Roche

DIG Application Manual

for Nonradioactive In Situ Hybridization

4th Edition

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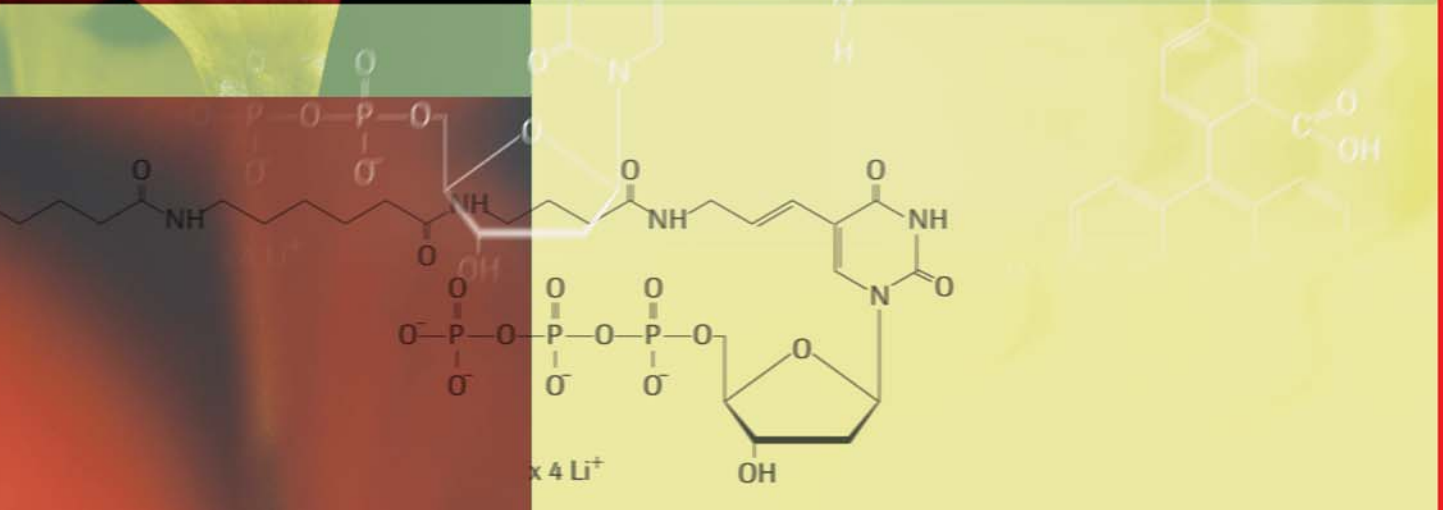

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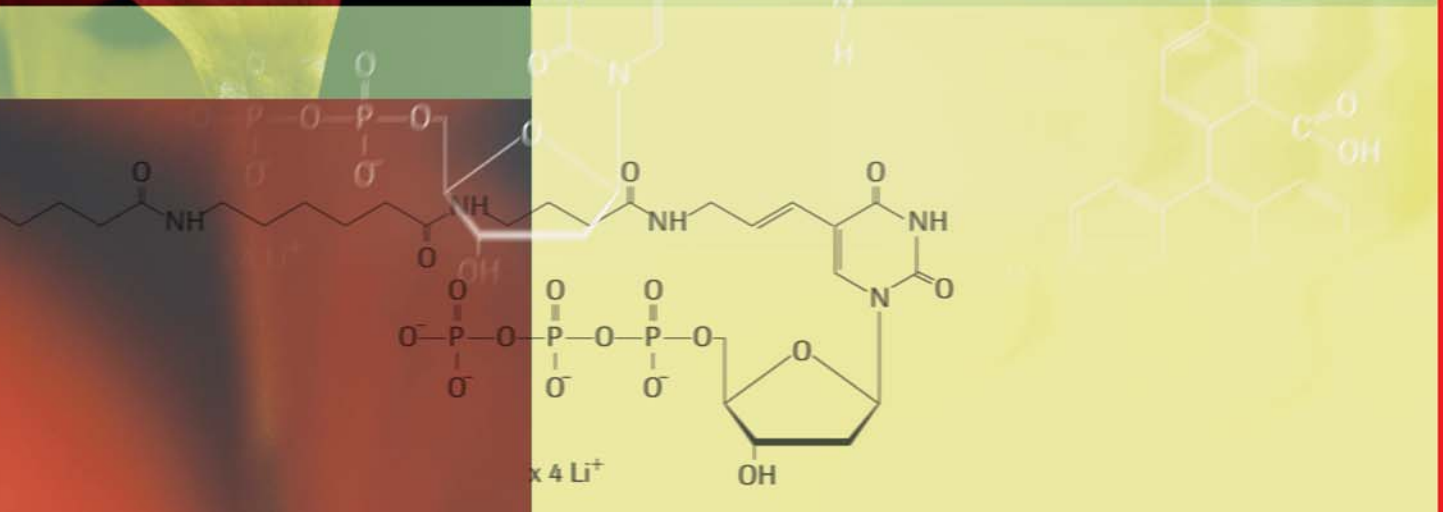

1

General Introduction to *In Situ* Hybridization

In situ hybridization techniques allow specific nucleic acid sequences to be detected in morphologically preserved chromosomes, cells or tissue sections. In combination with immunocytochemistry, *in situ* hybridization can relate microscopic topological information to gene activity at the DNA, mRNA, and protein level.

The technique was originally developed by Pardue and Gall (1969) and (independently) by John et al. (1969). At this time radioisotopes were the only labels available for nucleic acids, and autoradiography was the only means of detecting hybridized sequences. Furthermore, as molecular cloning was not possible in those days, *in situ* hybridization was restricted to those sequences that could be purified and isolated by conventional biochemical methods (*e.g.*, mouse satellite DNA, viral DNA, ribosomal RNAs).

Molecular cloning of nucleic acids and improved radiolabeling techniques have changed this picture dramatically. For example, DNA sequences a few hundred base pairs long can be detected in metaphase chromosomes by autoradiography (Harper et al., 1981; Jhanwag et al., 1984; Rabin et al., 1984; Schroeder et al., 1984). Also radioactive *in situ* techniques can detect low copy number mRNA molecules in individual cells (Harper et al., 1986). Some years ago, chemically synthesized, radioactively labeled oligonucleotides began to be used, especially for *in situ* mRNA detection (Coghlan et al., 1985).

In spite of the high sensitivity and wide applicability of *in situ* hybridization techniques, their use has been limited to research laboratories. This is probably due to the problems associated with radioactive probes, such as the safety measures required, limited shelf life, and extensive time required for autoradiography. In addition, the scatter inherent in radioactive decay limits the spatial resolution of the technique.

However, preparing nucleic acid probes with a stable nonradioactive label removes the major obstacles which hinder the general application of *in situ* hybridization. Furthermore, it opens new opportunities for combining different labels in one experiment. The many sensitive antibody detection systems available for such probes further enhances the flexibility of this method. In this manual, therefore, we describe nonradioactive alternatives for *in situ* hybridization.

Direct and indirect methods

There are two types of nonradioactive hybridization methods: direct and indirect. In the direct method, the detectable molecule (reporter) is bound directly to the nucleic acid probe so that probe-target hybrids can be visualized under a microscope immediately after the hybridization reaction. For such methods it is essential that the probe-reporter bond survives the rather harsh hybridization and washing conditions. Perhaps more important, however, is, that the reporter molecule does not interfere with the hybridization reaction. The terminal fluorochrome labeling procedure of RNA probes developed by Bauman et al. (1980, 1984), and the direct enzyme labeling procedure of nucleic acids described by Renz and Kurz (1984) meet these criteria. Roche Applied Science has introduced several fluorochrome-labeled nucleotides that can be used for labeling and direct detection of DNA or RNA probes.

If antibodies against the reporter molecules are available, direct methods may also be converted to indirect immunochemical amplification methods (Bauman et al., 1981; Lansdorp et al., 1984; Pinkel et al., 1986).

Indirect procedures require the probe to contain a reporter molecule, introduced chemically or enzymatically, that can be detected by affinity cytochemistry. Again, the presence of the label should not interfere with the hybridization reaction or the stability of the resulting hybrid. The reporter molecule should, however, be accessible to antibodies. A number of such hapten modifications has been described (Langer et al., 1981; Leary et al., 1983; Landegent et al., 1984; Tchen et al., 1984; Hopman et al., 1986; Hopman et al., 1987; Shroyer and Nakane, 1983; Van Prooijen et al., 1982; Viscidi et al., 1986; Rudkin and Stollar, 1977; Raap et al., 1989). One of the most popular system is offered by Roche Applied Science: the Digoxigenin (DIG) System which is described in detail later in this chapter.

Many years ago, the chemical synthesis of oligonucleotides containing functional groups (*e.g.*, primary aliphatic amines or sulfhydryl groups) was described. These can react with haptens, fluorochromes or enzymes to produce a stable probe which can be used for *in situ* hybridization experiments (Agrawal et al., 1986; Chollet and Kawashima, 1985; Haralambidis et al., 1987; Jablonski et al., 1986). Modified oligonucleotides can also be obtained with the DIG system (Mühlegger et al., 1990). Such oligonucleotide probes will undoubtedly be widely used as automated oligonucleotide synthesis makes them available to researchers not familiar with DNA recombinant technology.

This manual concentrates on two labeling systems:

- Indirect methods using digoxigenin (detected by specific antibodies) and biotin (detected by streptavidin)
- Direct methods using fluorescein or other fluorochromes directly coupled to the nucleotide

The ordering information in Chapter 6 lists all the kits and single reagents Roche Applied Science offers for nonradioactive labeling and detection.

1

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.

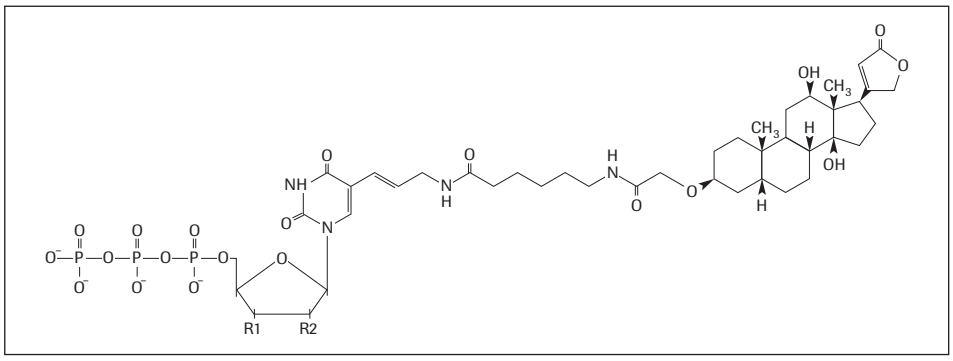

Figure 2: Digoxigenin-UTP/dUTP/ddUTP, alkali-stable.
Digoxigenin-UTP (R1 = OH, R2 = OH)
Digoxigenin-dUTP (R1 = OH, R2 = H)
Digoxigenin-ddUTP (R1 = H, R2 = H)

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.

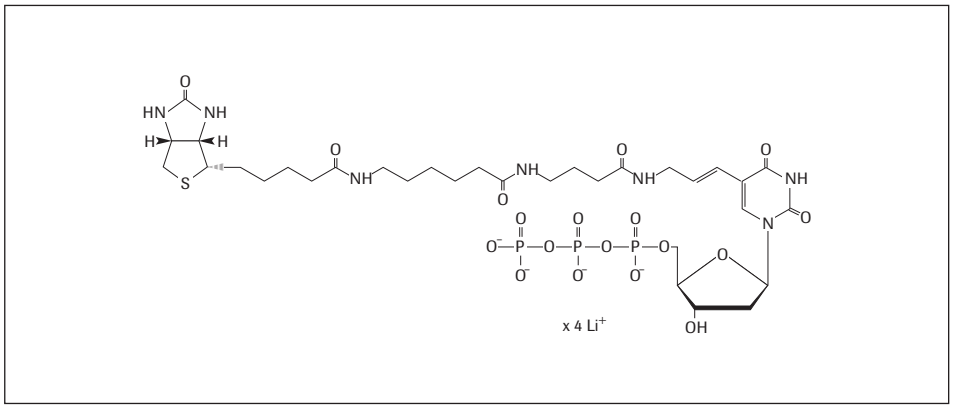

Figure 3: Biotin-dUTP.

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.

1

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.

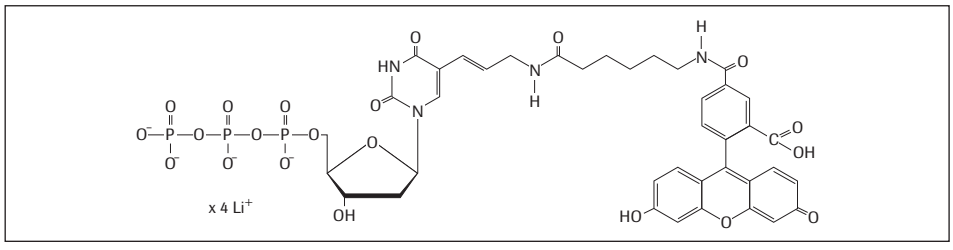

Figure 4: Fluorescein-dUTP.

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.

Choosing the Right Labeling Method for your Hybridization Experiment

1

For details on the labeling methods described here, see Chapter 4.

Homogeneous labeling methods for DNA

Probes prepared by random primed labeling are often preferred for blot applications because of the high incorporation rate of nucleotides and the high yield of labeled probe. In the random primed labeling reaction, the template DNA is linearized, denatured, and annealed to a primer. Starting from the 3'-OH end of the annealed primer, Klenow enzyme synthesizes new DNA along the single-stranded substrate. The size of the probe is about 200 to 1000 bp. With the help of a premixed labeling reagent (such as DIG-, Biotin-, or Fluorescein High Prime), the random primed reaction can produce from 30 – 70 ng (for 10 ng template and 1 h incubation) to 2.10 – 2.65 µg (for 3 µg template and 20 h incubation) of nonradioactively labeled DNA.

In the nick translation reaction the DNA template can be supercoiled or linear. After the DNA is nicked with DNase I, the 5'→3' exonuclease activity of DNA Polymerase I extends the nicks to gaps; then the polymerase replaces the excised nucleotides with labeled ones. The reaction produces optimal labeling after 60 to 90 min. The size of the probe after the reaction should be about 200 to 500 bp.

Probes can also be prepared by the Polymerase Chain Reaction (PCR). In PCR, two oligonucleotide primers hybridize to opposite DNA strands and flank a specific target sequence. Then, a thermostable polymerase (such as Taq DNA Polymerase) elongates the two PCR primers. A repetitive series of cycles (template denaturation, primer annealing, and extension of primers) results in an exponential accumulation of copies of the target sequence (about a million copies in twenty cycles). Incorporation of a labeled nucleotide during PCR can produce large amounts of labeled probe from minimal amounts (10 – 100 pg) of linearized plasmid or even from nanogram amounts of genomic DNA (1 – 50 ng). The length of the amplified probe is precisely defined by the 5' ends of the PCR primers, so PCR allows easy production of optimally sized hybridization probes.

A variant of PCR, in situ PCR, actually allows PCR amplification (and labeling) of target sequences inside intact cells or tissue slices. For details of the potential and problems of in situ PCR, see Komminoth and Long (1995) and "PCR Applications Manual" published by Roche Applied Science.

All three DNA labeling methods allow great flexibility with regard to length of the labeled fragments. In the nick translation reaction, changing the DNase concentration alters the fragment length. In the random primed labeling reaction, changing the primer concentration affects fragment length. In the PCR, changing the sequence of the PCR primers controls fragment length.

In both nick translation and random primed labeling, a heterogeneous population of probe strands, many of which have overlapping complementary regions, are produced. This may lead to a signal amplification in the hybridization experiment.

1

Homogeneous labeling methods for RNA

In contrast to DNA probes, RNA probes are generated by *in vitro* transcription from a linearized template. In this case a promoter for RNA polymerases must be available on the vector DNA containing the template. SP6, T3, or T7 RNA Polymerase is commonly used to synthesize RNA complementary to the DNA substrate. The synthesis is complete after 1 to 2 h. The synthesized transcripts are an exact copy of the sequence from the promoter site to the restriction site used for linearization. Thus, the size of the probe can be adjusted by choice of the linearizing restriction enzyme and probes all have the same length. RNA probes are inherently single-stranded. The amount of nonradioactively labeled RNA probe is about 10 µg for about 1 µg of input DNA.

Stability of probe–target interaction

In hybridization experiments the strength of the bond between probe and target plays an important role. The strength decreases in the order RNA-RNA, DNA-RNA, DNA-DNA (Wetmur et al., 1981). Stability of the hybrids is also influenced by the hybridization conditions, *e.g.*, the concentration of formamide, salt concentration, or the hybridization temperature (as detailed in Chapter 3).

Nonradioactive labeling of oligonucleotides

Synthetic oligonucleotides have several advantages. They are readily available through automated synthesis, small, and single-stranded. Their size gives them good penetration properties, which is considered one of the main factors for successful *in situ* hybridization.

The small size of oligonucleotides can also be a disadvantage because they usually cover less target than conventional cDNA probes. Recent studies, however, suggest that their good penetration properties largely compensate for the smaller target they cover. The fact that they are single-stranded also excludes the possibility of renaturation (see Chapter 3).

Oligonucleotides may be labeled either directly with a fluorochrome or enzyme, or with a hapten such as biotin and digoxigenin, which is visualized by affinity cytochemistry after hybridization. Such labeling can be carried out either chemically or enzymatically.

One chemical approach uses synthetic oligonucleotides which have reactive amine or thiol groups added in the final step of automated synthesis. After purification, the reactive oligonucleotide is modified with a hapten, *e.g.* DIG-NHS-ester, or with reporter molecules.

In this manual we describe only the enzymatic approach, which uses terminal deoxynucleotidyl transferase to label synthetic oligonucleotides enzymatically at the 3' end using digoxigenin-labeled deoxy- and dideoxyuridine triphosphate. Such methods are advantageous for small scale probe production because they do not require the synthesis and derivatization of oligonucleotides.

1

Double-stranded versus single-stranded probes

As detailed in Chapter 3, a number of competing reactions occur during *in situ* hybridization with double-stranded probes. Thus, single-stranded probes provide the following advantages:

- The probe is not exhausted by self-annealing in solution.
- Large concatenates are not formed in solution. Such concatenates would penetrate the section or chromosomes poorly.

With DNA-DNA *in situ* hybridization, the *in situ* renaturation of target DNA sequences cannot be prevented because *in situ* hybrids and renatured sequences have similar thermal stability. However, *in situ* renaturation is probably limited to repetitive sequences since they have the greatest chance of being in partial register. *In situ* renaturation of target DNA can, however, be prevented with the use of single-stranded RNA probes. Since DNA-RNA hybrids are more thermally stable than DNA-DNA hybrids, hybridization conditions can be designed in which DNA-DNA hybrid formation is not favored but DNA-RNA hybrid formation is.

References

Agrawal, S.; Christodoulow, C.; Gait, M. J. (1986) Efficient methods for attaching nonradioactive labels to the 5'-ends of synthetic oligodeoxyribonucleotides. *Nucleic Acids Res.* **14**, 6227–6245.

Baumann, J. G. J.; Van der Ploeg, M.; Van Duijn, P. (1984) Fluorescent hybridocytochemical procedures: DNA-RNA hybridization *in situ*. In: Chayen, J.; Bitensky, L. (Eds.) Investigative Microtechniques in Medicine and Microbiology. New York: Marcel Dekker, 41–48.

Baumann, J. G. J.; Wiegant, J.; Borst, P.; Van Duijn, P. (1980) A new method for fluorescence microscopical localization of specific DNA sequences by *in situ* hybridization of fluorochrome-labeled RNA. *Exp. Cell Res.* **138**, 485–490.

Baumann, J. G. J.; Wiegant, J.; Van Duijn, P. (1981) Cytochemical hybridization with fluorochrome-labeled RNA. III. Increased sensitivity by the use of anti-fluorescein antibodies. *Histochemistry* **73**, 181–193.

Chollet, A.; Kawashima, E. M. (1985) Biotin-labeled synthetic oligodeoxyribonucleotides: synthesis and uses as hybridization probes. *Nucleic Acids Res.* **13**, 1529–1541.

Coghlan, J. P.; Aldred, P.; Haralambridis, J.; Niall, H. D.; Penschow, J. D.; Tregear, G. W. (1985) Hybridization histochemistry. *Anal. Biochem.* **149**, 1–28.

Dirks, R. W.; Van Gijlswijk, R. P. M.; Vooijs, M. A.; Smit, A. B.; Bogerd, J.; Van Minnen, J.; Raap, A. K.; Van der Ploeg, M. (1991) 3'-end fluorochromized and haptenized oligonucleotides as *in situ* hybridization probes for multiple simultaneous RNA detection. *Exp. Cell Res.* **194**, 310–315.

Forster, A. C.; McInnes, J. L.; Skingle, D. C.; Symons, R. H. (1985) Nonradioactive hybridization probes prepared by the chemical labeling of DNA and RNA with a novel reagent, photobiotin. *Nucleic Acids Res.* **13**, 745–761.

Gebeyehu, G.; Rao, P. Y.; SooChan, P.; Simms, D.; Klevan, L. (1987) Novel biotinylated nucleotide analogs for labeling and colorimetric detection of DNA. *Nucleic Acids Res.* **15**, 4513–4534.

Gillam, I. C.; Tener, G. M. (1986) N4-(6-aminohexyl) cytidine and deoxycytidine nucleotides can be used to label DNA. *Anal. Biochem.* **157**, 199–207.

Haralambidis, J.; Chai, M.; Tregear, G. W. (1987) Preparation of base-modified nucleosides suitable for nonradioactive label attachment and their incorporation onto synthetic oligodeoxyribonucleotides. *Nucleic Acids Res.* **15**, 4857–4876.

Harper, M. E.; Marselle, L. M.; Gallo, R. C.; Wong-Stahl, F. (1986) Detection of lymphocytes expressing human T-lymphotropic virus type III in lymph nodes and peripheral blood from infected individuals by *in situ* hybridization. *Proc. Natl. Acad. Sci. USA* **83**, 772–776.

Harper, M. E.; Ullrich, A.; Saunders, G. F. (1981) Localization of the human insulin gene to the distal end of the short arm of chromosome 11. *Chromosoma* **83**, 431–439.

Höltke, H.-J.; Kessler, C. (1990) Nonradioactive labeling of RNA transcripts *in vitro* with the hapten digoxigenin (DIG); hybridization and ELISA-based detection. *Nucleic Acids Res.* **18**, 5843–5851.

Höltke, H.-J.; Seibl, R.; Burg, J.; Mühlegger, K.; Kessler, C. (1990) Nonradioactive labeling and detection of nucleic acids: II. Optimization of the digoxigenin system. *Biol. Chem. Hoppe-Seyler* **371**, 929–938.

Höltke, H.-J.; Sagner, G.; Kessler, C.; Schmitz, G. (1992) Sensitive chemiluminescent detection of digoxigenin-labeled nucleic acids: a fast and simple protocol and its applications. *BioTechniques* **12** (1), 104–114.

Hopman, A. H. N.; Wiegant, J.; Tesser, G. I.; Van Duijn, P. (1986) A nonradioactive *in situ* hybridization method based on mercurated nucleic acid probes and sulfhydryl-hapten ligands. *Nucleic Acids Res.* **14**, 6471–6488.

Hopman, A. H. N.; Wiegant, J.; Van Duijn, P. (1987) Mercurated nucleic acid probes, a new principle for nonradioactive *in situ* hybridization. *Exp. Cell Res.* **169**, 357–368.

Jablonski, E.; Moomaw, E. W.; Tullis, R. H.; Ruth, J. L. (1986) Preparation of oligonucleotide-alkaline phosphatase conjugates and their use as hybridization probes. *Nucleic Acids Res.* **14**, 6115–6129.

Jhanwag, S. C.; Neel, B. G.; Hayward, W. S.; Chaganti, R. S. K. (1984) Localization of the cellular oncogenes ABL, SIS and FES on human germ-line chromosomes. *Cytogenet. Cell Genet.* **38**, 73–75.

John, H.; Birnstiel, M.; Jones, K. (1969) RNA-DNA hybrids at the cytological level. *Nature* **223**, 582–587.

Kessler, C. (1990) The digoxigenin system: principle and applications of the novel nonradioactive DNA labeling and detection system. *BioTechnology Int.* **1990**, 183–194.

Kessler, C. (1991) The digoxigenin system: anti-digoxigenin technology – a survey on the concept and realization of a novel bioanalytic indicator system. *Mol. Cell. Probes* **5**, 161–205.

Kessler, C.; Höltke, H.-J.; Seibl, R.; Burg, J.; Mühlegger, K. (1990) Nonradioactive labeling and detection of nucleic acids: I. A novel DNA labeling and detection system based on digoxigenin: anti-digoxigenin ELISA principle. *Biol. Chem. Hoppe-Seyler* **371**, 917–927.

Komminoth, P.; Long, A. A. (1995) *in situ* Polymerase Chain Reaction – methodology, applications and nonspecific pathways. In: PCR Applications Manual. Mannheim, Germany: Roche Applied Science, 97–106.

Landegent, J. E.; Jansen in de Wal, N.; Baan, R. A.; Hoeijmakers, J. H. J.; Van der Ploeg, M. (1984) 2-Acetylaminofluorene-modified probes for the indirect hybridocytochemical detection of specific nucleic acid sequences. *Exp. Cell Res.* **153**, 61–72.

Langer, P. R.; Waldrop, A. A.; Ward, D. A. (1981) Enzymatic synthesis of biotin-labeled polynucleotides: novel nucleic acid affinity probes. *Proc. Natl. Acad. Sci. USA* **78**, 6633–6637.

Lansdorp, P. M.; Van der Kwast, T. H.; de Boer, M.; Zeijlemaker, W. P. (1984) Stepwise amplified immunoperoxidase (PAP) staining. I. Cellular morphology in relation to membrane markers. *J. Histochem. Cytochem.* **32**, 172–178.

Leary, J. L.; Brigati, D. J.; Ward, D. C. (1983) Rapid and sensitive colorimetric method for visualizing biotin-labeled DNA probes hybridized to DNA or RNA immobilized on nitrocellulose: Bio-blots. *Proc. Natl. Acad. Sci. USA* **80**, 4045–4049.

Martin, R.; Hoover, C.; Grimme, S.; Grogan, C.; Höltke, J.; Kessler, C. (1990) A highly sensitive, nonradioactive DNA labeling and detection system. *BioTechniques* **9**, 762–768.

Mühlegger, K.; Huber, E.; von der Eltz, H.; Rüger, R.; Kessler, C. (1990) Nonradioactive labeling and detection of nucleic acids: IV. Synthesis and properties of digoxigenin-modified 2'-deoxy-uridine-5'-triphosphates and a photoactivatable analog of digoxigenin (photodigoxigenin). *Biol. Chem. Hoppe-Seyler* **371**, 953–965.

Mühlegger, K.; Batz, H.-G.; Böhm, S.; von der Eltz, H.; Höltke, H.-J.; Kessler, C. (1990) Synthesis and use of new digoxigenin-labeled nucleotides in nonradioactive labeling and detection of nucleic acids. *Nucleosides & Nucleotides* **8**, 1161–1163.

Pardue, M. L.; Gall, J. G. (1969) Molecular hybridization of radioactive DNA to the DNA of cytological preparations. *Proc. Natl. Acad. Sci. USA* **64**, 600–604.

Pinkel, D.; Straume, T.; Gray, J. W. (1986) Cytogenetic analysis using quantitative, high sensitivity fluorescence hybridization. *Proc. Natl. Acad. Sci. USA* **83**, 2934–2938.

Raap, A. K.; Hopman, A. H. N., Van der Ploeg, M. (1989) Use of hapten modified nucleic acid probes in DNA *in situ* hybridization. *Techniques Immunocytochem.* **4**, 167–197.

Rabin, M.; Watson, M.; Barker, P. E.; Ryan, J.; Breg, W. R.; Ruddle, F. H. (1984) N-ras transforming gene maps to region p11–p13 on chromosome 1 by *in situ* hybridization. Cytogenet. *Cell Genet.* **38**, 70–72.

Reisfeld, A.; Rothenberg, J. M.; Bayer, E. A.; Wilchek, M. (1987) Nonradioactive hybridization probes prepared by the reaction of biotin hydrazide with DNA. *Biochem. Biophys. Res. Commun.* **142**, 519–526.

Renz, M.; Kurz, C. (1984) A colorimetric method for DNA hybridization. *Nucleic Acid Res.* **12**, 3435–3444.

Rüger, R.; Höltke, H.-J.; Reischl, U.; Sagner, G.; Kessler, C. (1990) Use of the Polymerase Chain Reaction for labeling specific DNA sequences with digoxigenin. *J. Clin. Chem. Clin. Biochem.* **28**, 566.

Rudkin, G. T.; Stollar, B. D. (1977) High resolution detection of DNA:RNA hybrids *in situ* by indirect immunofluorescence. *Nature* **265**, 472–473.

Schmitz, G. G.; Walter, T.; Seibl, R.; Kessler, C. (1991) Nonradioactive labeling of oligonucleotides *in vitro* with the hapten digoxigenin by tailing with terminal transferase. *Anal. Biochem.* **192**, 222–231.

Schroeder, W. T.; Lopez, L. C.; Harper, M. E.; Saunders, G. F. (1984) Localization of the human glucagon gene (GCG) to chromosome segment 2936–37. *Cytogenet. Cell Genet.* **38**, 76–79.

Seibl, R.; Höltke, H.-J.; Rüger, R.; Meindl, A.; Zachau, H. G.; Raßhofer, R.; Roggendorf, M.; Wolf, H.; Arnold, N.; Wienberg, J.; Kessler, C. (1990) Nonradioactive labeling and detection of nucleic acids: III. Applications of the digoxigenin system. *Biol. Chem. Hoppe-Seyler* **371**, 939–951.

Shroyer, K. R.; Nakane, P. K. (1983) Use of DNP-labeled cDNA in *in situ* hybridization. *J. Cell Biol.* **97**, 377a.

Sverdlov, E. D.; Monastyrskaya, G. S.; Guskova, L. I.; Levitan, T. L.; Scheichenko, V. I.; Budowski, E. I. (1974) Modification of cytidin residues with a bisulphite-O-methylhydroxylamine mixture. *Biochim. Biophys. Acta* **340**, 153–165.

Tchen, P.; Fuch, R. P. P.; Sage, E.; Leng, M. (1984) Chemically modified nucleic acids as immunodetectable probes in hybridization experiments. *Proc. Natl. Acad. Sci. USA* **81**, 3466–3470.

Van Prooijen-Knegt, A. C.; Van Hoek, J. F. M.; Baumann, J. G. J.; Van Duijn, P.; Wool, I. G.; Van der Ploeg, M. (1982) *in situ* hybridization of DNA sequences in human metaphase chromosomes visualized by an indirect fluorescent immunocytochemical procedure. *Exp. Cell Res.* **141**, 397–407.

Viscidi, R. P.; Connelly, C. J.; Yolken, R. H. (1986) Novel chemical method for the preparation of nucleic acids for non-isotopic hybridization. *J. Clin. Microbiol.* **23**, 311–317.

Wetmur, J. G.; Ruyechan, W. T.; Douthart, R. J. (1981) Denaturation and renaturation of *Penicillium chrysogenum* mycophage double-stranded ribonucleic acid in tetraalkylammonium salt solutions. *Biochemistry* **20**, 2999–3002.

Wiegant, J.; Ried, T.; Nederlof, P. M.; Van der Ploeg, M.; Tanke, H. J.; Raap, A. K. (1991) *in situ* hybridization with fluoresceinated DNA. *Nucleic Acids Res.* **19** (12), 3237–3241.

2

Guidelines for *In Situ* Hybridization

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Details of the Technique 18

2

Guidelines for *In Situ* Hybridization

An *in situ* hybridization protocol follows this general outline:

- Preparation of slides and fixation of material
- Pretreatments of material on slides, *e.g.*, permeabilization of cells and tissues
- Denaturation of *in situ* target DNA (not necessary for mRNA target)
- Preparation of probe
- *In situ* hybridization
- Posthybridization washes
- Immunocytochemistry
- Microscopy

All these steps are discussed below in detail. The information provided will make it easier to decide whether a certain step should or should not be included in a given *in situ* hybridization protocol.

Details of the Technique

Slide preparation

For chromosome spreads, alcohol/ether (1:1) cleaned slides are sufficient. However, since tissue sections may be lost during the procedure, use either polylysine or glutaraldehyde-activated gelatin chrome aluminum slides for these sections.

Fixation

To preserve morphology, the biological material must be fixed. From a chemical point of view, there is little limitation in the type of fixation used because one of the following will be true:

- The functional groups involved in base pairing are protected in the double helix structure of duplex DNA.
- RNA is fairly unreactive to crosslinking agents.
- The reaction is reversible (*e.g.*, with formaldehyde).

For metaphase chromosome spreads, methanol/acetic acid fixation is usually sufficient. For paraffin-embedded tissue sections, use formalin fixation. Cryostat sections fixed for 30 min with 4% formaldehyde or with Bouin's fixative have been used successfully, as well as paraformaldehyde vapor fixation. Tissues can also be freeze-dried.

It should be noted that the DNA and RNA target sequences are surrounded by proteins and that extensive crosslinking of these proteins masks the target nucleic acid. Therefore, permeabilization procedures are often required.

Unfortunately, a fixation protocol which can be used for all substrates has not yet been described. The fixation and pretreatment protocols must be optimized for different applications.

Pretreatments of material on the slide

Endogenous enzyme inactivation

When an enzyme is used as the label, the endogenous enzyme activity may have to be inactivated. For peroxidase, this is done by treating the sections with 1% H_2O_2 in methanol for 30 min. For alkaline phosphatase, levamisole may be added to the substrate solution, but this may be unnecessary since residual alkaline phosphatase activity is usually lost during hybridization.

RNase treatment

RNase treatment serves to remove endogenous RNA and may improve the signal-to-noise ratio in DNA-DNA hybridizations. This treatment can also be used as a control in hybridizations with (m)RNA as target. It is done by incubating the preparations in DNase-free RNase (100 µg/ml) in 2× SSC at 37°C for 60 min. (SSC = 150 mM NaCl, 15 mM sodium citrate, pH 7.4).

HCl treatment

In several protocols a 20–30 min treatment with 200 mM HCl is included. The precise action of the acid is not known, but extraction of proteins and partial hydrolysis of the target sequences may contribute to an improvement in the signal-to-noise ratio.

Detergent treatment

Preparations may be pretreated with Triton X-100, sodium dodecyl sulfate, or other detergents if lipid membrane components have not been extracted by other procedures such as fixation, dehydration, embedding, and endogenous enzyme inactivation procedures.

Protease treatment

Protease treatment serves to increase target accessibility by digesting the protein that surrounds the target nucleic acid. To digest the sample, incubate the preparations with up to 500 µg/ml Proteinase K (the optimal amount must be determined) in 20 mM Tris-HCl, 2 mM $CaCl_2$, pH 7.4, for 7.5–30 min at 37°C. For example, with chromosomes or isolated preparations of nuclei, a reasonable starting point for protease digestion is up to 1 µg/ml Proteinase K for 7.5 min. For formalin-fixed material, 5–15 µg/ml for 15–30 min usually gives good results.

The use of pepsin has been shown to give excellent results for formalin-fixed, paraffin-embedded tissue sections. Routine pepsin digestion involves incubating the preparations for 30 min at 37°C in 200 mM HCl containing 500 µg/ml pepsin. For the pretreatment of chromosome spreads with pepsin see page 69.

Other hydrolases, such as Collagenase and Dispase, may be tried if preparations of connective tissue and liver give high background reactions.

Also, freeze/thaw cycles have been used to improve the penetration of probes into tissues.

Prehybridization

A prehybridization incubation is often necessary to prevent background staining. The prehybridization mixture contains all components of a hybridization mixture except for probe and dextran sulfate.

2

Denaturation of probe and target

For *in situ* hybridization to chromosomal DNA, the DNA target must be denatured. In general such treatments may lead to loss of morphology, so in practice, a compromise must be found between hybridization signal and morphology. Alkaline denaturations have traditionally been used. Heat denaturations have also become popular, because of their experimental simplicity and greater effectiveness. Variations in time and temperature should be evaluated to find the best conditions for denaturation.

For heat denaturation, the probe and target chromosomal DNA may be denatured simultaneously. To accomplish this, put the probe on the slide and cover with a coverslip. Bring the slide to 80°C for 2 min in an oven for the denaturation and then cool to 37°C. For tissue sections, if necessary, extend the time of denaturation at 80°C to 10 min.

For competition *in situ* hybridization, where the labeled probe is allowed to reanneal with unlabeled competitor DNA, denature the chromosomal preparation and probe separately.

Hybridization

See Chapter 3 for the composition of the hybridization solution, and the factors affecting hybridization kinetics and hybrid stability.

Posthybridization washes

Labeled probe can hybridize nonspecifically to sequences which are partially but not entirely homologous to the probe sequence. Such hybrids are less stable than perfectly matched hybrids. They can be dissociated by performing washes of various stringencies (as described in Chapter 3). The stringency of the washes can be manipulated by varying the formamide concentration, salt concentration, and temperature. Often a wash in 2× SSC containing 50% formamide suffices. For some applications the stringency of the washes should be higher. However, we recommend hybridizing stringently rather than washing stringently.

Immunocytochemistry

In this manual immunocytochemical procedures using digoxigenin, biotin, and fluorochromes are described. These allow flexibility in the choice of the final reporter molecule and type of microscopy.

Blocking reaction

Use a blocking step prior to the immunological procedure to remove high background.

For example, perform biotin detection steps in PBS containing Tween 20 and BSA (when using monoclonal antisera) or normal serum (when using polyclonal antisera).

Perform routine digoxigenin detection in Tris-HCl buffer containing Blocking Reagent (instead of BSA or normal serum) to remove background. Also the addition of 0.4 M NaCl can help prevent background staining if the antigen/hapten bond can survive the high salt conditions.

In a standard reaction, block the target tissue, cell, or chromosome preparation and then incubate it with the antibody-conjugate solution for 30 min at 37°C (or 2 h at room temperature) in a moist chamber. Afterwards, wash the slide three times (5–10 min each) with buffer containing Tween 20.

Fluorescence

Useful fluorochromes for fluorescence *in situ* hybridization (FISH) include the blue fluorochrome AMCA (amino-methylcoumarin-acetic acid), the green fluorophore fluorescein, and the red fluorochromes CY3, rhodamine, and Texas Red. Recently, the FISH application of an infrared dye has been reported (Ried et al., 1992), although it can only be visualized with infrared-sensitive cameras. For routine experiments, several immunofluorophores with good spectral separation properties can be used for FISH analysis (Table 1). Chapter 5 contains detailed procedures for using all these fluorochromes.

	Color	Excitation max. (nm)	Emission max. (nm)
AMCA	Blue	399	446
Fluorescein	Green	494	523
CY3	Red	552	565
Rhodamine	Red	555	580
Texas Red	Red	590	615

Table 1: Spectral properties of fluorophores used in FISH analysis.

Rhodamine-, fluorescein-, and coumarin-based dyes cover the three primary colors of the visible part of the electro-magnetic spectrum. By using selective excitation and emission filters, and dichroic mirrors, these colors can be visualized without "crosstalk", making triple color FISH feasible. To increase the identification of targets by color, a combinatorial labeling approach has been developed (Niederlof et al., 1990; Ried et al., 1992; Wiegant et al., 1993). Multiplicity is then given by $2^n - 1$, where n is the number of colors.

After establishing that hybridized probes labeled with two haptens in different ratios still fluoresce at fairly constant ratios, laboratories extended the combinatorial approach to ratio-labeling (Nederlof et al., 1992). In ratio-labeling, the ratio of fluorescence intensities of the various color combinations is used for color identification of the probes. This approach allowed simultaneous identification of twelve different probes (Dauwerse et al., 1992). For detailed information, refer to the literature describing combinatorial and ratio labeling (Wiegant and Dauwerse, 1995).

Fluorescent DNA counterstaining is usually performed with red fluorescent propidium iodide (PI) or with blue fluorescent DAPI. Currently, new counterstains are being introduced, *e.g.*, green fluorescent YOYO-1 (Molecular Probes, Inc., USA).

An antifading agent should be added to the embedding medium to retard fading. When fluorescent labels are used, the recommended antifading agent is Vectashield (Vector Laboratories). The different DNA counterstains can be directly dissolved in the embedding medium (40 ng DAPI/ml; 100 ng PI/ml; or 0.1 µM YOYO-1/ml) or the fluorescent specimen can first be counterstained and then embedded in Vectashield. If desired, the coverslip may be sealed with nail varnish.

2

Enzymes

Use any enzyme commonly used in immunocytochemistry. We show examples for peroxidase and alkaline phosphatase.

With peroxidase (POD), use the diaminobenzidine (DAB)/imidazole reaction (Graham and Karnovsky, 1966). With alkaline phosphatase (AP), use the 5-Bromo-4-chloro-3-indolylphosphate/Nitro-blue tetrazolium (BCIP/NBT) reaction. The advantages of these colorimetric detection methods are good localization properties, high sensitivity (De Jong et al., 1985; Straus, 1982; Scopsi and Larson, 1986) and stability of the precipitates. As the DAB reaction produces a color which contrasts with the blue alkaline phosphatase staining reaction (and vice versa), one can use both enzymes in double hybridizations. Furthermore, both detection methods have bright reflective properties. They can be amplified by gold/silver (Gallyas et al., 1982; Burns et al., 1985) and the color can be modified with heavy metal ions (Hsu and Soban, 1982; Cremers et al., 1987).

When using brightfield microscopy with peroxidase, prepare the following peroxidase substrate solution: 0.5 mg/ml diaminobenzidine in 50 mM Tris-HCl, pH 7.4, containing 10 mM imidazole. Shortly before use, add 0.05% H_2O_2. Incubate with substrate from 2 – 30 min (depending on the signal-to-noise ratio). We advise inspecting sections microscopically during the peroxidase reaction. Stop the reaction by washing the sample with water. Counterstain with Giemsa if desired.

In the case of reflection contrast microscopy with peroxidase, prepare the following peroxidase substrate solution: 0.1 mg/ml diaminobenzidine in 50 mM Tris-HCl, pH 7.4. Shortly before use, add 0.01% H_2O_2. The usual reaction time is 10 min. After stopping the reaction with H_2O and dehydrating with ethanol, evaluate slides by oil-immersion microscopy without embedding.

When using Alkaline Phosphatase, prepare the following substrate: 0.16 mg/ml 5-Bromo-4-chloro-3-indolyl-phosphate (BCIP) and 0.33 mg/ml Nitro-blue tetrazolium salt (NBT) in 200 mM Tris-HCl, 10 mM $MgCl_2$, pH 9.2. Determine reaction times by evaluating the signal-to-noise ratio, which should be checked microscopically during the enzyme reaction. The reaction medium itself is stable (in the dark). The final product (NBT formazan) also has reflective properties.

A recently introduced alkaline phosphatase substrate (HNPP/Fast Red TR) also allows fluorescent detection of alkaline phosphatase label. For fluorescence microscopy, prepare the following substrate:

10 mg/ml HNPP (in dimethylformamide) and 25 mg/ml Fast Red TR in redist H_2O. For details on the use of HNPP/Fast Red TR, see the article in Chapter 5, page 105.

Microscopy

Brightfield microscopy

In brightfield microscopy the image is obtained by the direct transmission of light through the sample.

Evaluation of *in situ* hybridization results by brightfield microscopy is preferred for most routine applications because the preparations are permanent. However, the sensitivity demanded by many applications (*e.g.*, single copy gene localization requires the detection of a few attograms of DNA) may require more sophisticated microscopy.

Darkfield microscopy

In the darkfield microscope, the illuminating rays of light are directed from the side, so that only scattered light enters the microscope lenses. Consequently, the material appears as an illuminated object against a black background.

Darkfield microscopy is extensively used for radioactive *in situ* hybridization experiments because large fields can be examined at low magnification. The distribution of the silver grains can be seen with high contrast. In contrast, these types of microscopy are seldom used to localize reaction products of nonradioactive hybridization procedures (Heyting et al., 1985). However, Garson et al. (1987) shows the application of phase contrast microscopy to single copy gene detection.

Phase contrast microscopy

Phase contrast microscopy exploits the interference effects produced when two sets of waves combine. This is the case when light passing *e.g.*, through a relatively thick or dense part of the cell (such as the nucleus) is retarded and its phase consequently shifted relative to light that has passed through an adjacent (thinner) region of the cytoplasm. Phase contrast microscopy is often used for enzyme detection.

Reflection contrast microscopy

Reflection contrast microscopy is similar to phase contrast microscopy. This technique measures the shift of wavelength of the light reflected from the sample relative to that of directly emitted light.

Bonnet (personal communication) made the crucial observation that with reflection contrast microscopy (Ploem, 1975; Van der Ploeg and Van Duijn, 1979; Landegent et al., 1985a) the DAB/peroxidase product displays bright reflection when present in extremely low amounts (*i.e.*, low local absorption, typically $A<0.05$). This property has made reflection contrast microscopy instrumental in detecting the first single copy gene by nonradioactive means (Landegent et al., 1985b; Hopman et al., 1986b; Ambros et al., 1986). Studies of DAB image formation in reflection contrast microscopy have shown that best results require thin objects and low local absorbances of the DAB product (Cornelese ten Velde et al., 1988).

2

Fluorescence microscopy

A fluorescence microscope contains a lamp for excitation of the fluorescent dye and a special filter which transmits a high percentage of light emitted by the fluorescent dye. Usually antifading reagents have to be added before analysis.

