

Introduction to Hapten Labeling and Detection of Nucleic Acids

A wide variety of labels are available for *in situ* hybridization experiments. This manual presents (Chapter 5) examples for all the labels described below.

Digoxigenin (DIG) labeling

The Digoxigenin (DIG) System is emphasized in this manual. It was developed and continues to be expanded by Roche Applied Science (Kessler, 1990, 1991; Kessler et al., 1990; Mühlegger et al., 1989; Höltke et al., 1990; Seibl et al., 1990; Mühlegger, et al., 1990; Höltke and Kessler, 1990; Rüger et al., 1990; Martin et al., 1990; Schmitz et al., 1991; Höltke et al., 1992 and many more). The first kit, the DIG DNA Labeling and Detection Kit, was introduced in 1987.

The DIG labeling method is based on a steroid isolated from digitalis plants (*Digitalis purpurea* and *Digitalis lanata*, Figure 1). As the blossoms and the leaves of these plants are the only natural source of digoxigenin, the anti-DIG antibody does not bind to other biological material.

Digoxigenin is linked to the C-5 position of uridine nucleotides via a spacer arm containing eleven carbon atoms (Figure 2). The DIG-labeled nucleotides may be incorporated, at a defined density, into nucleic acid probes by DNA polymerases (such as *E.coli* DNA Polymerase I, T4 DNA Polymerase, T7 DNA Polymerase, Reverse Transcriptase, and Taq DNA Polymerase) as well as RNA Polymerases (SP6, T3, or T7 RNA Polymerase), and Terminal Transferase. DIG label may be added by random primed labeling, nick translation, PCR, 3'-end labeling/tailing, or *in vitro* transcription.

Nucleic acids can also be labeled chemically with DIG-NHS ester or with DIG Chem-Link.

Hybridized DIG-labeled probes may be detected with high affinity anti-digoxigenin (anti-DIG) antibodies that are conjugated to alkaline phosphatase, peroxidase, fluorescein, rhodamine, or colloidal gold. Alternatively, unconjugated anti-digoxigenin antibodies and conjugated secondary antibodies may be used.

Detection sensitivity depends upon the method used to visualize the anti-DIG antibody conjugate. For instance, when an anti-DIG antibody conjugated to alkaline phosphatase is visualized with colorimetric (NBT and BCIP) or fluorescent (HNPP) alkaline phosphatase substrates, the sensitivity of the detection reaction is routinely 0.1 pg (on a Southern blot).



Figure 1: *Digitalis purpurea*.

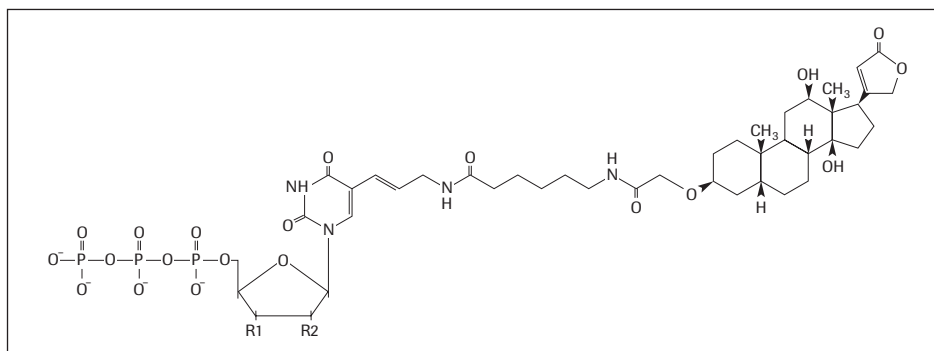


Figure 2: Digoxigenin-UTP/dUTP/ddUTP, alkali-stable.

Digoxigenin-UTP (R1 = OH, R2 = OH)

Digoxigenin-dUTP (R1 = OH, R2 = H)

Digoxigenin-ddUTP (R1 = H, R2 = H)

The labeling mixtures in the DIG labeling kits contain a ratio of DIG-labeled uridine to dTTP which produces an optimally sensitive hybridization probe. For most applications, that ratio produces a DNA with a DIG-labeled nucleotide incorporated every 20th to 25th nucleotide. This labeling density permits optimal steric interaction between the hapten and anti-DIG antibody conjugate; the antibody conjugate is large enough to cover about 20 nucleotides.

To see the full line of DIG kits and reagents, turn to Chapter 6.

Biotin labeling of nucleic acids

Enzymatic labeling of nucleic acids with biotin-dUTP (Figure 3) was developed by David Ward and coworkers at Yale University (Langer et al., 1981). More recently, laboratories have synthesized other biotinylated nucleotides such as biotinylated adenosine and cytosine triphosphates (Gebeyehu et al., 1987). Also, a photochemical procedure (Forster et al., 1985) and a number of chemical biotinylation procedures (Sverdlov et al., 1974) has been described (Gillam and Tener, 1986; Reisfeld et al., 1987; Viscidi et al., 1986):

Chemically nucleic acids can be labeled with the Biotin Chem-Link.

