensitive (false positives). One important variable to note is the difference between direct and indirect labeling & detection. Direct detection means you detect the label in the probe itself (i.e., radioactive nucleotides, fluorescently tagged nucleotides). Here, signal intensity is a direct function of how many labeled molecules are in the bound probe. Indirect detection detects a signal generated by an intermediate, such as an enzyme tagged to an antibody. **Indirect detection allows for amplification of signal** in ways not permitted by direct detection.