Fluorescence microscopy is of great value for nonradioactive *in situ* hybridization. It is highly sensitive. Furthermore, it can be used to excite three different immunofluorophores with spectrally well-separated emissions (which allows multiple detection). Also the option of using highly sensitive image acquisition systems and the relative ease of quantitation of fluorescence signals adds to its attractiveness.

Digital imaging microscopy

Digital imaging microscopes can detect signals that cannot be seen with conventional microscopes. Also, image processing technology provides enhancement of signal-to-noise ratios as well as measurement of quantitative data. The most sensitive system to date, the cooled CCD (charged coupled device) camera, counts emitted photons with a high efficiency over a broad spectral range of wavelengths, and thus is the instrument of choice for two-dimensional analysis. For three-dimensional analysis, confocal laser scanning microscopy is used.

Electron microscopy

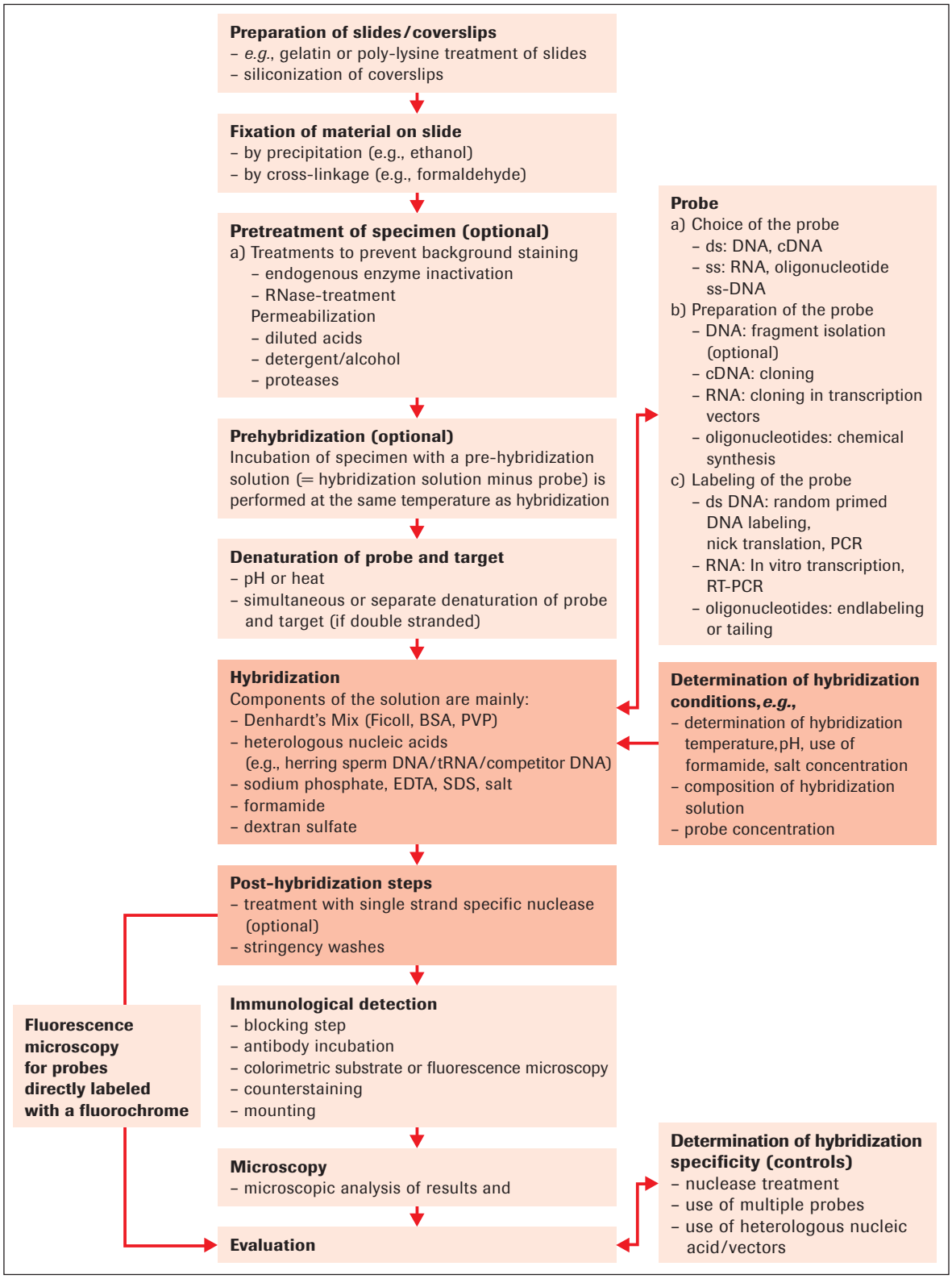
The resolution of microscopy is enhanced when electrons instead of light are used, since electrons have a much shorter wavelength (0.004 nm). The practical resolving power of most modern electron microscopes is 0.1 nm. With the electron microscope, the fine structure of a cell can be resolved. Such analysis requires special preparative procedures which are described in detail in Chapter 5.

Flow cytometry

The enormous speed with which fluorescence of individual cells can be measured with a flow cytometer has many advantages for quantitating *in situ* hybridization signals. Procedures for fluorescence *in situ* hybridization in suspension have been described (Trask et al., 1985) and further developments in this field will be very important.

2

Flow diagram for *in situ* hybridization



2

References

Ambros, P. F.; Matzke, M. A.; Matzke, A. J. M. (1986) Detection of a 17 kb unique (T-DNA) in plant chromosomes by *in situ* hybridization. *Chromosoma* **94**, 11–16.

Burns, J.; Chan, V. T. W.; Jonasson, J. A.; Fleming, K. A.; Taylor, S.; McGee, J. O. D. (1985) Sensitive system for visualizing biotinylated DNA probes hybridized *in situ*: rapid sex determination of intact cells. *J. Clin. Pathol.* **38**, 1085–1092.

Cornelese ten Velde, I.; Bonnet, J.; Tanke, H. J.; Ploem, J. S. (1988) Reflection contrast microscopy. Visualization of (peroxidase generated) diaminobenzidine polymer products and its underlying optical phenomena. *Histochemistry* **89**, 141–150.

Cremers, A. F. M.; Jansen in de Wal, N.; Wiegant, J.; Dirks, R. W.; Weisbeek, P.; Van der Ploeg, M.; Landegent, J. E. (1987) Nonradioactive *in situ* hybridization. A comparison of several immunocytochemical detection systems using reflection contrast microscopy and electron microscopy. *Histochemistry* **86**, 609–615.

Dauwerse, J. G.; Wiegant, J.; Raap, A. K.; Breuning, M. H.; Van Ommen, G. J. B. (1992) Multiple colors by fluorescence *in situ* hybridization using ratio-labeled DNA probes create a molecular karyotype. *Hum. Molec. Genet.* **1**, 593–598.

De Jong, A. S. H.; Van Kessel-Van Vark, M.; Raap, A. K. (1985) Sensitivity of various visualization methods for peroxidase and alkaline phosphatase activity in immunoenzyme histochemistry. *Histochem. J.* **17**, 1119–1130.

Gallyas, F.; Gorcs, T.; Merchenthaler, I. (1982) High grade intensification of the end-product of the diaminobenzidine reaction for peroxidase histochemistry. *J. Histochem. Cytochem.* **30**, 183–184.

Garson, J. A.; Van den Berghe, J. A.; Kemshead, J. T. (1987) Novel non-isotopic *in situ* hybridization technique detects small (1 kb) unique sequences in routinely G-banded human chromosomes: fine mapping of N-myc and b-NGF genes. *Nucleic Acids Res.* **15**, 4761–4770.

Graham, R. C.; Karnovsky, M. J. (1966) The early stages of absorption of injected horseradish peroxidase in the proximal tubules of mouse kidney: ultrastructural cytochemistry by a new technique. *J. Histochem. Cytochem.* **14**, 291–302.

Heyting, C.; Kroes, W. G. M.; Kriek, E.; Meyer, I.; Slater, R. (1985) Hybridization of N-acetoxy-N-acetyl-2-aminofluorene labeled RNA to Q-banded metaphase chromosomes. *Acta Histochem.* **77**, 177–184.

Hopman, A. H. N.; Wiegant, J.; Raap, A. K.; Landegent, J. E.; Van der Ploeg, M.; Van Duijn, P. (1986b) Bi-color detection of two target DNAs by nonradioactive *in situ* hybridization. *Histochemistry* **85**, 1–4.

Hsu, S. M.; Soban, E. (1982) Color modification of diaminobenzidine (DAB) precipitation by metallic ions and its application for double immunohistochemistry. *J. Histochem. Cytochem.* **30**, 1079–1082.

Landegent, J. E., Jansen in de Wal, N.; Ommen, G. J. B.; Baas, F.; De Vijlder, J. J. M.; Van Duijn, P.; Van der Ploeg, M. (1985b) Chromosomal localization of a unique gene by non-autoradiographic *in situ* hybridization. *Nature* **317**, 175–177.

Landegent, J. E.; Jansen in de Wal, N.; Ploem, J. S.; Van der Ploeg, M. (1985a) Sensitive detection of hybridocytochemical results by means of reflection contrast microscopy. *J. Histochem. Cytochem.* **33**, 1241–1246.

Nederlof, P .M.; Van der Flier, S.; Wiegant, J.; Raap, A. K.; Tanke, H. J.; Ploem, J. S.; Van der Ploeg, M. (1990) Multiple fluorescence *in situ* hybridization. *Cytometry* **11**, 126–131.

Nederlof, P. M.; Van der Flier, S.; Vrolijk, J.; Tanke, H. J.; Raap, A. K. (1992) Quantification of *in situ* hybridization signals by fluorescence digital imaging microscopy. III. Fluorescence ratio measurements of double labeled probes. *Cytometry* **13**, 838–845.

Ploem, J. S. (1975) Reflection contrast microscopy as a tool for investigation of the attachment of living cells to a glass surface. In: Van Furth, R. (Ed.) Mononuclear Phagocytes in Immunity, Infection and Pathology. Oxford: Blackwell, 405–421.

Reid, T.; Baldini, A.; Rand, T. C.; Ward, D. C. (1992) Simultaneous visualization of seven different probes by *in situ* hybridization using combinatorial fluorescence and digital imaging microscopy. *Proc. Natl. Acad. Sci. USA* **89**, 1388–1392.

Scopsi, L.; Larsson, L. I. (1986) Increased sensitivity in peroxidase immunocytochemistry. A comparative study of a number of peroxidase visualization methods employing a model system. *Histochemistry* **84**, 221–230.

Straus, W. (1982) Imidazole increases the sensitivity of the cytochemical reaction for peroxidase with diaminobenzidine at a neutral pH. *J. Histochem. Cytochem.* **30**, 491–493.

Trask, B.; Van den Engh, G.; Landegent, J.; Jansen in de Wal, N.; Van der Ploeg, M. (1985) Detection of DNA sequences in nuclei in suspension by *in situ* hybridization and dual beam flow cytometry. *Science* **230**, 1401–1403.

Van der Ploeg, M.; Van Duijn, P. (1979) Reflection versus fluorescence. *Histochemistry* **62**, 227–232.

Wiegant, J.; Wiesmeijer, C. C.; Hoovers, J. M. N.; Schuuring, E.; D'Azzo, A.; Vrolijk, J.; Tanke, H. J.; Raap, A. K. (1993) Multiple and sensitive *in situ* hybridization with rhodamine-, fluorescein-, and coumarin-labeled DNAs. *Cytogenet. Cell Genet.* **63**, 73–76.

Wiegant, J.; Dauwerse, J. G. (1995) Multiple-colored chromosomes by fluorescence *in situ* hybridization. In: Verma, R. S.; Babu, A. (Eds.) Human Chromosomes: Principles and Techniques, 2nd ed. New York: McGraw-Hill, Inc.

Nucleic Acid Hybridization – General Aspects

3

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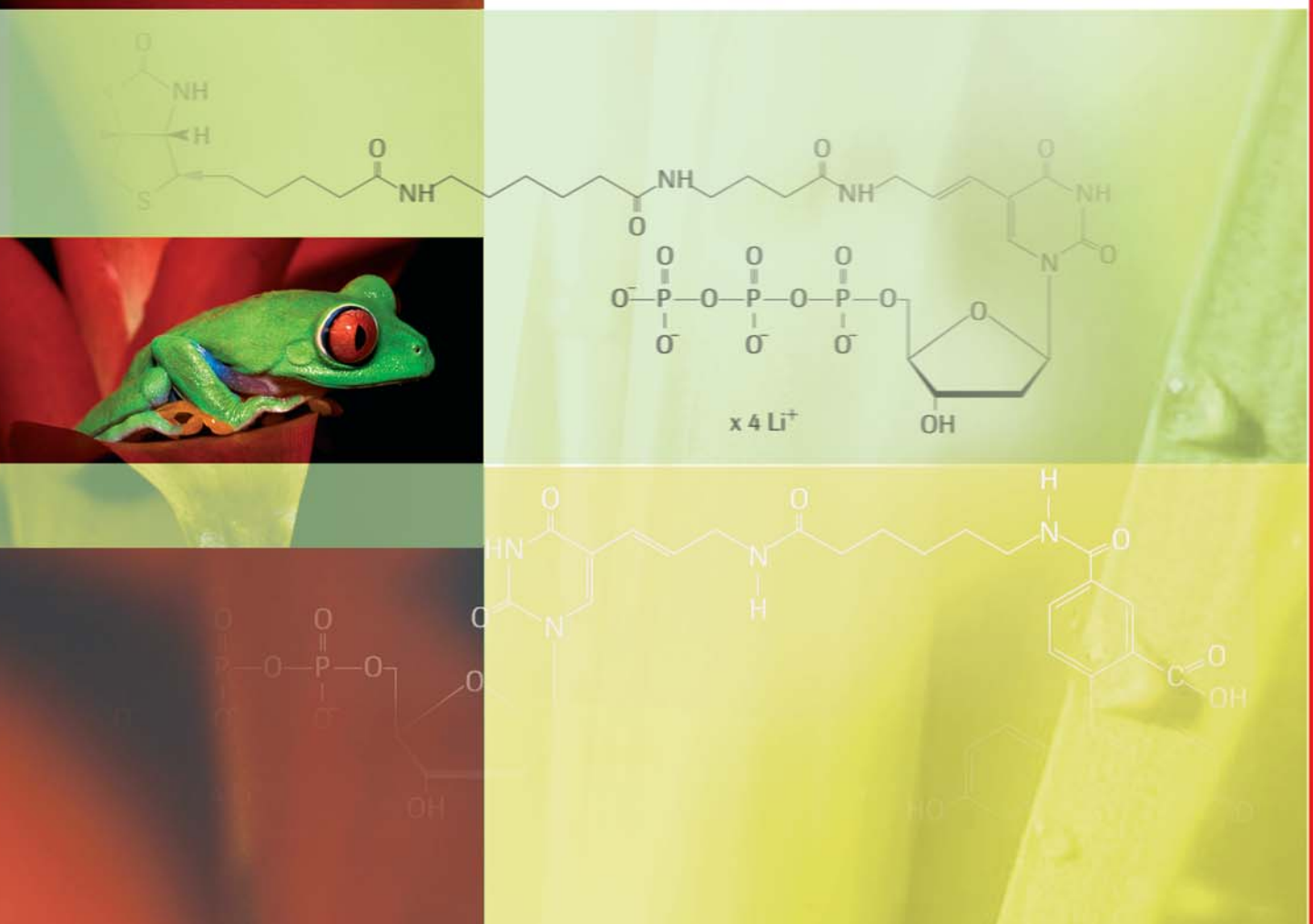

Nucleic Acid Hybridization – General Aspects

This chapter discusses the effects of various components of the hybridization solution on the rate of renaturation and thermal stability of DNA hybrids free in solution. The features will be more or less identical to those of immobilized nucleic acids, such as in filter and *in situ* hybridizations. The largest deviation probably occurs in the kinetics. The reader is referred to the following literature for more background information: Casey and Davidson (1976), Cox et al. (1984), Flavell et al. (1974), Hames and Higgins (1985), Maniatis et al. (1982), Raap et al. (1986), Schildkraut and Lifson (1965), Spiegelman et al. (1973), Wetmur and Davidson (1968), Wetmur (1975).

3

The main parameters that influence hybridization

Hybridization depends on the ability of denatured DNA to reanneal with complementary strands in an environment just below their melting point (T_m).

The T_m is the temperature at which half the DNA is present in a single-stranded (denatured) form. The T_m value is different for genomic DNA isolated from various organisms, *e.g.*, for *Pneumococcus* DNA it is 85°C, for *Serratia* DNA it is 94°C. The T_m can be calculated by measuring the absorption of ultraviolet light at 260 nm. The stability of the DNA is directly dependent on the GC content. The higher the molar ratio of GC pairs in a DNA, the higher the melting point.

T_m and renaturation of DNA are primarily influenced by four parameters:

- Temperature
- pH
- Concentration of monovalent cations
- Presence of organic solvents

Temperature

The maximum rate of renaturation (hybridization) of DNA is at 25°C. However, the bell-shaped curve relating renaturation rate and temperature is broad, with a rather flat maximum from about 16°C to 32°C below T_m.

pH

From pH 5 – 9, the rate of renaturation is fairly independent of pH. Buffers containing 20 – 50 mM phosphate, pH 6.5 – 7.5 are frequently used.

Higher pH can be used to produce more stringent hybridization conditions.

Monovalent cations

Monovalent cations (*e.g.*, sodium ions) interact electrostatically with nucleic acids (mainly at the phosphate groups) so that the electrostatic repulsion between the two strands of the duplex decreases with increasing salt concentration, *i.e.* higher salt concentrations increase the stability of the hybrid. Low sodium concentrations affect the T_m, as well as the renaturation rate, drastically.

Sodium ion (Na^+) concentrations above 0.4 M only slightly affect the rate of renaturation and the melting temperature.

The following equation has been given for the dependence of T_m on the GC content and the salt concentration (for salt concentrations from 0.01 to 0.20 M):

$$T_m = 16.6 \log M + 0.41 (GC) + 81.5$$

where M is the salt concentration (molar) and GC, the molar percentage of guanine plus cytosine. Above 0.4 M Na^+, the following formula holds:

$$T_m = 81.5 + 0.41 (GC)$$

Free divalent cations strongly stabilize duplex DNA. Remove them from the hybridization mixture or complex them (*e.g.*, with agents like citrate or EDTA).

Formamide

DNA melts (denatures) at 90°–100°C in 0.1–0.2 M Na^+. For *in situ* hybridization this implies that microscopic preparations must be hybridized at 65°–75°C for prolonged periods. This may lead to deterioration of morphology. Fortunately, organic solvents reduce the thermal stability of double-stranded polynucleotides, so that hybridization can be performed at lower temperatures in the presence of formamide.

Formamide has for years been the organic solvent of choice. It reduces the melting temperature of DNA-DNA and DNA-RNA duplexes in a linear fashion by 0.72°C for each percent formamide. Thus, hybridization can be performed at 30°–45°C with 50% formamide present in the hybridization mixture. The rate of renaturation decreases in the presence of formamide. The melting temperature of hybrids in the presence of formamide can be calculated according to the following equation:

For 0.01–0.2 M Na^+:

$$T_m = 16.6 \log M + 0.41 (GC) + 81.5 - 0.72 (\% \text{ formamide})$$

For Na^+ concentrations above 0.4 M:

$$T_m = 81.5 + 0.41 (GC) - 0.72 (\% \text{ formamide})$$

To obtain a large increase of *in situ* hybridization signal for rDNA, hybridize with rRNA in 80% formamide at 50°–55°C, instead of 70% formamide at 37°C.

Finally, it should be mentioned that during the *in situ* hybridization procedure, relatively large amounts of DNA can be lost (Raap et al., 1986).

3

Additional hybridization variables

Additional parameters must be considered when calculating the optimal hybridization conditions including the probe length, probe concentration, the inclusion of dextran sulfate, the extent of mismatch between probe and target, the washing conditions, and whether the probes will be single- or double-stranded.

Probe length

The rate of the renaturation of DNA in solution is proportional to the square root of the (single-stranded) fragment length. Consequently, maximal hybridization rates are obtained with long probes. However, short probes are required for *in situ* hybridization because the probe has to diffuse into the dense matrix of cells or chromosomes. The fragment length also influences thermal stability. The following formula, which relates the shortest fragment length in a duplex molecule to change in T_m, has been derived:

Change in $T_m \cdot n = 500$ (n = nucleotides).

Probe concentration

The probe concentration affects the rate at which the first few base pairs are formed (nucleation reaction). The adjacent base pairs are formed afterwards, provided they are in register (zippering). The nucleation reaction is the rate limiting step in hybridization. The kinetics of hybridization is considered to be a second order reaction [$r = k_2$ (DNA) (DNA)]. Therefore, the higher the concentration of the probe, the higher the reannealing rate.

Dextran sulfate

In aqueous solutions dextran sulfate is strongly hydrated. Thus, macromolecules have no access to the hydrating water, which causes an apparent increase in probe concentration and consequently higher hybridization rates.

Base mismatch

Mismatching of base pairs results in reduction of both hybridization rates and thermal stability of the resulting duplexes. To discriminate maximally between closely related DNA sequences, hybridize under fairly stringent conditions (*e.g.* at T_m -15°C). On the average, the T_m decreases about 1°C per % (base mismatch) for large probes. Mismatching in oligonucleotides greatly influences hybrid stability; this forms the basis of point mutation detection.

Stringency washes

During hybridization, duplexes form between perfectly matched sequences and between imperfectly matched sequences. The extent to which the latter occurs can be manipulated to some extent by varying the stringency of the hybridization reaction. (See above.)

To remove the background associated with nonspecific hybridization, wash the sample with a dilute solution of salt. The lower the salt concentration and the higher the wash temperature, the more stringent the wash.

In general, greater specificity is obtained when hybridization is performed at a high stringency and washing at similar or lower stringency, rather than hybridizing at low stringency and washing at high stringency.

Use of single-stranded versus double-stranded probes

A number of competing reactions occur during *in situ* hybridization with double-stranded probes. These include:

- Probe renaturation in solution
- *In situ* hybridization
- *In situ* renaturation (possibly, for ds targets)

Consequently, the use of single-stranded probes has advantages for *in situ* hybridization. Such probes can be made by using the single-stranded M13 (or like bacteriophage cloning vectors) as template, or by using transcription vectors which permit the production of large amounts of single-stranded RNA. (See Chapter 4 for a detailed description).

Competition *in situ* hybridization

Recombinant DNA isolated from eukaryotic DNA often contains genomic repetitive sequences (*e.g.*, the Alu sequence in humans). *In situ* hybridization to chromosomes with a probe which contains repetitive DNA usually results in uniform staining. However, unlabeled competitor DNA (usually total genomic DNA) prevents the repetitive probe sequences from annealing to the target, and leads to stronger *in situ* hybridization signals from the unique sequences in the probe. (This approach was first described for *in situ* hybridization by Landegent et al., 1987; Lichter et al., 1988a; Pinkel et al., 1988.) Obviously, the greater the complexity of probe (plasmids < phages < cosmids < yeast artificial chromosomes < chromosome libraries), the greater the need for competition *in situ* hybridization. This approach has proved particularly useful for *in situ* hybridization with DNA isolated from chromosome-specific libraries (CISS-hybridization); a specific chromosome can be fluorescently labeled over its full length (Lichter et al., 1988a,b; Cremer et al., 1988; Pinkel et al., 1988).

Oligonucleotide hybridization

The rules given for hybrid stability and kinetics of hybridization can probably not be extrapolated to hybridization with oligo-deoxynucleotides. For *in situ* hybridization, the advantages of oligonucleotides include their small size (good penetration properties) and their single-strandedness (to prevent probe reannealing, as outlined in Chapter 1).

The small size, however, is also a disadvantage because it covers less target. The nonradioactive label should be positioned at the 3' or the 5' end; internal labeling affects the T_m too much.

In an experiment with 20-mers of 40 – 60% GC content, start with the hybridization conditions described below. Depending on the results obtained, you may decide to use other stringency conditions.

3

Standard *in situ* hybridization conditions

Department of Cytochemistry and Cytometry, University of Leiden, Netherlands.

For "large" DNA probes (≥100 bp):

– 50% deionized formamide
– 2× SSC (see below)
– 50 mM NaH_2PO_4/Na_2HPO_4 buffer; pH 7.0
– 1 mM EDTA
– carrier DNA/RNA (1 mg/ml each)
– probe (approx. 20 – 200 ng/ml)

Optional components:

– 1× Denhardt's (see below)
– dextran sulfate, 5 – 10%
– Temperature: 37° – 42°C
– Hybridization time: 5 min – 16 h

For synthetic oligonucleotides:

– 25% formamide
– 4× SSC (see below)
– 50 mM NaH_2PO_4/Na_2HPO_4 buffer; pH 7.0
– 1 mM EDTA
– carrier DNA/RNA (1 mg/ml each)
– probe (approx. 20 – 200 ng/ml)
– 5× Denhardt's (see below)
– Temperature: room temperature
– Hybridization time: 2 – 16 h

Composition of SSC and Denhardt's solution

1× SSC: 150 mM NaCl, 15 mM sodium citrate; pH 7.0:
Make a 20x stock solution (3 M NaCl, 0.3 M sodium citrate).

50× Denhardt's:
1% polyvinylchloride, 1% pyrrolidone, 2% BSA.

References

Casey, J.; Davidson, N. (1976) Rates of formation and thermal stabilities of RNA-DNA and DNA-DNA duplexes at high concentrations of formamide. *Nucleic Acids Res.* **4**, 1539–1552.

Cox, K. H.; DeLeon, D. V.; Angerer, L. M.; Angerer, R. C. (1984) Detection of mRNAs in sea urchin embryos by *in situ* hybridization using asymmetric RNA probes. *Develop. Biol.* **101**, 485–503.

Cremer, T.; Lichter, P.; Borden, J.; Ward, D. C.; Manuelidis, L. (1988) Detection of chromosome aberrations in metaphase and interphase tumor cells by *in situ* hybridization using chromosome specific library probes. *Hum. Genet.* **80**, 235–246.

Flavell, R. A.; Birfelder, J. E.; Sanders, J. P. M., Borst, P. (1974) DNA-DNA hybridization on nitrocellulose filters. I. General considerations and non-ideal kinetics. *Eur. J. Biochem.* **47**, 535–543.

Hames, B. D.; Higgins, S. J. (1985) Nucleic Acid Hybridization: A Practical Approach. Oxford: IRL Press.

Landegent, J. E.; Jansen in de Wal; Dirks, R. W.; Baas, F.; van der Ploeg, M. (1987) Use of whole cosmid cloned genomic sequences for chromosomal localization by nonradioactive *in situ* hybridization. *Hum. Genet.* **77**, 366–370.

Lichter, P.; Cremer, T.; Borden, J.; Manuelidis, L.; Ward, D. C. (1988a) Delineation of individual human chromosomes in metaphase and interphase cells by *in situ* suppression hybridization using recombinant DNA libraries. *Hum. Genet.* **80**, 224–234.

Lichter, P.; Cremer, T.; Tang, C. C.; Watkins, P. C.; Manuelidis, L.; Ward, D. C. (1988b) Rapid detection of human chromosomes 21 aberrations by *in situ* hybridization. *Proc. Natl. Acad. Sci. USA* **85**, 9664–9668.

Maniatis, T.; Fritsch, E. F.; Sambrook, J. (1982) Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratories.

Pinkel, D.; Landegent, J.; Collins, C.; Fuscoe, J.; Segraves, R.; Lucas, J.; Gray, J. W. (1988) Fluorescence *in situ* hybridization with human chromosome specific libraries: detection of trisomy 21 and translocations of chromosome 4. *Proc. Natl. Acad. Sci. USA* **85**, 9138–9142.

Raap, A. K.; Marijnen, J. G. J.; Vrolijk, J.; Van der Ploeg, M. (1986) Denaturation, renaturation, and loss of DNA during *in situ* hybridization procedures. *Cytometry* **7**, 235–242.

Schildkraut, C.; Lifson, S. (1965) Dependence of the melting temperature of DNA on salt concentration. *Biopolymers* **3**, 195–208.

Spiegelman, G. B.; Haber, J. E.; Halvorson, H. O. (1973) Kinetics of ribonucleic acid-deoxyribonucleic acid membrane filter hybridization. *Biochemistry* **12**, 1234–1242.

Wetmur, J. G. (1975) Acceleration of DNA renaturation rates. *Biopolymers* **14**, 2517–2524.

Wetmur, J. G.; Davidson, N. (1986) Kinetics of renaturation of DNA. *J. Mol. Biol.* **31**, 349–370.

3

4

Procedures for Labeling DNA, RNA, and Oligonucleotides with DIG, Biotin, or Fluorochromes

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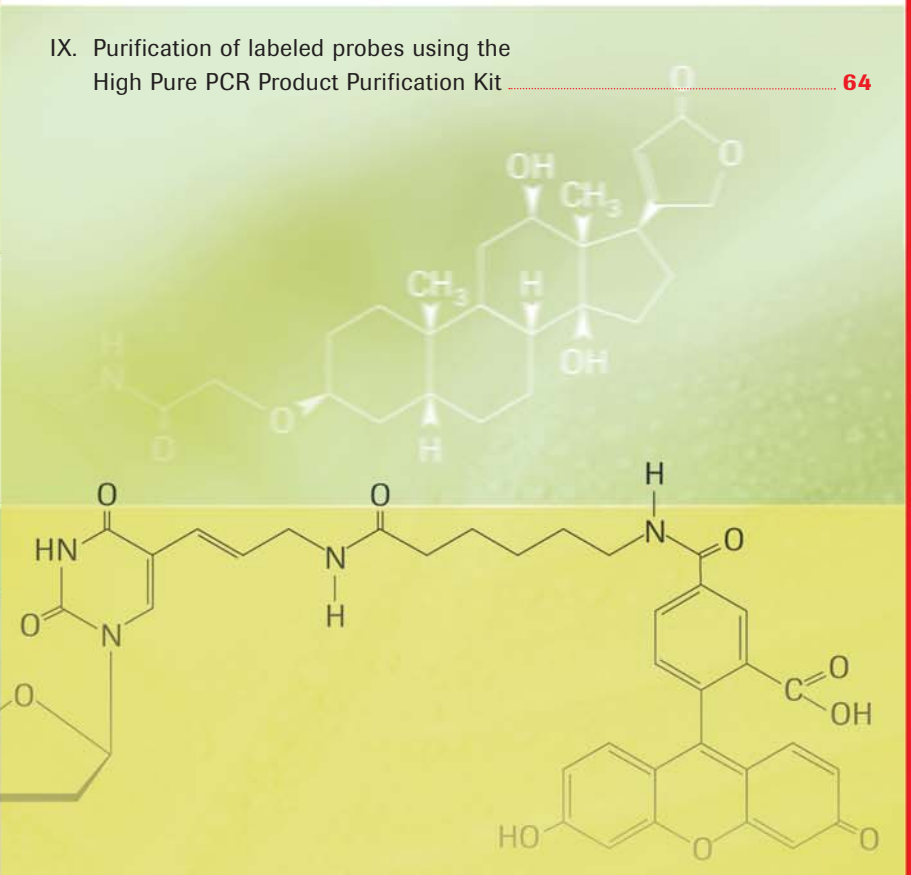

Procedures for Labeling DNA, RNA, and Oligonucleotides with DIG, Biotin, or Fluorochromes

Most of the following procedures have been taken from pack inserts of different kits available from Roche Applied Science. The exception is procedure IV, which is an optimized version of nick translation developed at the Department of Cytochemistry and Cytometry, University of Leiden, and the Department of Genetics, Yale University. The labeling procedures for digoxigenin, biotin, and fluorochromes are essentially identical. The determination of the labeling efficiency for DIG-labeled nucleic acids is described under IX "Estimating the Yield of DIG-labeled Nucleic Acids", page 59.

4

I. Random primed labeling of ds DNA with DIG-, Biotin- or Fluorescein-High Prime reaction mix

The random primed DNA labeling method (Feinberg and Vogelstein, 1984) allows efficient labeling of small (10 ng) and large (up to 3 µg) amounts of DNA. The labeling method also works with both short DNA (200 bp fragments) and long DNA (cosmid or λ DNA).

The standard labeling reaction is fast (1 h) and incorporates one modified nucleotide (DIG-, biotin-, or fluorescein-dUTP) at every 20–25th position in the newly synthesized DNA probe. This labeling density produces the most sensitive targets for indirect (immunological) detection.

The amount of newly synthesized labeled DNA depends on the amount and purity of the template DNA, the label used, and the incubation time (Table 1).

	DIG label		Fluorescein label		Biotin label	
Template DNA (ng)	Yield (ng) in 1 h	Yield (ng) in 20 h	Yield (ng) in 1 h	Yield (ng) in 20 h	Yield (ng) in 1 h	Yield (ng) in 20 h
10	45	600	30	250	70	850
30	130	1050	70	320	160	1350
100	270	1500	200	650	350	1650
300	450	2000	320	1350	700	2200
1000	850	2300	850	1700	1250	2600
3000	1350	2650	1200	2100	1600	2600

Table 1: Effect of template amount and labeling time on probe yield in the High Prime labeling reactions. Labeling reactions were performed with Biotin-, DIG-, or Fluorescein-High Prime labeling mix and varying amounts of purified template. The amounts of labeled DNA synthesized after a 1 h incubation and after a 20 h incubation were determined by the incorporation of a radioactive tracer and confirmed by a dot blot. Data shown for each label is the average of ten independent labeling assays. Experimental yield may vary from the above because of template purity, sequence, etc.

The size of the labeled DNA fragments obtained by random primed DNA labeling depends on the amount and size of the template DNA. With linear pBR328 plasmid DNA, the standard (1 h) reaction produces labeled DNA fragments which are approximately 200–1000 bp long.

Linear DNA is labeled more efficiently than circular and supercoiled DNA. In all cases the template DNA should be thoroughly heat-denatured prior to random primed labeling. DNA fragments in low melting point agarose are also labeled efficiently, but often with slower kinetics.

A. Standard labeling reaction for DNA in solution

1. To a 1.5 ml microcentrifuge tube, add 1 µg template DNA (linear or supercoiled) and sterile, double dist. water to a final volume of 16 µl.

   For this standard reaction, the amount of DNA in the 16 µl sample can vary from 10 ng to 3 µg of DNA. To label >3 µg of DNA, scale up the volume of the sample as well as the amounts and volumes of all reaction components below.

2. Denature the DNA by heating the tube in a boiling water bath for 10 min, then chilling **quickly** in an ice/water bath.

   Complete denaturation is essential for efficient labeling.

3. Mix thoroughly either DIG-High Prime or Biotin-High Prime or Fluorescein-High Prime and add 4 µl to the denatured DNA.

   Each High Prime reaction mix contains 5× concentrated reaction buffer; 50% glycerol; 1 U/µl Klenow enzyme, labeling grade; 5× concentrated random primer mix; 1 mM each of dATP, dCTP, and dGTP; 0.65 mM dTTP; and 0.35 mM X-dUTP [X = DIG (alkali-labile), biotin, or fluorescein].

4. Mix the reagents, then centrifuge briefly to collect the reaction mixture at the bottom of the tube.

5. Incubate the tube at least 1 h at 37°C.

   Longer incubations (up to 20 h) increase the yield of labeled DNA (Table 1).

6. To stop the reaction, add 2 µl 0.2 M EDTA (pH 8.0) to the reaction tube and/or heat the tube to 65°C for 10 min.

7. **Optional:** Precipitate the labeled probe by performing the following steps:

   As an alternative to the ethanol precipitation procedure below you may purify labeled probes which are 100 bp of longer with the High Pure PCR Product Purification Kit. See the procedure on page 65 in this chapter.

   - To the labeled DNA, add 2.5 µl 4 M LiCl and 75 µl prechilled (-15 to -25°C) 100% ethanol. Mix well.
   - Let the precipitate form for at least 30 min at -70°C or 2 h at -15 to -25°C.
   - Centrifuge the tube (at 13,000 × *g*) for 15 min at 2–8°C.
   - Discard the supernatant.
   - Wash the pellet with 50 µl ice-cold 70% (v/v) ethanol.
   - Centrifuge the tube (at 13,000 × *g*) for 5 min at 2–8°C.
   - Discard the supernatant.
   - Dry the pellet under vacuum.

     Drying the pellet is important because small traces of residual ethanol will cause precipitation if the hybridization mixture contains dextran sulfate. Trace ethanol can also lead to serious background problems.

   - Dissolve the pellet in a minimal amount of TE (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) buffer.

8. Do one of the following:

   - *If* you are not going to use the probe immediately, store the probe solution at -15 to -25°C.
   - *If* you are going to use the probe immediately, dilute an aliquot of the probe solution to a convenient stock concentration (*e.g.*, 10–40 ng/ml) in the hybridization buffer to be used for the *in situ* experiment (as described in Chapters 2 and 5 of this manual).

   The DIG-dUTP used in this procedure is alkali-labile. Avoid exposing DIG-labeled probes to strong alkali (e.g., 0.2 M NaOH). Standard in situ procedures should not detach the DIG label from the probe.

Reagents available from Roche Applied Science for this procedure

Reagent	Description	Cat. No.	Pack size
DIG-High Prime*,‡	5× solution with: 1 mM dATP, dCTP, dGTP (each); 0.65 mM dTTP; 0.35 mM DIG-11-dUTP, alkali-labile; random primer mixture; 1 U/µl Klenow enzyme, labeling grade, in reaction buffer, 50% glycerol (v/v).	11 585 606 910	160 µl (40 reactions)
Biotin-High Prime‡	5× solution with: 1 mM dATP, dCTP, dGTP (each); 0.65 mM dTTP; 0.35 mM biotin-16-dUTP; random primer mixture; 1 U/µl Klenow enzyme, labeling grade, in reaction buffer, 50% glycerol (v/v).	11 585 649 910	100 µl (25 reactions)
Fluorescein-High Prime‡	5× solution with: 1 mM dATP, dCTP, dGTP (each); 0.65 mM dTTP; 0.35 mM fluorescein-12-dUTP; random primer mixture; 1 U/µl Klenow enzyme, labeling grade, in reaction buffer, 50% glycerol (v/v).	11 585 622 910	100 µl (25 reactions)

‡ This product or the use of this product may be covered by one or more patents owned by Roche Diagnostics GmbH, including the following: US patent 5.814.502.

* The labeling of nucleic acids with DIG is covered by EP patents 0 324 474 and 0 371 262 as well as the following US patents 5.344.757, 5.354.657 and 5.702.888 owned by Roche Diagnostics GmbH.

4

II. PCR labeling of ds DNA with the PCR DIG Probe Synthesis Kit or PCR Labeling Mixes

The procedures given here are necessarily generalized. Optimal PCR reaction conditions are very dependent on the sequence of the template DNA and the primer. The optimal concentrations of template, primer, Mg^{2+} ion, and polymerase, as well as the optimal incubation times and temperatures should be determined empirically for each new primer/template combination (Innis et al., 1990, Rolfs, A. et al., 1992).

The three procedures in this section use PCR to produce probes that are directly labeled with digoxigenin (DIG) or fluorescein. The procedures will be especially useful for producing highly labeled probes from limited amounts of template DNA. Each procedure takes advantage of a premixed labeling solution or kit that has been optimized for the production of certain types of probes. They are:

- **PCR DIG Probe Synthesis Kit**, which contains a 2 + 1 ratio of dTTP:DIG-dUTP and is ideal for generating highly labeled hybridization probes containing unique sequences. Such probes can detect target sequences present at a low copy number in complex genomes.
- **PCR DIG Labeling Mix**, which contains a 19 + 1 ratio of dTTP:DIG-dUTP and is ideal for generating moderately labeled hybridization probes containing repetitive elements. Such probes can detect target sequences (*e.g.*, human alphoid sequences) which are present at a high copy number in complex genomes.
- **PCR Fluorescein Labeling Mix**, which contains a 3 + 1 ratio of dTTP:fluorescein-dUTP and produces optimally labeled hybridization probes suitable for direct *in situ* detection experiments.

A. PCR DIG labeling reaction for highly labeled probes containing unique sequences

The following labeling reaction produces 50 µl of DIG-labeled probe solution. In a control experiment, 50 µl of DIG-labeled probe was enough to perform 25 hybridization reactions.

To produce less probe, scale the reaction volume and components down proportionally.

1 Place a sterile microcentrifuge tube on ice and, for each PCR, add to the tube:

- 5 µl 10× concentrated PCR buffer with 15 mM $MgCl_2$ (vial 3).
  - Numbered vials are included in the PCR DIG Probe Synthesis Kit.
- 5 µl 10× concentrated PCR DIG probe synthesis mix, containing a 2:1 ratio of dTTP:DIG-dUTP (2 mM each of dATP, dCTP, and dGTP; 1.3 mM dTTP; 0.7 mM DIG-dUTP, alkali-labile; pH 7.0) (vial 2).
- Upstream primer 1–10 µM solution (0.1–1 µM final conc.).
- Downstraeam primer 1–10 µM solution (0.1–1 µM final conc.).
  - *The concentration of PCR primers must be determined empirically (Innis et al.; 1990). Initially, try a 0.3 mM concentration of each primer in the reaction mixture.*
- Template DNA:
  Plasmid DNA 10–100 pg (optimal amount, 10 pg).
  Genomic DNA 1–50 ng (optimal amount, 10 ng).
- 0.75 µl (2.6 U) Enzyme mix,Expand High Fidelity (vial 1).
- Add sterile double dist. water to a final reaction volume of 50 µl.

2 Mix reagents and centrifuge briefly to collect the sample at the bottom of the tube.

3 Overlay with 100 µl mineral oil to reduce evaporation of the mix during amplification.

If your thermal cycler has a top heater, the oil overlay is not necessary.

4 Place samples in a thermal cycler and start PCR.

Cycling conditions depend on the combination of template, primers and thermal cycler. The conditions below may not be optimal for your template/primer combination, but are a good starting point for initial experiments.

Use the following thermal profile:
Initial denaturation 2 min at 95°C, 30 s

1–10 cycles of:

Denaturation,	95°C, 30 s
Annealing,	60°C, 30 s
Elongation,	72°C, 40 s

11–33 cycles of:

Denaturation,	95°C, 30 s
Annealing,	60°C, 30 s
Elongation,	72°C, 40 s + additional 20 s for each successive cycle

The increased elongation time is only required for long (3 kb) fragments. For amplification of shorter fragments, continue to use the 40 s elongation time for all 30 cycles.

Final elongation step, 72°C, 7 min

Hold, 4°C

5 Check PCR labeleed probe via agarose gel (load an aliquot, *e.g.*, 5 µl) and compare to PCR product without DIG label.

6 The PCR labeled probe should be stored:

- Short term, at 2 – 8°C until the PCR product is used for hybridization
- Long term, at -15 to -25°C, stable for at least one year.

4

7 **Optional:** Precipitate the labeled probe by performing the following steps:

As an alternative to the ethanol precipitation procedure below, you may purify labeled probes which are 100 bp or longer with the High Pure PCR Product Purification Kit. See the procedure on page 65 in this chapter.

- To the labeled DNA, add 5 µl 4 M LiCl and 150 µl prechilled (-15 to -25°C) 100% ethanol. Mix well.
- Let the precipitate form for at least 30 min at -70°C or 2 h at -15 to -25°C.
- Centrifuge the tube (at 13,000 × *g*) for 15 min at 2 – 8°C.
- Discard the supernatant.
- Wash the pellet with 100 µl ice-cold 70% (v/v) ethanol.
- Centrifuge the tube (at 13,000 × *g*) for 5 min at 2 – 8°C.
- Discard the supernatant.
- Dry the pellet under vacuum.

  Drying the pellet is important because small traces of residual ethanol will cause precipitation if the hybridization mixture contains dextran sulfate. Trace ethanol can also lead to serious background problems.

- Dissolve the pellet in 50 µl TE (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) buffer.

  If the hybridization buffer (used in Step 7 below) contains a high percentage formamide, dissolve the probe pellet in a smaller amount of TE buffer to form a more concentrated probe stock solution.

8 Do one of the following:

- *If* you are not going to use the probe immediately, store the probe solution at -15 to -25°C.

  Avoid repeated freezing and thawing of the probe.

- *If* you are going to use the probe immediately, dilute an aliquot of the probe solution in the hybridization buffer to be used for the *in situ* experiment (as described in Chapters 2 and 5 of this manual).

  The amount of probe solution to use in the hybridization reaction must be determined empirically. Initially, try using 2 µl of probe solution (out of the original 50 µl total) in 20 µl hybridization solution for each hybridization reaction (under a 24 × 24 mm coverslip). If the probe was dissolved in <50 µl TE (in Step 6 above), add correspondingly less of the concentrated probe stock to the hybridization buffer.

  The DIG-dUTP used in this labeling reaction is alkali-labile. Avoid exposing the probe to strong alkali (e.g., 0.2 M NaOH). Standard in situ procedures should not detach the DIG label from the probe.

B. PCR DIG labeling reaction for moderately labeled probes

This labeling reaction produces 100 µl of DIG-labeled probe solution. In a control experiment, 100 µl of DIG-labeled probe was enough to perform 20 – 50 indirect *in situ* detections of repetitive (human alphoid) sequences in metaphase chromosomes. To produce less probe, scale the reaction volume and components down proportionally.

1 Briefly centrifuge all reagents before starting.

2 Prepare two mixes of reagents in sterile microcentrifuge tubes (on ice):

MIX 1 (final volume 25 µl)

- PCR DIG Labeling Mix 10×, containing a 19:1 ratio of dTTP:DIG-dUTP (2 mM each)
- Upstream primer 0.1 – 0.6 µM
- Dowmstream Primer 0.1 – 0.6 µM
- Template DNA:

  Plasmid DNA 0.1 ng – 15 ng.