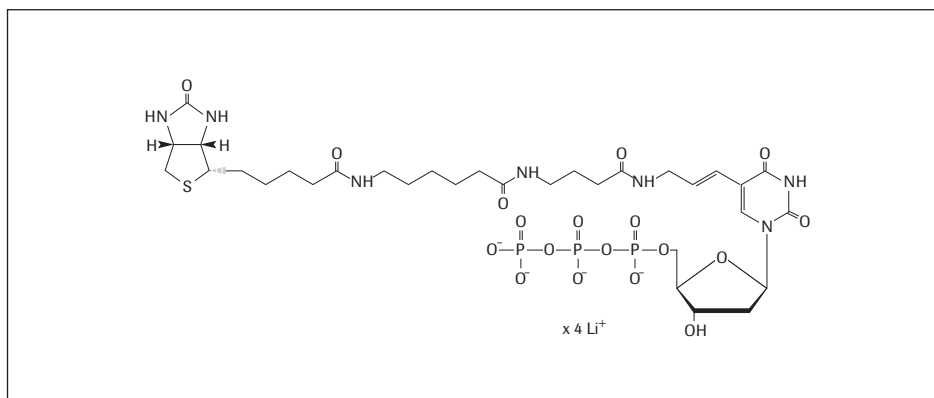


Figure 3: Biotin-dUTP.

In principle biotin can be used in the same way as digoxigenin; it can be detected by anti-biotin antibodies. However, streptavidin or avidin is more frequently used because these molecules have a high binding capacity for biotin. Avidin, from egg white, is a 68 kd glycoprotein with a binding constant of 10^{15} M^{-1} at 25°C .

To see the selection of biotin reagents offered by Roche Applied Science, turn to Chapter 6.

Fluorescent labeling of nucleic acids

Fluorescein-labeled nucleotides (Figure 4) were released by Roche Applied Science in 1991 as a new nonradioactive labeling alternative. Fluorescein nucleotide analogues can be used for direct as well as indirect *in situ* hybridization experiments (Dirks et al., 1991; Wiegant et al., 1991).

Fluorescein-dUTP/UTP/ddUTP can be incorporated enzymatically into nucleic acids according to standard techniques (as listed in Chapter 4). Since fluorescein is a direct label, no immunocytochemical visualization procedure is necessary and the background is low. General drawbacks of direct methods are, however, that they can be less sensitive than the indirect methods described above.

Alternatively, fluorescein-labeled nucleotides can be detected with an anti-fluorescein antibody-enzyme conjugate or with an unconjugated antibody and a fluorescein-labeled secondary antibody. The sensitivity of such experiments corresponds to that of other indirect methods.

Other fluorochrome-labeled nucleotides, such as Tetramethylrhodamine-5-dUTP (red fluorescent dye) are also available from Roche Applied Science.

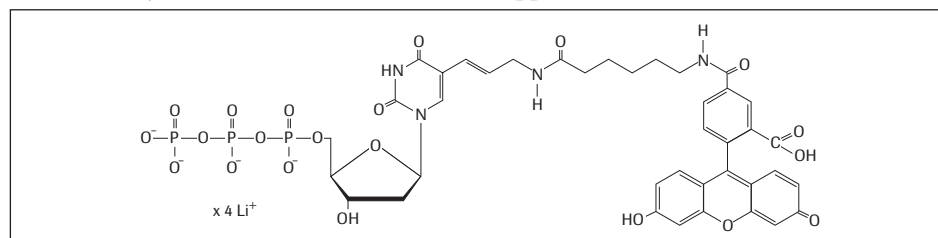


Figure 4: Fluorescein-dUTP.

Multiple labeling and detection

By using combinations of digoxigenin-, biotin- and fluorochrome-labeled probes, laboratories can perform multiple simultaneous hybridizations to localize different chromosomal regions or different RNA sequences in one preparation. Such multi-probe experiments are made possible by the availability of different fluorescent dyes coupled to antibodies; these include fluorescein or FITC (fluorescein isothiocyanate; yellow), rhodamine or TRITC (tetramethylrhodamine isothiocyanate; red) and AMCA (amino-methylcoumarin acetic acid; blue).

Chapter 5 contains several detailed examples of such multiple labeling and detection experiments. These use two, three, and even twelve different probes in a single experiment.

Antibody conjugates

Various reporter molecules can be coupled to detecting antibodies to visualize the specific probe-target hybridization. Commonly used conjugates include:

- ▶ Enzyme-coupled antibodies require substrates which usually generate a precipitating, colored product. Alternatively, Roche Applied Science recently introduced an alkaline phosphatase substrate (HNPP) that produces a precipitating, fluorescent product. These conjugates are most commonly used for *in situ* hybridization experiments.
- ▶ Fluorochrome-labeled antibodies require the availability of a fluorescent microscope and specific filters which allow visualization of the wavelength emitted by the fluorescent dye.
- ▶ Antibodies coupled to colloidal gold are mainly used for electron microscopy on cryostatic sections.