  Human genomic DNA 10 – 250 ng.

  The optional buffer for template DNA is either sterile double dist. water or 5 – 10 mM Tris (ph 7 – 8). Avoid TE -buffer, because EDTA chelates Mg^{2+}.

MIX 2 (final volume 25 µl)

- 19.75 µl sterile double dist.water
- 5 µl PCR reaction buffer 10×, with 15 mM $MgCl_2$ (delivered with the Taq DNA Polymerase)
- 0.25 µl Taq DNA Polymerase

3 Combine Mix 1 and Mix 2 in a thin-walled PCR tube (on ice).

Gently vortex the mixture to produce a homogeneous reaction, then centrifuge briefly to collect the sample at the bottom of the tube.

Continue to thermal cycling immediatly.

Carefull overlay the reaction with mineral oil if required by your type of thermal cycler.

4 Thermal cycling

Place samples in the thermal cycler and start cycling using the thermal profiles mentioned in the Taq DNA Polymerase pack insert (Cat. No. 1 146 165 001) or also available at

http://www.roche-applied-science.com/pack-insert/1146165a.pdf .

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5 **Optional:** Precipitate the labeled probe by performing the following steps:

As an alternative to the ethanol precipitation procedure below, you may purify labeled probes which are 100 bp or longer with the High Pure PCR Product Purification Kit. See the procedure on page 64 in this chapter.

- To the labeled DNA, add 10 µl 4 M LiCl and 300 µl prechilled (-15 to -25°C) 1 00% ethanol. Mix well.
- Let the precipitate form for at least 30 min at -70°C or 2 h at -15 to -25°C.
- Centrifuge the tube (at 13,000 × *g*) for 15 min at 2 – 8°C.
- Discard the supernatant.
- Wash the pellet with 100 µl ice-cold 70% (v/v) ethanol.
- Centrifuge the tube (at 13,000 × *g*) for 5 min at 2 – 8°C.
- Discard the supernatant.
- Dry the pellet under vacuum.

  Drying the pellet is important because small traces of residual ethanol will cause precipitation if the hybridization mixture contains dextran sulfate. Trace ethanol can also lead to serious background problems.

- Dissolve the pellet in 100 µl TE (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) buffer.

  If the hybridization buffer (used in Step 6 below) contains a high percentage formamide, dissolve the probe pellet in a smaller amount of TE buffer to form a more concentrated probe stock solution.

6 Do one of the following:

- *If* you are not going to use the probe immediately, store the probe solution at -15 to -25°C for at least 1 year.

  Avoid repeated freezing and thawing of the probe.

- *If* you are going to use the probe immediately, dilute an aliquot of the probe solution in the hybridization buffer to be used for the *in situ* experiment (as described in Chapters 2 and 5 of this manual).

  The amount of probe solution to use in the hybridization reaction must be determined empirically. Initially, try using 2 – 5 µl of probe solution (out of the original 100 µl total) in 20 µl hybridization solution for each hybridization reaction (under a 24 × 24 mm coverslip). If the probe was dissolved in <100 µl TE (in Step 3 above), add correspondingly less of the concentrated probe stock to the hybridization buffer.

C. PCR fluorescein labeling reaction for direct *in situ* probes

This labeling reaction produces 100 µl of fluorescein-labeled probe solution. In a control experiment, 100 µl of fluorescein-labeled probe was enough to perform 20 – 50 direct *in situ* detections of repetitive (human alphoid) sequences in metaphase chromosomes. To produce less probe, scale the reaction volume and components down proportionally.

1 Add the following components to a sterile 1.5 ml microcentrifuge tube on ice:

- 10 µl 10× concentrated PCR buffer without $MgCl_2$ (100 mM Tris-HCl, 500 mM KCl, pH 8.3) (vial 2).

  Numbered vials are included with the PCR Fluorescein Labeling Mix.

- 12 – 20 µl 25 mM $MgCl_2$ (vial 3).

  The concentration of $MgCl_2$ must be determined empirically. We use 4 mM $MgCl_2$ (16 µl vial 3) as our standard concentration.

- 10 µl 10× concentrated PCR Fluorescein Labeling Mix (2 mM each of dATP, dCTP, and dGTP; 1.5 mM dTTP; 0.5 mM fluorescein-dUTP; pH 7.0) (vial 1).
- 0.1 – 1.0 µM PCR primer 1.
- 0.1 – 1.0 µM PCR primer 2.

  The concentration of PCR primers must be determined empirically (Innis et al., 1990). Initially, try a 0.3 mM concentration of each primer in the reaction mixture.

- Template DNA (1 – 100 ng human genomic DNA or 10 – 100 pg plasmid DNA).

  The amount of template DNA must be determined empirically (Innis et al., 1990). Initially, try 50 ng human genomic DNA or 50 pg plasmid DNA.

- 1 – 5 U Taq DNA Polymerase.

  The amount of polymerase must be determined empirically. As a starting amount, try 2.5 units.

- Add sterile, double dist. water to a total volume of 100 µl.

2 Mix the reagents, overlay with oil, and perform PCR exactly as in Steps 2 – 5 of Procedure IIA above (for labeling of probes containing unique sequences).

3 **Optional:** Precipitate the labeled probe by performing the following steps:

As an alternative to the ethanol precipitation procedure below, you may purify labeled probes which are 100 bp or longer with the High Pure PCR Product Purification Kit. See the procedure on page 65 in this chapter.

- To the labeled DNA, add 10 µl 4 M LiCl and 300 µl prechilled (-15 to -25°C) 100% ethanol. Mix well.
- Let the precipitate form for at least 30 min at -70°C or 2 h at -15 to -25°C.
- Centrifuge the tube (at 13,000 × *g*) for 15 min at 2 – 8°C.
- Discard the supernatant.
- Wash the pellet with 100 µl ice-cold 70% (v/v) ethanol.
- Centrifuge the tube (at 13,000 × *g*) for 5 min at 2 – 8°C.
- Discard the supernatant.
- Dry the pellet under vacuum.

  Drying the pellet is important because small traces of residual ethanol will cause precipitation if the hybridization mixture contains dextran sulfate. Trace ethanol can also lead to serious background problems.

- Dissolve the pellet in 100 µl TE (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) buffer.

  If the hybridization buffer (used in Step 4 below) contains a high percentage formamide, dissolve the probe pellet in a smaller amount of TE buffer to form a more concentrated probe stock solution.

4 Do one of the following:

- *If* you are not going to use the probe immediately, store the probe solution at -15 to -25°C.

  ⚠ *Avoid repeated freezing and thawing of the probe.*

- *If* you are going to use the probe immediately, dilute an aliquot of the probe solution in the hybridization buffer to be used for the *in situ* experiment (as described in Chapters 2 and 5 of this manual).

  ! *The amount of probe solution to use in the hybridization reaction must be determined empirically. Initially, try using 2–5 µl of probe solution (out of the original 100 µl total) in 20 µl hybridization solution for each hybridization reaction (under a 24 × 24 mm coverslip). If the probe was dissolved in <100 µl TE (in Step 3 above), add correspondingly less of the concentrated probe stock to the hybridization buffer.*

Reagents available from Roche Applied Science for these procedures

Reagent	Description	Cat. No.	Pack size
PCR DIG Probe Synthesis Kit*	Kit for 25 labeling reactions which incorporate alkali-labile DIG-dUTP by PCR	11 636 090 910	1 kit (25 reactions)
PCR DIG Labeling Mix*	dATP, dCTP, dGTP, 2 mM each; dTTP 1.9 mM; alkali-stable DIG-dUTP, 0.1 mM; pH 7.0	11 585 550 910	500 µl (for 2 × 25 PCR reactions)
PCR Fluorescein Labeling Mix (for rapid and reliable synthesis of fluorescein-dUTP labeled probes that are particular useful for fluorescence in situ hybridization [FISH])	Contents: dATP, dCTP, dGTP 2 mM each; dTTP, 1.5 mM; fluorescein-dUTP, 0.5 mM; premixed in 100 µl water; ph 7.0 10× PCR buffer without $MgCl_2$ 25 mM $MgCl_2$ stock solution	11 636 154 910	for 10 PCR reactions
Taq DNA Polymerase, 5 U/µl#	PCR buffer included	11 146 165 001 11 146 173 001 11 418 432 001 11 596 594 001 11 435 094 001	100 units 500 units 4 × 250 units 10 × 250 units 20 × 250 units
Taq DNA Polymerase 1 U/µl#	PCR buffer included	11 647 679 001 11 647 687 001	250 units 4 × 250 units

* The labeling of nucleic acids with DIG is covered by EP patents 0 324 474 and 0 371 262 as well as the following US patents 5.344.757, 5.354.657 and 5.702.888 owned by Roche Diagnostics GmbH.

\# Use of this product is covered by one or more of the following US patents and corresponding patent claims outside the US: 5,079,352, 5,789,224, 5,618,711, 6,127,155 and claims outside the US corresponding to US Patent No. 4,889,818. The purchase of this product includes a limited, non-transferable immunity from suit under the foregoing patent claims for using only this amount of product for the purchaser's own internal research. No right under any other patent claim (such as the patented 5' Nuclease Process claims in US Patents Nos. 5,210,015 and 5,487,972), no right to perform any patented method, and no right to perform commercial services of any kind, including without limitation reporting the results of purchaser's activities for a fee or other commercial consideration, is conveyed expressly, by implication, or by estoppel. This product is for research use only. Diagnostic uses under Roche patents require a separate license from Roche. Further information on purchasing licenses may be obtained by contacting the Director of Licensing, Applied Biosystems, 850 Lincoln Centre Drive, Foster City, California 94404, USA.

III. Nick-translation labeling of ds DNA with Nick Translation Mixes for *in situ* Probes

The nick translation procedure was originally described by Rigby et al. (1977) and used for incorporating nucleotide analogs by Langer et al. (1981). The procedure described here incorporates one modified nucleotide (DIG-, biotin-, fluorescein-, AMCA-, or tetramethylrhodamine-dUTP) at approximately every 20–25th position in the newly synthesized DNA. This labeling density allows optimal enzymatic incorporation of the modified nucleotide and produces the most sensitive targets for indirect (immunological) detection.

For *in situ* hybridization procedures, the length of the labeled fragments obtained from this procedure should be about 200–500 bases.

DNA does not need to be denatured before it is labeled by nick translation.

A. Labeling reaction with DIG-dUTP or Biotin-dUTP

1. Place a 1.5 ml microcentrifuge tube on ice and add to the tube:
   - 16 µl sterile double dist. water containing 1 µg template DNA (either linear or supercoiled).
   - 4 µl of *either* DIG-Nick Translation Mix for *in situ* Probes *or* Biotin-Nick Translation Mix for *in situ* Probe.

     Each Nick Translation Mix contains 5× concentrated reaction buffer; 50% glycerol; DNA Polymerase I; DNase I; 0.25 mM each of dATP, dCTP, and dGTP; 0.17 mM dTTP; and 0.08 mM X-dUTP (X = DIG or biotin).
2. Mix ingredients and centrifuge tube briefly.
3. Incubate at 15°C for 90 min.
4. Chill the reaction tube to 0°C.
5. Take a 3 µl aliquot from the tube and analyze it as follows:
   - Mix the aliquot with enough gel loading buffer to make a sample which will fit in one well of an agarose minigel.
   - Denature the sample (DNA aliquot + gel loading buffer) for 3 min at 95°C.
   - Place the denatured sample on ice for 3 min.
   - Run the sample on an agarose minigel with a DNA molecular weight marker.
6. Depending on the average size of the probe, do one of the following:
   - *If* the probe is between 200 and 500 nucleotides long, go to Step 7.
   - *If* the probe is longer than 500 nucleotides, incubate the reaction tube further at 15°C until the fragments are the proper size.

     If the fragment is too long, the labeled probe can also be sonicated to the proper size.
7. Stop the reaction as follows:
   - Add 1 µl 0.5 M EDTA (pH 8.0) to the tube.
   - Heat the tube to 65°C for 10 min.

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B. Labeling reaction with Fluorescein-dUTP, or Tetramethylrhodamine-dUTP

1. Prepare 50 µl of a 5x fluorophore labeling mixture (enough for about 12 labeling reactions) by mixing the following in a 1.5 ml microcentrifuge tube on ice:
   - 5 µl 2.5 mM dATP.
   - 5 µl 2.5 mM dCTP.
   - 5 µl 2.5 mM dGTP.
   - 3.4 µl 2.5 mM dTTP.
   - 4 µl of either 1 mM Fluorescein-dUTP *or* 1 mM Tetramethylrhodamine-dUTP.
   - 27.6 µl sterile double dist. water.

2. Place a 1.5 ml microcentrifuge tube on ice and add to the tube:
   - 12 µl sterile double dist. water containing 1 µg template DNA (either linear or supercoiled).
   - 4 µl 5× concentrated fluorophore labeling mixture (from Step 1).
   - 4 µl of Nick Translation Mix for *in situ* Probes.

     Nick Translation Mix contains 5× concentrated reaction buffer; 50% glycerol; DNA Polymerase I; and DNase I.

3. Mix, incubate and stop the reaction as in Steps 2 – 7 for DIG- and biotin-labeling above.

■

C. Purification of labeled probe (optional)

1. Precipitate the labeled probe (from either procedure above) by performing the following steps. *As alternative to the ethanol precipitation procedure below, you may purify labeled probes which are 100 bp or longer with the High Pure PCR Product Purification Kit. See the procedure on page 64 in this chapter.*
   - To the labeled DNA, add 2.5 µl 4 M LiCl and 75 µl prechilled (-15 to -25°C) 100% ethanol. Mix well.
   - Let the precipitate form for at least 30 min at -70°C or 2 h at -15 to -25°C.
   - Centrifuge the tube (at 13,000 × *g*) for 15 min at 2 – 8°C.
   - Discard the supernatant.
   - Wash the pellet with 50 µl ice-cold 70% (v/v) ethanol.
   - Centrifuge the tube (at 13,000 × *g*) for 5 min at 2 – 8°C.
   - Discard the supernatant.
   - Dry the pellet under vacuum.

     Drying the pellet is important because small traces of residual ethanol will cause precipitation if the hybridization mixture contains dextran sulfate. Trace ethanol can also lead to serious background problems.

2. Do one of the following:
   - *If* you are not going to use the probe immediately, dissolve the pellet in a minimal amount of TE (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) buffer and store the probe solution at -15 to -25°C.

     Avoid repeated freezing and thawing of the probe.

   - *If* you are going to use the probe immediately, dissolve the pellet in a minimal amount of an appropriate buffer (TE, sodium phosphate, etc.), then dilute the probe solution to a convenient stock concentration (*e.g.*, 10 – 40 ng/µl) in the hybridization buffer to be used for the *in situ* experiment (as described in Chapters 2 and 5 of this manual).

■

Reagents available from Roche Applied Science for this procedure

Reagent	Description	Cat. No.	Pack size
DIG-Nick Translation Mix*	5× conc. stabilized reaction buffer in 50% glycerol (v/v) and DNA Polymerase I, DNase I, 0.25 mM dATP, 0.25 mM dCTP, 0.25 mM dGTP, 0.17 mM dTTP and 0.08 mM alkali-stable DIG-11-dUTP.	11 745 816 910	160 µl (40 labeling reactions)
Biotin-Nick Translation Mix*	5× conc. stabilized reaction buffer in 50% glycerol (v/v) and DNA Polymerase I, DNase I, 0.25 mM dATP, 0.25 mM dCTP, 0.25 mM dGTP, 0.17 mM dTTP and 0.08 mM biotin-16-dUTP.	11 745 824 910	160 µl (40 labeling reactions)
Nick Translation Mix*	5× conc. stabilized reaction buffer in 50% glycerol, DNA Polymerase I and DNase I.	11 745 808 910	200 µl (50 labeling reactions)
dNTP Set	Set of dATP, dCTP, dGTP, dTTP 100 mM solutions, lithium salts.	11 277 049 001	4 × 10 µmol (100 µl)
Fluorescein-12-dUTP	Tetralithium salt, 1 mM solution	11 373 242 910	25 nmol (25 µl)
Tetramethyl-rhodamine-5-dUTP	Tetralithium salt, 1 mM solution	11 534 378 910	25 nmol (25 µl)

* The labeling of nucleic acids with DIG is covered by EP patents 0 324 474 and 0 371 262 as well as the following US patents 5.344.757, 5.354.657 and 5.702.888 owned by Roche Diagnostics GmbH.

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IV. Nick-translation labeling of ds DNA with DIG-, Biotin-, or Fluorochrome-labeled dUTP

Department of Cytochemistry and Cytometry, University of Leiden, Netherlands, and Department of Genetics, Yale University, U.S.A.

For in situ hybridization procedures, the length of the labeled fragments obtained from this procedure should be about 200 – 400 bases.

1. Place a 1.5 ml microcentrifuge tube on ice and add to the tube:
   - 27 µl sterile,double dist. water.
   - 5 µl 10× concentrated nick translation buffer [500 mM Tris-HCl (pH 7.8), 50 mM $MgCl_2$, 0.5 mg/ml Bovine Serum Albumin (nuclease-free)].
   - 5 µl 100 mM Dithiothreitol.
   - 4 µl Nucleotide Mixture (0.5 mM each of dATP, dGTP, and dCTP; 0.1 mM dTTP).
   - 2 µl 1 mM DIG-dUTP *or* 1 mM Biotin-dUTP *or* 1 mM fluorochrome-labeled dUTP.
   - 1 µg template DNA.
   - 5 µl (5 ng) DNase I.
   - 1 µl (10 U) DNA Polymerase I.
2. Mix ingredients and centrifuge tube briefly.
3. Incubate at 15°C for 2 h.
4. Chill the reaction tube to 0°C.

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5 Take a 3 µl aliquot from the tube and run the sample on an agarose minigel with a DNA Molecular Weight Marker.

6 Depending on the average size of the probe, do one of the following:

- *If* the probe is between 200 and 400 nucleotides long, go to Step 7.
- *If* the probe is longer than 400 nucleotides, incubate the reaction tube further at 15°C until the fragments are the proper size.

  If the fragment is too long, the labeled probe can also be sonicated to the proper size.

7 Stop the reaction as follows:

- To the tube, add carrier DNA (*e.g.*, 50-fold excess of fragmented herring sperm DNA) and carrier RNA (*e.g.*, 50-fold excess of yeast tRNA).
- Add 0.1 volume 3 M sodium acetate, pH 5.6.
- Add 2.5 volumes prechilled (-20°C) 100% ethanol.
- Mix reagents well.

  Do **not** *use a pipette tip to mix.*

- Place tube on ice for 30 min.
- Centrifuge tube for 30 min in a microcentrifuge at 2–8°C.
- Discard the supernatant.
- Dry the pellet under vacuum.

  Drying the pellet is important because small traces of residual ethanol will cause precipitation if the hybridization mixture contains dextran sulfate. Trace ethanol can also lead to serious background problems.

8 Do one of the following:

- *If* you are not going to use the probe immediately, dissolve the pellet in a minimal amount of TE (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) buffer and store the probe solution at -15 to -25°C.

  Avoid repeated freezing and thawing of the probe.

- *If* you are going to use the probe immediately, dissolve the pellet in a minimal amount of an appropriate buffer (TE, sodium phosphate, etc.), then dilute an aliquot of the probe solution to a convenient stock concentration (*e.g.*, 10–40 ng/µl) in the hybridization buffer to be used for the *in situ* experiment (as described in Chapters 2 and 5 of this manual).

Reagents available from Roche Applied Science for this procedure

Reagent	Description	Cat. No.	Pack size
Digoxigenin-11-dUTP, alkali-stable*	Tetralithium salt, 1 mM solution	11 093 088 910	25 nmol (25 µl)
		11 558 706 910	125 nmol (125 µl)
		11 570 013 910	5 × 125 nmol (5 × 125 µl)
Fluorescein-12-dUTP	Tetralithium salt, 1 mM solution	11 373 242 910	25 nmol (25 µl)
Tetramethylrhodamine-5-dUTP	Tetralithium salt, 1 mM solution	11 534 378 910	25 nmol (25 µl)
Biotin-16-dUTP	Tetralithium salt, 1 mM solution	11 093 070 910	50 nmol (50 µl)
DNase I	Grade II, lyophylizate	10 104 159 001	100 mg
DNA Polymerase I	Endonuclease-free	10 642 711 001	250 U
		10 642 720 001	1000 U

* EP Patent 0371262 and US 5,198,537 owned by Roche Diagnostics GmbH.

V. RNA labeling by *in vitro* transcription of DNA with DIG, Biotin or Fluorescein RNA Labeling Mix

The DNA to be transcribed should be cloned into the polylinker site of a transcription vector which contains a promoter for SP6, T7, or T3 RNA Polymerase (Dunn and Studier, 1983; Kassavetis, 1982). To synthesize "run-off" transcripts, use a restriction enzyme that creates a 5'-overhang to linearize the template before transcription. Alternatively, use the circular vector DNA as template to create "run-around" transcripts.

A PCR fragment that has the appropriate promoter ligated to its 5'-ends can also serve as a transcription template.

The procedure described here incorporates one modified nucleotide (DIG-, Biotin-, or Fluorescein-UTP) at approximately every 20 – 25th position in the transcripts. Since the nucleotide concentration does not become limiting in the following reaction, 1 µg linear plasmid DNA (with a 1 kb insert) can produce approximately 10 µg of full-length labeled RNA transcript in a 2 h incubation. Larger amounts of labeled RNA can be synthesized by scaling up the reaction components.

The amount of newly synthesized labeled RNA depends on the amount, size, site of linearization, and purity of the template DNA.

4

1 Purify template DNA in one of the following ways:

- Use phenol/chloroform extraction and ethanol precipitation to purify *linearized plasmid* DNA after the linearizing restriction digestion. Resuspend the pellet in 10 mM Tris-HCl, pH 8.0.
- Ethanol precipitate *circular plasmid* DNA and resuspend it in 10 mM Tris-HCl, pH 8.0.
- Use acrylamide gel electrophoresis and elution (in 10 mM Tris-HCl, pH 8.0) to purify a PCR *fragment* which has an RNA polymerase promoter ligated to its 5'-ends.

2 Add the following to a 1.5 ml microcentrifuge tube on ice:

- 1 µg purified, linearized plasmid DNA *or* 1 µg purified, circular plasmid DNA *or* 100 – 200 ng purified PCR fragment.
- 2 µl of *either* 10× concentrated DIG RNA Labeling Mix *or* 10× concentrated Biotin RNA Labeling Mix *or* 10× concentrated Fluorescein RNA Labeling Mix.

  Concentrated RNA Labeling Mix contains 10 mM each of ATP, CTP, and GTP; 6.5 mM UTP; 3.5 mM X-UTP (X = DIG, Biotin or Fluorescein); pH 7.5 (20°C)].
- 2 µl 10× concentrated Transcription Buffer [400 mM Tris-HCl (pH 8.0, 20°C), 60 mM $MgCl_2$, 100 mM Dithiothreitol (DTT), 20 mM spermidine].

  10x concentrated Transcription Buffer is supplied with SP6, T3, or T7 RNA Polymerase.
- 2 µl RNA Polymerase (SP6, T7, or T3).
- Enough sterile, redistilled water to make a total reaction volume of 20 µl.

3 Mix the components and centrifuge the tube briefly.

4 Incubate the tube for 2 h at 37°C.

Longer incubations do not increase the yield of labeled RNA. To produce larger amounts of RNA, scale up the reaction components.

4

5 Do one of the following:

- **Optional:** *If* you want to remove the template DNA, add 2 U DNase I, RNase-free to the tube and incubate for 15 min at 37°C. Then, go to Step 6.
- *If* you do not want to remove the template DNA, go to Step 6.

  Since the amount of labeled RNA transcript is far in excess of the template DNA (by a factor of approx. 10), it is usually not necessary to remove the template DNA by DNase treatment before an in situ hybridization experiment.

6 Add 2 µl 0.2 M EDTA (pH 8.0) to the tube to stop the polymerase reaction.

7 **Optional:** Precipitate the labeled RNA transcript by performing the following steps.

As an alternative to the ethanol procedure below you may purify labeled probes which are 100 bp or longer with the High Pure PCR Purification Kit. See the procedure on page 47 in this chapter.

- To the reaction tube, add 2.5 µl 4 M LiCl and 75 µl prechilled (-15 to -25°C) 100% ethanol. Mix well.
- Let the precipitate form for at least 30 min at -70°C or 2 h at -15 to -25°C.
- Centrifuge the tube (at 13,000 × *g*) for 15 min at 2–8°C.
- Discard the supernatant.
- Wash the pellet with 50 µl ice-cold 70% (v/v) ethanol.
- Centrifuge the tube (at 13,000 × *g*) for 5 min at 2–8°C.
- Discard the supernatant.
- Dry the pellet under vacuum.
- Dissolve the RNA pellet for 30 min at 37°C in 100 µl DEPC (diethylpyrocarbonate)-treated (or sterile) double dist. water.

8 To estimate the yield of the transcript, do the following:

- Run an aliquot of the transcript on an agarose or acrylamide gel beside an RNA standard of known concentration.
- Stain with ethidium bromide.
- Compare the relative intensity of staining between the labeled transcripts and the known standard.

9 Do one of the following:

- *If* you are not going to use the labeled probe immediately, store the probe solution at -70°C.

  Avoid repeated freezing and thawing of the probe.

- *If* you are going to use the probe immediately, dilute an aliquot of the probe solution to its working concentration (*e.g.*, 0.2–10 ng/µl) in the hybridization buffer to be used for the *in situ* experiment (as described in Chapter 5 of this manual).

Regulation of RNA Probe Length by Alkaline Hydrolysis

Some applications require shorter RNA probes than other techniques. When performing *in situ* hybridizations, probes must be short enough to allow diffusion into and out of the tissue. Alkaline hydrolysis allows you to regulate the size of RNA probes.

Procedure

The following procedure is a modification of the protocol regulating the size of RNA probes by alkaline hydrolysis described by Cox, et al. (1984, Develop. Biol. 101, 485 – 502). This protocol was adapted for use with DIG-UTP-labeled RNA probes.

1. Hydolyze 1 µg RNA by adding an equal volume of DMPC-treated H_2O and two volumes of carbonate buffer. Incubate for 10 – 60 min at +60°C.
   Calculate the incubation time of according to the formula below, however the optimal incubation time must be determined empirically. We have found that hydrolysis starts as early as 30 s after the addition of the carbonate buffer.
2. Add an equal volume of hydolysis-neutralization buffer to stop the hydolysis.
3. Add 3 volumes of chilled ethanol to precipitate the RNA. Mix well and incubate at -70°C for 30 min.
4. Centrifuge at 13,000 × *g* for 15 min at +4°C in a microcentrifuge.
5. Decant the ethanol, and wash the pellet with 100 µl of cold 70% ethanol. Centrifuge at 13,000 × *g* for 5 min at +4°C in the microcentrifuge, then remove the 70% ethanol.
6. Dry the pellet and resuspend in 100 µl DMPC-treated H_2O. If not used immediatly, store the probe at -70°C.
7. Check the resulting probe length by electrophoresis of 10 µl hydrolyzed RNA on a 1% ethidium bromide-stained agarose gel.

Additionally required solution	Description
DMPC-treated H_2O	sterile, distilled water treated with 0.1% dimethylpyrocarbonate
Carbonate buffer	60 mM Na_2CO_3; 40 mM $NaHCO_3$; pH 10.2
Hydrolysis-neutralization buffer	3 M sodium acetate; 1% (v/v) acetic acid; pH 6.0
Ethanol	Absolute ethanol, chilled at -20°C; when 70% ethanol is indicated, dilute the ethanol with DMPC-treated water.

Limited alkaline hydrolysis (Optional)

Calculate the incubation time as follows:

$$t = \frac{L_O - L_f}{k \times L_O \times L_f}$$

L_o = initial lenght of transcript (in kb)

L_f = desired probe lenght (in kb)

k = constant = 0.11 kb/min

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Reagents available from Roche Applied Science for this procedure

Reagent	Description	Available as
DIG RNA Labeling Kit (SP6, T7)*	Kit for 2 × 10 labeling reactions	Cat. No. 11 175 025 910
DIG RNA Labeling Mix*	10 mM ATP, 10 mM CTP, 10 mM GTP, 6.5 mM UTP, 3.5 mM DIG-UTP; in Tris-HCl, pH 7.5 (+20°C)	▶ Vial 7, DIG RNA Labeling Kit (SP6/T7) (Cat. No. 11 175 025 910) ▶ DIG RNA Labeling Mix (Cat. No. 11 277 073 910)
or One of the following		
Biotin RNA Labeling Mix	10 mM ATP, 10 mM CTP, 10 mM GTP, 6.5 mM UTP, 3.5 mM biotin-16-UTP; in Tris-HCl, pH 7.5 (+20°C)	Cat. No. 11 685 597 910
Fluorescein RNA Labeling Mix	10 mM ATP, 10 mM CTP, 10 mM GTP, 6.5 mM UTP, 3.5 mM fluorescein-12-UTP	Cat. No. 11 685 619 910
10× Transcription buffer	400 mM Tris-HCl, pH 8.0; 60 mM $MgCl_2$, 100 mM dithioerythritol (DTE), 20 mM spermidine, 100 mM NaCl, 1 U/ml RNase inhibitor	▶ Vial 8, DIG RNA Labeling Kit (SP6/T7) (Cat. No. 11 175 025 910) ▶ Supplied with the RNA Polymerase
DNase I, RNase-free	10 U/µl DNase I, RNase-free	▶ Vial 9, DIG RNA Labeling Kit (SP6/T7) (Cat. No. 11 175 025 910)
Protector RNase Inhibitor	2.000 U 10.000 U	Cat. No. 03 335 399 001 Cat. No. 03 335 402 001
One of the following		
SP6 RNA Polymerase	20 U/µl SP6 RNA Polymerase	▶ Vial 11, DIG RNA Labeling Kit (SP6/T7) (Cat. No. 11 175 025 910) ▶ SP6 RNA Polymerase (Cat. Nos. 10 810 274 001, 11 487 671 001)
T7 RNA Polymerase	20 U/µl T7 RNA Polymerase	▶ Vial 12, DIG RNA Labeling Kit (SP6/T7) (Cat. No. 11 175 025 910) ▶ T7 RNA Polymerase (Cat. Nos. 10 881 767 001, 10 881 775 001)
T3 RNA	20 U/µl T3 RNA Polymerase	▶ T3 RNA Polymerase (Cat. Nos. 11 031 163 001, 11 031 171 001)

* The labeling of nucleic acids with DIG is covered by EP patents 0 324 474 and 0 371 262 as well as the following US patents 5.344.757, 5.354.657 and 5.702.888 owned by Roche Diagnostics GmbH.

VI. Oligonucleotide 3'-end labeling with DIG-ddUTP or Biotin-ddUTP

Terminal Transferase is used to add a single modified dideoxyuridine-triphosphate (*e.g.*, DIG-ddUTP) to the 3' ends of an oligonucleotide (Schmitz et al., 1990).

HPLC- or gel-purified oligonucleotides from 14 – 100 nucleotides long can be labeled in this procedure.

1. Dissolve the purified oligonucleotide in sterile double dist. water.

2. Prepare a 1 mM solution of X-ddUTP (X = DIG or Biotin) in double dist. water.

3. Add the following to a microcentrifuge tube on ice:

   The 5× reaction buffer and the Terminal Transferase contain toxic material. Handle with care.

   - 4 µl of 5x concentrated reaction buffer [1 M potassium cacodylate, 0.125 M Tris-HCl, 1.25 mg/ml Bovine Serum Albumin; pH 6.6 (25°C)] (vial 1).

     Numbered vials are included in the DIG Oligonucleotide 3'-End Labeling Kit.

   - 4 µl of 25 mM $CoCl_2$ (vial 2).
   - 100 pmol oligonucleotide.

     Do not increase the concentration of oligonucleotide in this standard reaction. To make larger amounts of labeled oligonucleotide, scale up all reaction components and volumes.

   - 1 µl of either 1 mM DIG-ddUTP(vial 3) or 1 mM Biotin-ddUTP.
   - 1 µl (50 U) Terminal Transferase [supplied in 200 mM potassium cacodylate, 1 mM EDTA, 200 mM KCl, 0.2 mg/ml Bovine Serum Albumin, 50% glycerol; pH 6.5 (25°C)] (vial 4).
   - Add double dist. water to a final volume of 20 µl.

4. Mix the reaction components well and centrifuge briefly.

5. Incubate at 37°C for 15 min, then place on ice.

6. **Optional:** Stop the reaction by doing the following:

   - Mix 200 µl 0.2 M EDTA (pH 8.0) with 1 µl of glycogen solution (20 mg/ml, in double dist. water) (vial 8).
   - Add 2 µl of the glycogen-EDTA mixture to the reaction mixture.

     Do not use phenol/$CHCl_3$ extraction to stop the reaction, since the labeled oligonucleotide will migrate to the organic layer during such extraction.

7. **Optional:** Precipitate the labeled oligonucleotide by performing the following steps:

   - To the reaction tube, add 2.5 µl 4 M LiCl and 75 µl prechilled (-15 to -25°C) 100% ethanol. Mix well.
   - Let the precipitate form for at least 30 min at -70°C or 2 h at -15 to -25°C.
   - Centrifuge the tube (at 13,000 × *g*) for 15 min at 2 – 8°C.
   - Discard the supernatant.
   - Wash the pellet with 50 µl ice-cold 70% (v/v) ethanol.
   - Centrifuge the tube (at 13,000 × *g*) for 5 min at 2 – 8°C.
   - Discard the supernatant.
   - Dry the pellet under vacuum.

▶▶▶

8 Do one of the following:

- *If* you are not going to use the labeled oligonucleotide probe immediately, dissolve the pellet in a minimal amount of sterile, double dist. water and store the probe solution at -15 to -25°C for last 1 year.

  ⚠ *Avoid repeated freezing and thawing of the probe.*

- *If* you are going to use the labeled oligonucleotide probe immediately, dissolve the pellet in a minimal amount of sterile, double dist. water, then dilute an aliquot of the probe solution to a convenient stock concentration (*e.g.*, 1–7 ng/µl) in the hybridization buffer to be used for the *in situ* experiment (as described in Chapters 2 and 5 of this manual).

9 The efficiency of the labeling reaction can be checked by comparison with the labeled control-oligonucleotide (vial 6) in hybridization or direct detection. It is recommended to routinely check the labeling efficiency by direct detection (see page 60).

10 The labeled oligonucleotide can be analyzed by polyacrylamide-gel electrophoresis and subsequent silver staining in comparison to the unlabeled oligonucleotide. DIG labeled oligonucleotides are shifted to higher molecular weight due to the addition of the label. The control oligonucleotide labeled in the standard reaction is completely shifted to the end labeled form.

It is not recommended to increase the amount of oligonucleotide in the labeling reaction. Larger amounts of oligonucleotide can be labeled by increasing the reaction volume and all components proportionally.

Reagents available from Roche Applied Science for this procedure

Reagent	Description	Available as
DIG Oligonucleotide 3'-End Labeling Kit, 2nd generation*		▶ (Cat. No. 03 353 575 910) kit for 25 labeling reactions of 100 pmol oligonucleotides
5× Reaction buffer	1 M potassium cacodylate, 125 mM Tris-HCl, 1.25 mg/ml bovine serum albumin, pH 6.6 (+25°C)	▶ Vial 1, DIG Oligonucleotide 3'-End Labeling Kit, 2nd generation (Cat. No. 03 353 575 910) ▶ Supplied with Terminal Transferase
$CoCl_2$ solution	25 mM cobalt chloride ($CoCl_2$)	▶ Vial 2, DIG Oligonucleotide 3'-End Labeling Kit, 2nd generation (Cat. No. 03 353 575 910) ▶ Supplied with Terminal Transferase
DIG-ddUTP*	1 mM Digoxigenin-11-ddUTP (2', 3'-dideoxyuridine-5'-triphosphate, coupled to digoxigenin via an 11-atom spacer arm) in double dist. water	▶ Vial 3, DIG Oligonucleotide 3'-End Labeling Kit, 2nd generation (Cat. No. 03 353 575 910) ▶ DIG-ddUTP (Cat. No. 11 363 905 910)
Biotin-16-ddUTP	1 mM tetralithium salt, solution	▶ Cat. No. 11 427 598 910, 25 nmol (25 µl)
Terminal Transferase recombinant from *E.coli*	400 U/µl Terminal Transferase, in 60 mM K-phosphate (pH 7.2 at 4°C), 150 mM KCE 1 mM 2-Mercaptoethanol 0.5% Triton X-100 50% Glycerol	▶ Vial 4, DIG Oligonucleotide 3'-End Labeling Kit, 2nd generation (Cat. No. 03 353 575 910) ▶ Terminal Transferase Cat. No. 03 333 566 001 8.000 U for 20 tailing or 3'-end labeling reactions ▶ Terminal Transferase Cat. No. 03 333 574 001 24.000 U for 60 tailing or 3'-end labeling reactions

* The labeling of nucleic acids with DIG is covered by EP patents 0 324 474 and 0 371 262 as well as the following US patents 5.344.757, 5.354.657 and 5.702.888 owned by Roche Diagnostics GmbH.

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VII. Oligonucleotide tailing with a DIG-dUTP, Biotin-dUTP, or Fluorescein-dUTP mixture

Terminal Transferase is used to add a mixture of labeled dUTP and dATP to the 3' ends of an oligonucleotide in a template-independent reaction (Schmitz et al., 1990). In the tailing reaction, the concentrations of labeled dUTP and unlabeled dATP are adjusted to produce the highest hapten incorporation, optimal spacing of the hapten, and (ultimately) the highest sensitivity.

This procedure produces a tail which ranges in length from 10 – 100 nucleotides (average: 50), and contains, on average, 5 labeled dUTP molecules. Alternatively, this procedure may be modified to add only 2 – 3 labeled dUTP molecules, without any intervening dATP. (For details on modifying the length and composition of the tail, see the pack insert from the DIG Oligonucleotide Tailing Kit).

HPLC- or gel-purified oligonucleotides from 14 – 100 nucleotides long can be labeled in this procedure.

1. Dissolve the purified oligonucleotide in sterile double dist. water.

2. Prepare a 1 mM solution of X-dUTP (X = DIG, Biotin, or Fluorescein) in double dist. water.

3. Add the following to a microcentrifuge tube on ice:

   The 5× reaction buffer and the Terminal Transferase contain toxic material. Handle with care.

   - 4 µl of 5× concentrated reaction buffer [1 M potassium cacodylate, 0.125 M Tris-HCl, 1.25 mg/ml Bovine Serum Albumin; pH 6.6 (25°C)] (vial 1)

     Numbered vials are included in the DIG Oligonucleotide Tailing Kit.

   - 4 µl of 25 mM $CoCl_2$ (vial 2).
   - 100 pmol oligonucleotide.

     Do not increase the concentration of oligonucleotide in this standard reaction. To make larger amounts of labeled oligonucleotide, scale up all reaction components and volumes.

   - 1 µl of either 1 mM DIG-dUTP (vial 3) *or* 1 mM Biotin-dUTP *or* 1 mM Fluorescein-dUTP.
   - 1 µl of 10 mM dATP [in Tris buffer, pH 7.5 (25°C)] (vial 4).
   - 1 µl (50 U) Terminal Transferase [supplied in 200 mM potassium cacodylate, 1 mM EDTA, 200 mM KCl, 0.2 mg/ml Bovine Serum Albumin, 50% glycerol; pH 6.5 (25°C)] (vial 5).
   - Add double dist. water to a final volume of 20 µl.

4. Mix the reaction components well and centrifuge briefly.

5. Incubate at 37°C for 15 min, then place on ice.

6. **Optional:** Stop the reaction by doing the following:

   - Mix 200 µl 0.2 M EDTA (pH 8.0) with 1 µl of glycogen solution (20 mg/ml, double dist. water) (vial 9).
   - Add 2 µl of the glycogen-EDTA mixture to the reaction mixture.

     Do not use phenol/$CHCl_3$ extraction to stop the reaction, since the labeled oligonucleotide will migrate to the organic layer during such extraction.

▶▶▶

7 **Optional:** Precipitate the labeled oligonucleotide by performing the following steps:

- To the reaction tube, add 2.5 µl 4 M LiCl and 75 µl prechilled (-15 to -25°C) 100% ethanol. Mix well.
- Let the precipitate form for at least 30 min at –70°C or 2 h at -15 to -25°C.
- Centrifuge the tube (at 13,000 × *g*) for 15 min at 2 – 8°C.
- Discard the supernatant.
- Wash the pellet with 50 µl ice-cold 70% (v/v) ethanol.
- Centrifuge the tube (at 13,000 × *g*) for 5 min at 2 – 8°C.
- Discard the supernatant.
- Dry the pellet under vacuum.

8 Do one of the following:

- *If* you are not going to use the labeled oligonucleotide probe immediately, dissolve the pellet in a minimal amount of sterile, double dist. water and store the probe solution at -15 to -25°C.

  ⚠ *Avoid repeated freezing and thawing of the probe.*

- *If* you are going to use the labeled oligonucleotide probe immediately, dissolve the pellet in a minimal amount of sterile, double dist. water, then dilute an aliquot of the probe solution to a convenient stock concentration (*e.g.*, 1 – 7 ng/µl) in the hybridization buffer to be used for the *in situ* experiment (as described in Chapters 2 and 5 of this manual).

9 The efficiency of the tailing reaction can be checked by comparison with the tailed control-oligonucleotide (vial 8) in hybridization or direct detection. It is recommended to routinely check the tailing efficiency by direct detection (see DIG Nucleic Acid Detection Kit).

10 The tailed oligonucleotide can be analyzed by polyacrylamide-gel electrophoresis and subsequent silver staining in comparison to the untailed oligonucleotide (vial 7). DIG-tailing of oligonucleotides results in a heterogeneous shift to higher molecular weight and is detectable as a smear in polyacrylamide gels. The control oligonucleotide tailed in the standard reaction is completely shifted to the labeled form.

❗ *It is not recommended to increase the amount of oligonucleotide in the tailing reaction. Larger amounts of oligonucleotide may be labeled by increasing the reaction volume and all components proportionally.*

■

Stability of labeled probes

DIG-labeled probes can be stored at -15 to -25°C for at least 1 year.

4

Reagents available from Roche Applied Science for this procedure

Reagent	Description	Available as
DIG Oligonucleotide Tailing Kit, 2nd generation*		▶ (Cat. No. 03 353 583 910) kit for 25 tailing reactions of 100 pmol oligonucleotides
5× Reaction buffer	1 M potassium cacodylate, 125 mM Tris-HCl, (Cat. No. 1 417 231) 1.25 mg/ml bovine serum albumin, pH 6.6 (+25°C)	▶ Vial 1, DIG Oligonucleotide Tailing Kit, 2nd generation (Cat. No. 03 353 583 910) ▶ Supplied with Terminal Transferase
$CoCl_2$ solution	25 mM cobalt chloride ($CoCl_2$)	▶ Vial 2, DIG Oligonucleotide Tailing Kit, 2nd generation (Cat. No. 03 353 583 910) ▶ Supplied with Terminal Transferase
DIG-dUTP	1 mM Digoxigenin-11-dUTP (2'-dideoxyuridine-5'- triphosphate, coupled to digoxigenin via an 11-atom spacer arm) in double dist. water	▶ Vial 3, DIG Oligonucleotide Tailing Kit, 2nd generation (Cat. No. 03 353 583 910) ▶ DIG-dUTP, alkali-labile (Cat. Nos. 11 573 152 910, 11 573 179 910) ▶ DIG-dUTP, alkali-stable (Cat. Nos. 11 093 088 910, 11 558 706 910, 11 570 013 910)
dATP	Lithium salt, 10 mM dATP solution; in Tris buffer, pH 7.5	▶ Vial 4, DIG Oligonucleotide Tailing Kit, 2nd generation (Cat. No. 03 353 583 910) ▶ dATP [Cat. No. 11 051 440 001 (sold as a 100 mM solution; must be diluted before use)]
Terminal Transferase recombinant from *E.coli*	400 U/µl Terminal Transferase, in 60 mM K-phosphate (pH 7.2 at 4°C), 150 mM KCl 1 mM 2-Mercaptoethanol 0.5% Triton X-100 50% Glycerol	▶ Vial 5, DIG Oligonucleotide Tailing Kit, 2nd generation (Cat. No. 03 353 583 910) ▶ Terminal Transferase Cat. No. 03 333 566 001 8.000 U for 20 tailing or 3'-end labeling reactions ▶ Terminal Transferase Cat. No. 03 333 574 001 24.000 U for 60 tailing or 3'-end labeling reactions
Glycogen from mussels	20 mg/ml, aqueous solution	▶ Cat. No. 10 901 393 001

* The labeling of nucleic acids with DIG is covered by EP patents 0 324 474 and 0 371 262 as well as the following US patents 5.344.757, 5.354.657 and 5.702.888 owned by Roche Diagnostics GmbH.

References

Dunn, J. J.; Studier, F. W. (1983) Complete nucleotides sequence of bacteriophage T7 DNA and the locations of T7 genetic elements. *J. Mol. Biol.* **166**, 477–535.

Feinberg, P.; Vogelstein, B. (1984) A technique for radiolabeling DNA restriction enzyme fragments to high specific activity. *Anal. Biochem.* **137**, 266–267.

Innis, M. A.; Gelfand, D. H.; Sninsky, J. J.; White, T. J. (Eds.) (1990) PCR Protocols: A Guide to Methods and Applications. San Diego, CA: Academic Press.

Kassavetis, G. A.; Butler, E. T.; Roulland, D.; Chamberlin, M. J. (1982) Bacteriophage SP6-specific RNA polymerase. *J. Biol. Chem.* **57**, 5779–5788.

Langer, P. R.; Waldrop, A. A.; Ward, D. C. (1981) Enzymatic synthesis of biotin-labeled polynucleotides: Novel nucleic acid affinity probes. *Proc. Natl. Acad. Sci. USA* **78**, 6633–6637.

Rigby, P. W. J.; Dieckmann, M.; Rhodes, C.; Berg, P. (1977) Labeling deoxyribonucleic acid to high specific activity *in vitro* by nick translation with DNA polymerase I. *J. Mol. Biol.* **113**, 237–241.

Schmitz, G. G.; Walter, T.; Seibl, R.; Kessler, C. (1991) Nonradioactive labeling of oligonucleotides *in vitro* with the hapten digoxigenin by tailing with terminal transferase. *Anal. Biochem.* **192**, 222–231.

VIII. Estimating the yield of DIG-labeled nucleic acids

An accurate quantification of DIG-labeled DNA obtained in the labeling reaction is most important for optimal and reproducible results in various membrane or *in situ* hybridization techniques. Too high of a probe concentration in the hybridization mix usually causes background, while too low of a concentration leads to weak signals.

Estimate the yield of DIG-labeling the following way:

Dilution series of the labeling reaction and dilutions of an appropriate standard are both spotted on nylon membranes. The membrane is then processed in a short detection procedure.

Exception: PCR labeled DIG probes are checked via agarose gel (compare to page 39)

Estimating the yield in a spot test with a DIG-labeled control

The estimation of yield can should also be performed in a side by side comparison of the DIG-labeled sample nucleic acid with a DIG-labeled control, that is provided in the labeling kits. Dilution series of both are prepared and spotted on a piece of membrane. Subsequently, the membrane is colorimetrically detected. Direct comparison of the intensities of sample and control allows the estimation of labeling yield.

Products required

DIG-labeled controls for estimating the yield of DNA, RNA are available as separate reagents or in the respective labeling kits. The DIG-dUTP/dATP-tailed and 3'-End labeled Oligonucleotide Control is only available in the DIG Oligonucleotide Tailing Kit, 2nd generation, and the DIG Oligonucleotide 3'-End Labeling Kit, 2nd generation.

4

Reagents available from RAS for this procedure

Reagent	Description	Available as
Labeled Control DNA	Digoxigenin-labeled pBR328 DNA that has been random primed labeled according to the standard labeling procedure; the total DNA concentration in the vial is 25 µg/ml, but only 5 µg/ml of it is DIG-labeld DNA.	▶ DIG-labeled Control DNA* (Cat. No. 11 585 738 910)
Control Oligonucleotide, DIG-ddUTP-labeled	2.5 pmol/µl oligonucleotide, labeled with Digoxigenin-11-ddUTP according to the standard labeling procedure.	▶ Vial 6, DIG Oligonucleotide 3'-End Labeling Kit, 2nd generation* (Cat. No. 03 353 575 910)
Control Oligonucleotide, DIG-dUTP/dATP tailed	2.5 pmol/µl oligonucleotide, tailed with Digoxigenin-11-dUTP and dATP according to the standard labeling procedure.	▶ Vial 7, DIG Oligonucleotide Tailing Kit, 2nd generation* (Cat. No. 03 353 583 910)
Labeled Control RNA	Digoxigenin-labeled "antisense"-Neo RNA, transcribed with T7RNA polymerase from 1 µg template DNA, according to the standard labeling procedure. The solution contains approx. 100 µg/ml DIG-labeled RNA and 10 µg/ml unlabeled DNA template.	▶ DIG-labeled Control RNA* (Cat. No. 11 585 746 910)
DNA Dilution buffer	10 mM Tris-HCl, pH 8.0 (20°C), 50 µg/ml DNA from fish sperm.	▶ Vial 9, DIG Oligonucleotide 3'-End Labeling Kit, 2nd generation* (Cat. No. 03 353 575 910) ▶ Vial 10, DIG Oligonucleotide Tailing Kit, 2nd generation* (Cat. No. 03 353 583 910)
RNA Dilution buffer	DMPC-treated H_2O, 20× SSC and formaldehyde, mixed in a volume ratio of 5 + 3 + 2.	
Blocking Reagent	Blocking reagent for nucleic acid hybridization; white powder.	▶ Blocking Reagent (Cat. No. 11 096 176 001)
Anti-Digoxigenin-AP	Anti-digoxigenin [Fab] conjugated to alkaline phosphatase.	▶ Anti-Digoxigenin-AP, Fab fragments* (Cat. No. 11 093 274 910)
NBT solution	75 mg/ml nitroblue tetrazolium salt in dimethylformamide.	▶ NBT [Cat. No. 11 383 213 001) (dilute from 100 mg/ml)]
BCIP solution	50 mg/ml 5-bromo-4-chloro-3-indolyl phosphate (BCIP), toluidinium salt in dimethylformamide.	▶ BCIP (Cat. No. 11 383 221 001)

* The labeling of nucleic acids with DIG is covered by EP patents 0 324 474 and 0 371 262 as well as the following US patents 5.344.757, 5.354.657 and 5.702.888 owned by Roche Diagnostics GmbH.

Additionally required solutions	Description
Washing Buffer (Bottle 1; dilute 1:10 with double dist. water)	100 mM Maleic acid, 150 mM NaCl; pH 7.5 (+20°C); 0.3% (v/v) Tween 20
Maleic acid Buffer (Bottle 2; dilute 1:10 with double dist. water)	100 mM Maleic acid, 150 mM NaCl; pH 7.5 (+20°C)
Blocking Solution (Bottle 3; dilute 1:10 with 1× Maleic acid Buffer)	1% (w/v) Blocking Reagent for nucleic acid hybridization, dissolved in Maleic acid Buffer. Blocking Solution is cloudy and should not be filtered. It is stable for at least two weeks when stored at 2–8°C, but must then be brought to 15–25°C temperature before use
Detection Buffer (Bottle 4; dilute 1:10 with double dist. water)	100 mM Tris-HCl, 100 mM NaCl; pH 9.5 (20°C)
TE Buffer	10 mM Tris-HCl, 0.1 mM EDTA; pH 8.0 (20°C)

4

Procedure

1. Prepare a 1 ng/µl working solution of DIG-labeled Control DNA (original conc. 5 ng/µl) in DNA Dilution Buffer, by mixing 5 µl DIG-labeled Contol DNA with 20 µl DNA Dilution Buffer.

   Prepare a 10 ng/µl working solution of DIG-labeled Control RNA (original conc. 100 ng/µl) in RNA Dilution Buffer, by mixing 2 µl DIG-labeled Control RNA with 18 µl RNA Dilution Buffer.

   For the DIG 3' -end labeled or tailed control oligonucleotide a predilution is not required.

2. Make serial dilutions of the (prediluted) controls, according to the appropriate dilution scheme. Mix thoroughly between dilution steps.

4

Dilution Scheme A (for DNA probes)

Tube	DNA (µl)	From Tube #	DNA Dilution Buffer (µl)	Overall Dilution (from Tube D1)	Final Concentration
D1*		-		None	1 ng/µl
D2	2	D1	198	1 : 100	10 pg/µl
D3	15	D2	35	1 : 330	3 pg/µl
D4	5	D2	45	1 : 1000	1 pg/µl
D5	5	D3	45	1 : 3300	0.3 pg/µl
D6	5	D4	45	$1 : 10^4$	0.1 pg/µl
D7	5	D5	45	1 : 33,000	0.03 pg/µl
D8	5	D6	45	$1 : 10^5$	0.01 pg/µl
D9	0	-	50	-	0

*working solution of labeled probe or control DNA.

Dilution Scheme B (for Oligonucleotide probes)

Tube	Oligo (µl)	From Tube #	DNA Dilution (from Tube N1)	OverallDilution (from Tube N1)	Final Concentration
N1*			-	None	100 fmol/µl
N2	3	N1	7	1 : 3.3	30 fmol/µl
N3	2	N1	18	1 : 10	10 fmol/µl
N4	2	N2	18	1 : 33	3 fmol/µl
N5	2	N3	18	1 : 100	1 fmol/µl
N6	0	-	20	-	0

* working solution of labeled probe or control oligonucleotide

Dilution Scheme C (for RNA probes)

Tube	RNA (µl)	From Tube #	RNA Dilution Buffer (µl)	Overall Dilution (from Tube R1)	Final Concentration
R1*		-		None	10 ng/µl
R2	2	R1	18	1:10	1 ng/µl
R3	2	R2	198	1:1000	10 pg/µl
R4	15	R3	35	1:3300	3 pg/µl
R5	5	R3	45	$1:10^4$	1 pg/µl
R6	5	R4	45	$1:3.3 \times 10^4$	0.3 pg/µl
R7	5	R5	45	$1:10^5$	0.1 pg/µl
R8	5	R6	45	$1:3.3 \times 10^5$	0.03 pg/µl
R9	5	R7	45	$1:10^6$	0.01 pg/µl
R10	0	-	50	-	0

* working solution of labeled probe or control RNA

Highly diluted solutions of RNA in H_2O are not very stable. Spots have to be made immediately after preparing the dilutions. Alternatively the RNA can be diluted in RNA dilution buffer (DMPC-treated H_2O, 20× SSC and formaldehyde, mixed in a volume ratio of 5 + 3 + 2) for greater stability.

3 Use table 1 on page 36 to estimate the expected yield of DNA labeling reactions. Predilute an aliquot of the newly labeled experimental **DNA probe** to an expected final concentration of approx. 1 ng/µl.

or

Predilute an aliquot of the newly labeled experimental **oligonucleotide probe** to a final concentration of 100 fmol/µl.

or

Predilute an aliquot of the newly synthesized experimental **RNA probe** to an expected final concentration of approx. 10 ng/µl. In a standard RNA labeling reaction approx. 10 g newly synthesized DIG-RNA probe is transcribed from 1 µg DNA template.

4 Make serial dilutions of the prediluted experimental probe, according to the appropriate dilution scheme:

- for DNA probes, use dilution scheme A
- for oligonucleotide probes, use dilution scheme B
- for RNA probes, use dilution scheme C

5 On a narrow strip (approx. 3 × 5 cm) of Positively Charged Nylon Membrane (Cat. No. 11 209 272 001), apply 1 µl spots from probe dilutions:

- for DNA probes D2–D9
- for oligonucleotide probes N2–N6
- for RNA probes R3–R10

6 In a row parallel to the probe dilutions, apply 1 µl spots of the corresponding control dilutions (*i.e.*, D2–D9, N2–N6 or R3–R10, made from the appropiate control).

Mark location of each probe and control spot with a pencil.

7 Fix the nucleic acid spots to the membrane by doing one of the following:

- Crosslink with Stratalinker 120 mJ, or
- Crosslink with UV light for 3–5 min, or
- Bake the membrane at 120°C for 30 min, or
- Bake the membrane at 80°C for 2 h

8 Transfer the membrane to a plastic container (*e.g.*, a petri dish) containing 20 ml Washing Buffer.

Incubate for 2 min with shaking.

Discard the Washing Buffer.

9 Incubate membrane for 30 min in 10 ml Blocking Solution.

Discard the Blocking Solution.

10 Prepare 10 ml antibody solution by dilution of Anti-DIG alkaline phosphatase 1:5000, recommended for NBT/BCIP detection, in Blocking Solution. Therefore centrifuge the Anti-DIG-AP for 5 min at 10 000 rpm in the original vial and pipet the necessary amount from the surface.

11 Incubate the membrane for 30 min in 10 ml Antibody Solution.

12 Wash membrane twice (2 × 15 min) with 10 ml portions of Washing Buffer.

13 Equilibrate membrane 2–5 min in 10 ml Detection Buffer.

▶▶▶

14	Mix 45 µl NBT solution and 35 µl BCIP solution in 10 ml of Detection Buffer. This color substrate solution must be prepared freshly. *Alternatively, chemiluminescent detection can be performed, using the DIG Luminescent Detection Kit.*
15	Pour off the Detection Buffer and add the color substrate solution. Allow the color development to occur in the dark. The color precipitate starts to form within a few minutes and continues for approx. 16 h. Do not shake while the color is developing.
16	When the spots appear in sufficient intensity, stop the reaction by washing the membrane with TE buffer or double dist. water for 5 min.
17	Compare spot intensities of the control and experimental dilutions to estimate the concentration of the experimental probe (See Figure 1).

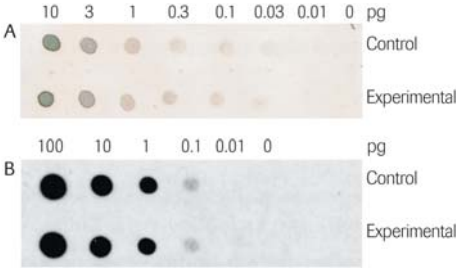

Figure 1: Estimating the Yield of DIG-labeled DNA. Dilutions of the Labeled Control DNA and the newly labeled (experimental) DNA were spotted on, fixed to, and directly detected on a Roche Applied Science Nylon Membrane, with colorimetric (Panel A) or chemiluminescent detection (Panel B).

IX. Purification of labeled probes using the High Pure PCR Product Purification Kit

The High Pure PCR Product Purification Kit is designed for the efficient and convenient isolation of PCR products from amplification reactions, but is also suited for the removal of unincorporated nucleotides from DIG DNA and RNA labeling reactions. The DNA binds specifically to the surface of glass fibres in the presence of chaotrope salts. Primers, unincorporated nucleotides, contaminating agarose particles and proteins are removed by a simple washing step. The bound DIG-labeled DNA is subsequently eluted in a low-salt buffer.

A minimum length of approx. 100 bp is required for efficient binding. The kit can therefore not be used for the removal of unincorporated nucleotides from oligonucleotide labeling reactions.

Products required

Reagent	Description	Available as
High Pure PCR Product Purification Kit	Kit for 50 purifications Kit for 250 purifications	Cat. No. 11 732 668 001 Cat. No. 11 732 676 001
consisting of:		
▶ **Binding Buffer, green cap**	Nucleic acids Binding Buffer; 3 M guanidine-thiocyanate, 10 mM Tris-HCl, 5% (v/v) ethanol, pH 6.6 (25°C)	Vial 1
▶ **Wash Buffer, blue cap**	Wash Buffer; **add 4 volumes of absolute ethanol before use!** Final concentrations; 20 mM NaCl, 2 mM Tris-HCl, pH 7.5 (25°C), 80% ethanol	Vial 2
▶ **Elution Buffer**	Elution Buffer; 10 mM Tris-HCl, pH 8.5 (25°C)	Vial 3
▶ **High Pure Filter Tubes**	Polypropylene tubes, containing two layers of a specially pre-treated glass fibre fleece; maximum sample volume: 700 µl	
▶ **Collection Tubes**	2 ml Polypropylene tubes	

4

Procedure

Make sure that 4 volumes ethanol have been added to the Wash Buffer (vial 2, blue cap). The Binding Buffer (vial 1, green cap) contains guanidine-thiocyanate which is an irritant. Wear gloves and follow laboratory safety conditions during handling.

1. Fill up the labeling reaction to 100 µl with double dist. water.
2. Add 500 µl Binding Buffer (vial 1, green cap) and mix well.

   It is important that the volume ratio between sample and binding buffer is 1:5. When using other sample volumes than 100 µl, adjust the volume of Binding Buffer accordingly.
3. ▶ Insert one High Pure filter tube into one collection tube.
   ▶ Transfer the sample from step 2 using a pipette to the upper reservoir of the filter tube.
   ▶ Centrifuge 30 – 60 seconds at maximum speed in a standard table top centrifuge at 15 – 25°C.
4. ▶ Disconnect the filter tube, and discard the flowthrough solution.
   ▶ Re-connect the filter tube to the same collectiuon tube.
5. ▶ Add 500 µl Wash Buffer to the upper reservoir.
   ▶ Centrifuge 1 minute at maximum speed (as above).
6. ▶ Discard the flowthough solution.
   ▶ Re-combine the filter tube with the same collection tube.
   ▶ Add 200 µl Wash Buffer.
   ▶ Centrifuge 1 minute at maximum speed (as above).

   This second 200 µl wash step ensures optimal purit and complete removal of Wash Buffer from the glass fibers.
7. ▶ Discard the flowthough solution and collection tube.
   ▶ Re-connect the filter tube to a clean 1.5 ml microcentrifuge tube.
8. ▶ Add 50 –100 µl Elution Buffer to the upper reservoir of the filter tube.
   ▶ Centrifuge 1 minute at maximum speed.

   Do not use water for elution since alkaline pH is required for optimal yield.
9. The microcentrifuge tube now contains the purified DNA.

   The elution efficiency is increased with higher volume of elution buffer applied. At least 68% and 79% rcovery are found with 50 and 100 µl elution buffer, respectively. Normally almost quantitative recovery can be found, as can be determined in a direct detection assay.

IF YOU WANT TO...	THEN...
go ahead	use the eluted purified DNA right away
to stop	store DNA at 2 – 8°C or -15 to -25°C for later analysis

5

Procedures for *In Situ* Hybridization to Chromosomes, Cells, and Tissue Sections

ISH to whole chromosomes

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Procedures for *In Situ* Hybridization to Chromosomes, Cells, and Tissue Sections

This chapter includes contributions from several leading scientists, from researchers in molecular biology to clinical pathologists, who practice *in situ* hybridization. Protocols are given for *in situ* hybridizations on widely varying substrates, *e.g.*, chromosome spreads, chromosomes in suspension, single cells, paraffin-embedded tissue sections, ultrathin tissue sections, and whole mount preparations. Hybridization methods are described for both DNA and RNA targets.

Applications covered include gene mapping, gene expression, developmental biology, tumor biology, cell sorting, clinical cytogenetics, and analysis of infectious diseases.

These procedures use digoxigenin, biotin, and fluorochromes for labeling DNA, RNA and oligonucleotides. Labeling techniques include the classical methods, such as random primed DNA labeling, nick translation, and oligonucleotide tailing with terminal transferase, as well as PCR.

Please note that the protocols have been optimized by members of the individual laboratories and can be varied if necessary.

5

In situ hybridization to human metaphase chromosomes using DIG-, biotin-, or fluorochrome-labeled DNA probes and detection with fluorochrome conjugates

J. Wiegant, Department of Cytochemistry and Cytometry, Leiden University, Netherlands.

In recent years, a number of improvements in chromosomal *in situ* hybridization protocols have been achieved. These have allowed a fairly high success rate for single copy gene localization as well as competition *in situ* hybridization. Here we present a detailed outline of the procedure that has been successfully applied in various laboratories.

The procedures have been optimized for fluorescent detection. For the second edition of this manual, we have included new procedures and new illustrations for multicolor, multitarget fluorescent detection.

The procedures have also been used with slight modifications by the groups of Dr. P. Lichter, Dr. J. Wienberg, Dr. T. Cremer and Dr. D. Ward. The illustrative material they have provided below demonstrates the flexibility and wide applicability of the technique.

At the end of the article, Dr. Lichter's group has provided a troubleshooting guide to help solve hybridization problems that may occur.

I. Pretreatment of metaphase spreads on slides (optional)

5

1. Incubate metaphase spreads with 100 µl of 100 µg/ml RNase A (in 2× SSC) under a coverslip for 1 h at 37°C.
2. Wash slides 3 × 5 min with 2× SSC.
3. Dehydrate slides in an ethanol series (increasing ethanol concentrations).
4. Incubate with 0.005–0.02% pepsin in 10 mM HCl for 10 min at 37°C.
5. Wash slides as follows:
   - 2 × 5 min with PBS.
   - Once with PBS containing 50 mM $MgCl_2$.
6. Post-fix for 10 min at room temperature with a solution of PBS containing 50 mM $MgCl_2$ and 1% formaldehyde.
7. Wash with PBS and dehydrate (as in Step 3 above).

II. Denaturation and hybridization

For denaturation and hybridization three alternative protocols are given:

A. For probes recognizing repetitive targets such as the alphoid sequences

B. For probes recognizing unique sequences

C. For probes or probe cocktails which contain repetitive sequences occurring throughout the genome (*e.g.*, Alu sequences) [competition hybridization]

A. For repetitive probes

1. Using digoxigenin- (DIG-), biotin-, or any fluorochrome-labeled nucleotide, label 1 µg of DNA probe according to the procedures described in Chapter 4 of this manual.
2. Precipitate the labeled probe with ethanol.
3. Prepare a probe stock solution as follows:
   - Resuspend the precipitated probe at a concentration of 10 ng/µl in a solution containing 60% deionized formamide, 2× SSC, 50 mM sodium phosphate; pH 7.0.
   - Incubate the tube for 15 min at 37°C with occasional vortexing until the precipitated DNA dissolves.
4. Dilute the probe from the stock to the desired concentration (typically 2 ng/µl) in a solution containing 60% deionized formamide, 2× SSC, 50 mM sodium phosphate; pH 7.0.
5. Apply 5 µl diluted probe under a coverslip on the object slide and place the slide in an oven at 80°C for 2–4 min to denature the probe and target.

   ! *Sealing with rubber cement is not mandatory.*
6. To hybridize, place the slides in a moist chamber at 37°C overnight.
7. Wash the slide as follows:
   - 3 × 5 min at 37°C with 2× SSC containing 60% formamide.
   - 1 × 5 min with the buffer to be used for immunological detection.

■

B. For unique probes

1. Prepare the probe stock solution as described under Procedure IIA, but use a solution containing 50% deionized formamide, 2× SSC, 10% dextran sulfate, 50 mM sodium phosphate; pH 7.
2. Dilute 2–5 µl of the probe stock solution to 10 µl with a solution containing 50% deionized formamide, 2× SSC, 10% dextran sulfate, 50 mM sodium phosphate; pH 7. Mix well!
3. To denature probe and target, do the following:
   - Apply 10 µl of the dilute hybridization solution under a coverslip on the object slide.
   - Seal coverslip to slide with rubber cement.
   - Place the slide in an oven at 80°C for 2–4 min.
4. To hybridize, place the slides in a moist chamber at 37°C overnight.
5. Wash the slide as follows:
   - 3 × 5 min at 45°C with 2× SSC containing 50% formamide.
   - 5 × 2 min with 2× SSC.
   - 1 × 5 min with the buffer used in detection.

■

C. For probes containing repetitive elements [competitive hybridization]

1. Using DIG-, biotin-, or any fluorochrome-labeled nucleotides, label 1 µg of DNA probe according to the procedures described in Chapter 4 of this manual.

2. To precipitate the labeled probe, do the following:
   - Add a 50-fold excess of human COT-1 DNA (or 500-fold excess fragmented human placenta DNA), a 50-fold excess of fragmented herring sperm DNA, a 50-fold excess of yeast tRNA, 1/10th volume of 3 M sodium acetate (pH 5.6), and 2.5 volumes of cold (-20°C) 100% ethanol.
   - Incubate for 30 min on ice.
   - Centrifuge for 30 min at 4°C at 13,000 × *g*.
   - Discard the supernatant and dry the pellet.

3. Prepare a probe stock solution by dissolving the pellet in a solution of 50% deionized formamide, 2× SSC, 10% dextran sulfate, 50 mM sodium phosphate; pH 7. Depending on the probe cocktail, the final concentration ranges from 10–40 ng/µl. Mix well!

   Examples: *Prepare a 10 ng/µl stock solution of cosmid probes, a 20 ng/µl stock of chromosome specific libraries, or a 40 ng/µl stock of YAC probes.*

4. For hybridization, dilute the probe from the stock to the desired concentration in a solution of 50% deionized formamide, 2× SSC, 10% dextran sulfate, 50 mM sodium phosphate; pH 7.

   Examples: *For hybridization, use 2–5 ng/µl of cosmid probes, 10–20 ng/µl of chromosome specific libraries, or 20–40 ng/µl of YAC probes.*

5. Denature the probe at 75°C for 5 min, chill on ice, and allow annealing of the repetitive elements at 37°C for *either* 30 min (if COT HUMAN DNA is used as competitor) *or* 2 h (if total human placenta DNA is used as competitor).

6. Prepare the chromosomes on the slide by performing the following steps:
   - To the chromosomes, add a solution of 70% formamide, 2× SSC, 50 mM sodium phosphate; pH 7. Cover with a coverslip.
   - Incubate in an oven at 80°C for 2–4 min to denature the chromosomes.
   - Remove the coverslip and quench the chromosomes in chilled 70% ethanol.
   - Dehydrate the slide by passing it through 90% ethanol, then 100% alcohol.
   - Air dry the slide, preferably on a metal plate at 37°C.

7. Hybridize the chromosomes at 37°C overnight with 10 µl of the pre-annealed probe (from Step 5) under a sealed coverslip.

8. Wash the slide as follows:
   - 3 × 5 min at 45°C with 2× SSC containing 50% formamide.
   - 3 × 5 min at 60°C with 0.1× SSC.
   - 1 × 5 min with the buffer to be used in immunological detection.

III. Single color fluorescent detection with immunological amplification

A1. Biotin-labeled probe, low sensitivity

1. Wash slides briefly with TNT buffer [100 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.05% Tween 20].
2. Incubate for 30 min at 37°C with TNB buffer [100 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.5% blocking reagent].
3. Incubate for 30 min at 37°C with the proper dilution (typically 10 µg/ml) of a fluorescently labeled streptavidin conjugate in TNB.
4. Wash the slides (3 × 5 min) with TNT.
5. Prepare the slides for viewing by performing the following steps:
   - Dehydrate through an ethanol series (70%, then 90%, then 100% ethanol; 5 min each).
   - Air dry.
   - Stain with the appropriate DNA counterstain.
   - Embed in Vectashield (Vector).
6. View the slides by fluorescence microscopy, using appropriate filters.

A2. Biotin-labeled probe, high sensitivity

1. Wash slides briefly with TNT buffer [100 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.05% Tween 20].
2. Incubate for 30 min at 37°C with TNB buffer [100 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.5% blocking reagent].
3. Incubate for 30 min at 37°C with the proper dilution (typically 10 µg/ml) of a fluorescently labeled streptavidin conjugate in TNB.
4. Wash the slides (3 × 5 min) with TNT.
5. Incubate the slides for 30 min at 37°C with the proper dilution of biotinylated goat anti-streptavidin (5 µg/ml) in TNB.
6. Repeat the washes as in Step 4.
7. Repeat Step 3.
8. Repeat the washes as in Step 4.
9. Prepare the slides for viewing as in the biotin low sensitivity procedure (Procedure IIIA1), Step 5.

B1. Digoxigenin-labeled probe, low sensitivity

1. Wash slides briefly with TNT buffer [100 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.05% Tween 20].
2. Incubate for 30 min at 37°C with TNB buffer [100 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.5% blocking reagent].
3. Incubate for 30 min at 37°C with the proper dilution (typically 2 µg/ml, in TNB) of a fluorescently labeled sheep anti-DIG antibody.
4. Wash the slides (3 × 5 min) with TNT.
5. Prepare the slides for viewing as in the biotin low sensitivity procedure (Procedure IIIA1), Step 5.

B2. Digoxigenin-labeled probe, high sensitivity

1. Wash slides briefly with TNT buffer [100 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.05% Tween 20].
2. Pipette 100 µl of TNB buffer [100 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.5% blocking reagent] onto each slide and cover with a 24 × 50 mm coverslip. Incubate for 30 min at 37°C in a moist chamber (1 L beaker containing moistened tissues, covered with aluminum foil).
3. Immerse the slides for 5 min in TNT to loosen the coverslips.
4. Prepare fresh working solutions of three antibodies:
   - Antibody 1 working solution: mouse monoclonal anti-DIG, 0.5 µg/ml in TNB.
   - Antibody 2 working solution: DIG-conjugated sheep anti-mouse Ig, 2 µg/ml in TNB.
   - Antibody 3 working solution: fluorescein- or rhodamine-conjugated sheep anti-DIG, 2 µg/ml in TNB.

   The three antibodies used in this procedure are also available in the Fluorescent Antibody Enhancer Set for DIG Detection.
5. Pipette 100 µl of antibody 1 working solution onto each slide and cover with a 24 × 50 mm coverslip. Incubate for 30 min at 37°C in a moist chamber.
6. Wash the slides (3 × 5 min) at room temperature with TNT.
7. Pipette 100 µl of antibody 2 working solution onto each slide and add a 24 × 50 mm coverslip. Incubate for 30 min at 37°C in a moist chamber.
8. Repeat the washes as in Step 6.
9. Pipette 100 µl of antibody 3 working solution onto each slide and add a 24 × 50 mm coverslip. Incubate for 30 min at 37°C in a moist chamber.

   For even higher sensitivity, repeat Steps 5–8 before performing the incubation with antibody 3 (Step 9).
10. Repeat the washes as in Step 6.
11. Prepare the slides for viewing as in the biotin low sensitivity procedure (Procedure IIIA1), Step 5.

C1. Fluorescently labeled probes (fluorescein, coumarin, CY3, rhodamine, Texas Red), low sensitivity

After the posthybridization washes (Procedure II), prepare the slides for viewing as in the biotin low sensitivity procedure (Procedure IIIA1), Step 5.

C2. Fluorescein-labeled probes, high sensitivity

1	Wash slides briefly with TNT buffer [100 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.05% Tween 20].
2	Incubate for 30 min at 37°C with TNB buffer [100 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.5% blocking reagent].
3	Incubate for 30 min at 37°C with the proper dilution of an anti-fluorescein antibody (either polyclonal or monoclonal) in TNB.
4	Wash the slides (3 × 5 min) with TNT.
5	Depending on the antibody used in Step 3, incubate with the proper dilution of a fluorescently labeled rabbit anti-mouse or goat anti rabbit antibody in TNB for 30 min at 37°C.
6	Repeat the washes as in Step 4.
7	Prepare the slides for viewing as in the biotin low sensitivity procedure (Procedure IIIA1), Step 5.

IV. Multicolor fluorescence *in situ* hybridization (Multicolor FISH)

For multicolor FISH, several sets of probes with different labels must be hybridized simultaneously to the target and visualized with combinations of antibodies.

Table 1 lists an antibody-probe matrix which allows a triple color detection of three differently labeled chromosome specific libraries (so-called chromosome painting probes).

Incubation	Probe 1 Biotin	Probe 2 Digoxigenin	Probe 3 Fluorescein
1	Streptavidin-Texas Red	Mouse anti-DIG antibody	Rabbit anti-fluorescein antibody
2	Biotin-labeled anti-streptavidin	Sheep anti-mouse antibody	FITC-labeled goat anti-rabbit antibody
3	Streptavidin-Texas Red	AMCA-labeled sheep anti-DIG antibody	

Table 1: Probes and antibodies for a triple color FISH.

Be careful that the selected antibodies do not cross-react.

Outline of protocol for multicolor FISH

1. Simultaneously hybridize all probes listed in Table 1 to the target.
2. For each of the 3 detection incubations, perform the following steps:
   - Prepare the antibodies needed in the incubation (as listed in Table 1) at the proper dilution in the correct buffer.

     Use the procedures in Section III above as a guideline.
   - Mix all antibodies needed in the incubation and incubate simultaneously with the slide.
   - Perform blocking and washing steps as in Section III above.
3. Repeat Step 2 until all incubations are complete.
4. Do not counterstain the slides, but dehydrate, air dry, and mount the slides as in the biotin low sensitivity procedure (Procedure IIIA1), Step 5.

V. Results obtained with human metaphase chromosome spreads

The following examples (from our laboratory and other laboratories) show different applications of the procedures described in this section .

A. Multicolor FISH of human lymphocyte metaphase and interphase cells

from the Department of Cytochemistry and Cytometry, Leiden University, Netherlands

The techniques described above allow the detection of two, three, or many different probes simultaneously (Figures 1 – 4).

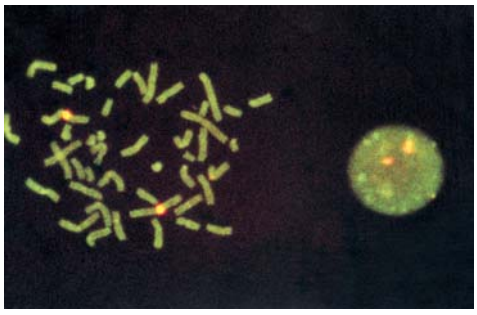

Figure 1: FISH of CY3-labeled satellite III probe pUC1.77 (chromosome 1) to human lymphocyte metaphase and interphase cells. The slides were counterstained with YOYO-1. The photomicrograph was taken with a double band-pass fluorescence filter to allow simultaneous visualization of fluorescein and Texas Red.

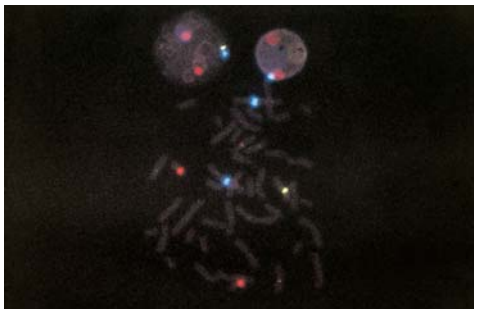

Figure 2: Triple color FISH of coumarin-labeled satellite III probe pUC1.77 (chromosome 1), fluorescein-labeled alphoid probe pBamX5 (chromosome X), and rhodamine-labeled alphoid probe p17H8 (chromosome 17) to human lymphocyte metaphase and interphase cells. The photomicrograph was taken by superimposing the coumarin, fluorescein, and rhodamine images.

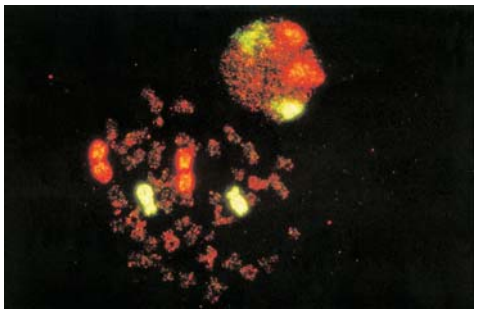

Figure 3: Double color FISH of a biotin-labeled chromosome 1-specific library and a digoxigenin-labeled chromosome 4-specific library to human lymphocyte metaphase and interphase cells. The libraries were visualized by a combined immunocytochemical reaction using fluorescein-labeled anti-digoxigenin and Texas Red-labeled streptavidin. The photomicrograph was taken with a double band-pass fluorescence filter to allow simultaneous visualization of fluorescein and Texas Red.

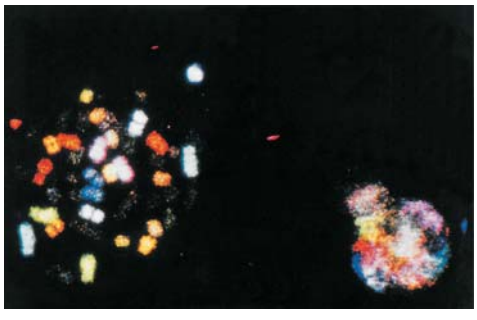

Figure 4: Twelve color FISH of 12 different ratio-labeled chromosome-specific libraries to human lymphocyte metaphase and interphase cells. For details of the experiment, see Dauwerse et al. (1992). The photomicrograph was created by superimposing the coumarin image and the image obtained with a double band-pass fluorescence filter (to allow simultaneous visualization of fluorescein and Texas Red).

B. Detection of a trisomy in a leukemia patient in interphase nuclei using double *in situ* hybridization

from U. Mathieu and Dr. P. Lichter, German Cancer Research Center, Heidelberg, Germany.

Nick translation was used to label a chromosome 17-specific alphoid DNA (with digoxigenin) and a chromosome 18-specific alphoid DNA (with biotin). Both the digoxigenin-labeled and the biotin-labeled probes were hybridized to a chromosome sample from a leukemia patient. The procedures described above for repetitive DNA (Procedure IIA of this article) were used for slide preparation, labeling, and hybridization, except the following minor modifications were necessary for alphoid DNA:

1. After nick translation labeling, concentrate the probe (approx. 10–30 ng DNA) by drying the DNA directly in the tube.

   ! *Ethanol precipitation is not necessary.*

2. Dissolve the dried DNA pellet in 60% formamide, 2× SSC.

   ! *Do not use dextran sulfate in the resuspension buffer. Omission of dextran sulfate reduces the chance of cross-hybridization.*

3. After hybridization, use the following washes:

   ► 3 × 5 min at 42°C with 60% formamide, 2× SSC; pH 7.

   ! *Compared to the standard protocol the concentration of formamide is increased up to 60%.*

■

Figure 5 shows the result of the hybridization. The digoxigenin-labeled probe was visualized with anti-DIG-rhodamine; the biotin-labeled probe, with avidin-FITC.

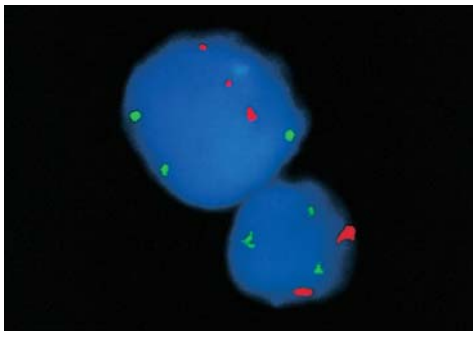

Figure 5: Double color FISH of a digoxigenin-labeled chromosome 17-specific probe and a biotin-labeled chromosome 18-specific probe to a human chromosome preparation containing a trisomy. Each alphoid DNA probe was specific for the centromere of its target chromosome. In the metaphase and in the interphase, the probes detected three signals (green) from chromosome 18 and two signals (red) from chromosome 17. DAPI was used for counterstaining.

C. Detection of a trisomy and a tetrasomy in interphase nuclei of a leukemia patient with two different cosmid probes

from U. Mathieu and Dr. P. Lichter

A digoxigenin-labeled cosmid probe specific for chromosome 14 and a biotin-labeled cosmid probe specific for chromosome 21 were used to detect polyploidy in a leukemia patient (Figure 6). Probes were labeled by nick translation and visualized with either anti-DIG-rhodamine (chromosome 14-specific probe) or avidin-FITC (chromosome 21-specific probe).

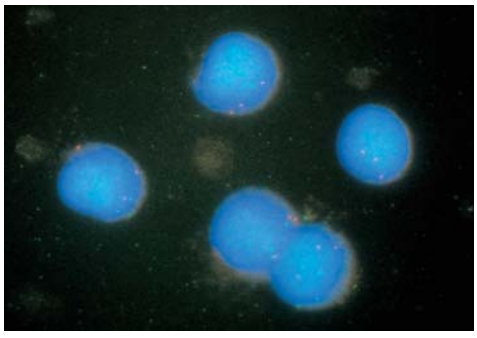

Figure 6: Double color FISH of a digoxigenin-labeled chromosome 14-specific cosmid probe and a biotin-labeled chromosome 21-specific cosmid probe to a human chromosome preparation of a leukemia patient containing a trisomy and a tetrasomy. In the interphase nuclei, the cosmid DNA probes detected three copies (red) of chromosome 14 and four copies (green) of chromosome 21. DAPI was used for counterstaining.

D. Interphase nuclei of a patient with Ph1-positive ALL (acute lymphoblastic leukemia)

from M. Bentz, P. Lichter, H. Döhner and G. Cabot.

The Philadelphia chromosome (Ph1) is the derivative of a translocation between chromosome 9 and chromosome 22 [(t9;22) (q34;q11)]. Figure 7 shows the use of two probes, one specific for chromosome 9 and the other specific for chromosome 22, to detect Ph1.

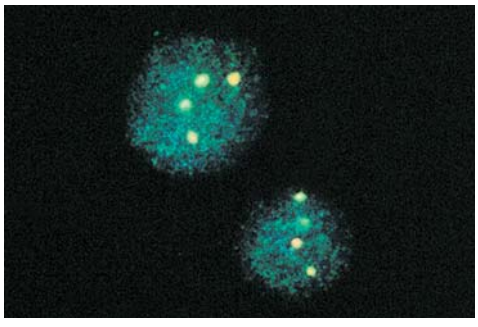

Figure 7: Double color FISH to detect the Philadelphia chromosome (Ph1) in a patient with ALL. A cosmid probe specific for chromosome 9 and a YAC probe specific for chromosome 22 were hybridized to interphase nuclei (Bentz et al., 1994). These two probes detected two red (rhodamine) signals from chromosomes 22, one green (FITC) signal from chromosome 9 and one yellow signal caused by the overlapping of red (chromosome 22) and green (chromosome 9) signals on Ph1. DAPI was used for counterstaining.

Reprinted from Bentz et al. (1994) Detection of Chimeric BCR–ABL genes on Bone Marrow Samples and Blood Smears in Chronic Myeloid and Acute Lymphoblastic Leukemia by in situ Hybridization, Blood 83 (7); 1922–1928 with permission of the Journal Permissions Department, W. B. Saunder's Company.

E. Chromosomal *in situ* suppression (CISS) hybridization of the human DNA library specific for chromosome 2 to chromosomes of *Gorilla gorilla*

from Dr. J. Wienberg and Dr. N. Arnold, Institute for Anthropology and Human Genetics, University of Munich, Germany.

The hybridization pattern (Figure 8) obtained with specific DNA probe sets (Weinberg et al., 1990) confirms the assumed fusion of two submetacentric chromosomes during human evolution.

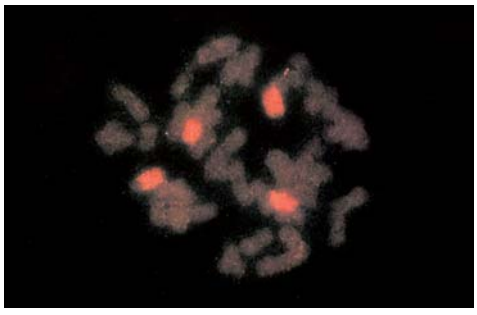

Figure 8: FISH of a chromosome 2-specific library to chromosomes of *Gorilla gorilla*. Standard chromosome preparations were obtained from EBV-transformed lymphoblastoid cell lines. DNA probes were from flow-sorted human chromosomes cloned to phages or plasmids. Probes were labeled with digoxigenin by nick translation. Detection was performed with monoclonal mouse anti-DIG antibody, rabbit anti-mouse-TRITC, and goat anti-rabbit-TRITC.

F. Illustration of a chromosomal translocation from a patient with chronic myeloid leukemia

from S. Popp and Dr. T. Cremer, Institute for Human Genetics and Anthropology, University of Heidelberg, Germany.

The chromosomal translocation was detected by using library probes from sorted chromosomes 9 and 22 (Figure 9). In Panel a, the chromosome 22-specific probe has clearly painted the normal chromosome 22 (bottom), the Philadelphia chromosome (triangle) and the translocated material 22q11 → 22qter contained in the derivative chromosome 9 (arrow). In Panel b, the chromosome 9-specific probe has stained the normal chromosome 9 blue except for the centromeric region, while in the derivative chromosome 9, the region 9pter → 9q34 is also painted. The arrow points to the breakpoint on chromosome 9.

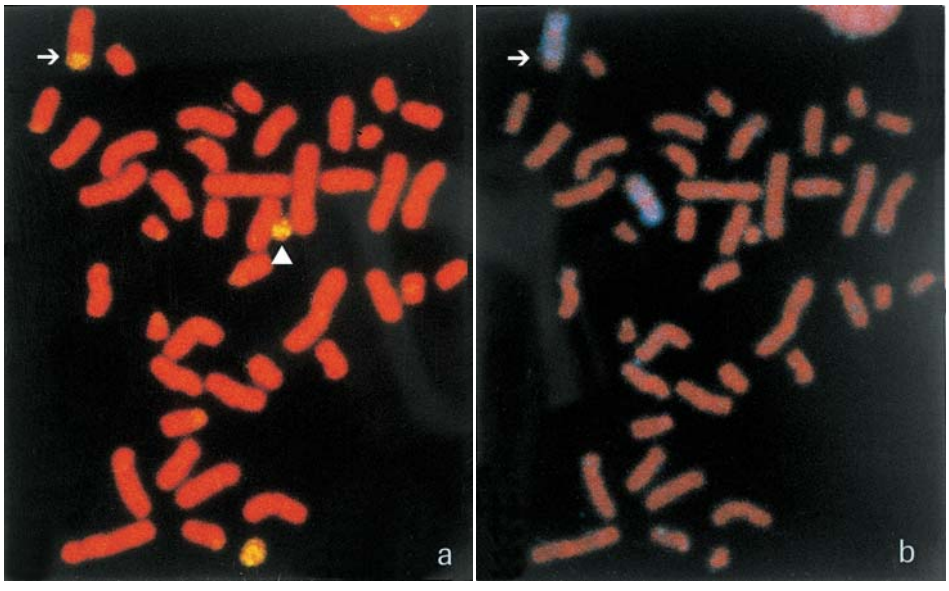

Figure 9: Two-color chromosomal *in situ* suppression (CISS)-hybridization of a metaphase spread obtained from a patient with chronic myeloid leukemia 46, XY, t(9;22) (q34;q11). In Panel a, the probe was a digoxigenin-labeled chromosome 22-specific library, which was detected with a mouse anti-DIG antibody and a FITC-conjugated sheep anti-mouse antibody. The same metaphase spread was simultaneously painted (Panel b) with a biotin-labeled chromosome 9-specific library, which was detected with avidin conjugated to the fluorochrome AMCA (aminocoumarin). Chromosomes were counterstained with propidium iodide. Chromosomal analysis using GTG-banded chromosomes was kindly performed by R. Becher, Westdeutsches Tumorzentrum, Essen.

G. Digitized images of female mouse chromosomes visualized with a Zeiss epifluorescent microscope equipped with a cooled CCD camera

from Dr. D. C. Ward, Human Genetics Department, Yale University, New Haven, CT, USA.

In Figure 10, a probe specific for Na^+/K^+ AT-Pase alpha subunits and a probe containing an L1-repetitive sequence were hybridized to female mouse chromosomes.

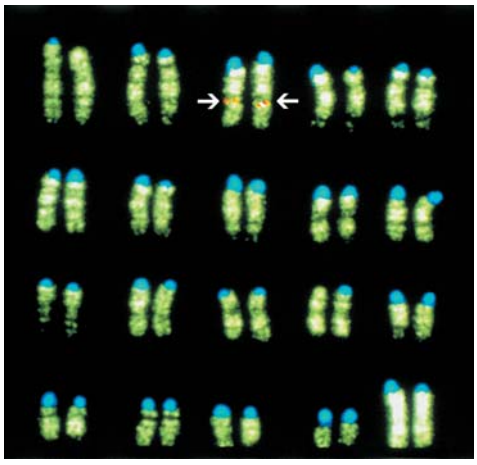

Figure 10: FISH of an ATPase-specific probe and a probe containing L1-repetitive sequences to female mouse chromosomes. Yellow color (banding) is due to the biotin-labeled probe containing L1-repetitive sequences, which was detected with FITC. Red "dots" indicate a signal from each of the four (look closely) chromatids obtained with the digoxigenin-labeled ATPase-specific probe, which was detected with anti-DIG Fab fragments (from sheep) and Texas Red-conjugated anti-sheep Ig Fab. The blue color is the DAPI counterstain.

Reagents available from Roche Applied Science for these procedures

Reagent	Description	Cat. No.	Pack size
DIG-Nick Translation Mix*	5× conc. stabilized reaction buffer in 50% glycerol (v/v) and DNA Polymerase I, DNase I, 0.25 mM dATP, 0.25 mM dCTP, 0.25 mM dGTP, 0.17 mM dTTP and 0.08 mM DIG-11-dUTP.	11 745 816 910	160 µl (40 labeling reactions)
Biotin-Nick Translation Mix*	5× conc. stabilized reaction buffer in 50% glycerol (v/v) and DNA Polymerase I, DNase I, 0.25 mM dATP, 0.25 mM dCTP, 0.25 mM dGTP, 0.17 mM dTTP and 0.08 mM biotin-16-dUTP.	11 745 824 910	160 µl (40 labeling reactions)
Nick Translation Mix*	5× conc. stabilized reaction buffer in 50% glycerol, DNA Polymerase I and DNase I.	11 745 808 910	200 µl (50 labeling reactions)
dNTP Set	Set of dATP, dCTP, dGTP, dTTP, 100 mM solutions, lithium salts.	11 277 049 001	4 × 10 µmol (100 µl)
Fluorescein-12-dUTP	Tetralithium salt, 1 mM solution	11 373 242 910	25 nmol (25 µl)
Tetramethyl-rhodamine-5-dUTP	Tetralithium salt, 1 mM solution	11 534 378 910	25 nmol (25 µl)
RNase A	Pyrimidine specific endoribonuclease which acts on single-stranded RNA.	10 109 142 001 10 109 169 001	25 mg 100 mg
Pepsin	Aspartic endopeptidase with broad specificity.	10 108 057 001	1 g
COT Human DNA	COT Human DNA is used in chromosomal *in situ* suppression [CISS] hybridization.	11 581 074 001	500 µg (500 µl)
DNA, MB-grade from fish sperm	The ready-to-use solution is directly added to the hybridization mix.	11 467 140 001	500 mg (50 ml)
tRNA from baker's yeast	Lyophilizate, 1 mg of dry substance corresponds to 16 A_{260} units.	10 109 495 001 10 109 509 001	100 mg 500 mg
Tween 20	Aqueous solution, 10% (w/v)	11 332 465 001	5 × 10 ml
Tris	Powder	10 708 976 001 11 814 273 001	1 kg 5 kg
Blocking Reagent, for nucleic acid hybridization	Powder	11 096 176 001	50 g
Anti-Digoxigenin*, clone 1.71.256, mouse IgG1, χ	For the detection of digoxigenin-labeled compounds	11 333 062 910	100 µg
Anti-Digoxigenin-Fluorescein, Fab fragments* from sheep	For the detection of digoxigenin-labeled compounds	11 207 741 910	200 µg
Anti-Digoxigenin-Rhodamine, Fab fragments* from sheep	For the detection of digoxigenin-labeled compounds	11 207 750 910	200 µg
DAPI 4',6-Diamidine-2'-Phenylindole Dihydrochloride	Fluorescence dye for staining of chromosomes	10 236 276 001	10 mg

* The labeling of nucleic acids with DIG is covered by EP patents 0 324 474 and 0 371 262 as well as the following US patents 5.344.757, 5.354.657 and 5.702.888 owned by Roche Diagnostics GmbH.

VI. Troubleshooting guide for *in situ* hybridization on chromosome spreads

from Stefan Joos and Peter Lichter, German Cancer Research Center, Heidelberg, Germany.

Use the following tips to diagnose and correct commonly occurring problems in the FISH protocols described above.

A. *If* no signal is observed, then:

1 Amplify signal.

2 Check probe labeling by performing the following dot blot assay:

- Spot serial dilutions of labeled control.
- Spot serial dilutions of labeled sample.
- Compare intensities of the spots.

For the detailed procedure on how to estimate the labeling efficiency please refer to Chapter 4, XIII, page 59.

3 After labeling the probe according to Steps 1–4 of the nick translation procedure (Procedure III) in Chapter 4 of this manual, check the size of the labeled probe molecules as follows:

- To a 5–10 µl aliquot of probe, add gel loading buffer and denature the probe by incubating it in a boiling water bath for 3 min, then cool it on ice for 3 min.

  Keep the remainder of the probe sample on ice while running the gel.
- Load the aliquot on a standard 1–2% agarose minigel along with a suitable size marker.
- Quickly run the gel (*e.g.*, 15 volts per cm for 30 min) to avoid renaturation of the probe in the gel.
- Visualize DNA in the gel, *e.g.*, by staining gel in 0.5 µg/ml ethidium bromide, and take photograph during UV illumination.

  The probe molecules will be visible as a smear. The smear should contain only fragments smaller than 500 nucleotides (nt) and larger than 100 nt. A peak intensity at 250–300 nt seems optimal.
- Depending on the size of the probe molecules, do one of the following:
  If the DNA is between 100–500 nt, proceed with the EDTA inactivation of the reaction mixture (as in Step 7 of the nick translation procedure, Chapter 4).
- *If* the probe is larger than 500 nt, add more DNase I (roughly about 1–10 ng) to the probe sample kept on ice, then incubate longer at 15°C.

  Usually higher concentrations of DNase must be added for an additional 30 min incubation. After the incubation, analyze the probe on a gel as above.
- *If* the DNA is almost or completely undigested, purify the probe and repeat the labeling step.
  If part of the DNA is smaller than 100 nt, repeat the labeling step using less DNase I.
  If all the DNA is smaller than 100 nt, repurify the probe (to remove any possible contaminating DNase) and repeat the labeling step.

4 Check whether the fluorochromes have separated from the detecting molecules by passing the solution of fluorochrome-conjugated molecules through a Quick Spin column (G-50). Then determine the state of the molecules:

- *If* color remains in flow through, fluorochromes are still conjugated.
- *If* color remains in the upper part of the column, fluorochromes have separated from the detecting molecules.

5 Modify chromosome denaturation procedure (Procedure II in this article) by either varying the time and temperature of the denaturation step or by further pepsin digestion according to the following protocol:

Digestion with pepsin can significantly improve probe penetration, but overdigestion can also occur. Pepsin treatment is often useful when specimen preparations are suboptimal, e.g., in many clinical samples.

- Mark the area of interest on the back of the slide using a diamond stylus.
- Wash slide in 2× SSC at 37°C for 5 min (in a Coplin jar).
- Apply 120 µl RNase A solution (100 µg/ml RNase A in 2× SSC) to slide, cover with larger coverslip (*e.g.*, 22 × 50 mm), and incubate for 1 h at 37°C in a moist chamber.
- Wash slide in 2× SSC (or PBS) for 3 × 5 min (Coplin jar).
- Wash slide in prewarmed PBS for 5 min at 37°C.
- Incubate slide in pepsin solution (10 mg pepsin in 100 ml 10 mM HCl) for 10 min at 37°C (in a water bath).
- Wash slide in PBS for 5 min at room temperature (RT).
- Incubate slide for 10 min at RT in a solution of 1% acid-free formaldehyde, PBS, 50 mM $MgCl_2$.
- Wash slide again in PBS for 5 min at RT.
- Dehydrate slide in a series of ethanol baths (70%, then 90%, then 100%; ≥5 min each) and air dry.

6 Check quality of the formamide. Deionize formamide (several batches from several sources should be tested) using ion exchange resin (*e.g.*, Dowex XG8).

7 Check microscope. Be sure that mercury (Hg_2) lamp of the microscope is in good condition and well adjusted. Old lamps don't have good excitation properties.

8 Perform control experiments with alphoid DNA probes.

B. *If* the hybridization signal fades, then:

1 Change to a different batch of fluorochrome-conjugated antibody.

2 Amplify with a secondary and/or tertiary antibody to increase the signal.

Be aware of the background, which will also be amplified. Before amplification, remove embedding medium by washing (2× SSC, 37°– 42°C, 4 × 10 min).

3 Try different antifading solutions [*e.g.* 1,4-diazobicyclo-(2,2,2)-octane (DABCO) or Vectastain (Vector)].

C. *If* there is a milky background fluorescence, then:

Possible cause	Remedy
Inadequate quality of chromosome preparation	Treat with pepsin to remove cell debris (see protocol in Step A5 above).
Insufficient blocking	Try to block with different reagents such as 3% BSA, human serum, or 3–5% dry milk.
Dirty glass slides	Wash the slide with ethanol and rinse with water before spreading chromo somes.
Bad quality of embedding medium	Change embedding medium.

D. *If* there is a starry background fluorescence, *then*:

Possible cause	Remedy
Agglutination of fluoro-chromes coupled to anti-bodies	Perform a control experiment in the absence of probe to see if you get a non-specific signal from the fluorochrome-conjugated antibody alone. *If* you do, spin the detection solution briefly and take only the supernatant. Alternatively pass the detection solution through a G-50 Quick Spin column to remove the agglutinates.
Labeled probe molecules are too long	Check the size of the probe as described in Step A3 above and label again.

5

E. *If* there is a general strong staining of chromosomes and nuclei, *then*:

- Check the size of the labeled probe (as in Step A3 above). It is most likely too small.
- *If* the probe is too small, repurify (to remove any contaminating DNase) and relabel the probe.

References

Bentz, M.; Cabot, G.; Moos, M.; Speicher, M. R., Ganser, A.; Lichter, P.; Döhner, H. (1994) Detection of chimeric BCR-ABL genes on bone marrow samples and blood smears in chronic myeloid and acute lymphoblastic leukemia by *in situ* hybridization. *Blood* **83**, 1922–1928.

Dauwerse, J. G.; Wiegant, J.; Raap, A. K.; Breuning, M. H.; Van Ommen, G. J. B. (1992) Multiple colors by fluorescence *in situ* hybridization using ratio-labeled DNA probes create a molecular karyotype. *Hum. Mol. Genet.* **1**, 593–598.

Wienberg, J.; Jauch, A.; Stanyon, R.; Cremer, T. (1990) Molecular cytotaxonomy of primates by chromosomal *in situ* suppression hybridization. *Genomics* **8**, 347–350.

Fluorescence in situ hybridization of a repetitive DNA probe to human chromosomes in suspension

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Fluorescence *in situ* hybridization (FISH) has found widespread application in the analysis of chromosomes and interphase nuclei fixed on slides (Anastasi et al., 1990; Cremer et al., 1988, 1990; Dekken et al., 1990; Devilee et al., 1988; Kolluri et al., 1990; Lichter et al., 1991; Pinkel et al., 1988; Schardin et al., 1985; Wienberg et al., 1990).

However, hybridization of specific DNA probes to isolated metaphase chromosomes in suspension offers a new approach to chromosome analysis and chromosome separation. Initial investigations were done on chromosomes obtained from a (Chinese hamster X human) hybrid cell line with biotinylated human genomic DNA as the probe (Dudin et al., 1987, 1988; Hausmann et al., 1991).

So far the technique for FISH in suspension has been a modification of FISH techniques used for metaphase chromosomes and interphase nuclei fixed on slides. Formamide (and to some extent dextran sulfate) are obligatory components of this method.

Thus, the technique requires a certain number of washing steps after hybridization. The washing steps for FISH in suspension, however, are based on centrifugal steps. These steps are responsible for a considerable reduction in the final amount of chromosomal material.

Additionally, the existing method for FISH in suspension appears to favor an aggregation of the chromosomal material in suspension.

We report here a hybridization technique which does not need formamide and dextran sulfate. As a model system, we used the repetitive specific human DNA probe pUC 1.77 (Cooke and Hindley, 1979; Emmerich et al., 1989), labeled it with digoxigenin-11-dUTP by nick-translation, and hybridized it to metaphase chromosomes in suspension. These chromosomes were isolated by standard techniques from human lymphocytes.

In preliminary experiments, this technique produced a large number of isolated metaphase chromosomes with satisfactory morphology and clear hybridization signals (Figure 1).

Adapting the same method and probe to metaphase spreads (Figure 2) allowed us to determine hybridization efficiency.

Procedure

The DNA probe pUC 1.77 is a clone of the plasmid vector pUC 9 that contains a 1.77 kb human Eco RI fragment. The inserted DNA was isolated from human satellite DNA fraction II/III. This insert contains mainly a tandemly organized repetitive sequence in the region q12 of chromosome 1 (Cooke and Hindley, 1979; Gosden et al., 1981).

1. Label the DNA probe pUC 1.77 with Digoxigenin-11-dUTP according to standard nick translation procedures (as described in Chapter 4 of this manual).
2. Cultivate human lymphocytes from peripheral blood.
3. Prepare chromosomes from the lymphocytes by standard techniques.
4. Resuspend the chromosomes and interphase nuclei in methanol/acetic acid (3:1) and store at -20°C.
5. Perform fluorescence detection with Anti-DIG-Fluorescein, Fab fragments as described (Lichter et al., 1990), with the following modifications:
   - Incubate for approx. 1 h at 37°C.
   - After the blocking step, do not use bovine serum albumin in the rest of the procedure.
   - Modify as necessary to allow FISH in suspension.
6. After labeling and FISH procedures, pipette 10 µl of the suspension on a slide for microscopic analysis. Counterstain with 15 µM propidium iodide (PI) and 5 µM DAPI.
7. Analyze with an epifluorescence microscope (Orthoplan equipped with a Planapo oil 63× objective and a 1.40 aperture, Leitz, Wetzlar, Germany).
8. Photograph with, for instance, Fujichrome P1600 D film at a final image magnification of about 630×, using Leitz filter system "I 2/3" for detection of FITC fluorescence.

Results

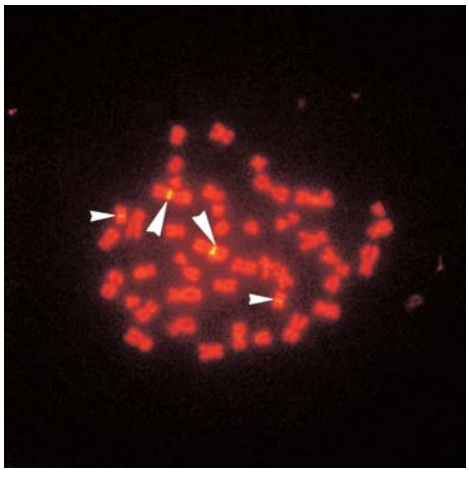

Figure 1: FISH of a human metaphase spread according to the same hybridization technique used in Figure 1. In this case the pUC 1.77 DNA probe binds to major hybridization sites (q12 region of human chromosome 1) that appear as two large yellowish-green "spots" on two of the largest chromosomes (large arrowheads). Additionally, a minor binding site of the DNA probe (chromosome 16; Gosden et al., 1981) is indicated by two minor yellowish-green "spots" on two of the smaller chromosomes (small arrowheads).

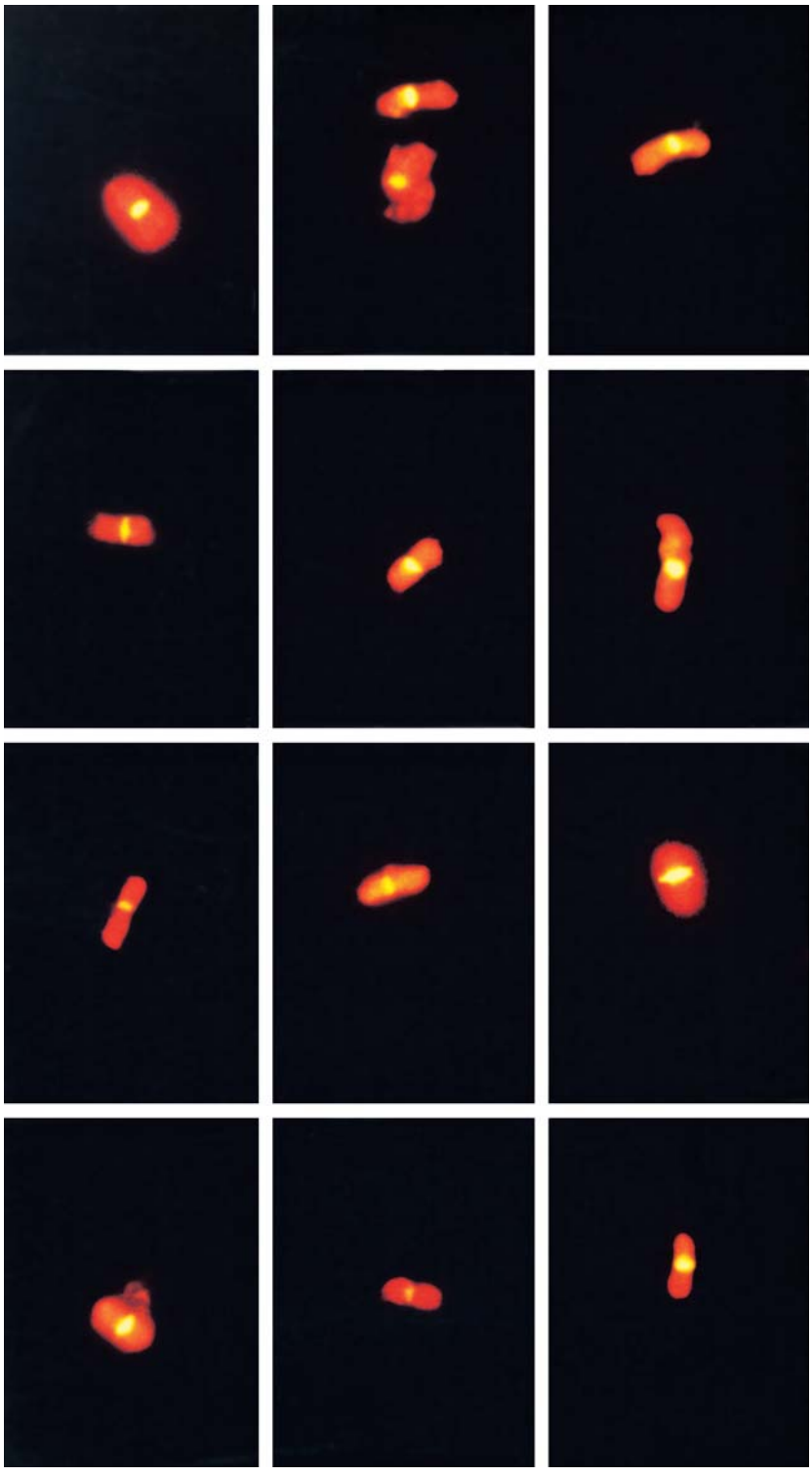

Figure 2: FISH of digoxigenin-labeled DNA probe pUC 1.77 to isolated human chromosomes in suspension. For details of the hybridization procedure, see the text. The sites of hybridization on the chromosomes appear as yellowish-green "spots" in the centromeric regions. Counterstaining was with propidium iodide (PI) and DAPI.

Reagents available from Roche Applied Science for this procedure

Reagent	Description	Cat. No.	Pack size
DIG-Nick Translation Mix* (for *in situ* probes)	5× conc. stabilized reaction buffer in 50% glycerol (v/v) and DNA Polymerase I, DNase I, 0.25 mM dATP, 0.25 mM dCTP, 0.25 dGTP, 0.17 mM dTTP and 0.08 mM DIG-11-dUTP	11 745 816 910	160 µl (40 labeling reactions)
Anti-Digoxigenin-Fluorescein*	Fab Fragments from sheep	11 207 741 910	200 µg
DAPI	Fluorescence dye for staining of chromosomes	10 236 276 001	10 mg

* The labeling of nucleic acids with DIG is covered by EP patents 0 324 474 and 0 371 262 as well as the following US patents 5.344.757, 5.354.657 and 5.702.888 owned by Roche Diagnostics GmbH

Acknowledgments

We thank Prof. Dr. T. Cremer, Intitute of Human Genetics and Anthropology, University of Heidelberg, for providing the plasmid pUC 9 and the human lymphocyte preparations. The work was supported by the Deutsche Forschungsgemeinschaft.

References

Anastasi, J.; Le Beau M. M.; Vardiman, J. W.; Westbrook, C. A. (1990) Detection of numerical chromosomal abnormalities in neoplastic hematopoietic cells by *in situ* hybridization with a chromosome-specific probe. *Am. J. Pathol.* **136**, 131–139.

Cooke, H. J.; Hindley, J. (1979) Cloning of human satellite III DNA: different components are in different chromosomes. *Nucleic Acids Res.* **10**, 3177–3197.

Cremer, T.; Lichter, P.; Borden, J.; Ward, D. C.; Manuelidis, L. (1988) Detection of chromosome aberrations in metaphase and interphase tumor cells by *in situ* hybridization using chromosome-specific library probes. *Hum. Genet.* **80**, 235–246.

Cremer, T.; Popp, S.; Emmerich, P.; Lichter, P.; Cremer, C. (1990) Rapid metaphase and interphase detection of radiation-induced chromosome aberrations in human lymphocytes by chromosomal suppression *in situ* hybridization. *Cytometry* **11**, 110–118.

Dekken van, H.; Pizzolo, J. G.; Reuter, V. E.; Melamed, M. R. (1990) Cytogenetic analysis of human solid tumors by *in situ* hybridization with a set of 12 chromosome specific DNA probes. Cytogenet. *Cell Genet.* **54**, 103–107.

Devilee, P.; Thierry, R. F.; Kievits, T.; Kolluri, R.; Hopman, A. H. N.; Willard, H. F.; Pearson, P. L.; Cornellisse, C. J. (1988) Detection of chromosome aneuploidy in interphase nuclei from human primary breast tumors using chromosome-specific repetitive DNA probes. *Cancer Res.* **48**, 5825–5830.

Dudin, G.; Cremer, T.; Schardin, M.; Hausmann, M.; Bier, F.; Cremer, C. (1987) A method for nucleic acid hybridization to isolated chromosomes in suspension. *Hum. Genet.* **76**, 290–292.

Dudin, G.; Steegmayer, E. W.; Vogt, P.; Schnitzer, H.; Diaz, E.; Howell, K. E.; Cremer, T.; Cremer, C. (1988) Sorting of chromosomes by magnetic separation. *Human. Genet.* **80**, 111–116.

Emmerich, P.; Loos, P.; Jauch, A.; Hopman, A. H. N.; Wiegant, J.; Higgins, M. J.; White, B. N.; van der Ploeg, M.; Cremer, C.; Cremer, T. (1989) Double *in situ* hybridization in combination with digital image analysis: a new approach to study interphase chromosome topography. *Exp. Cell Res.* **181**, 126–149.

Gosden, J. R.; Lawrie, S. S.; Cooke, H.J. (1981) A cloned repeated DNA sequence in human chromosome heteromorphisms. *Cytogenet. Cell Genet.* **29**, 32–39.

Hausmann, M.; Dudin, G.; Aten, J. A.; Heilig, R.; Diaz, E.; Cremer, C. (1991) Slit scan flow cytometry of isolated chromosomes following fluorescence hybridization: an approach of online screening for specific chromosomes and chromosome translocations. *Z. Naturforsch.* **46c**, 433–441.

Kolluri, R. V.; Manuelidis, L.; Cremer, T.; Sait, S.; Gezer, S.; Raza, A. (1990) Detection of monosomy 7 in interphase cells for patients with myeloid disorders. *Am. J. Hermatol.* **33** (2), 117–122.

Lichter, P.; Boyle, A. L.; Cremer, T.: Ward, D. C. (1991) Analysis of genes and chromosomes by non-isotopic *in situ* hybridization. Genet. *Anal. Techn. Appl.* **8**, 24–35.

Lichter, P.; Tang, C. C.; Call, K.; Hermanson, G.; Evans, H. J.; Housman, D.; Ward, D. C. (1990) High resolution mapping of human chromosome 11 by *in situ* hybridization with cosmid clones. *Science* **247**, 64–69.

Pinkel, D.; Landegent, J.; Collins, C.; Fuscoe, J.; Segraves, R.; Lucas, J.; Gray, J. W. (1988) Fluorescence *in situ* hybridization with human chromosome-specific libraries: detection of trisomy 21 and translocations of chromosome 4. *Proc. Natl. Acad. Sci. USA* **85**, 9138–9142.

Schardin, M.; Cremer, T.; Hager, H. D.; Lang, M. (1985) Specific staining of human chromosomes in Chinese hamster X human cell lines demonstrates interphase territories. *Hum. Genet.* **71**, 281–287.

Wienberg, J.; Jauch, A.; Stanyon, R.; Cremer, T. (1990) Molecular cytotaxonomy of primates by chromosomal *in situ* suppression hybridization. *Genomics* **8**, 347–350.

A simplified and efficient protocol for nonradioactive *in situ* hybridization to polytene chromosomes with a DIG-labeled DNA probe

Prof. Dr. E. R. Schmidt, Institute for Genetics, Johannes Gutenberg-University of Mainz, Germany.

The protocol given here is a derivative of several published methods (Langer-Safer et al., 1982; Schmidt et al., 1988) with some minor modifications that make the method of *in situ* hybridization easier, faster, more reliable, and available to anyone who can operate a microscope.

I. Labeling the hybridization probe

For best labeling results, follow the random primed DNA labeling procedure (Procedure IA) in Chapter 4 page 36 of this manual. We suggest a modification of this procedure as described below:

1. Use 0.5–1 µg linearized DNA in 16 µl redistilled H_2O.
2. Denature in a boiling water bath for 10 min and chill on ice.
3. Add 4 µl DIG-High Prime reaction mix.
4. Mix, centrifuge and incubate either 2 h at 37°C or overnight at room temperature.
5. Stop the labeling procedure by heating in boiling water for 10 min.
6. Add water and 20× SSC to give a final volume of 100 µl with a final concentration of 5× SSC.

   If a larger number of preparations is to be hybridized, increase the volume to 200 µl.
7. Add 1 volume of 10% SDS to 100 volumes of the mixture in Step 6 (*i.e.*, 1 µl SDS to 100 µl of mixture) to give a final concentration of 0.1% SDS.
8. The mixture is ready for hybridization and can be stored at -20°C for at least several years without any significant decrease in hybridization efficiency.

   Comment: *In my experience, it is absolutely unnecessary to remove unincorporated dNTPs from the mixture before using the labeled probe. On the contrary, purification steps lead to the loss of hybridizable probe DNA and thus to a decrease in hybridization efficiency.*

II. Preparation and denaturation of polytene chromosomes from *Drosophila*, *Chironomus*, or other species

Squash larval salivary glands in 40% acetic acid according to standard procedure. For long term storage, place slides with squashed chromosomes in 100% 2-propanol at -20°C.

Prior to the *in situ* hybridization, denature the chromosomal DNA according to this procedure:

1	Rehydrate the slides by incubating them, for 2 min each, in solutions containing decreasing amounts of ethanol: 70% ethanol, 50% ethanol, 30% ethanol, 0.1× SSC – 2× SSC.
2	Digest with RNase if necessary. *This is usually not necessary.*
3	For better preservation of the chromosomes, include a "heat stabilization" step by incubating slides in 2× SSC for at least 30 min at 80°C.
4	Incubate the slides for 90 s in 0.1 N NaOH, at room temperature.
5	Wash slides for 30 s in 2× SSC.
6	Dehydrate slides by incubating them, for 2 min each, in solutions containing increasing amounts of ethanol: 30% ethanol, 50% ethanol, 70% ethanol, 95% ethanol.
7	Air dry the slides for 5 min. The preparations are then ready for hybridization. ***Comment:*** *If "denatured" chromosome preparations are to be stored for a long time, store them in 95% ethanol or 2-propanol at -20°C.*

III. Hybridization and detection

1. Add 5 µl of the hybridization mixture containing the digoxigenin-labeled probe DNA (from Procedure I) to the chromosome preparation and cover with a coverslip (18 × 18 mm).
2. Seal coverslip with rubber cement.
3. Incubate slides at the appropriate hybridization temperature between 50°C and 65°C (depending on the probe, AT-content, sequence homology, etc.) for 4–6 h (for single copy sequences) or for 1 h (for repetitive sequences).

   Longer incubations do not markedly increase the hybridization signal!
4. Remove rubber cement, then wash off the coverslip in 2× SSC for 2–5 min at room temperature .
5. Wash the slide in PBS for 2 min. Remove excess buffer from the slide by wiping with soft paper around the chromosome preparation.

   Do not let the preparation dry completely; this produces background.
6. Apply 5 µl of a 1:10 diluted solution of fluorescein- or rhodamine-labeled anti-DIG antibody. The antibodies should be diluted in PBS containing 1 mg/ml bovine serum albumin.
7. Incubate 30 min with anti-DIG antibody at 37°C under a coverslip.
8. Wash 5 min in PBS.
9. Blot off excess buffer with paper.
10. Finally, mount the chromosomal preparation in "glycerol-para-phenylenediamine-mixture" [1 mg p-phenylenediamine dissolved in 1 ml phosphate buffered saline (1 mM sodium phosphate, pH 8.0; 15 mM NaCl) containing 50% glycerol].
11. Inspect with a fluorescence microscope with the appropriate set of filters.

    Comments: *Instead of antibodies labeled with fluorescent dyes, any other antibody conjugates (enzyme conjugated, gold-labeled) can be used. In the case of biotin-labeled DNA, streptavidin can be used instead of antibodies to detect the hybridized DNA. I have tested a number of these possibilities, and all work very well (including gold-labeled anti-digoxigenin antibody followed by silver-enhancement). However, in my experience, the most precise localization is obtained with immunofluorescence. It seems to me that the sensitivity is also higher (or at least the signal/background ratio is improved) with fluorescently labeled antibodies. Admittedly, this might be a matter of personal preference.*

Results

Figure 1: Double *in situ* hybridization of a polytene chromosome of Chironomus with two different clones from a chromosomal walk within the sex-determining region of Chironomus piger. The two l-clones contain genomic DNA fragments which are 60 kbp apart. One clone was labeled with digoxigenin and detected with rhodamine-labeled anti-DIG antibody (red); the second clone was labeled with biotin and detected with anti-biotin antibody and fluorescein-labeled secondary antibody (green). The red and green signals can be seen simultaneously if the fluorescence microscope is equipped with a filter system for simultaneous blue and green excitation (filter no. 513803, Leica, Wetzlar). For the hybridization, the two probes are mixed 1:1, and the detection is performed with mixed antibody solutions. The method allows a very clear orientation for a chromosomal walk. We were able to discriminate the location of two clones which were only approximately 30 kb apart.

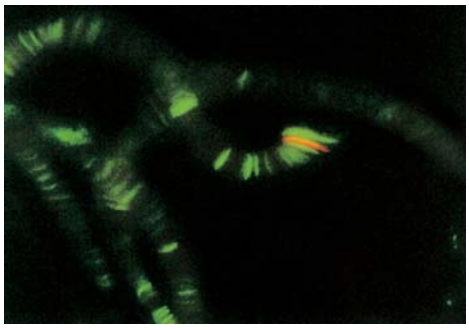

Figure 2: Double hybridization of a single copy clone from the sex-determining region together with a highly repetitive DNA element. The single copy DNA fragment was labeled with digoxigenin and the repetitive element with biotin. The single copy DNA (red) hybridizes to a single band, while the repetitive element (green) produces signals over numerous chromosomal sites throughout the entire chromosome. Hybridization and detection of the hybridized DNA was essentially the same as in Figure 1.

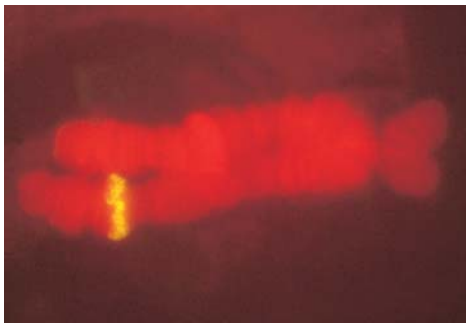

Figure 3: Double *in situ* hybridization of biotin- and digoxigenin-labeled DNA probes from two different "DNA puffs" in the salivary gland polytene chromosome C of *Trichosia pubescens* (Sciaridae, Diptera). The probes were hybridized simultaneously as described in Figure 1. The two sites of hybridization were detected with rhodamine-labeled anti-DIG and anti-biotin antibodies plus fluorescein-labeled secondary antibody. The two differentially labeled sites are so-called "DNA puffs", which are chromosomal regions with a developmentally regulated DNA amplification. The double in situ hybridization method allows very rapid mapping of the chromosomes, even when the banding structure of the chromosome is not analyzed in detail.

5

References

Langer-Safer, P. R.; Levine, M.; Ward, D. C. (1982) Immunological method for mapping genes on *Drosophila* polytene chromosomes. *Proc. Natl. Acad. Sci. USA* **79**, 4381–4385.

Schmidt, E. R.; Keyl, H.-G.; Hankeln, T. (1988) *in situ* localization of two haemoglobin gene clusters in the chromosomes of 13 species of Chironomus. *Chromosoma (Berl.)* **96**, 353–359.

Reagents available from Roche Applied Science for this procedure

Reagent	Description	Cat. No.	Pack size
DIG-High Prime‡	Complete reaction mixture for random primed labeling of DNA with DIG-dUTP 5× solution with: 1 mM dATP, dCTP, dGTP (each), 0.65 mM dTTP, 0.35 mM DIG-11-dUTP, alkali-labile; random primer mixture; 1 unit/µl Klenow enzyme labeling grade, in reaction buffer, 50% glycerol (v/v)	11 585 606 910	160 µl (40 reactions)
Biotin High Prime‡	Complete reaction mixture for random primed labeling of DNA with Biotin-dUTP 5× solution with: 1 mM dATP, dCTP, dGTP (each), 0.65 mM dTTP, 0.35 mM biotin-16-dUTP, random primer mixture, 1 unit/µl Klenow enzyme labeling grade, in reaction buffer, 50% glycerol (v/v)	11 585 649 910	100 µl (25 reactions)
RNase, DNase-free	RNase, DNase-free, can be used directly without prior heat treatment in any DNA isolation technique	11 119 915 001	500 µg (1 ml)
Anti-Digoxigenin-Rhodamine*	Fab Fragments from sheep	11 207 750 910	200 µg

‡ This product or the use of this product may be covered by one or more patents owned by Roche Diagnostics GmbH, including the following: US patent 5.814.502.

* The labeling of nucleic acids with DIG is covered by EP patents 0 324 474 and 0 371 262 as well as the following US patents 5.344.757, 5.354.657 and 5.702.888 owned by Roche Diagnostics GmbH.

Multiple-target DNA *in situ* hybridization with enzyme-based cytochemical detection systems

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Fluorescence *in situ* hybridization (ISH) is widely utilized because of its high sensitivity, resolution, and ability to detect multiple cellular nucleic acid sequences in different colors. Fluorescence ISH, however, has disadvantages, such as:

- Fluorescence signals fade when they are exposed to light.
- Autofluorescence in, *e.g.*, tissue sections can interfere with target analysis.

Here we outline a multicolor enzyme-based cytochemical detection protocol for nucleic acids *in situ*. This protocol produces permanent cell preparations with non-diffusible, nonfading reaction products. The reaction products can be analyzed with brightfield (Speel et al., 1994a), reflection-contrast (Speel et al., 1993), or, in one case (alkaline phosphatase-Fast Red reaction), fluorescence microscopy (Speel et al., 1992).

We show examples of single- and multiple-target ISH experiments on standard human lymphocyte metaphase spreads, as well as on interphase cell preparations. These results demonstrate the potential of this detection methodology for metaphase and interphase cytogenetics, *e.g.*, for studying chromosome aberrations in different cell types (Martini et al., 1995). In addition, this methodology can be applied in the area of pathology, since the universal detection protocol described here can be combined with other sample preparation procedures, *e.g.*, for tissue sections (Hopman et al., 1991, 1992).

The procedures given below are modifications of previously published procedures (Speel et al., 1992, 1993, 1994a, 1994b).

5

I. Cell preparations

Lymphocytes: Prepare chromosomes from peripheral blood lymphocytes by standard methods. Fix in methanol:acetic acid (3:1, v/v), and drop chromosomes onto glass slides that have been cleaned with a 1:1 mix of ethanol and ether.

Cultured cells: Make preparations from cultured normal diploid cells or tumor cell lines by one of the following methods:

- Ethanol suspension: Trypsinize cells (if necessary), harvest, wash in PBS, fix in cold 70% ethanol (-20°C), and drop onto glass slides that have been coated with poly-L-lysine.
- Slide and coverslip preparations: Grow cells on glass slides or coverslips. Fix in cold methanol (-20°C) for 5 s, then in cold acetone (4°C) for 3 × 5 s. Air dry samples and store at -20°C.

  ! *Alternatively, use other fixatives for the slide and coverslip preparations.*
- Cytospins: Cytospin floating cells onto glass slides at 1000 rpm for 5 min. Air dry samples for 1 h at room temperature. Fix and store as with slide and coverslip preparations above.

II. Cell processing

1. Decide whether samples need to be treated with RNase. Then do one of the following:
   - *If* the cells need RNase, go to Step 2.
   - *If* the cells do not need RNase, go to Step 3.

2. Treat slides with RNase as follows:
   - Overlay each sample with 100 µl RNase solution (100 µg/ml RNase A in 2× SSC) and a coverslip.
   - Incubate cell samples for 1 h at 37°C.
   - Remove coverslip and wash samples 3 × 5 min with 2× SSC.
   - Go to Step 3.

3. Treat slides with pepsin as follows:
   - Overlay each sample with 100 µl pepsin solution (50–100 µg/ml pepsin in 10 mM HCl) and a coverslip.
   - Incubate cell samples for 10–20 min at 37°C.
   - Wash samples as follows:
     - 2 min with 10 mM HCl
     - 2 × 5 min with PBS

4. Post-fix samples as follows:
   - Incubate samples with PBS containing 1% (para)formaldehyde for *either* 20 min at 4°C or 10 min at room temperature.
   - Wash slides 2 × 5 min with PBS.
   - Dehydrate samples by passing slides through a series of ethanol solutions (70%, then 96%, then 100% ethanol), incubating 10–60 s in each solution.

■

5

III. Probe preparation

1. Label the DNA probes (containing either repetitive or unique sequences) with Biotin-, Digoxigenin-, or Fluorescein-dUTP according to the nick translation procedure in Chapter 4 of this manual.

2. Just before use, prepare hybridization buffer containing:
   - 50% or 60% formamide.
   - 10% dextran sulfate.
   - 2× SSC.
   - 0.2 µg/µl sonicated herring sperm DNA.
   - 0.2 µg/µl yeast tRNA.
   - 1–2 ng labeled probe DNA/µl hybridization buffer [*if* the probe contains unique or highly repetitive (*e.g.*, centromere probe) sequences]
     or
     2–4 ng labeled probe DNA/µl hybridization buffer, together with an excess (100–1000 fold) of total human DNA or Cot HUMAN DNA [if the probe contains repetitive (*e.g.*, *Alu*) elements].

3. Perform ISH by one of the following methods:
   - *If* the probe contains unique or highly repetitive (*e.g.*, centromere probe) sequences, follow procedure IVA.
   - *If* the probe contains repetitive (*e.g.*, *Alu*) elements, follow procedure IVB.
   - For multiple-target *in situ* hybridization, prepare DNA probes labeled with different haptens (biotin, digoxigenin, or fluorescein), mix them together, and follow either Procedure IVA or Procedure IVB (depending on the nature of the probes).

■

IV. Multiple-target *in situ* hybridization (ISH)

IVA. ISH with simultaneous probe and target denaturation [for probes with unique or highly repetitive (*e.g.*, centromere probe) sequences]

1. On each sample, place 10 µl of hybridization buffer containing labeled probe DNA (prepared as in Procedure III, Step 2).
2. Cover sample with a 20 × 20 mm coverslip and (if you wish) seal the coverslip to the slide with rubber cement.
3. Denature probe and cellular DNA simultaneously by placing slides at 70°–75°C for 3–5 min on the bottom of a metal box.
4. Incubate hybridization samples overnight at 37°C.
5. Go to Procedure V.

■

IVB. ISH with separate probe and target denaturation [for probes with repetitive (*e.g.*, Alu) elements]

1. Incubate the probe mixture (labeled probe DNA, human or COT Human DNA, hybridization buffer; prepared as in Procedure III, Step 2) for 5 min at 75°C.
2. Chill the denatured probe mixture on ice.
3. Incubate the denatured probe mixture for 1–4 h at 37°C to pre-anneal the repetitive sequences in the mixture.
4. Denature the cell samples as follows:
   - Overlay the cell samples with 70% formamide in 2× SSC.
   - Incubate the slides for 2 min at 70°C to denature the cells.
   - Dehydrate the cell samples in a series of chilled (-20°C) ethanol solutions (70%, then 96%, then 100% ethanol), incubating them for 5 min in each solution.
   - Air dry the samples.
5. On each denatured cell sample (from Step 4), place 10 µl denatured, pre-annealed probe mixture (from Step 3).
6. Incubate hybridization samples overnight at 37°C.
7. Go to Procedure V.

■

V. Post-hybridization washes

1. Perform the following stringent washes of the samples (from either Procedure IVA or Procedure IVB) at 42°C:
   - 2 × 5 min with 2× SSC containing 50% (or 60%) formamide and 0.05% Tween 20.
   - 2 × 5 min with 2× SSC.
2. Depending upon the nature of the probe, do one of the following:
   - *If* the probe contains repetitive (*e.g.*, *Alu*) elements, wash the samples 2 × 5 min at 60°C with 0.01× SSC.
   - *If* the probe does not contain repetitive elements, skip this step.

■

VI. Enzyme-based cytochemical detection

VIA. Single color detection

Probe label	Incubation		
	1	2	3
Biotin	Avidin-E[1]		
Biotin	Avidin-E	Biotinylated anti-avidin Ab	Avidin-E
Hapten[2]	Anti-hapten Ab-E		
Hapten	Mouse anti-hapten Ab	Anti-mouse Ab-E	
Hapten	Mouse anti-hapten Ab	Rabbit anti-mouse Ab-E	Anti-rabbit Ab-E
Hapten	Mouse anti-hapten Ab	Biotinylated anti-mouse Ab	ABC
Hapten	Mouse anti-hapten Ab	DIG-labeled anti-mouse Ab	Anti-DIG Ab-E

Table 1: Frequently used detection systems for enzyme-based *in situ* hybridization.

1 Abbreviations used: Ab, antibody; ABC, avidin-biotinylated enzyme (horseradish peroxidase or alkaline phosphatase) complex; E, enzyme (horseradish peroxidase or alkaline phosphatase).

2 Hapten = biotin, digoxigenin, FITC, or DNP.

3 Anti-hapten Ab raised in another species (*e.g.*, rabbit, goat, swine) can also be used as primary Ab in probe detection schemes.

1. Wash samples briefly with 4× SSC containing 0.05% Tween 20.
2. Incubate samples for 10 min at 37°C with 4× SSC containing 5% nonfat dry milk.
3. Choose an appropriate enzyme-based detection system (Table 1).
4. Dilute detecting molecules as follows:
   - Dilute avidin conjugates in 4× SSC containing 5% nonfat dry milk.
   - Dilute antibody conjugates in PBS containing 2–5% normal serum and 0.05% Tween 20.
5. For the first incubation in the detection system (Table 1), do the following:
   - Incubate samples with diluted detecting molecule for 30 min at 37°C.
   - Wash samples 2 × 5 min in the appropriate wash buffer (4× SSC for avidin; PBS for antibodies) containing 0.05% Tween 20.
6. Repeat Step 5 with the next incubation in the detection system (Table 1) until all incubations are complete.
7. After all incubations in the detection system are complete, wash samples 5 min with PBS.
8. Visualize according to one of the procedures in Section VII.
9. If the detection procedure uses the same enzyme (peroxidase or alkaline phosphatase) to detect two different probes, do the following:
   - Inactivate the enzyme on the first detecting molecule by incubating the sample for 10 min at room temperature with 10 mM HCl.
   - Repeat Steps 5–8 with a second detection system that recognizes the second probe (Speel et al., 1994b).

VIB. Multiple target, multicolor detection

To detect multiple probes labeled with different haptens, use a combination of detection systems. For example, Table 2 outlines a protocol for triple-target *in situ* hybridization. For protocols with double-target *in situ* hybridizations, see Hopman et al. (1986), Emmerich et al. (1989), Mullink et al. (1989), Herrington et al. (1989), Kerstens et al. (1994), and Speel et al. (1995).

Alternatively, if two peroxidase or phosphatase reactions are used in the detection protocol (Speel et al., 1994b), inactivate the enzyme after the first detection reaction in 10 M HCl for 10 min at room temperature, then perform the second detection reaction (as in Procedure VIA above).

	Detection step	Incubation time[2]	Incubation temperature
1.	Detect biotin with AvPO[1] (diluted 1:50)	20 min	37°C
2.	Visualize PO by Procedure VIIA (PO-DAB, brown signal)	5 min	37°C
3.	Inactivate residual AvPO with 10 mM HCl.	10 min	RT
4.	Detect digoxigenin and FITC with MADIG and RAFITC (each diluted 1:2000)	30 min	37°C
5.	Detect anti-digoxigenin and anti-FITC with GAMAPase (diluted 1:25) and SWARPO (diluted 1:100)	30 min	37°C
6.	Visualize APase activity by Procedure VIIC (APase-Fast Red, red signal)	5 –10 min	37°C
7.	Visualize PO activity by Procedure VIIB (PO-TMB, green signal)	1 – 2 min	37°C
8.	Counterstain with hematoxylin	1 sec	RT
9.	Air dry	10 min	RT
10.	Embed in a protein matrix[3]	10 min	37°C

Table 2: Detection protocol for triple-target *in situ* hybridization with a biotin-, digoxigenin-, and a FITC-labeled probe.

[1] Abbreviations used: Ab, antibody; APase, alkaline phosphatase; AvPO, PO-conjugated avidin (Vector); DAB, diaminobenzidine; GAMAPase, APase-conjugated goat anti-mouse Ab (DAKO); MADIG, mouse anti-digoxigenin Ab; PO, horseradish peroxidase; RAFITC, rabbit anti-FITC Ab (DAKO); RT, room temperature; SWARPO, PO-conjugated swine anti-rabbit Ab (DAKO).

[2] For details of detection reactions, see Procedure VIA. For details of visualization reactions, see Procedures VIIA–VIID.

[3] For details of protein matrix, see Procedure VIII.

VII. Visualization

Enzyme label	Substrate	Precipitate colors in microscopy		
		Brightfield	Reflection-contrast	Fluorescence
PO[2]	H_2O_2/DAB H_2O_2/TMB	Brown Green	White Pink/Red	– –
APase	N-ASMX-P/FR BCIP/NBT	Red Purple	Yellow Yellow/Orange	Red –

Table 3: Enzyme reaction protocols and colors in different types of light microscopy[1].

[1] Other enzyme reactions that have been used for ISH are described in Speel et al. (1993, 1995).

[2] Abbreviations used: APase, alkaline phosphatase; BCIP, bromo-chloro-indolyl phosphate; DAB, diaminobenzidine; FR, Fast Red TR; N-ASMX-P, naphthol-ASMX-phosphate; NBT, nitroblue tetrazolium; PO, horseradish peroxidase; TMB, tetramethylbenzidine.

Depending upon the type of microscopy to be used for analysis (Table 3) and the detecting molecule, choose one of the following procedures to visualize the hybrids. In our hands, each of these procedures is optimal for *in situ* hybridization.

VIIA. Horseradish peroxidase-diaminobenzidine (PO-DAB)

1. Mix color reagent just before use:
   - 1 ml 3,3-diaminobenzidine tetrachloride (DAB; Sigma) stock (5 mg DAB/ml PBS).
   - 9 ml PBS containing 0.1 M imidazole, pH 7.6.
   - 10 µl 30% H_2O_2.
2. Overlay each sample with 100 µl color reagent and a coverslip.
3. Incubate samples for 5-15 min at 37°C.
4. Wash samples 3 × 5 min with PBS and (if you wish) dehydrate them.
5. Coverslip with an aqueous or organic mounting medium.

VIIB. Horseradish peroxidase-tetramethylbenzidine (PO-TMB)

1. Dissolve 100 mg sodium tungstate (Sigma) in 7.5 ml 100 mM citrate-phosphate buffer (pH 5.1). Adjust the pH of the tungstate solution to pH 5.0–5.5 with 37% HCl.
2. Just before use, dissolve 20 mg dioctyl sodium sulfosuccinate (Sigma) and 6 mg 3,3',5,5'-tetramethylbenzidine (TMB, Sigma) in 2.5 ml 100% ethanol at 80°C.
3. Prepare 10 ml color reagent by combining the tungstate solution (Step 1), the TMB solution (Step 2), and 10 µl 30% H_2O_2.
4. Overlay each sample with 100 µl color reagent and a coverslip.
5. Incubate samples for 1–2 min at 37°C.
6. Wash samples 3 × 1 min with ice-cold 100 mM phosphate buffer (pH 6.0) and (if you wish) dehydrate them.
7. Coverslip with an organic mounting medium or immersion oil.

VIIC. Alkaline phosphatase-Fast Red (APase-Fast Red)

1. Mix color reagent just before use:
   - 4 ml TM buffer [200 mM Tris-HCl (pH 8.5), 10 mM $MgCl_2$] containing 5% polyvinyl alcohol (PVA, MW 40,000; Sigma).
   - 250 µl TM buffer containing 1 mg naphthol-ASMX-phosphate (Sigma).
   - 750 µl TM buffer containing 5 mg Fast Red TR salt (Sigma).
2. Overlay each sample with 100 µl color reagent and a coverslip.
3. Incubate samples for 5–15 min at 37°C.
4. Wash samples 3 × 5 min with PBS.
5. Coverslip with an aqueous mounting medium.

VIID. Alkaline phosphatase-bromochloroindolyl phosphate (APase-BCIP/NBT)

Follow the standard procedure given in Chapter 2 of this manual.

VIII. Embedding and light microscopy

1. Prepare samples for microscopy by doing either of the following:
   - *If* samples require a single mounting medium, embed stained samples as described in Procedure VII.
   - *If* multiple precipitation reactions would require different (aqueous or organic) embedding mediums, apply instead a protein embedding layer by smearing 50 µl of a 1:1 mixture of BSA solution (40 mg/ml in deionized H_2O) and 4% formaldehyde onto the slides. Air dry for 10 min at 37°C.

     This protein embedding layer can be used to prevent solubilization of enzyme precipitates during all types of light microscopy (Speel et al., 1993, 1994a).

2. Perform brightfield, fluorescence and reflection contrast microscopy as described in the literature (Speel et al., 1992, 1993, 1994b; Cornelese-Ten Velde et al., 1989).

Results

Figure 1: Single-target ISH on a normal human lymphocyte metaphase spread with a peroxidase detection system. The probe was a biotinylated cosmid that recognizes 40 kb of chromosome 11q23. The detection system included monoclonal anti-biotin Ab (DAKO), rabbit anti-mouse Ab-PO, and the PO-DAB reaction. The sample was counterstained with hematoxylin and viewed by brightfield microscopy.

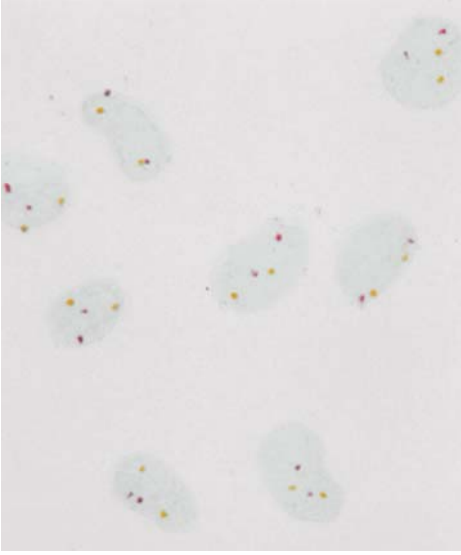


Figure 2: Double-target ISH on normal human umbilical vein endothelial cells with brightfield viewing. The centromere of chromosome 1 (brown) was detected with a biotinylated probe; that of chromosome 7 (red), with a digoxigenin-labeled probe. The detection steps included (1) avidin-PO, (2) monoclonal anti-digoxigenin Ab and rabbit anti-mouse Ab-APase, (3) the APase-Fast Red reaction, and (4) the PO-DAB reaction. The sample was counterstained with hematoxylin and viewed by brightfield microscopy.

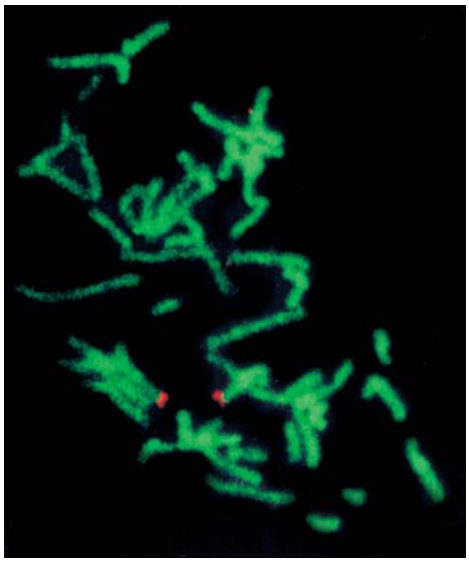


Figure 3: Same ISH experiment as in Figure 1, but with a fluorescent alkaline phosphatase detection system. The detection system for the biotinylated cosmid probe included monoclonal anti-biotin, horse anti-mouse Ab-biotin, avidin-biotinylated APase complex, and the APase-Fast Red reaction (Speel et al., 1994a). The sample was counterstained with Thiazole Orange (Molecular Probes) and viewed by fluorescence microscopy.

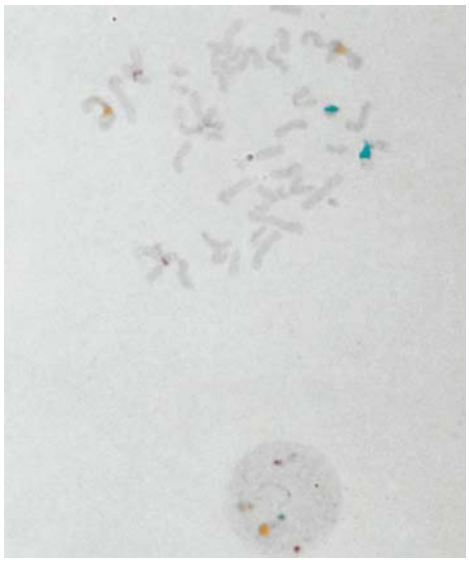


Figure 4: Triple-target ISH on a normal human lymphocyte metaphase spread. Probes specific for the centromeres of chromosomes 1 (brown), 7 (red), and 17 (green) were labeled with biotin, digoxigenin, and FITC, respectively. Detection, counterstaining, and embedding in a BSA protein layer was as outlined in the text (Table 2). The sample was viewed by brightfield microscopy.

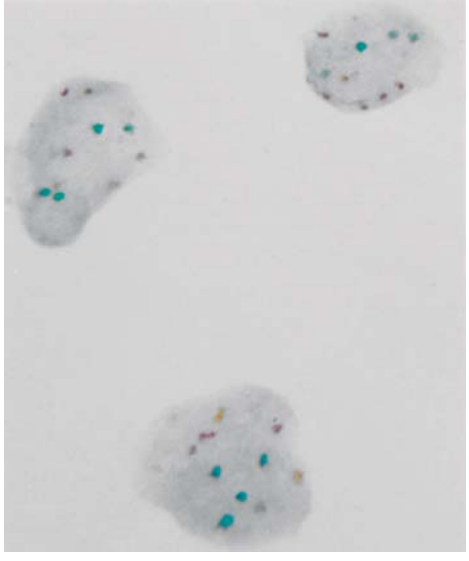


Figure 5: Triple-target ISH on human bladder tumor cell line T24. The probes and experimental procedures were the same as in Figure 4.

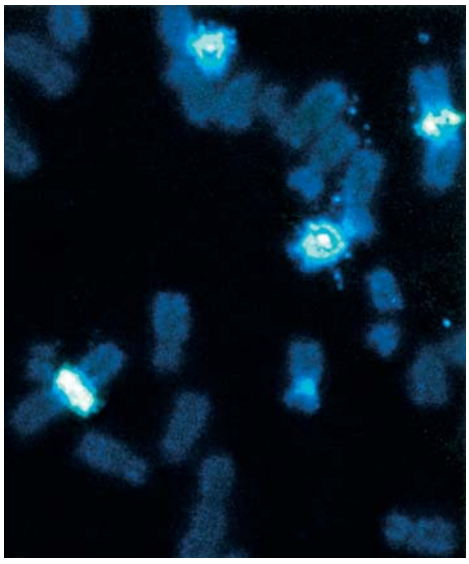


Figure 6: Double-target ISH on a normal human lymphocyte metaphase spread with reflection contrast viewing. The centromere of chromosome 1 (yellow) was detected with a biotinylated probe; that of chromosome 17 (white), with a digoxigenin-labeled probe. The detection steps included (1) monoclonal anti-biotin Ab and rabbit anti-digoxigenin Ab, (2) horse anti-mouse Ab-biotin and swine anti-rabbit Ab-PO, (3) streptavidin-biotinylated APase complex, (4) the APase-Fast Red reaction, and (5) the PO-DAB reaction (Speel et al., 1993). The sample was not counterstained, but was embedded in a BSA protein layer and viewed by reflection contrast microscopy.

Reprinted from Speel, E. J. M.; Kamps, M.; Bonnet, J.; Ramaekers, F. C. S.; Hopman, A. H. N.; (1993) Multicolor preparations for *in situ* hybridization using precipitating enzyme cytochemistry in combination with reflection contrast microscopy. *Histochemistry* 100, 357–366 with permission of Springer-Verlag GmbH & Co. KG.

References

Cornelese-Ten Velde, I.; Wiegant, J.; Tanke, H. J.; Ploem, J. S. (1989) Improved detection and quantification of the (immuno) peroxidase product using reflection contrast microscopy. *Histochemistry* **92**, 153–160.

Emmerich, P.; Loos, P.; Jauch, A.; Hopman, A. H. N.; Wiegant, J.; Higgins, M. J.; White, B. N.; Van der Ploeg, M.; Cremer, C.; Cremer, T. (1989) Double *in situ* hybridization in combination with digital image analysis: a new approach to study interphase chromosome topography. *Exp. Cell Res.* **181**, 126–140.

Herrington, C. S.; Burns, J.; Graham, A. K.; Bhatt, B.; McGee, J. O'D. (1989) Interphase cytogenetics using biotin and digoxygenin labeled probes II: simultaneous differential detection of human and papilloma virus nucleic acids in individual nuclei. *J. Clin. Pathol.* **42**, 601–606.

Hopman, A. H. N.; Wiegant, J.; Raap, A. K.; Landegent, J. E.; Van der Ploeg, M.; Van Duijn, P. (1986) Bi-color detection of two target DNAs by non-radioactive *in situ* hybridization. *Histochemistry* **85**, 1–4.

Hopman, A. H. N.; Van Hooren, E.; Van de Kaa, C. A.; Vooijs, G. P.; Ramaekers, F. C. S. (1991) Detection of numerical chromosome aberrations using *in situ* hybridization in paraffin sections of routinely processed bladder cancers. *Modern Pathol.* **4**, 503–513.

Hopman, A. H. N.; Poddighe, P. J.; Moesker, O.; Ramaekers, F. C. S. (1992) Interphase cytogenetics: an approach to the detection of genetic aberrations in tumours. In: McGee, J. O'D.; Herrington, C. S. (Eds.) Diagnostic Molecular Pathology, A Practical Approach, 1st ed. New York: IRL Press, 141–167.

Kerstens, H. M. J.; Poddighe, P. J.; Hanselaar, A. G. J. M. (1994) Double-target *in situ* hybridization in brightfield microscopy. *J. Histochem. Cytochem.* **42**, 1071–1077.

Martini. E.; Speel, E. J. M.; Geraedts, J. P. M.; Ramaekers, F. C. S.; Hopman, A. H. N. (1995) Application of different *in situ* hybridization detection methods for human sperm analysis. *Hum. Reprod.* **10**, 855–861.

Mullink, H.; Walboomers, J. M. M.; Raap, A. K.; Meyer, C. J. L. M. (1989) Two colour DNA *in situ* hybridization for the detection of two viral genomes using nonradioactive probes. *Histochemistry* **91**, 195–198.

Speel, E. J. M.; Schutte, B.; Wiegant, J.; Ramaekers, F. C. S.; Hopman, A. H. N. (1992) A novel fluorescence detection method for *in situ* hybridization, based on the alkaline phosphatase-Fast Red reaction. *J. Histochem. Cytochem.* **40**, 1299–1308.

Speel, E. J. M.; Kamps, M.; Bonnet, J.; Ramaekers, F. C. S.; Hopman, A. H. N. (1993) Multicolour preparations for *in situ* hybridization using precipitating enzyme cytochemistry in combination with reflection contrast microscopy. *Histochemistry* **100**, 357–366.

Speel, E. J. M.; Herbergs, J.; Ramaekers, F. C. S.; Hopman, A. H. N. (1994a) Combined immunocytochemistry and fluorescence *in situ* hybridization for simultaneous tricolor detection of cell cycle, genomic, and phenotypic parameters of tumor cells. *J. Histochem. Cytochem.* **42**, 961–966.

Speel, E. J. M.; Jansen, M. P. H. M.; Ramaekers, F. C. S.; Hopman, A. H. N. (1994b) A novel triple-color detection procedure for brightfield microscopy, combining *in situ* hybridization with immuo-cytochemistry. *J. Histochem. Cytochem.* **42**, 1299–1307.

Speel, E. J. M.; Ramaekers, F. C. S.; Hopman, A. H. N. (1995) Detection systems for *in situ* hybridization, and the combination with immunocytochemistry. Histochem. J., in press.

Reagents available from Roche Applied Science for this procedure

Reagent	Description	Cat. No.	Pack size
RNase A	Dry powder	10 109 142 001 10 109 169 001	25 mg 100 mg
Pepsin	Aspartic endopeptidase with broad specificity	10 108 057 001	1 g
Digoxigenin-11-dUTP, alkali-stable▽	Tetralithium salt, 1 mM solution	11 093 088 910 11 558 706 910 11 570 013 910	25 nmol (25 µl) 125 nmol (125 µl) 5× 125 nmol (5× 125 µl)
Fluorescein-12-dUTP	Tetralithium salt, 1 mM solution	11 373 242 910	25 nmol (25 µl)
Tetramethyl-rhodamine-5-dUTP	Tetralithium salt, 1 mM solution	11 534 378 910	25 nmol (25 µl)
Biotin-16-dUTP	Tetralithium salt, 1 mM solution	11 093 070 910	50 nmol (50 µl)
DNase I	Lyophilizate	10 104 159 001	100 mg
DNA Polymerase I	Nick Translation Grade	10 104 485 001 10 104 493 001	500 units 1000 units
tRNA	From baker's yeast	10 109 495 001 10 109 509 001	100 mg 500 mg
COT Human DNA	Solution in 10 mM Tris-HCl, 1 mM EDTA, pH 7.4, COT Human DNA is used in chromosomal *in situ* suppression (CISS).	11 581 074 001	500 µg (500 µl)
Tween 20		11 332 465 001	5× 10 ml
Anti-Digoxigenin*	Clone 1.71.256, mouse IgG 1, :	11 333 062 910	100 µg
Anti-Digoxigenin-POD*	Fab fragments from sheep	11 207 733 910	150 U
Anti-Digoxigenin-AP*	Fab fragments from sheep	11 093 274 910	150 U
NBT/BCIP, (stock solution)	Solution of 18.75 mg/ml nitro-blue tetrazolium chloride and 9.4 mg/ml 5-bromo-4-chloro-3-indolyl phosphate, toluidine-salt in 67% DMSO (v/v)	11 681 451 001	8 ml
Fast Red	1 tablet contains 0.5 mg naphtol substrate, 2 mg Fast Red chromogen and 0.4 mg levamisole (inhibitor of endogenous alkaline phosphatase activity)	11 496 549 001	20 tablets
Tris	Powder	10 708 976 001 11 814 273 001	1 kg 1 kg
BM Purple AP Substrate, precipitating	Ready to use solution	11 442 074 001	100 ml

▽ EP Patent 0371262 and US 5,198,537 owned by Roche Diagnostics GmbH.

* The labeling of nucleic acids with DIG is covered by EP patents 0 324 474 and 0 371 262 as well as the following US patents 5.344.757, 5.35 4.657 and 5.702.888 owned by Roche Diagnostics GmbH.

DNA *in situ* hybridization with an alkaline phosphatase-based fluorescent detection system

Dr. G. Sagner, Research Laboratories, Roche Applied Science GmbH, Penzberg, Germany.

We describe here a high sensitivity indirect detection procedure for DIG-labeled hybridization probes. The procedure uses the components of the HNPP Fluorescent Detection Set to form a fluorescent precipitate of HNPP (2-hydroxy-3-naphthoic acid-2'-phenylanilide phosphate) and Fast Red TR at the site of hybridization.

This procedure can be used to detect single copy sequences as small as 1 kb on human metaphase chromosomes.

I. *In situ* hybridization with DIG-labeled probes

Prepare chromosomal spreads, label hybridization probes with digoxigenin (DIG), hybridize probes to chromosomes, and perform posthybridization washes of the sample according to standard procedures, *e.g.*, those listed in Chapters 4 and 5 of this manual.

II. Detection of DIG-labeled probes

1 Prepare a fresh 1:500 dilution of alkaline phosphatase-conjugated sheep anti-DIG antibody in blocking buffer [100 mM Tris-HCl, 150 mM NaCl, 0.5% blocking reagent; pH 7.5 (20°C)].

Do not store the diluted antibody conjugate more than 12 h at 4°C.

2 Pipette 100 µl of diluted anti-DIG antibody conjugate onto each slide and add a coverslip.

3 Incubate slides for 1 h at 37°C in a moist chamber.

4 Wash the slides at room temperature as follows:

- 3 × 10 min with washing buffer [100 mM Tris-HCl, 150 mM NaCl, 0.05% Tween 20; pH 7.5 (20°C)].
- 2 × 10 min with detection buffer [100 mM Tris-HCl,100 mM NaCl, 10 mM $MgCl_2$; pH 8.0 (20°C)].

5 Prepare fresh HNPP/Fast Red TR mix as follows:

Numbered vials are components of the HNPP Fluorescent Detection Set.

- Prepare Fast Red TR stock solution by dissolving 5 mg Fast Red TR (vial 2) in 200 µl distilled H_2O.
- Mix 10 µl HNPP stock solution (premixed, vial 1), 10 ml Fast Red TR stock solution, and 1 ml detection buffer [100 mM Tris-HCl, 100 mM NaCl, 10 mM $MgCl_2$; pH 8.0 (20°C)].
- Filter the HNPP/Fast Red TR mix through a 0.2 µm nylon filter.

The Fast Red TR stock solution should be no more than 4 weeks old. The HNPP/Fast Red TR mix should be no more than a few days old. Always filter the mix just before use.

▶▶▶

6. Cover each sample with 100 µl filtered HNPP/Fast Red TR mix and a coverslip, then incubate slides for 30 min at room temperature.
7. Wash the slides 1× at room temperature with washing buffer [100 mM Tris-HCl, 150 mM NaCl, 0.05% Tween 20; pH 7.5 (20°C)].
8. Repeat Steps 6 and 7 three times.

   If you are detecting abundant or multiple copy sequences, skip Step 8. One incubation is enough.
9. Wash the slides for 10 min with distilled H_2O.

III. Fluorescence microscopy

1. In a Coplin jar, counterstain slides with 50 ml DAPI solution (100 ng DAPI/ml PBS) for 5 min in the dark at room temperature.
2. Wash slides under running water for 2–3 min.
3. Air dry slides in the dark.
4. Add 20 µl DABCO antifading solution (PBS containing 50% glycerol and 2% DABCO) to each slide.
5. Cover each slide with a 24 × 24 mm coverslip.

   For long term storage of the slides, seal the edges of the coverslip with nail polish and store in the dark at -20°C.
6. View the slides by fluorescence microscopy, using appropriate filters.

   The maximum emission wavelength of dephosphorylated HNPP/Fast Red TR is 562 nm. However, since the emission peak is broad (540–590 nm), you may use the filter sets for either fluorescein or rhodamine to analyze the HNPP/Fast Red TR product.

Results

The fluorescent signal obtained with the HNPP/Fast Red procedure described above is more intense than a direct signal from fluorescein. In a comparison experiment (Figure 1), the HNPP/Fast Red reaction product was visible after a 70-fold shorter exposure (0.2 sec exposure vs. 15 s exposure) than the fluorescein signal. Even when the HNPP/Fast Red signal is viewed under a less than optimal filter (Figure 2), the fluorescence is clearly visible.

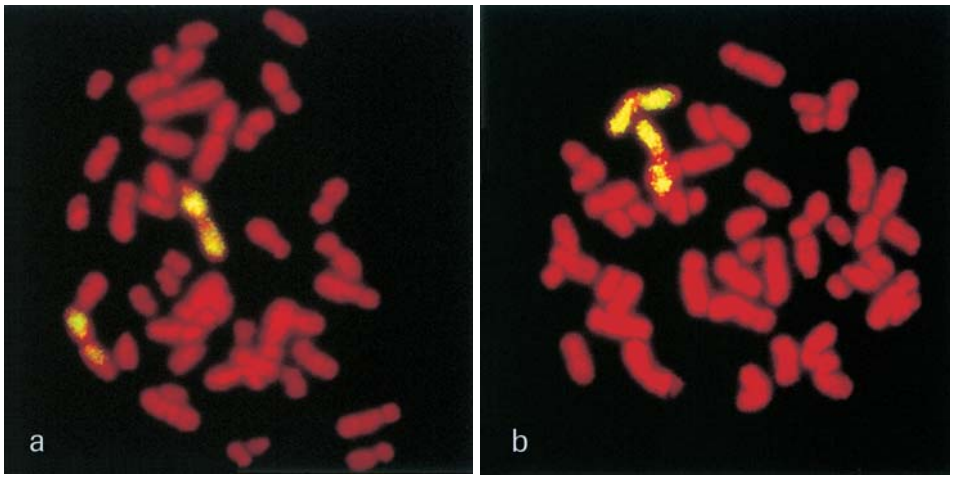


Figure 1: Comparison of fluorescein and HNPP signal intensity. A DIG-labeled painting probe specific for human chromosome 1 was hybridized to metaphase chromosomal spreads. The DIG-labeled probe was detected with (Panel a) anti-DIG-fluorescein conjugate or (Panel b) anti-DIG-alkaline phosphate conjugate and the HNPP/Fast Red detection reaction described in the text. The fluorescein signal (Panel a) required a 15 s exposure while the HNPP/Fast Red signal (Panel b) required a 0.2 s exposure. Fluorescent images were pseudo-colored with a CCD camera and a digital imaging system. Chromosomes were counter-stained with DAPI.

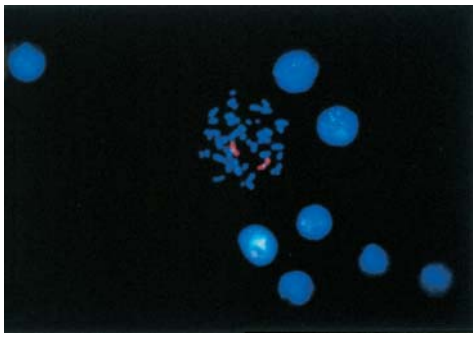


Figure 2: HNPP/Fast Red fluorescence viewed with a filter for DAPI. A DIG-labeled painting probe specific for human chromosome 1 was hybridized to metaphase chromosomal spreads, then detected with anti-DIG-alkaline phosphate conjugate and the HNPP/Fast Red detection reaction as in Figure 1. The HNPP/Fast Red signal was viewed under a filter that was optimal for DAPI (emission maximum, 470 nm), but not for HNPP (emission maximum, 562 nm). The fluorescence was photographed directly without digital manipulation. Chromosomes were counter-stained with DAPI.

Reagents available from Roche Applied Science for this procedure

Reagent	Description	Cat. No.	Pack size
Tris	Powder	10 708 976 001 11 814 273 001	1 kg 5 kg
Blocking Reagent	Powder	11 096 176 001	50 g
Anti-Digoxigenin-AP*	Fab Fragments from sheep	11 093 274 910	150 U
Tween 20		11 332 465 001	5 × 10 ml
HNPP Fluorescent Detection Set	For sensitive fluorescent detection of nonradioactively labeled nucleic acids in fluorescence *in situ* hybridization (FISH) and membrane hybridization	11 758 888 001	Set for 500 FISH reactions
DAPI	Fluorescence dye for staining of chromosomes	10 236 276 001	10 mg

* The labeling of nucleic acids with DIG is covered by EP patents 0 324 474 and 0 371 262 as well as the following US patents 5.344.757, 5.35 4.657 and 5.702.888 owned by Roche Diagnostics GmbH.

Combined DNA *in situ* hybridization and immunocytochemistry for the simultaneous detection of nucleic acid sequences, proteins, and incorporated BrdU in cell preparations

E. J. M. Speel, F. C. S. Ramaekers, and A. H. N. Hopman, Department of Molecular Cell Biology & Genetics, University of Limburg, Maastricht, The Netherlands.

The combination of *in situ* hybridization (ISH) and immunocytochemistry (ICC) enables us, *e.g.*, to simultaneously demonstrate mRNA and its protein product in the same cell, to immunophenotype cells containing a specific chromosomal aberration or viral infection, or to characterize cytokinetic parameters of tumor cell populations that are genetically or phenotypically aberrant. The factors that determine the success and sensitivity of a combination ICC and nonradioactive ISH procedure include:

- Preservation of cell morphology and protein epitopes
- Accessibility of nucleic acid targets
- Lack of cross-reaction between the different detection procedures
- Good color contrast
- Stability of enzyme cytochemical precipitates and fluorochromes

Since several steps in the ISH procedure (enzymatic digestion, post-fixation, denaturation at high temperatures, and hybridization in formamide) may destroy antigenic determinants, ICC usually precedes ISH in a combination procedure. A variety of such combined ICC/ISH procedures have been reported with either enzyme precipitation reactions (Mullink et al., 1989; Van den Brink et al., 1990; Knuutila et al., 1994; Speel et al., 1994b), fluorochromes (Van den Berg et al., 1991; Weber-Matthiesen et al., 1993), or a combination of both (Strehl & Ambros, 1993; Zheng et al., 1993; Herbergs et al., 1994; Speel et al., 1994a). The procedures can be subdivided into two groups, those which use fluorochromes for ICC, and those which use enzyme reactions.

Fluorochromes have been used mainly on acetone-fixed cell preparations, since the material can be mildly post-fixed (usually with paraformaldehyde) after antigen detection and used directly for fluorescence ISH without any further pretreatment (Weber-Matthiesen et al., 1993). However, amplification steps for both ICC and ISH signals are often necessary for clear visualization. In such cases, enzymatic ISH pre-treatment after ICC lowers fluorescent ICC staining dramatically (Speel et al., 1994a).

Enzyme precipitation reactions have also been used efficiently for combined ICC/ISH staining of proteins in cell preparations and tissue sections. Enzyme precipitation products that withstand the proteolytic digestion and denaturation steps used in the ISH procedure include:

- Several precipitates (Fast Red, New Fuchsin, and BCIP/NBT) formed by alkaline phosphatase
- The diaminobenzidine precipitate formed by horseradish peroxidase
- The BCIG (X-Gal) precipitate formed by β-galactosidase

In these cases, the digestion and denaturation steps of the ISH procedure remove the antibody and enzyme detection layers, but the precipitate remains firmly in place. The stability of the ICC precipitate thus prevents unwanted cross-reaction between the detection procedures for ISH and ICC.

Here we present a combined ICC/ISH procedure which describes compatible detection systems for fluorescence or brightfield microscopy (Table 1). Additionally, this procedure allows the localization of incorporated BrdU by fluorescence microscopy.

The procedures given below are modifications of previously published procedures (Speel et al., 1994a, 1994b).

		For analysis by	
		Fluorescence microscopy	**Brightfield microscopy**
ICC[2]	**Detection by**	Anti-antigen Ab-APase	Anti-antigen-β-gal
	Visualization by	APase-Fast Red	β-Gal-BCIG
ISH	**Probe**	FITC- or AMCA-labeled nucleic acid	DIG- or biotin-labeled nucleic acid
	Detection and visualization by	Direct viewing	PO- or APase-labeled anti-DIG Ab or anti-biotin Ab plus PO-DAB, PO-TMB, or APase-Fast Red reaction
BrdU labeling	**Detection by**	AMCA- or FITC-labeled[3] anti-BrdU Ab	–
	Visualization by	Direct viewing	–

Table 1: Detection systems for combined ICC/ISH[1].

[1] Amplification steps may be necessary for detection of low amounts of antigen, nucleic acid target, or incorporated BrdU.

[2] Abbreviations used: Ab, antibody; AMCA, aminomethylcoumarin acetic acid; APase, alkaline phosphatase; BCIG, bromochloroindolyl-galactoside; BrdU, bromodeoxyuridine; DAB, diaminobenzidine; DIG, digoxigenin; FITC, fluorescein isothiocyanate; β-Gal, β-galactosidase; ICC, immunocytochemistry; ISH, *in situ* hybridization; PO, peroxidase; TMB, tetramethylbenzidine

[3] The fluorochrome used in the BrdU visualization step should be different from the one used in the ISH visualization step.

5

I. Cell preparations and BrdU labeling

1. (Optional) To label cells, add BrdU (final concentration, 10 μM) to the culture medium 30 min before harvesting the cells (Speel et al., 1994a).

2. Prepare cultured normal diploid cells or tumor cell lines (labeled or unlabeled) by one of the following methods:
   - Slide and coverslip preparations: Grow cells on glass slides or coverslips. Fix in cold methanol (-20°C) for 5 s, then in cold acetone (4°C) for 3 × 5 s. Air dry samples and store at -20°C.

     Alternatively, use other fixatives for the slide and coverslip preparations.
   - Cytospins: Cytospin floating cells onto glass slides at 1000 rpm for 5 min. Air dry samples for 1 h at room temperature. Fix and store as with slide and coverslip preparations above.

II. Detection of antigen by immunocytochemistry (ICC)

1. Incubate slides for 10 min at room temperature with PBS-Tween-NGS (PBS buffer containing 0.05% Tween 20 and 2–5% normal goat serum).
2. Incubate slides for 45 min at room temperature with an appropriate dilution of antigen-specific primary antibody in PBS-Tween-NGS.
3. Wash slides for 2 × 5 min with PBS containing 0.05% Tween 20.
4. Incubate slides for 45 min at room temperature with an appropriate secondary antibody conjugate. Use the following to decide which antibody conjugate to use:
   - *If* you wish to detect the ICC antigen, the ISH antigen, and (optionally) BrdU labeling by fluorescence microscopy, use a secondary antibody that is conjugated to alkaline phosphatase (APase).
   - *If* you wish to detect the ICC antigen and the ISH antigen under brightfield microscopy, use a secondary antibody that is conjugated to β-galactosidase (β-Gal).
5. Wash slides as follows:
   - 5 min with PBS containing 0.05% Tween 20.
   - 5 min with PBS.

   For amplification of the ICC signal, you may add a third antibody step after this wash. For details of possible antibodies to use in an amplified three-antibody detection procedure, see Table 1 of the article "Multiple target DNA in situ hybridization with enzyme-based cytochemical detection systems" on page 94 of this manual.
6. Visualize the antibody-antigen complexes according to either Procedure IIIA (for APase conjugates) or Procedure IIIB (for β-Gal conjugates).

■

5

III. Visualization of ICC antigen

IIIA. APase-Fast Red reaction (for producing a red precipitate visible under either fluorescence or brightfield microscopy)

1. Mix color reagent just before use:
   - 4 ml TM buffer [200 mM Tris-HCl (pH 8.5), 10 mM $MgCl_2$] containing 5% polyvinyl alcohol (PVA, MW 40,000; Sigma).
   - 250 µl TM buffer containing 1 mg naphthol-ASMX-phosphate (Sigma).
   - 750 µl TM buffer containing 5 mg Fast Red TR salt (Sigma).
2. Overlay each sample with 100 µl color reagent and a coverslip.
3. Incubate samples for 5–15 min at 37°C.

   Monitor the enzyme reaction under a microscope and adjust the reaction time to keep the precipitate from becoming so dense that it shields nucleic acid sequences from the ISH detection step.
4. Wash samples 3 × 5 min with PBS.

   Do not dehydrate samples after washing.

■

IIIB β-Gal-BCIG reaction (for producing a blue precipitate visible under brightfield microscopy)

1. Mix color reagent:
   - 2.5 µl 5-bromo-4-chloro-3-indolyl-β-D-galactoside (BCIG; X-Gal) stock solution [20 mg/ml BCIG (X-Gal) in N,N-dimethylformamide].
   - 100 µl diluent (PBS containing 0.9 mM $MgCl_2$, 3 mM potassium ferricyanide, and 3 mM potassium ferrocyanide).
2. Overlay each sample with 100 µl color reagent and a coverslip.
3. Incubate samples for 15 – 60 min at 37°C.

   ⚠ *Monitor the enzyme reaction under a microscope and adjust the reaction time to keep the precipitate from becoming so dense that it shields nucleic acid sequences from the ISH detection step.*
4. Wash samples 3 × 5 min with PBS.

   ! *If you wish, dehydrate the samples after washing.*

■

IV. Cell processing for *in situ* hybridization

1. Wash slides for 2 min at 37°C with 10 mM HCl.
2. Digest samples with pepsin as follows:
   - Overlay each sample with pepsin solution (100 µg/ml pepsin in 10 mM HCl).
   - Incubate cell samples for 10 – 20 min at 37°C.
3. Wash samples for 2 min at 37°C with 10 mM HCl.

   ! *For cells stained with the β-Gal-BCIG reaction, you may (if you wish) dehydrate the slides after washing them.*
4. Post-fix samples for 20 min at 4°C with PBS containing 1% paraformaldehyde.
5. Wash samples as follows:
   - 5 min with PBS.
   - 5 min with 2× SSC.

■

V. *In situ* hybridization (ISH)

1. Perform a standard ISH detection and visualization procedure on the slides as described elsewhere in this manual. Use either:
   - Fluorescence-based procedures (FITC, AMCA) (when APase-conjugated antibodies were used for ICC).
   - Enzyme-based procedures (PO-DAB, PO-TMB, APase-Fast Red) (when β-Gal-conjugated antibodies were used for ICC).

   Example: *For a detailed description of enzyme-based ISH procedures, see "Multiple target DNA in situ hybridization with enzyme-based cytochemical detection systems" on page 107 of this manual.*

2. Include appropriate controls in the ISH procedure to ensure the lack of cross-reaction between ICC and ISH.

   The ICC precipitate remains firmly in place during ISH. The stability of the ICC precipitate usually prevents unwanted cross-reaction between the detection procedures for ISH and ICC.

■

VI. Fluorescence detection of BrdU (optional)

1. After performing the APase-Fast Red ICC (Procedure IIIA) and fluorescence ISH (Procedure V) reactions on BrdU-labeled cells, incubate the cells for 45 min at room temperature with a specific anti-BrdU antibody.
2. Wash slides for 2 × 5 min with PBS containing 0.05% Tween 20.
3. Incubate samples with a secondary antibody that is conjugated to a fluorochrome not used in the ICC or ISH reactions.

   Example: *Use an alkaline-phosphatase conjugated antibody and the APase-Fast Red for ICC; a FITC-conjugated antibody for ISH; and an AMCA-conjugated antibody for BrdU detection.*

   If necessary, use an amplified three-antibody detection procedure as outlined in the article "Multiple target DNA in situ hybridization with enzyme-based cytochemical detection systems" on page 94 of this manual.

4. Before embedding, wash slides as follows:
   - 2 × 5 min with PBS containing 0.05% Tween 20.
   - 5 min with PBS.
5. Include appropriate controls to ensure that the BrdU detection step does not cross-react with ISH detection.

■

VII. Embedding

For fluorescence microscopy (for detection of APase-Fast Red ICC, fluorescence ISH, and BrdU):

Embed specimens in a Tris-glycerol mixture [1:9 (v/v) mix of 0.2 M Tris-HCl (pH 7.6) and glycerol] containing 2% DABCO (Sigma) and (optionally) 0.5 µg/ml blue DAPI (Sigma).

For brightfield microscopy (for detection of β-Gal-BCIG ICC and enzyme-based ISH):

Depending on the enzyme reactions used, embed specimens in one of the following:

- Aqueous, organic, or protein embedding medium, as described in the article "Multiple target DNA *in situ* hybridization with enzyme-based cytochemical detection systems" on page 94 of this manual.
- Entellan (Merck) organic embedding medium and immersion oil (Zeiss) (for peroxidase-DAB or peroxidase-TMB reactions only).
- Tris-glycerol mixture [1:9 (v/v) mix of 200 mM Tris-HCl (pH 7.6) and glycerol] (for peroxidase-DAB or phosphatase-Fast Red reactions only).

Results

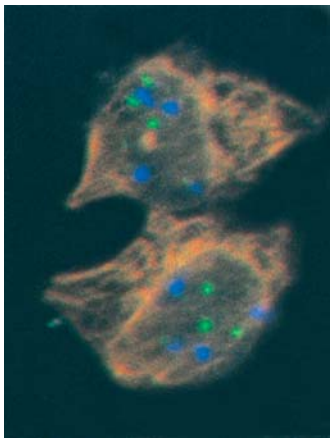


Figure 1: Combined ICC and fluorescence ISH on lung tumor cell line EPLC 65, showing cytokeratin filaments, chromosome 1, and chromosome 17. The cytokeratins (red) were visualized with a monoclonal anti-cytokeratin antibody (Ab), an alkaline phosphatase-conjugated goat anti-mouse Ab, and the APase-Fast Red reaction. The centromere of chromosome 1 (blue) was visualized with a biotinylated probe, avidin-AMCA, a biotinylated goat anti-avidin Ab, and avidin-AMCA. The centromere of chromosome 17 (green) was visualized directly with a FITC-labeled probe.

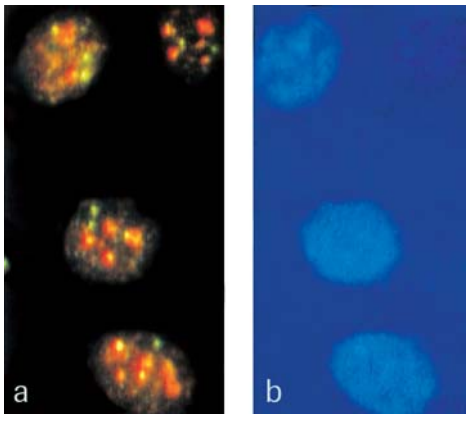


Figure 2: Combined ICC and fluorescence ISH on EPLC 65 cells, showing the nuclear proliferation marker Ki67, chromosome 7, and incorporated BrdU.

Panel a: The Ki67 antigen (red) was visualized with a rabbit anti-Ki67 Ab, alkaline phosphatase-conjugated swine anti-rabbit Ab, and the APase-Fast Red reaction. The centromere of chromosome 7 (green) was visualized directly with a FITC-labeled probe.
Panel b: Incorporated BrdU (blue) was visualized with monoclonal anti-BrdU Ab, biotinylated horse anti-mouse Ab, and avidin-AMCA.

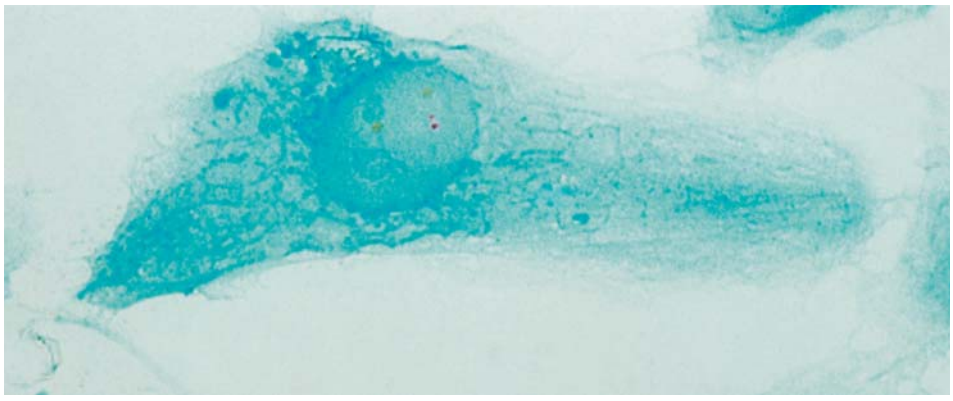


Figure 3: Combined ICC and enzyme-based ISH on a human umbilical vein endothelial cell, showing the intermediate filament protein vimentin, chromosome 1, and chromosome 7. Vimentin (blue) was visualized with monoclonal anti-vimentin Ab, β-galactosidase-conjugated goat anti-mouse Ab, and the β-galactosidase-BCIG reaction. The centromeres of chromosome 1 (biotinylated probe, brown) and chromosome 7 (digoxigenin-labeled probe, red) were visualized with avidin-peroxidase, rabbit anti-digoxigenin Ab and alkaline phosphatase-conjugated swine anti-rabbit Ab, the APase-Fast Red reaction, and the PO-DAB reaction. Samples were embedded in a Tris-glycerol mixture, but were not counterstained. Slides were viewed by brightfield microscopy.

References

Herbergs, J.; De Bruine, A. P.; Marx, P. T. J.; Vallinga, M. I. J.; Stockbrügger, R. W.; Ramaekers, F. C. S.; Arends, J. W.; Hopman, A. H. N. (1994) Chromosome aberrations in adenomas of the colon. Proof of trisomy 7 in tumor cells by combined interphase cytogenetics and immunocytochemistry. *Int. J. Cancer* **57**, 781–785.

Knuutila, S.; Nylund, S. J.; Wessman, M.; Larramendy, M. L. (1994) Analysis of genotype and phenotype on the same interphase or mitotic cell. A manual of MAC (morphology antibody chromosomes) methodology. *Cancer Genet. Cytogenet.* **72**, 1–15.

Mullink, H.; Walboomers, J. M. M.; Tadema, T. M.; Jansen, D. J.; Meijer, C. J. L. M. (1989) Combined immuno- and non-radioactive hybridocytochemistry on cells and tissue sections: influence of fixation, enzyme pre-treatment, and choice of chromogen on detection of antigen and DNA sequences. *J. Histochem. Cytochem.* **37**, 603–609.

Speel, E. J. M.; Herbergs, J.; Ramaekers, F. C. S.; Hopman, A. H. N. (1994a) Combined immunocytochemistry and fluorescence *in situ* hybridization for simultaneous tricolor detection of cell cycle, genomic, and phenotypic parameters of tumor cells. *J. Histochem. Cytochem.* **42**, 961–966.

Speel, E. J. M.; Jansen, M. P. H. M.; Ramaekers, F. C. S.; Hopman, A. H. N. (1994b) A novel triple-color detection procedure for brightfield microscopy, combining *in situ* hybridization with immunocytochemistry. *J. Histochem. Cytochem.* **42**, 1299–1307.

Speel, E. J. M.; Ramaekers, F. C. S.; Hopman, A. H. N. (1995) Detection systems for *in situ* hybridization, and the combination with immunocytochemistry. *Histochem. J.*, in press.

Strehl, S.; Ambros, P. F. (1993) Fluorescence *in situ* hybridization combined with immunohistochemistry for highly sensitive detection of chromosome 1 aberrations in neuroblastoma. *Cytogenet. Cell Genet.* **63**, 24–28.

Van Den Berg, H.; Vossen, J. M.; Van Den Bergh, R. L.; Bayer, J.; Van Tol, M. J. D. (1991) Detection of Y chromosome by *in situ* hybridization in combination with membrane antigens by two-color immunofluorescence. *Lab. Invest.* **64**, 623–628.

Van Den Brink, W.; Van Der Loos, C.; Volkers, H.; Lauwen, R.; Van Den Berg, F.; Houthoff, H.; Das, P. K. (1990) Combined β-galactosidase and immunogold/silver staining for immunohistochemistry and DNA *in situ* hybridization. *J. Histochem. Cytochem.* **38**, 325–329.

Weber-Matthiesen, K.; Deerberg, J.; Müller-Hermelink, A.; Schlegelberger, B.; Grote, W. (1993) Rapid immunophenotypic characterization of chromosomally aberrant cells by the new fiction method. *Cytogenet. Cell. Genet.* **63**, 123–125.

Zheng, Y.-L.; Carter, N. P.; Price, C. M.; Colman, S. M.; Milton, P. J.; Hackett, G. A.; Greaves, M. F.; Ferguson-Smith, M. A. (1993) Prenatal diagnosis from maternal blood: simultaneous immunophenotyping and FISH of fetal nucleated erythrocytes isolated by negative magnetic cell sorting. *J. Med. Genet.* **30**, 1051–1056.

Reagents available from Roche Applied Science for this procedure

Reagent	Description	Cat. No.	Pack size
Pepsin	Aspartic endopeptidase with broad specificity	10 108 057 001	1 g
Digoxigenin-11-dUTP, alkali-stable▽	Tetralithium salt, 1 mM solution	11 093 088 910 11 558 706 910 11 570 013 910	25 nmol (25 µl) 125 nmol (125 µl) 5 × 125 nmol (5 × 125 µl)
Fluorescein-12-dUTP	Tetralithium salt, 1 mM solution	11 373 242 910	25 nmol (25 µl)
Tetramethyl-rhodamine-5-dUTP	Tetralithium salt, 1 mM solution	11 534 378 910	25 nmol (25 µl)
Biotin-16-dUTP	Tetralithium salt, 1 mM solution	11 093 070 910	50 nmol (50 µl)
DNase I	Lyophilizate	10 104 159 001	100 mg
DNA Polymerase I	Nick Translation Grade	10 104 485 001 10 104 493 001	500 units 1000 units
Anti-Digoxigenin-AP*	Fab Fragments from sheep	11 093 274 910	150 U (200 µl)
Fast Red	1 tablet contains 0.5 mg naphtol substrate, 2 mg Fast Red chromogen and 0.4 mg levamisole (inhibitor of endogenous alkaline phosphatase activity)	11 496 549 001	20 tablets

▽ EP Patent 0371262 and US 5,198,537 owned by Roche Diagnostics GmbH.

* The labeling of nucleic acids with DIG is covered by EP patents 0 324 474 and 0 371 262 as well as the following US patents 5.344.757, 5.35 4.657 and 5.702.888 owned by Roche Diagnostics GmbH.

In situ hybridization to mRNA in *in vitro* cultured cells with DNA probes

Department of Cytochemistry and Cytometry, University of Leiden, The Netherlands.

The protocol given below has been developed with a cell line (rat 9G) which has integrated into its genome the major immediate early transcription unit 1 of human cytomegalovirus (Boom et al., 1986). The protocol is written in general terms and is applicable to abundantly expressed mRNA species. More information concerning this specific *in situ* hybridization application can be found in Raap et al. (1991).

I. Cell preparation, fixation and permeabilization

All solutions must be treated with RNase inhibitors.

1. Culture cells at 37°C in a 5% CO_2 atmosphere on poly-L-lysine coated microscopic slides. As culture medium, use Dulbecco's minimal essential medium without phenol red.
2. Wash the cells with PBS at 37°C, then fix at room temperature for 30 min in a solution of 4% (w/v) formaldehyde, 5% (v/v) acetic acid, and 0.9% (w/v) NaCl.
3. Wash the fixed cells with PBS at room temperature and store them in 70% ethanol at 4°C.
4. Before *in situ* hybridization, treat the fixed cells as follows:
   - Dehydrate by incubating successively in 70%, 90%, and 100% ethanol.
   - Wash in 100% xylene to remove residual lipids.
   - Rehydrate by incubating successively in 100%, 90%, and 70% ethanol.
   - Finally, incubate in PBS.
5. Treat the fixed cells at 37°C with 0.1% (w/v) pepsin in 0.1 N HCl, to increase permeability to macromolecular reagents.
6. Finally, treat the fixed cells as follows:
   - Wash with PBS for 5 min.
   - Post-fix with 1% formaldehyde for 10 min.
   - Wash again with PBS.

II. *In situ* hybridization

1. Label a suitable probe DNA with digoxigenin (DIG), using any of the protocols given elsewhere in this manual.
2. Prepare hybridization solution which contains 60% deionized formamide, 300 mM NaCl, 30 mM sodium citrate, 10 mM EDTA, 25 mM NaH_2PO_4 (pH 7.4), 5% dextran sulfate, and 250 ng/µl sheared salmon sperm DNA.
3. Denature the DIG-labeled probe DNA at 80°C shortly before use and add it to the hybridization solution at a concentration of 5 ng/µl.
4. Add 10 µl of the hybridization mixture (hybridization solution plus denatured probe) to the fixed, permeabilized cells and cover with an 18 × 18 mm coverslip.

   An in situ denaturation step is optional. Inclusion of such a step may intensify the RNA signal since it makes the sample more accessible to the probe.
5. Hybridize at 37°C for 16 h.

III. Washes

1. After hybridization, remove coverslips by shaking the slides at room temperature in a solution of 60% formamide, 300 mM NaCl, and 30 mM sodium citrate .
2. Using the same formamide-salt solution as in Step 1, wash the slides as follows:
   - Wash 3× at room temperature.
   - Wash 1× at 37°C.
3. Finally, wash the slides 1 × 5 min in PBS.

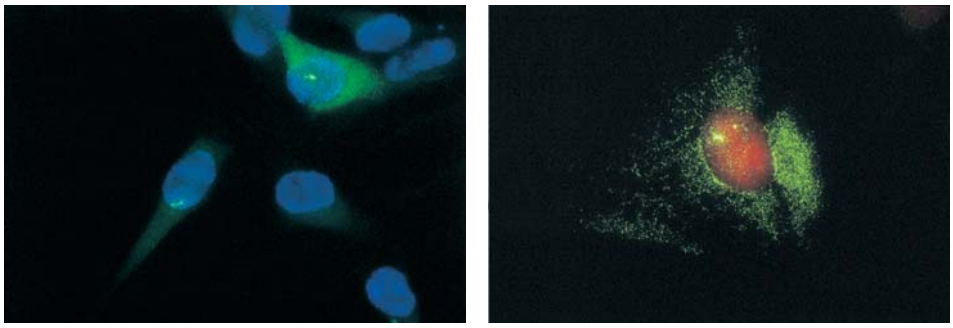
IV. Immunofluorescent detection

Other protocols for amplifying the signal may also be used. See the protocols under "Single color fluorescent detection with immunological amplification" on page 72.

1. Block non-specific binding by the following steps:
   - Add 100 µl blocking solution [100 mM Tris-HCl; pH 7.5, 150 mM NaCl, 0.5% (w/v) blocking reagent] to each slide.
   - Cover with a 24 × 50 mm coverslip.
   - Place slide in a moist chamber.
2. To loosen the coverslips, wash the slides briefly with the buffer used for immunological detection.
3. Detect the hybridized DIG-labeled probe by incubating the slides in a moist chamber for 45 min with a 1:500 dilution of anti-DIG-fluorescein in blocking solution (from Step 1) .
4. Wash slides with a solution of 100 mM Tris-HCl, pH 7.5; 150 mM NaCl; 0.05% Tween 20.
5. To dehydrate the cell samples, incubate the slides for 5 min in each of a series of ethanol solutions: 70%, 90%, then 100% ethanol.
6. Air dry the slides.
7. Embed the cell samples in an anti-fading solution which contains:
   - 9 parts glycerol

   plus
   - 1 part staining mixture: 1 M Tris-HCl (pH 7.5), 2% 1,4-diaza-bicyclo-[2,2,2]-octane (DABCO), and a DNA counterstain [either propidium iodide (500 ng/ml) or DAPI (75 ng/µl)].

Results



Panel a: PI counterstain. Panel b: V-30b

Figure 1: Nuclear staining of integrated IE viral DNA after induction with cycloheximide. The signal throughout the cytoplasm represents viral mRNA. The two panels show the same signal with different counterstains.

References

Boom, R.; Geelen, J. L.; Sol, C. J.; Raap, A. K.; Minnaar, R. P.; Klaver, B. P.; Noordaa, J. v. d. (1986) Establishment of a rat cell line inducible for the expression of human cytomegalovirus immediate – early gene products by protein synthesis inhibition. *J. Virol.* **58**, 851–859.

Raap, A. K.; Van de Rijke, F .M.; Dirks, R. W.; Sol, C. J.; Boom, R.; Van der Ploeg, M. (1991) Bicolor fluorescence *in situ* hybridization to intron- and exon mRNA sequences. *Exp. Cell Res.* **197**, 319–322.

Reagents available from Roche Applied Science for this procedure

Reagent	Description	Cat. No.	Pack size
Pepsin	Aspartic endopeptidase with broad specificity	10 108 057 001	1 g
Tween 20		11 332 465 001	5 × 10 ml
Blocking Reagent	Powder	11 096 176 001	50 g
Anti-Digoxigenin Fluorescein*	Fab Fragments from sheep	11 207 741 910	200 µg
DAPI	Fluorescence dye for staining of chromosomes	10 236 276 001	10 mg
Digoxigenin-11-dUTP, alkali-stable▽	Tetralithium salt, 1 mM solution	11 093 088 910 11 558 706 910 11 570 013 910	25 nmol 25 µl) 125 nmol (125 µl) 5 × 125 nmol (5 × 125 µl)
Fluorescein-12-dUTP	Tetralithium salt, 1 mM solution	11 373 242 910	25 nmol (25 µl)
Tetramethyl-rhodamine-5-dUTP	Tetralithium salt, 1 mM solution	11 534 378 910	25 nmol (25 µl)
Biotin-16-dUTP	Tetralithium salt, 1 mM solution	11 093 070 910	50 nmol (50 µl)
DNase I	Lyophilizate	10 104 159 001	100 mg
DNA Polymerase I	Nick Translation Grade	10 104 485 001 10 104 493 001	500 units 1000 units

* The labeling of nucleic acids with DIG is covered by EP patents 0 324 474 and 0 371 262 as well as the following US patents 5.344.757, 5.354.657 and 5.702.888 owned by Roche Diagnostics GmbH.

▽ EP Patent 0371262 and US 5,198,537 owned by Roche Diagnostics GmbH.

Identification of single bacterial cells using DIG-labeled oligonucleotides

B. Zarda, Dr. R. Amann, and Prof. Dr. K.-H. Schleifer, Institute for Microbiology, Technical University of Munich, Germany.

An earlier version of this procedure has been published (Zarda et al., 1991).

I. Organisms and growth conditions

1. To prepare cells needed for this experiment, do the following:
   - Allow *Escherichia coli* (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, DSM 30083) to grow aerobically in YT broth (tryptone, 10 g/L; yeast extract, 5 g/L; glucose, 5 g/L; sodium chloride, 8 g/L; pH 7.2) at 37°C.
   - Cultivate *Pseudomonas cepacia* (DSM 50181) aerobically in M1 broth (peptone, 5 g/L; malt extract, 3 g/L; pH 7.0) at 30°C.

   Cells from Methanococcus vannielii (DSM1224) were a generous gift of Dr. R. Huber (Dept. of Microbiology, University of Regensburg, FRG).
2. To guarantee a high cellular rRNA content, harvest all cells at mid-logarithmic phase by centrifugation in a microcentrifuge (5000 × *g*, 1 min).
3. Discard the growth medium from the cell pellet and resuspend cells thoroughly in phosphate buffered saline (130 mM sodium chloride, 10 mM sodium phosphate; pH 7.2).

II. Cell fixation and preparation of cell smears

This procedure was adapted from Amann et al., 1990.

1. Fix the cells by adding 3 volumes of paraformaldehyde solution (4% paraformaldehyde in PBS) to 1 volume of suspended cells.
2. After 3 h incubation, do the following:
   - Pellet cells by centrifugation.
   - Remove the supernatant.
   - Wash the cell pellet with PBS.
   - Resuspending the cells in an aliquot of PBS.

   After adding 1 volume ethanol to the resuspended cells, you may store the cell suspension at -20°C for up to 3 months without apparent influence on the hybridization results.
3. Spot these fixed cell suspensions onto thoroughly cleaned glass slides and allow to air dry for at least 2 h.
4. Dehydrate the cell samples by immersing the slides successively in solutions of 50% ethanol, then 80% ethanol, then 98% ethanol (3 min for each solution).

III. DIG labeling of oligonucleotides with DIG-ddUTP

Label oligonucleotides either according to the protocols in Chapter 4 of this manual or at the 5' end according to the protocol of the DIG Oligonucleotide 5'-End Labeling Set.

IV. *In situ* hybridization using digoxigenin-labeled oligonucleotides

1. Prepare hybridization solution (900 mM sodium chloride, 20 mM Tris-HCl, 0.01% sodium dodecyl sulfate; pH 7.2).
2. Depending on the method of analysis, do either of the following:
   - *If* you will use fluorescently-labeled antibodies (Procedure Va below), proceed directly to Step 3.
   - *If* you will use peroxidase-conjugated antibodies (Procedure Vb below), then.
   - Prior to hybridization, incubate fixed cells for 10 min at 0°C with 1 mg/ml of lysozyme in TE (100 mM Tris-HCl, 50 mM EDTA; pH 8.0).
   - Remove lysozyme by thoroughly rinsing the slide with sterile H_2O.
   - Air dry the slide.
   - Proceed to Step 3.
3. Add 8 µl hybridization solution containing 50 ng of labeled oligonucleotide probe to the prepared slide (from Procedure II).
4. Incubate the slide for 2 h at 45°C in an isotonically equilibrated humid chamber.

■

Va. Detection of DIG-labeled oligonucleotides with fluorescently labeled anti-DIG Fab fragments

1. Dilute fluorescein- or rhodamine-labeled anti-DIG Fab fragments 1:4 in blocking solution (150 mM sodium chloride; 100 mM Tris-HCl, pH 7.5; 0.5% bovine serum albumin; and 0.5% blocking reagent).
2. Add 10 µl of the diluted antibody to the slide and incubate the slide for another hour at 27°C in the humid chamber.
3. Remove the slide from the humid chamber and immerse in 40 ml of a washing solution (150 mM sodium chloride, 100 mM Tris-HCl, 0.01% SDS; pH 7.4) at 29°C for 10 min.
4. Prepare the slides for viewing by doing the following:
   - Rinse the slides briefly with sterile water (which has been filtered through a 0.2 µm filter).
   - Air dry.
   - Mount in Citifluor (Citifluor Ltd., London, U.K.).
5. View the cell smears with a Plan-Neofluar 100x objective (oil immersion) on the following setup: a Zeiss Axioplan microscope fitted for epifluorescence microscopy with a 50 W mercury high pressure bulb and Zeiss filter sets 09 and 15 (Zeiss, Oberkochen, FRG).
6. For photomicrographs, use Kodak Ektachrome P1600 color reversal film and an exposure time of 15 s for epifluorescence or 0.01 s for phase contrast micrographs.

■

Vb. Detection of DIG-labeled oligonucleotide

1. Use the same conditions for hybridization and antibody binding as for fluorescent antibodies (Procedures IV and Va), except include a lysozyme pretreatment of the cell smears prior to hybridization. For details, see Procedure IV above.

2. Visualize the bound antibody as follows:
   - Prepare peroxidase substrate-nickel chloride solution [1.3 mM diaminobenzidine, 0.02% (v/v) H_2O_2, 0.03% (w/v) nickel chloride, 5 mM Tris-HCl (pH 7.4)].
   - Incubate slide with peroxidase substrate-nickel chloride solution until a purplish-blue precipitate forms.

3. View slides under a light microscope and photograph with Kodak Ektachrome P1600 color reversal film.

Results

Using the techniques in this article, we could detect *P. cepacia* cells in a mixed sample of three bacteria (Figure 1). Additional experiments with a DIG-labeled oligonucleotide not complementary to rRNA show no significant levels of non-specifically bound DIG-labeled nucleic acid probes and anti-DIG antibodies.

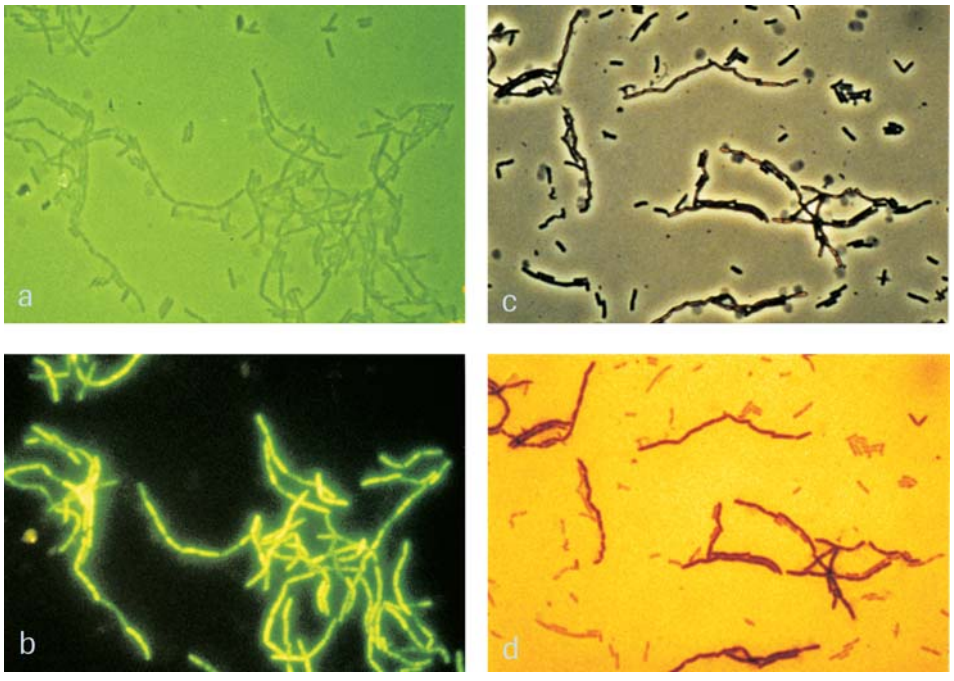


Figure 1: Specific identification of whole fixed cells of *P. cepacia* in a mixture of *P. cepacia* (chains of rods), *E. coli* (rods) and M. van-nielii (cocci) with a DIG-labeled oligonucleotide probe. Phase contrast (panel a) and epifluorescence (panel b) photos show detection with fluorescently labeled anti-DIG Fab fragments (Zarda et al., 1991). Phase contrast (panel c) and brightfield (panel d) photos show detection with peroxidase-conjugated anti-DIG Fab fragments

Reprinted from Zarda, B.; Amann, R.; Wallner, G.; Schleifer, K. H. (1991) Identification of single bacterial cells using digoxigenin-labeled rRNA-targeted oligonucleotides. *J. Gen. Microbiol.* **137**, 2823–2830 with permission of the society for general microbiology.

References

Amann, R. I.; Binder, B. J.; Olson, R. J.; Chisholm, S. W.; Devereux, R.; Stahl, D. A. (1990) Combination of 16 S rRNA-targeted oligonucleotide probes with flow cytometry for analyzing mixed microbial populations. *Appl. Environ. Microbiol.* **56**, 1919–1925.

Mühlegger, K.; Huber, E.; von der Eltz, H.; Rüger, R.; Kessler, C. (1990) Nonradioactive labeling and detection of nucleic acids. *Biol. Chem. Hoppe-Seyler* **371**, 953–965.

Zarda, B.; Amann, R.; Wallner, G.; Schleifer, K. H. (1991) Identification of single bacterial cells using digoxigenin-labeled rRNA-targeted oligonucleotides. *J. Gen. Microbiol.* **137**, 2823–2830.

Reagents available from Roche Applied Science for this procedure

Reagent	Description	Cat. No.	Pack size
DIG-Oligo-nucleotide 3'-End Labeling Kit, 2nd generation*	3'-end labeling of oligonucleotides from 14 to 100 nucleotides in length with DIG-11-ddUTP and rec. Terminal Transferase	03 353 575 910	1 Kit (25 labeling reactions)
DIG-Oligo-nucleotide 5'-End Labeling Set*	With the DIG Oligonucleotide 5'-End Labeling Set, oligonucleotides can be labeled with digoxigenin at the 5'-end after synthesis that includes the addition of a phosphoramidite	11 480 863 910	1 Set (10 labeling reactions)
Anti-Digoxigenin-Fluorescein*	Fab Fragments from sheep	11 207 741 910	200 µg
Anti-Digoxigenin-Rhodamine*	Fab Fragments from sheep	11 207 750 910	200 µg
Anti-Digoxigenin-POD*	Fab Fragments from sheep	11 207 733 910	150 U
Tris	Powder	10 708 968 001 10 708 976 001	500 g 1 kg
Blocking Reagent	Powder	11 096 176 001	50 g

* The labeling of nucleic acids with DIG is covered by EP patents 0 324 474 and 0 371 262 as well as the following US patents 5.344.757, 5.354.657 and 5.702.888 owned by Roche Diagnostics GmbH.

Detection of HPV 11 DNA in paraffin-embedded laryngeal tissue with a DIG-labeled DNA probe

Dr. J. Rolighed and Dr. H. Lindeberg, ENT-department and Institute for Pathology, Aarhus University Hospital, Denmark.

The technique of tissue *in situ* hybridization has become a powerful tool in pathology, although it is still mainly used for research purposes. The most important application at the moment is probably the demonstration of viral DNA (or RNA) in biopsies. This technique has made it possible to demonstrate infection with CMV (Grody et al., 1987; Unger et al., 1988), HBV (Blum et al., 1983; Negro et al., 1985), parvovirus (Proter et al., 1988), coxsackie B (Archard et al., 1987) EBV and HIV (Pezzella et al., 1989). *In situ* hybridization has been widely used to demonstrate HPV sequences in biopsies from the uterine cervix as well as from the head and neck (Lindeberg et al., 1990; Lindeberg et al., 1989; Shah et al., 1988). Although some of these viruses can be demonstrated by other methods, *in situ* hybridization is much faster than methods using infection of tissue cultures; for HPV tissue culture methods do not exist.

Radioactively labeled probes were used originally, but in recent years nonradioactive probes have become increasingly popular for obvious reasons.

In general, the use of radioactively labeled probes is considered superior to other methods. However, this is probably not correct (Nuevo and Richart, 1989). For instance, HPV type 16 was demonstrated in SiHa cells by *in situ* hybridization with digoxigenin-labeled probes (Heiles et al., 1988). As SiHa-cells contain only 1–2 HPV copies per cell, one can hardly ask for a further improvement in sensitivity.

There is an increasing demand from histopathologists for simple *in situ* hybridization methods, so that laboratory technicians can perform, for example, typing of HPV as a daily routine. To answer this demand, we present here a protocol for the detection of HPV DNA type 11 in laryngeal papillomas as well as in genital condylomatous lesions. The method is used on routine material which has been fixed in formalin and embedded in paraffin. The method described has the following advantages:

- The method is fast; results are obtained in about 5–6 h, and the hands-on time is about 2 h.
- The method is economical; the DIG Labeling and Detection Kit is sufficient for preparing 10 ml of DIG-labeled probe cocktail (1 µl labeled DNA/ml). This is enough for hybridizing 1000 sections.
- Problems linked to endogenous biotin are avoided, since endogenous digoxigenin apparently does not exist.

The main steps in performing *in situ* hybridization on formalin-fixed, paraffin-embedded specimens are:

1. Exposure of target DNA. This is done with a carefully controlled proteolytic digestion step.
2. Denaturation of ds DNA, followed by hybridization.
3. Washing and detection of DNA hybrids.

In our experience the most difficult step is optimization of the proteolytic digestion.

I. Probe preparation

1. Using standard methods, remove the viral insert from its vector by restriction enzyme cleavage and separate it on a submerged agarose gel.
2. Recover the insert from the gel by electroelution or other methods.
3. Label the 8 kb viral insert nonradioactively with digoxigenin acccording to the procedures given in Chapter 4 of this manual.
4. To a tube, add the following ingredients of the probe cocktail:
   - 10 µl 50× Denhart's solution.
   - 50 µl dextran sulfate 50% (w/v).
   - 10 µl sonicated salmon sperm DNA (10 mg/µl).
   - 100 µl 20× SSC.
   - 500 ng digoxigenin-labeled probe in 50 µl TE.
   - Enough distilled water to make a total volume of 250 µl.
5. To complete the probe cocktail, add 250 µl formamide to the tube.
   Tip: *Use gloves when handling solu-tions containing formamide. Formamide should be handled in a fume hood.*
6. Mix the probe cocktail (by vortexing) and store it at -20°C.

II. Pretreatment of slides

1. Wash slides overnight in a detergent, followed by an intensive wash in tap water, and finally, a wash in demineralized water.
2. After drying the slides, wash them for 3 min in acetone.
3. Transfer the washed slides to a 2% solution of 3-aminopropyltrithoxysilane in acetone and incubate for 5 min.
4. Wash the slides briefly in distilled water and allow to dry.
   The coated slides can be stored dry under dust-free conditions for several months.

III. Preparation of tissue sections

1. Fix biopsies from condylomatous lesions and from laryngeal papillomas in 4% phosphate-buffered formaldehyde and embed them in paraffin.
2. Cut routine sections, float them on demineralized water and place them on the pretreated slides.
3. Treat the sections on the slides as follows:
   - Bake the sections for 30 min at 60°C.
   - Dewax in xylene.
   - Rehydrate them by dipping them in a graded ethanol series (2× in 99% ethanol, 2× in 96% ethanol, 1× in 70% ethanol, 1× in H_2O).
4. Immediately before use, prepare a working Proteinase K solution as follows:
   - Thaw an aliquoted frozen stock solution of Proteinase K, 10 mg/ml, in H_2O.
   - Dilute the stock Proteinase K to the working concentration of 100 µg/ml in TES [50 mM Tris-HCl (pH 7.4), 10 mM EDTA, 10 mM NaCl].
5. Treat each section with 20–30 µl of the working solution of Proteinase K for 15 min at 37°C. During the proteolytic digestion cover the sections with coverslips and place them in a humid chamber.

   Tip: *We use only siliconized coverslips.*

   The proteolytic treatment is crucial and may need to be varied with different tissues. When too much Proteinase K is used, the tissue totally disintegrates, and if too little is used positive signals may be totally absent.

IV. Hybridization

1. After the treatment with Proteinase K, remove the coverslips and fix the sections in 0.4% formaldehyde for 5 min at 4°C.
2. Immerse the sections in distilled water for 5 min.
3. Allow the sections to drip and air dry for 5 min.
4. Distribute about 5–10 µl of the probe cocktail over each section.

   Large biopsies may require larger volumes of probe cocktail.
5. Prepare a negative control by covering one section from each block with a "blind" probe cocktail, *i.e.* a probe cocktail containing all the ingredients in Procedure I, except the labeled HPV DNA.
6. Place coverslips over each section and denature the DNA by placing the slides on a heating plate at 95°C for 6 min.

   Tip: *We use only siliconized coverslips.*
7. Cool the slides for 1 min on ice.
8. Place the slides in a humid chamber and incubate for 3 h at 42°C in an oven.

   Tip: *The hybridization time may be prolonged overnight when convenient.*

V. Washes

Remove the coverslips and wash the sections as follows:

- 2 × 5 min in 2× SSC at 20°C.
- 1 × 10 min in 0.1× SSC at 42°C.

VI. Detection of hybrids

1. Dip each section into buffer 1 [100 mM Tris-HCl, 150 mM NaCl; pH 7.5 (20°C)].
2. Cover each section with 20–40 µl buffer 2 [0.5% (w/v) blocking reagent in buffer 1], then add a coverslip.
3. Incubate sections at 20°C for 15 min.
4. Remove the coverslips and dip the sections in buffer 1.
5. Dilute the antibody conjugate 1:500 in buffer 2.
6. Distribute 10–20 µl of the diluted conjugate over each section, including the negative controls.
7. Place a coverslip over each section and place all sections in a humid chamber at room temperature for 1 h.
8. Remove the coverslips, wash the sections 2× 10 min in buffer 1, then equilibrate for 5 min in buffer 3.
9. Distribute approx. 20 µl color-solution (NBT/BCIP) onto each section, cover with a coverslip and leave in the dark overnight.

   You may shorten the color-reaction to 30–60 min if you wish. In fact, a positive reaction may be seen after a few minutes in the dark when HPV is present in a high copy-number.

   Hint: *Use gloves when handling the color solution.*
10. After the incubation, do the following:
    - Remove the coverslips.
    - Wash the sections gently.
    - Stain a few seconds with neutral red.
    - Mount.

    Avoid dehydrating procedures since ethanol may remove or weaken the signal.

■

Results

The described method is applied to biopsies of multiple and solitary laryngeal papillomas and on a flat condylomatous lesion of the uterine cervix (Figure 1).

The positive results are obvious, and little background is observed in these examples. The procedure is extremely straightforward and can be performed in 5–6 h, including the 3 h hybridization. Consequently, the whole procedure can be performed in 1 day and the results recorded the next morning. If needed, the hybridization can be shortened; in our initial experiments, we were able to detect HPV type 11 after only 1 h of hybridization.

Acknowledgment

Plasmids with HPV type 11 were kindly supplied by Prof. zur Hausen and co-workers, DKFZ, Heidelberg, Germany.

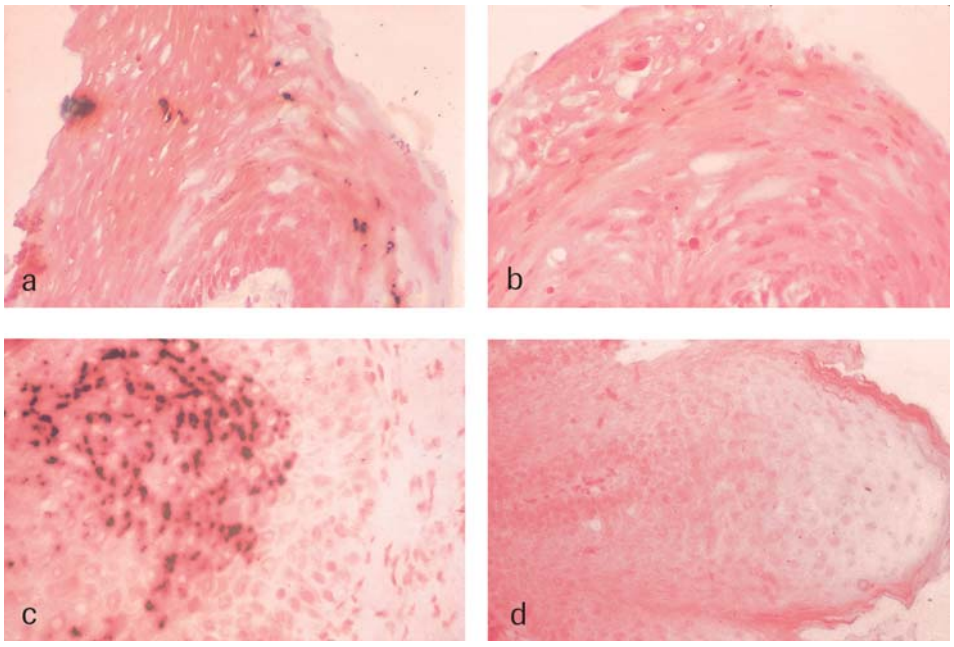


Figure 1: Detection of HPV 11 DNA in biopsies of multiple and solitary laryngeal papillomas and on a flat condylomatous lesion of the uterine cervix. Positive results are shown in Panels a and c. The negative controls (Panels b and d) show no signal.

Reagents available from Roche Applied Science for this procedure

Reagent	Description	Cat. No.	Pack size
DIG DNA Labeling and Detection Kit*	Random primed labeling of DNA probes with DIG-11-dUTP, alkali-labile and color detection of DIG-labeled hybrids.	11 093 657 910	1 Kit (25 labeling reactions and detection of 50 blots of 100 cm^2)
Proteinase K, rec., PCR Grade	Lyophilizate	03 115 836 001 03 115 879 001 03 115 801 001 03 115 852 001	25 mg 100 mg 2 × 250 mg 4 × 250 mg
Tris	Powder	10 708 968 001 10 708 976 001	500 g 1 kg
Blocking Reagent	Powder	11 096 176 001	50 g
Anti-Digoxigenin-AP*	Fab Fragments from sheep	11 093 274 001	150 U (200 µl)
NBT/BCIP	Stock solution	11 681 451 001	8 ml

* The labeling of nucleic acids with DIG is covered by EP patents 0 324 474 and 0 371 262 as well as the following US patents 5.344.757, 5.354.657 and 5.702.888 owned by Roche Diagnostics GmbH.

References

Archard, L. C.; Bowles, N. E.; Olsen, E. G. J.; Richardson, P. J. (1987) Detection of persistent coxsachie B virus RNA in dilated cardiomyopathy and myocarditis. *Eur. Heart J. (Suppl.)* **8**, 437–440.

Blum, H. E.; Stowringer, L.; Figue, A. et al. (1983) Detection of Hepatitis B virus DNA in hepatocytes, bile duct epithelium, and vascular elements by *in situ* hybridization. *Proc. Natl. Acad. Sci. (USA)* **80**, 6685–6688.

Grody, W. W.; Cheng, L.; Lewin, K. J. (1987) *in situ* viral DNA hybridization in diagnostic surgical pathology. *Hum. Pathol.* **18**, 535–543.

Heiles, H. B. J.; Genersch, E.; Kessler, C.; Neumann, R.; Eggers, H. J. (1988) *in situ* hybridization with digoxigenin-labeled DNA of human papillomaviruses (HPV 16/18) in HeLa and SiHa cells. *BioTechniques* **6** (10), 978–981.

Lindeberg, H.; Johansen, L. (1990) The presence of human papillomavirus (HPV) in solitary adult laryngeal papillomas demonstrated by *in situ* DNA hybridization with sulphonated probes. *Clin. Otolaryngol.* **15**, 367–371.

Lindeberg, J.; Syrjänen, S.; Karja, J.; Syrjänen, K. (1989) Human papillomavirus type 11 DNA in squamous cell carcinomas and preexisting multiple laryngeal papillomas. *Acta Oto-Laryngol.* **107**, 141–149.

Negro, F.; Berninger, M.; Chaiberge, E. et al. (1985) Detection of HBV-DNA by *in situ* hybridization using a biotin-labeled probe. *J. Med. Virol.* **15**, 373–382.

Nuevo, G. J.; Richart, R. M. (1989) A comparison of Biotin and 35S-based *in situ* hybridization methodologies for detection of human papillomavirus DNA. *Lab. Invest.* **61**, 471–475.

Pezzella, M.; Pezzella, F.; Rapicetta, M. et al. (1989) HBV and HIV expression in lymph nodes of HIV positive LAS patients: histology and *in situ* hybridization. *Mol. Cell Probes* **3**, 125–132.

Proter, H. J.; Quantrill, A. M.; Fleming, K. A. (1988) B19 parvovirus infection of myocardial cells. *Lancet* **1**, 535–536.

Shah, K. V.; Gupta, J. W.; Stoler, M. H. (1988) The *in situ* hybridization test in the diagnosis of human papillomaviruses. In: De Palo, G.; Rilke, F.; zur Hausen, H. (Eds.) Serono Symposia 46: Herpes and Papillomaviruses. New York: Raven Press, 151–159.

Unger, E. R.; Budgeon, L. R.; Myorson, B.; Brigati, D. J. (1988) Viral diagnosis by *in situ* hybridization. Description of a rapid simplified colorimetric method. *Am J. Surg. Pathol.* **18**, 1–8.

Detection of mRNA in tissue sections using DIG-labeled RNA and oligonucleotide probes

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The protocols given below are modifications of recently published methods for nonisotopic *in situ* hybridization with digoxigenin-labeled probes (Komminoth, 1992; Komminoth et al., 1992). In those reports we demonstrated:

- Digoxigenin-labeled prolabeled probes are as sensitive as ^{35}S-labeled probes.
- Protocols with digoxigenin-labeled probes may also be applied to diagnostic *in situ* hybridization procedures.

The following protocols have been successfully used in our laboratories to detect mRNA in frozen and paraffin-embedded tissue sections with either RNA or oligonucleotide probes.

RNA probes are best for high sensitivity detection procedures because:

- RNA *probes* are easily generated and labeled by *in vitro* transcription procedures.
- Hybrids between mRNA and RNA probes are highly stable.
- Protocols using stringent washing conditions and RNase digestion steps yield highly specific signals with low background.

Synthetic oligonucleotide probes are an attractive alternative to RNA probes for the detection of abundant mRNA sequences in tissue sections, because:

- Any known nucleic acid sequence can rapidly be made by automated chemical synthesis.
- Such probes are more stable than RNA probes.

Oligonucleotide probes are labeled during synthesis or by addition of reporter molecules at the 5' or 3' end after synthesis. The most efficient labeling method is addition of a "tail" of labeled nucleotides to the 3' end. Oligonucleotide probes are generally less sensitive than RNA probes since fewer labeled nucleotides can be incorporated per molecule of probe.

The protocols below are written in general terms. Additional information concerning the *in situ* hybridization methods and important technical details are provided in Komminoth (1992), Komminoth et al. (1992), Komminoth et al. (1995), Sambrook et al. (1989), and the Roche Applied Science DIG Application Manual for Filter Hybridization.

I. Preparation of slides

Gelatin-coated slides are excellent for large tissue sections obtained from frozen or paraffin-embedded specimens. Alternatively, silane-coated slides can be used, but are better suited for small tissue samples or cell preparations.

IA. Gelatin-coated slides

1. Clean slides by soaking for 10 min in Chromerge solution (Merck).
2. Wash slides in running hot water.
3. Rinse slides in distilled water.
4. Prepare gelatin solution as follows:
   - Dissolve 10 g gelatin (Merck) in 1 liter of distilled water which has been heated to 40°–50°C.
   - Add 4 ml 25% chromium potassium sulfate (CPS) solution (Merck) to the gelatin to make a final concentration of 0.1% CPS.
5. Dip slides in gelatin solution for 10 min.
6. Let the slides air dry.
7. Soak slides for 10 min in PBS (pH 7.4) containing 1% paraformaldehyde.
8. Let the slides air dry.
9. Bake slides at 60°C overnight.

IB. Silane-coated slides

1. Soak clean glass slides for 60 min in silane solution [5 ml of 3-aminopropyltriethoxysilane (Sigma) in 250 ml of acetone].
2. Wash the slides 2 × 10 min in distilled water.
3. Dry slides overnight at 60°C.

Both gelatin- and silane-coated slides can be stored for several months under dry and dust free conditions.

II. Tissue preparation

General guidelines: Fix or freeze tissue as soon as possible after surgical excision to prevent degradation of mRNA.

If possible, use cryostat sections of paraformaldehyde-fixed tissues which have been immersed in sucrose (as in Procedure IIA). These provide excellent conditions for mRNA localization and preservation of sample morphology.

However, in surgical pathology, most tissues are routinely fixed in formalin. For mRNA localization, do not fix in formalin for more than 24 h. Prolonged storage of samples in formalin will cause covalent linkages between mRNA and proteins, making target sequences less accessible.

Prepare all solutions for procedures IIA and IIB with distilled, deionized water (ddH_2O) that has been treated with 0.1% diethylpyrocarbonate (DEPC) (Sambrook et al., 1989).

IIA. Frozen sections

1. Cut tissues into 2 mm thick slices.
2. Incubate cut tissues at 4°C for 2–4 h with freshly made, filtered fixative (DEPC-treated PBS containing 4% paraformaldehyde; pH 7.5).
3. Decant fixative and soak tissues at 4°C overnight in sucrose solution [DEPC-treated PBS containing 30% sucrose (RNase-free)].

   During this step, the tissues should sink to the bottom of the container.
4. Store tissue samples in a freezing compound at -80°C, or, for long time storage, at -140°C (Naber et al., 1992).
5. For sectioning, warm the samples to -20°C and cut 10 µm sections in a cryostat.
6. Place the sections on pretreated glass slides (from Procedure I).
7. Dry slides in an oven at 40°C overnight.
8. Do either of the following:
   - ▶ Use the prepared slides immediately.

   or

   - ▶ Store the slides in a box at -80°C. Before processing, warm the stored slides to room temperature and dry them in an oven at 40°C for a minimum of 2 h.

     Slides with cryostat sections may be stored at -80°C for several weeks.

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IIB. Paraffin sections

1. Prepare formaldehyde-fixed and paraffin-embedded material according to standard procedures.
2. Cut 7 µm sections from the paraffin-embedded material and place the sections onto coated glass slides (from Procedure I).
3. Dry slides in an oven at 40°C overnight.
4. Dewax sections 2 × 10 min with fresh xylene.
5. Rehydrate sections in the following solutions:
   - 1 × 5 min in 100% ethanol.
   - 1 × 5 min in 95% ethanol.
   - 1 × 5 min in 70% ethanol.
   - 2 × in DEPC-treated ddH_2O.

IIIA. ISH protocol for detection of mRNA with DIG-labeled RNA probes

Prepare all solutions for procedures below (probe labeling through posthybridization) with distilled, deionized water (ddH$_2$O) that has been treated with 0.1% diethylpyrocarbonate (DEPC) (Sambrook et al., 1989). To avoid RNase contamination, wear gloves throughout the procedures and use different glassware for pre- and posthybridization steps.

Perform all procedures below at room temperature unless a different temperature is stated.

Probe labeling

To ensure tissue penetration, we prefer to work with RNA probes that are ≤500 bases long.

1. Clone a cDNA insert into an RNA expression vector (plasmid) according to standard procedures (Sambrook et al., 1989).
2. Linearize the RNA expression vector with appropriate restriction enzymes to allow *in vitro* run-off synthesis of both sense- and antisense-oriented RNA probes (Valentino et al., 1987).

   As an alternative to linearized plasmids, use PCR-generated templates containing RNA polymerase promoter sequences for in vitro transcription (Young et al., 1991).
3. Purify linearized plasmid by phenol-chloroform extraction and ethanol precipitation.

   Some plasmid purification kits contain RNase digestion steps for removing bacterial RNA. Traces of RNase may destroy transcribed probes. To ensure removal of residual RNase, perform multiple phenol-chloroform extractions of the linearized plasmid.
4. To avoid RNA polymerase inhibition, resuspend the plasmid in EDTA-free buffer or DEPC-treated ddH$_2$O.
5. Generate digoxigenin-labeled RNA probes in both the sense and antisense direction by *in vitro* transcription with the DIG RNA Labeling Kit or according to procedures in Chapter 4 of this manual.
6. Purify labeled probes according to Chapter 4, of this manual.

   Probes longer than 500 bases may not penetrate tissue. If probes are longer than 500 bases, shorten them by alkaline hydrolysis according.
7. Estimate the yield of labeled probes by direct blotting procedures as described in Chapter 4 of this manual.
8. Aliquot labeled probes and store them at -80°C.

   Probes are stable for up to one year.

Prehybridization

1. Incubate sections as follows:
   - ▶ 2 × 5 min with DEPC-treated PBS (pH 7.4) (Sambrook et al., 1989).
     - *PBS contains 140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4.*
   - ▶ 2 × 5 min with DEPC-treated PBS containing 100 mM glycine.
2. Treat sections for 15 min with DEPC-treated PBS containing 0.3% Triton X-100.
3. Wash 2 × 5 min with DEPC-treated PBS.
4. Permeabilize sections for 30 min at 37°C with TE buffer (100 mM Tris-HCl, 50 mM EDTA, pH 8.0) containing

   either

   1 µg/ml RNase-free Proteinase K (for frozen sections)

   or

   5–20 µg/ml RNase-free Proteinase K (for paraffin sections).

   - ⚠ *Permeabilization is the most critical step of the entire in situ hybridization procedure. In paraffin-embedded archival materials, optimal tissue permeabilization differs for each case, depending upon duration and type of fixation. We recommend titration of the Proteinase K concentration. Alternative permeabilization protocols for improved efficiency of digestion include incubation of slides for 20–30 min at 37°C with 0.1% pepsin in 0.2 M HCl.*
5. Post-fix sections for 5 min at 4°C with DEPC-treated PBS containing 4% paraformaldehyde.
6. Wash sections 2 × 5 min with DEPC-treated PBS.
7. To acetylate sections, place slide containers on a rocking platform and incubate slides 2 × 5 min with 0.1 M triethanolamine (TEA) buffer, pH 8.0, containing 0.25% (v/v) acetic anhydride (Sigma).
   - ⚠ *Acetic anhydride is highly unstable. Add acetic anhydride to each change of TEA-acetic anhydride solution immediately before incubation.*
8. Incubate sections at 37°C for at least 10 min with prehybridization buffer [4× SSC containing 50% (v/v) deionized formamide].
   - *Deionize formamide with Dowex MR 3 (Sigma) according to protocols described in Sambrook et al. (1989).*
   - *1× SSC = 150 mM NaCl, 15 mM sodium citrate, pH 7.2.*

In situ hybridization

1. Prepare hybridization buffer containing:
   - 40% deionized formamide.
   - 10% dextran sulfate.
   - 1× Denhardt's solution [0.02% Ficoll, 0.02% polyvinylpyrrolidone, 0.2 mg/ml RNase-free bovine serum albumin].
   - 4× SSC.
   - 10 mM DTT.
   - 1 mg/ml yeast t-RNA.
   - 1 mg/ml denatured and sheared salmon sperm DNA.

   ⚠ *Denature and add salmon sperm DNA to buffer shortly before hybridization.*

   ! *Hybridization buffer (without salmon sperm DNA) can be stored at -20°C for several months.*
2. Drain prehybridization buffer from the slides and overlay each section with 30 µl of hybridization buffer containing 5–10 ng of digoxigenin-labeled RNA probe.
3. Cover samples with a 24 × 30 mm hydrophobic plastic coverslip (*e.g.* cut from Gel Bond Film, FMC BioProducts, Rockland, ME, USA).
4. Incubate sections at 42°C overnight in a humid chamber.

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Posthybridization

⚠ *Use a separate set of glassware for posthybridization and prehybridization procedures to avoid RNase contamination in prehybridization steps.*

1. Remove coverslips from sections by immersing slides for 5–10 min in 2× SSC.

   ⚠ *Do not place samples which are hybridized with different probes in the same container.*
2. In a shaking water bath at 37°C, wash sections as follows:
   - 2 × 15 min with 2× SSC.
   - 2 × 15 min with 1× SSC.
3. To digest any single-stranded (unbound) RNA probe, incubate sections for 30 min at 37°C in NTE buffer [500 mM NaCl, 10 mM Tris, 1 mM EDTA, pH 8.0] containing 20 µg/ml RNase A.

   ⚠ *Be particularly careful with RNase. This enzyme is extremely stable and difficult to inactivate. Avoid contamination of any equipment or glassware which might be used for probe preparation or prehybridization procedures. Use separate glassware for RNase-contaminated solutions.*
4. In a shaking water bath at 37°C, wash sections 2 × 30 min with 0.1× SSC.

   ! *If this procedure gives high background or nonspecific signals, try posthybridization washings at 52°C with 2× SSC containing 50% formamide.*

■

Immunological detection

This detection procedure uses components of the DIG Nucleic Acid Detection Kit. Alternative detection procedures include the immunogold method with silver enhancement for enzyme-independent probe detection (Komminoth et al. 1992; Komminoth et al., 1995) and other detection procedures described in Chapter 5 of this manual.

1. Using a shaking platform, wash sections 2 × 10 min with buffer 1 [100 mM Tris-HCl (pH 7.5), 150 mM NaCl].
2. Cover sections for 30 min with blocking solution [buffer 1 containing 0.1% Triton X-100 and 2% normal sheep serum (Sigma)].
3. Decant blocking solution and incubate sections for 2 h in a humid chamber with buffer 1 containing 0.1% Triton X-100, 1% normal sheep serum, and a suitable dilution of sheep anti-DIG-alkaline phosphatase [Fab fragments].

   For optimal detection, incubate several sections (from the same sample) with different dilutions of the antibody (1:100; 1:500, and 1:1000).
4. Using a shaking platform, wash sections 2 × 10 min with buffer 1.
5. Incubate sections for 10 min with buffer 2 [100 mM Tris-HCl (pH 9.5), 100 mM NaCl, 50 mM $MgCl_2$].
6. Prepare a color solution containing:
   - 10 ml of buffer 2.
   - 45 µl nitroblue tetrazolium (NBT) solution (75 mg NBT/ml in 70% dimethylformamide).
   - 35 µl 5-bromo-4-chloro-3-indolyl-phosphate (BCIP or X-phosphate) solution (50 mg X-phosphate/ml in 100% dimethylformamide).
   - 1 mM (2.4 mg/10 ml) levamisole (Sigma).

     For convenience, prepare a 1 M stock solution of levamisole in ddH_2O (stable for several weeks, when stored at 4°C) and add 10 µl of the stock to 10 ml of the color solution. If endogenous phosphatase activity is high, increase the concentration of levamisole to 5 mM (50 µl 1 M stock/10 ml solution) in the color solution.

   NBT/BCIP produces a blue precipitating product. For other colors, use other alkaline phosphatase substrates. For example, use either Fast Red or INT/BCIP for red/brown precipitates.
7. Cover each section with approximately 200 µl color solution and incubate slides in a humid chamber for 2–24 h in the dark.
8. When color development is optimal, stop the color reaction by incubating the slides in buffer 3 [10 mM Tris-HCl (pH 8.1), 1 mM EDTA].
9. Dip slides briefly in distilled water.
10. Counterstain sections for 1–2 min with 0.02% fast green FCF or 0.1% nuclear Fast Red (Aldrich Chemical Corp., Milwaukee, WI, USA) in distilled H_2O.
11. Wash sections 2 × 10 min in tap water.
12. Mount sections using an aqueous mounting solution (*e.g.*, Immu-Mount Katalog Nr. 99900402, Shandon).

    Do not use xylene-based mounting solutions. These lead to crystal formation of color precipitates.

IIIB. ISH protocol for detecting mRNA with DIG-labeled oligonucleotide probes

Probe labeling

1. Label 100 pmol of oligonucleotide probe (20–30 mer) by tailing with the DIG Oligonucleotide Tailing Kit according to the protocol described in Chapter 4 of this manual.
2. Purify labeled probes according to procedures in the DIG Application Manual for Filter Hybridization or in Chapter 4 of this manual.
3. Estimate the yield of labeled probes by direct blotting procedures as described in Chapter 4 of this manual.
4. Aliquot the labeled probes and store them at -20°C.

   Probes are stable for up to one year.

To increase the sensitivity of in situ hybridization procedures, prepare several labeled probes that are complementary to different regions of the target RNA, then use mixtures of the probes for the hybridization step below.

Prehybridization

1. Follow Steps 1–7 from the prehybridization section of Procedure IIIA (for RNA probes).
2. Overlay each section with 40 µl prehybridization buffer (identical to the hybridization buffer to be used for *in situ* hybridization below, but containing no labeled probe).
3. Add a 24 × 30 mm coverslip to each section and incubate slides in a humid chamber at 37°C for 2 h.
4. Remove coverslips by immersing slides for 5 min in 2× SSC.

In situ hybridization

To increase the sensitivity of in situ hybridization procedures, use mixtures of oligonucleotide probes that are complementary to different regions of the target RNA.

1 Prepare hybridization buffer containing:

- 2× SSC.
- 1× Denhardt's solution [0.02% Ficoll, 0.02% polyvinylpyrrolidone, 0.2 mg/ml RNase-free bovine serum albumin].
- 10% dextran sulfate.
- 50 mM phosphate buffer (pH 7.0).
- 50 mM DTT.
- 250 µg/ml yeast t-RNA.
- 5 µg/ml polydeoxyadenylic acid.
- 100 µg/ml polyadenylic acid.
- up to 50 pM/ml Randomer Oligonucleotide Hybridization Probe (NEN).
- 500 µg/ml denatured and sheared salmon sperm DNA.

  Denature and add salmon sperm DNA to buffer shortly before hybridization
- Enough deionized formamide (%dF, as calculated by the formula below) to produce stringent hybridization conditions at 37°C
  [that is, to make 37°C = T_m (oligonucleotide probe) -10°C] (Long et al., 1992).

  %dF should not exceed 47% of the total volume of the hybridization buffer. If dF is less than 47%, add ddH_2O so that ddH_2O plus dF equals 47% of the volume.

Use the following formula to calculate the formamide concentration (%dF) in the hybridization buffer for each oligonucleotide probe:
where, in this case:
-7.9 = 16.6 log10 [Na^+] (for 2× SSC)
%GC = %[G + C] base content of the oligonucleotide
L = oligonucleotide length (in bases)
% mismatch = %(bases in oligonucleotide not complementary to target)
47 = hybridization temperature + 10°C (for 37°C)

Hybridization buffer (minus dF, ddH_2O, and salmon sperm DNA) can be stored at -20°C for several months.

2 Drain 2× SSC (Step 4, Prehybridization) from the slides and overlay each section with 30 µl of hybridization buffer containing 10 – 30 ng of digoxigenin-labeled oligonucleotide probe.

For frozen sections or for detection of abundant mRNAs probe concentrations of 1-5 ng per 30 µl (per slide) are sufficient.

3 Cover sections with a 24 × 30 mm hydrophobic plastic coverslip (*e.g.*, cut from Gel Bond Film, FMC BioProducts, Rockland, ME, USA).

4 Incubate samples at 37°C overnight in a humid chamber.

$$\%dF = \frac{(-7.9) + 81.5 + 0.41\,(\%GC) - 675/L - \%\ \text{mismatch} - (47)}{0.65}$$

where, in this case:
-7.9 = 16.6 log10 [Na^+] (for 2× SSC)
%GC = %[G + C] base content of the oligonucleotide
L = oligonucleotide length (in bases)
% mismatch = %(bases in oligonucleotide not complementary to target)
47 = hybridization temperature +10°C (for 37°C)

Posthybridization

1. Remove coverslips from sections by immersing slides for 5–10 min in 2× SSC.
2. In a shaking water bath at 37°C, wash sections as follows:
   - 2 × 15 min with 2× SSC.
   - 2 × 15 min with 1× SSC.
   - 2 × 15 min with 0.25× SSC.

Immunological detection

Use the same immunological detection protocol as in Procedure IIIA (for RNA probes) above.

Controls for *in situ* hybridizations

Adequate controls must always be included to ensure the specificity of detection signals. Controls should include positive and negative samples as well as technical controls to detect false positive and negative results (Herrington and McGee, 1992).

Positive controls

1. Positive sample: Tissue or cell line known to contain mRNA of interest.
2. Technical control to test quality of tissue mRNA and the procedure reagents: Labeled poly(dT) probe (for oligonucleotide *in situ* hybridization only); labeled RNA or oligonucleotide probes complementary to abundant "housekeeping genes" (such as α-tubulin) to test quality of tissue mRNA and the procedure reagents (should give positive results).

Negative controls

1. Negative sample: Tissue or cell line known to lack the sequence of interest.
2. Technical controls:
   - Target: Digestion of mRNA with RNase prior to *in situ* hybridization (should give negative results).
   - Hybridization:
     - Hybridization with sense probe
     - Hybridization with irrelevant probe (*e.g.*, probe for viral sequences)
     - Hybridization in the presence of excess unlabeled antisense probe (all should give negative results).
   - Detection:
     - Hybridization without probe–omission of anti-DIG antibody (both should give negative results).

Results

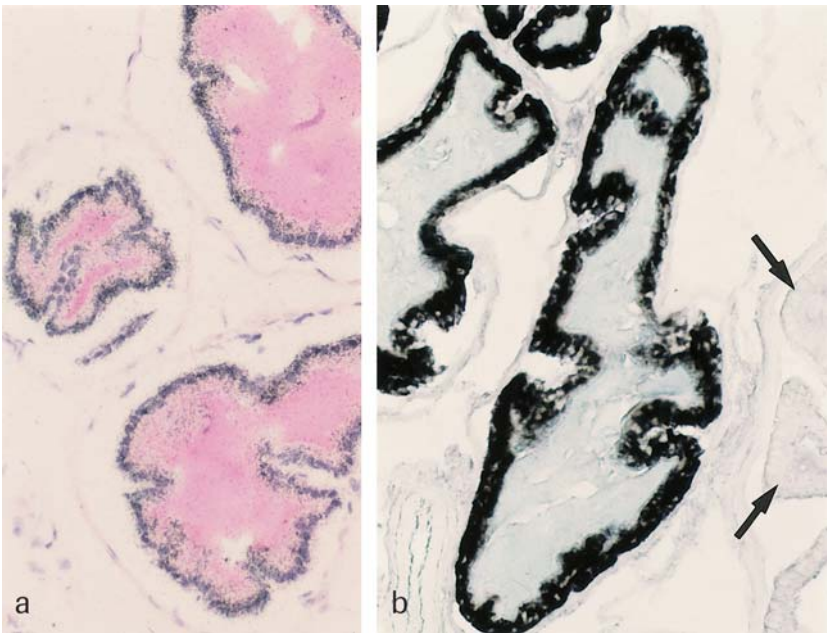

Figure 1: Comparison of ^{35}S- and digoxigenin-labeled cRNA probes for the detection of seminal vesicle secretion protein II (SVS II) mRNA in cryostat sections of the dorsolateral rat prostate. Note the equal intensity of signals in acini of the lateral lobe obtained with isotopic (Panel A) and non-isotopic (Panel B) *in situ* hybridization procedures. Also note the absence of signal in the coagulating glands (arrows in Panel B) which serves as an internal negative control.

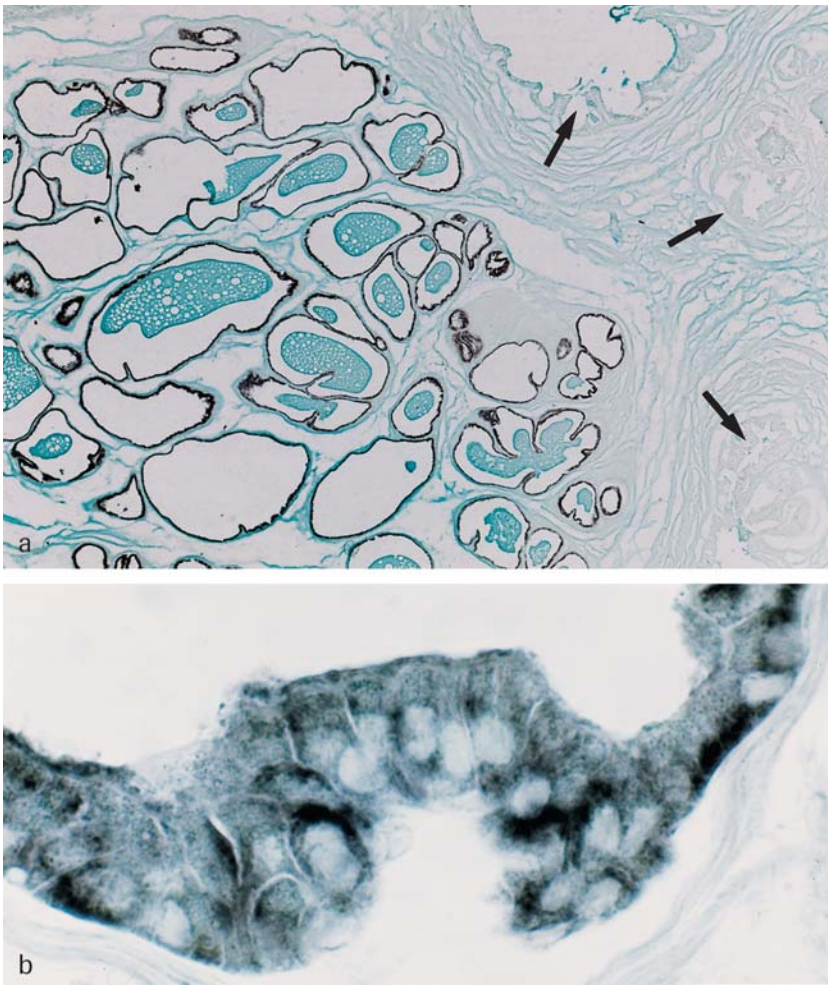

Figure 2: SVS II mRNA detection in contiguous glands of the rat prostate with a digoxigenin-labeled antisense RNA probe. Panel A: Strong signals are present in epithelia of the ampullary gland and no signals are encountered in adjacent duct epithelia of the coagulating gland (arrow). Panel B: A higher magnification shows that the hybridization signal is restricted to the cytoplasmic portion of the glandular epithelial cells (cryostat sections).

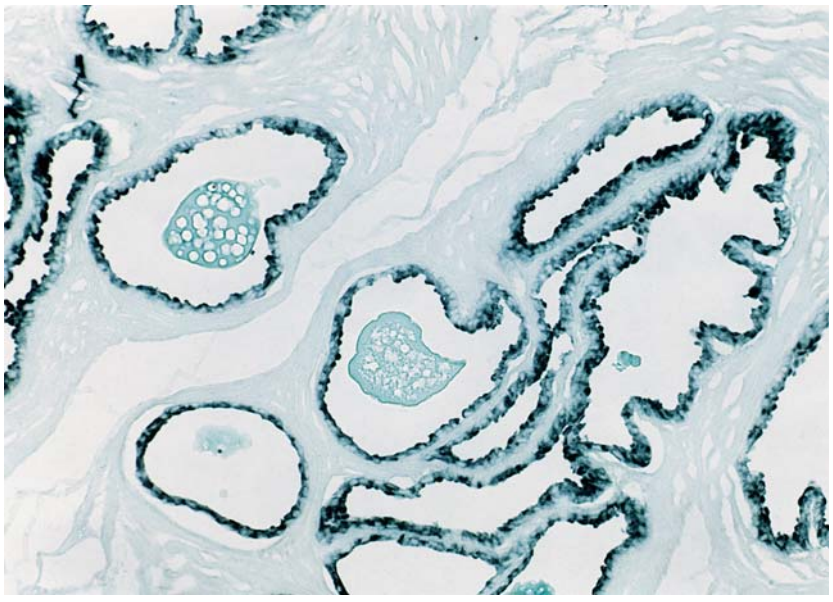

Figure 3: SVS II mRNA detection in contiguous glands of the rat prostate with a digoxigenin-labeled oligonucleotide antisense probe. Hybridization signals appear to be slightly less strong than with the SVS II RNA probe (Figure 2)**.**

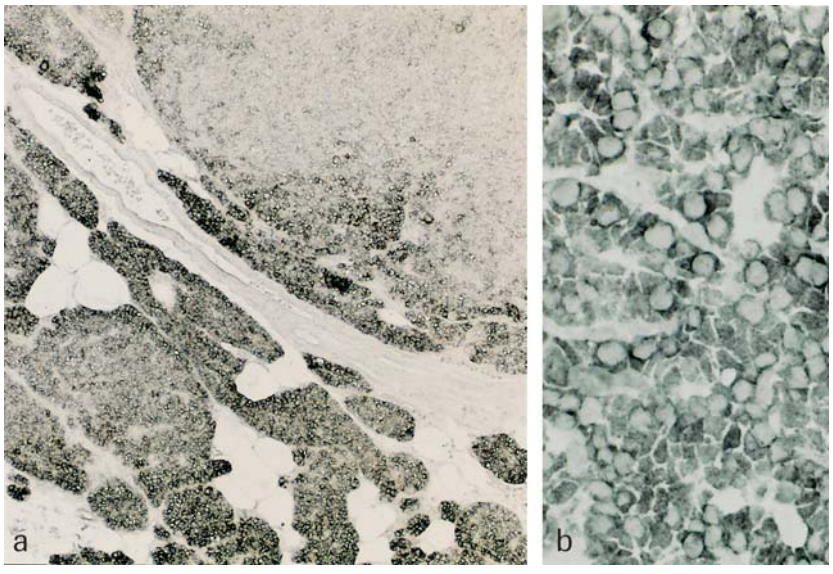

Figure 4: Detection of parathyroid hormone (PTH) mRNA in formaldehyde-fixed, paraffin-embedded tissue of a parathyroid gland with a digoxigenin-labeled antisense RNA probe. Panel A: Note the weaker signal in cells of an adenomatous lesion (upper right part) compared with the normal parathyroid tissue. Panel B: Higher magnification shows the excellent resolution of hybridization signals, which are confined to the cytoplasmic portion of cells.

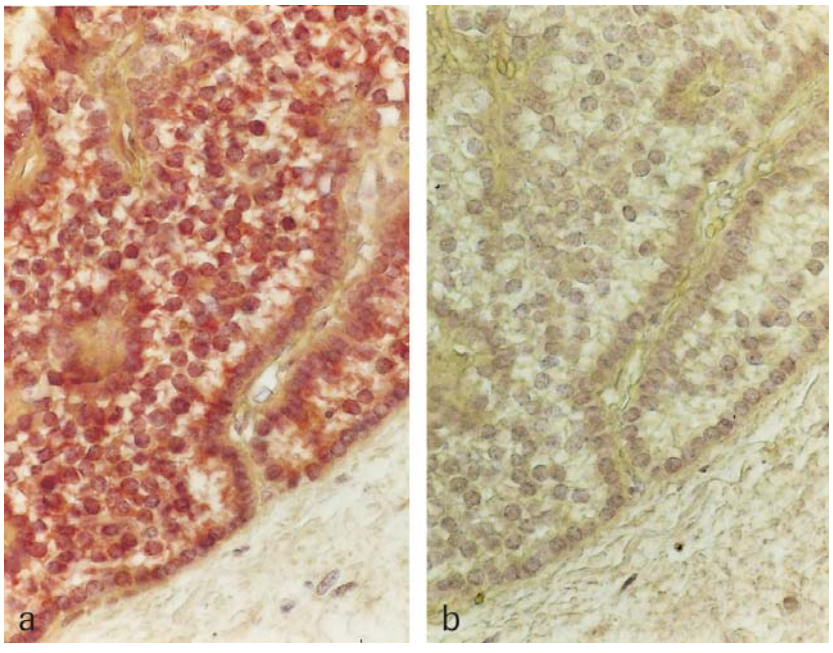

Figure 5: Use of a digoxigenin-labeled antisense RNA probe and Fast Red chromogen to detect PTH mRNA in formaldehyde-fixed, paraffin-embedded tissue of a parathyroid gland with chief cell hyperplasia. Panel A: PTH mRNA is marked by red precipitates. Panel B: Only weak signals are present when a sense PTH probe is used as a control.

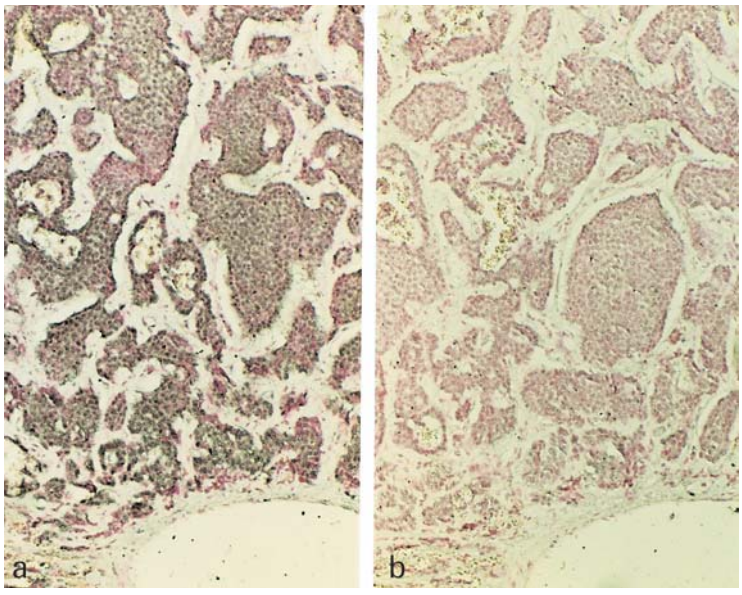

Figure 6: Detection of synaptophysin mRNA in formaldehyde-fixed, paraffin-embedded tissue of a neuroendocrine carcinoma of the gut with digoxigenin-labeled oligonucleotide probes and immunogold-silver enhancement method for visualization. Note the strong hybridization signals (consisting of silver precipitates) over tumor cells in Panel A (where an antisense probe was used) and the much weaker signal in Panel B (where the appropriate sense probe was used as a control).

Reagents available from Roche Applied Science for this procedure

Reagent	Description	Cat. No.	Pack size
DIG RNA Labeling Kit (SP6/T7)*	For RNA labeling with digoxigenin-UTP by *in vitro* transcription with SP6 and T7 RNA polymerase.	11 175 025 910	1 Kit (2×10 labeling reactions)
DIG Oligonucleotide Tailing Kit, 2nd generation*	For tailing oligonucleotides with digoxigenin-dUTP.	03 353 583 910	1 Kit (25 tailing reactions)
DIG Nucleic Acid Detection Kit*	For detection of digoxigenin-labeled nucleic acids by an enzyme-linked immunoassay with a highly specific anti-DIG-AP antibody conjugate and the color substrate NBT and BCIP.	11 175 041 910	1 Kit
Triton X-100	Vicous, liquid	10 789 704 001	100 ml
Proteinase K rec., PCR Grade	Solution	03 115 887 001 03 115 828 001 03 115 844 001	1.25 ml 5 ml 25 ml
Pepsin	Lyophilizate	10 108 057 001	1 g
DTT	Purity: >97%	10 197 777 001 10 708 984 001 11 583 786 001 10 708 992 001 10 709 000 001	2 g 10 g 25 g 50 g 100 g
Tris	Powder	10 708 976 001 11 814 273 001	1 kg 5 kg
RNase A	From bovine pancreas, dry powder	10 109 142 001 10 109 169 001	25 mg 100 mg
tRNA	From baker's yeast, lyophilizate	10 109 495 001 10 109 509 001	100 mg 500 mg
Poly(A)	Polyadenylic acid	10 108 626 001	100 mg
Poly(dA)	Poly-deoxy-adenylic acid	10 223 581 001	5 A_{260} units

* The labeling of nucleic acids with DIG is covered by EP patents 0 324 474 and 0 371 262 as well as the following US patents 5.344.757, 5.354.657 and 5.702.888 owned by Roche Diagnostics GmbH.

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References

Herrington, C. S.; McGee, J. O. (1992) Principles and basic methodology of DNA/RNA detection by *in situ* hybridization. In: Herrington, C. S.; McGee, J. O. (Eds.) *Diagnostic molecular pathology:* A practical approach. New York: IRL Press, Vol. 1, pp. 69–102.

Komminoth, P. (1992) Digoxigenin as an alternative probe labeling for *in situ* hybridization. *Diagn. Mol. Pathol.* **1**, 142–150.

Komminoth, P.; Merk, F. B.; Leav, I.; Wolfe, H. J.; Roth, J. (1992) Comparison of 35S- and digoxigenin-labeled RNA and oligonucleotide probes for *in situ* hybridization. Expression of mRNA of the seminal vesicle secretion protein II and androgen receptor genes in the rat prostate. *Histochemistry* **98**, 217–228.

Komminoth, P.; Roth, J.; Saremaslani, P.; Schrödel, S., Heitz, P. U. (1995) Overlapping expression of immunohistochemical markers and synaptophysin mRNA in pheochromocytomas and adrenocortical carcinomas. Implications for the differential diagnosis of adrenal gland tumors. *Lab. Invest.* **72**, 424–431.

Long, A. A.; Mueller, J.; Andre-Schwartz, J.; Barrett, K.; Schwartz, R., Wolfe, H. J. (1992) High-specificity *in situ* hybridization: Methods and application. *Diagn. Mol. Pathol.* **1**, 45–57.

Naber, S.; Smith, L.; Wolfe, H. J. (1992) Role of the frozen tissue bank in molecular pathology. *Diagn. Mol. Pathol.* **1**, 73–79.

Sambrook, J.; Fritsch, E.; Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Valentino, K. L.; Eberwine, J. H.; Barchas, J. D. (1987) *in situ* Hybridization: Applications To Neurobiology. Oxford, England: Oxford University Press.

Young, I. D.; Ailles, L.; Deugau, K.; Kisilevsky, R. (1991) Transcription of cRNA for *in situ* hybridization from Polymerase Chain Reaction-amplified DNA. *Lab. Invest.* **64**, 709–712.

Detection of mRNA on paraffin embedded material of the central nervous system with DIG-labeled RNA probes

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The following protocol was developed to show mRNA in paraffin embedded material of the central nervous system. It also allows double staining of protein and mRNA. This method is sensitive enough to show mRNA expressed at very low levels (Breitschopf et al., 1992).

I. Probe preparation

1. Prepare plasmids by standard methods (Sambrook et al., 1989).
2. Prepare RNA probes from the plasmids and label them with digoxigenin (DIG) either according to the methods in Chapter 4 of this manual or according to the instructions in the DIG RNA Labeling Kit.
3. Remove unincorporated nucleotides from the labeled probes by passing the labeling mixture over a Quick Spin column for radiolabeled RNA preparation (Sephadex G-50).

   ⚠ *Omission of this column step may lead to background problems.*
4. Determine the amount of DIG-labeling with a dot blot according to the method in Chapter 4 of this manual.

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II. Tissue preparation

1. Use samples fixed by standardized methods, such as:
   - Perfusion-fixed samples.

     ⚠ *Before using perfusion-fixed samples, treat them further by immersion-fixing for 3–4 h at room temperature in 100 mM phosphate buffer containing 4% paraformaldehyde.*

     ! *Samples fixed with 4% paraformaldehyde give the best results in this procedure.*
   - Routinely fixed autopsy samples.
   - Samples fixed with 2.5% glutaraldehyde.
2. After fixation, wash the samples in phosphate buffered saline (PBS).
3. Embed the fixed samples in paraffin.
4. Coat slides with a 2% solution of 3-aminopropyltriethoxy-silane (Sigma) in acetone, then air dry them.

   ⚠ *Perform Step 4 under RNase-free conditions.*
5. Cut 4 µm thick sections from the fixed samples with disposable knifes and attach them to the prepared slides.

   ⚠ *Perform Step 5 under RNase-free conditions.*
6. Dry the sections overnight at 55°C.
7. Store the sample slides at room temperature until they are used.

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III. Pretreatment of sections

Perform pretreatment and hybridization steps under RNase-free conditions. Prepare all solutions and buffers for these steps with DEPC-treated water (Sambrook et al., 1989). Bake all glassware for 4 h at 180°C. Assume that disposable plasticware is RNase-free.

1. Dewax slides extensively by treating with xylene (preferably overnight) and a graded series of ethanol solutions.
2. To preserve the mRNA during the following procedure, fix the sections once again with 4% paraformaldehyde (in 100 mM phosphate buffer) for 20 min.
3. Rinse the sections 3 – 5 times with TBS buffer [50 mM Tris-HCl (pH 7.5); 150 mM NaCl].
4. Treat the sections for 10 min with 200 mM HCl to denature proteins.
5. Rinse the sections 3 – 5 times with TBS.
6. To reduce nonspecific background (Hayashi et al., 1978), incubate the sections for 10 min on a magnetic stirrer with a freshly mixed solution of 0.5% acetic anhydride in 100 mM Tris (pH 8.0).
7. Rinse the sections 3 – 5 times with TBS.
8. Treat the sections for 20 min at 37°C with Proteinase K (10 – 500 µg per ml in TBS which contains 2 mM $CaCl_2$). The concentration of Proteinase K depends upon the degree of fixation. Generally, start with 20 µg/ml Proteinase K for paraformaldehyde-fixed tissue.

   The concentration needed for glutaraldehyde-fixed or overfixed tissue is generally higher than for paraformaldehyde-fixed tissue.

   Determine the optimal concentration of Proteinase K empirically for each type of fixation. One of the most critical steps in the whole procedure is finding the balance between prior fixation and the proper concentration of Proteinase K. Proteinase K concentrations which are too low or too high may lead to false negative results. (See Figure 3 under "Results".) Our experience shows that autolysis causes fewer problems than overfixation does. Even after 30 h autolysis, we could demonstrate mRNA. (See Figure 4 under "Results".) In overfixed material, we were not always successful in demonstrating mRNA, even when we increased Proteinase K to very high concentrations (up to 500 mg/ml).
9. Rinse the sections 3 – 5 times with TBS.
10. Incubate the sections at 4°C for 5 min with TBS (pH 7.5) to stop the Proteinase K digestion.

    Do not postfix the sections with paraformaldehyde after protease digestion, since this reduces signal intensity.
11. Dehydrate the sections in a graded series of ethanol solutions (increasing concentrations of ethanol).
12. Rinse the sections briefly with chloroform.

    At this point, you may, if necessary, store the sections under dust- and RNase-free conditions for several days.

IV. Hybridization

Perform the hybridization step under RNase-free conditions. Prepare all solutions and buffers for this step with DEPC-treated water (Sambrook et al., 1989). Bake all glassware for 4 h at 180°C. Assume that disposable plasticware is RNase-free.

1. Place the sections in a humid chamber at 55°C for 30 min.

2. Prepare a hybridization buffer by mixing the following components, then vortexing vigorously:
   - 2× SSC (Sambrook et al., 1989).
   - 10% dextran sulfate.
   - 0.01% sheared salmon sperm DNA (Sambrook et al., 1989).
   - 0.02% SDS.
   - 50% formamide.

   The concentration of dextran sulfate is critical. Without appropriate amounts of dextran sulfate, the method loses sensitivity. However, excessive concentrations of dextran sulfate causes higher viscosity of the hybridization buffer. To prevent uneven distribution of the probe and uneven signals, always vortex the hybridization buffer extensively.

3. Dilute the labeled antisense RNA probe to an appropriate degree in hybridization buffer. The diluted probe solution may contain as much as 1 part labeled probe to 4 parts hybridization buffer.

   The amount of probe needed depends upon the degree of probe labeling (as determined in Procedure I, Step 4 above). Generally, use the lowest probe concentration that gives optimal response in that dot blot procedure.

   Example: *In a serial dilution of the probe on a dot blot , if a 1:160 dilution gives a significantly stronger response than a 1:320 dilution, perform the initial hybridization experiments with both a 1:200 and a 1:300 dilution of the probe.*

4. Pipette the diluted antisense probe solution onto each section at a volume of 10 µl/cm^2. Cover with a coverslip.

   In our experience, a prehybridization step (with hybridization buffer alone) did not improve results.

5. As a control serve sections treated in the same way whereby in steps 3 and 4 a labeled sense RNA probe is used.

   Control hybridization with sense probes is necessary to prove the specificity of the reaction. However, hybridization with sense probes sometimes gives unexpected hybridization signals or background staining.

6. Place the slides on a hot plate at 95°C for 4 min.

   This step increases the signal from RNA/RNA hybrids.

7. Incubate the slides in a humid chamber for 4–6 h at 55°–75°C.

   Hybridization specificity can be increased by increasing the temperature of hybridization, if necessary, as high as 75°C. Increasing the temperature can help to differentiate mRNAs of highly homologous proteins (Taylor et al., 1994).

V. Washes and detection of mRNA

Stringency washings, often described as a method to remove nonspecifically bound probes, are helpful in reducing diffuse background staining, but do not significantly improve the specificity of the hybridization.

1. Incubate the slides in 2× SSC overnight.
   The coverslips will float off during this incubation.
2. Wash the slides as follows:
   - 3 × 20 min at 55°C with 50% deionized formamide in 1× SSC.
   - 2 × 15 min at room temperature with 1× SSC .
3. Rinse the sections 3–5 times with TBS.
4. Incubate the slides for 15 min with blocking mixture (blocking reagent containing 10% fetal calf serum).
5. Incubate the slides for 60 min with alkaline-phosphatase-conjugated anti-DIG antibody [diluted 1:500 in blocking mixture (from Step 4 above)].
6. Rinse the sections 3 – 5 times with TBS.
7. Prepare NBT/BCIP color reagent as recommended by the manufacturer.
8. In a Coplin jar in the refrigerator, incubate the sections with color reagent until sufficient color develops.
   Incubation can be extended up to 120 h if the color reagent is replaced every time it precipitates or changes color.
9. Stop the color reaction by rinsing the slides several times with tap water.
10. Finally, rinse the slides with distilled water.
11. Mount the slides directly with any water-soluble mounting medium.
    Optionally, counterstain the slides or use immunocytochemistry (as in Procedure VI below) to visualize proteins on the slides.

VI. Detection of proteins by immunocytochemistry

This protocol describes a triple APAAP (alkaline phosphatase anti-alkaline phosphatase) reaction (Vass et al., 1989), which allows visualization of proteins on the same slide with the mRNA. See Figure 1 under "Results" for an example of double staining obtained with this technique.

1. Incubate slide overnight at 4°C with primary antibody (either polyclonal or mouse monoclonal), diluted as necessary in TBS containing 10% fetal calf serum (TBS-FCS).

   Example: *The polyclonal antibody for proteolipid protein used in Figure 1 was diluted 1:1000.*

2. Rinse the slide 3 – 5 times with TBS at room temperature.

   Perform Step 2 as well as all the remaining incubation and wash steps of Procedure VI at room temperature.

3. Depending upon the type of primary antibody used in Step 1, do one of the following:
   - *If* the primary antibody was a polyclonal antibody from rabbit, incubate the slide for 60 min with anti-rabbit serum from mouse [Dakopatts], diluted 1:100 in TBS-FCS, then rinse the slide 3 – 5 times with TBS. Go to Step 4.
   - *If* the primary antibody was a mouse monoclonal antibody, skip this step and go to Step 4.

4. Incubate slide for 60 min with anti-mouse serum (from rabbit [Dakopatts], diluted 1:100 in TBS-FCS), then rinse the slide 3 – 5 times with TBS.

5. Incubate slide for 60 min with APAAP complex (mouse) (diluted 1:100 in TBS-FCS), then rinse the slide 3 – 5 times with TBS.

6. Incubate slide for 30 min with anti-mouse serum (from rabbit) (diluted 1:100 in TBS-FCS), then rinse the slide 3 – 5 times with TBS.

7. Incubate slide for 30 min with APAAP complex (mouse) (diluted 1:100 in TBS-FCS), then rinse the slide 3 – 5 times with TBS.

8. Repeat Steps 6 and 7.

   Repeating the APAAP steps will enhance the signal.

9. Prepare APAAP substrate by dissolving 200 mg naphthol-ASMX-phosphate, 20 ml dimethylformamide, and 1 ml of 1 M levamisole (all from Sigma) in 980 ml Tris-HCl (pH 8.2).

10. Dissolve 50 mg Fast Red TR Salt in 50 ml of APAAP substrate, then filter the solution.

11. In a Coplin jar at room temperature, incubate the slides with the Fast Red-APAAP substrate solution until the color develops.

    If you substitute PAP complex for APAAP complex, modify the above procedure as follows:
    - Perform all rinses and all dilutions with PBS rather than TBS.
    - For the color reaction, use filtered diaminobenzidine reagent (50 mg diaminobenzidine dissolved in 100 ml PBS which contains 0.01% H_2O_2)

    For an example of immunostaining with PAP complex, see Figure 2 under "Results".

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Results

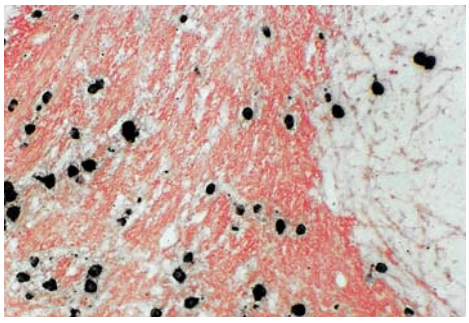

Figure 1: Double staining of mRNA and protein (APAAP method) in a normal rat brain. The mRNA (black-blue) for proteolipid protein (PLP) was detected by the procedure described in the text and stained with NBT/BCIP color reagent. Proteolipid protein (red) was detected with a 1:1000-diluted rabbit polyclonal antibody and visualized with APAAP and Fast Red TR Salt.

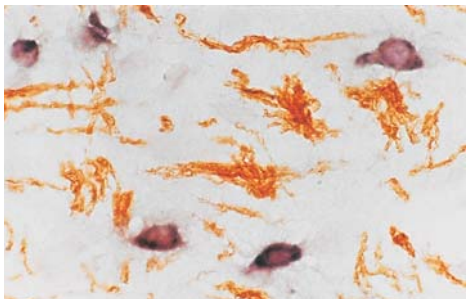

Figure 2: Double staining of mRNA and protein (PAP method) in a normal rat brain. The experiment is identical to that in Figure 1, except PLP (brown) was visualized with PAP and diaminobenzidine reagent.

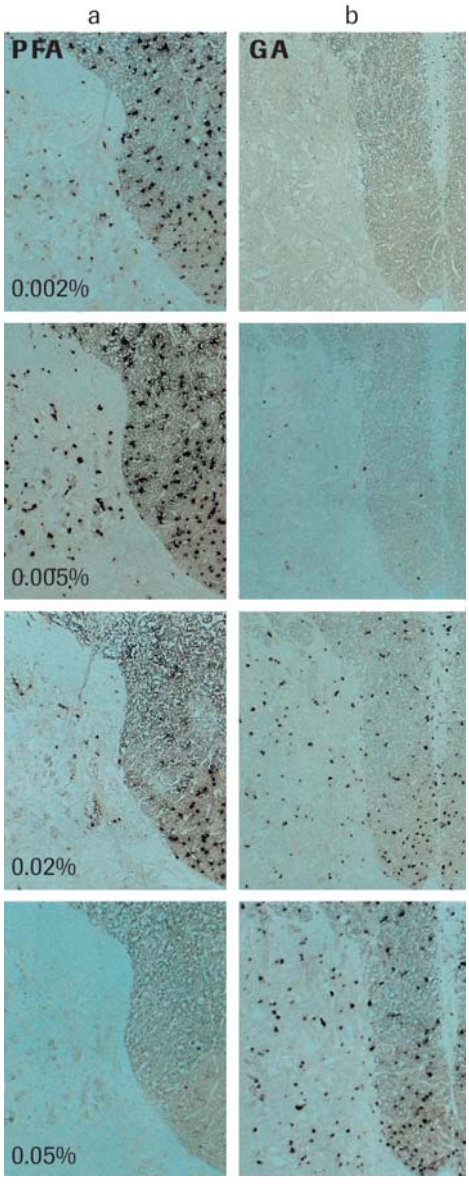

Figure 3: Effect of paraformaldehyde and glutaraldehyde fixation on *in situ* hybridization of mRNA. PLP mRNA was detected in sections of rat spinal cord which had been fixed by different methods. Panel a: Sections were fixed with 4% paraformaldehyde and then treated with different concentrations of Proteinase K. From top to bottom the Proteinase K concentrations were: 0.002%, 0.005%, 0.02%, 0.05%. Panel b: Sections were fixed in 2.5% glutaraldehyde, then treated with the same concentrations of Proteinase K as in Panel a.

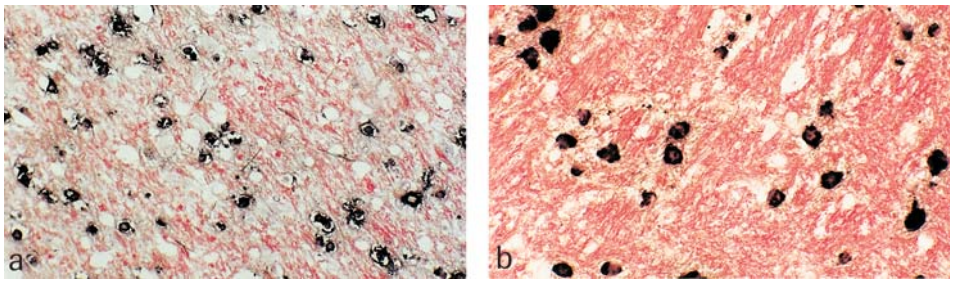

Figure 4: Effect of autolysis and overfixation on *in situ* hybridization of mRNA and immunocytochemical staining. In spite of pronounced autolytic changes that occur postmortem, a good PLP mRNA signal can be seen in human brain autopsy samples (Panel a). Although in well preserved and fixed experimental tissue (Panel b) both, the signal for *in situ* hybridization as well as the signal for immunocytochemistry is much more distinct. Protein (red) and mRNA (blue-black) were stained as in Figure 1.

Acknowledgments

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References

Breitschopf, H; Suchanek, G; Gould, R. M.; Colman, D. R.; Lassmann, H. (1992) *in situ* hybridization with digoxigenin-labeled probes: Sensitive and reliable detection method applied to myelinating rat brain. *Acta Neuropathol.* **84**, 581–587.

Hayashi, S; Gillam, I. C.; Delaney, A. D.; Tener, G. M. (1978) Acetylation of chromosome squashes of *Drosophila* melanogaster decreases the background in autoradiographs from hybridization with 125I-labeled RNA. *J. Histochem. Cytochem.* **26**, 677–679.

Sambrook, J; Fritsch, E. F.; Maniatis, T. (1989) Molecular cloning: A Laboratory Manual, 2nd ed. Cold Spring Harbor, N.Y.: Cold Spring Harbor Laboratory Press.

Taylor, V.; Miescher, G. C.; Pfarr, S.; Honegger, P.; Breitschopf, H.; Lassmann, H.; Steck, A. J. (1994) Expression and developmental regulation of Ehk-1, a neuronal Elk-like receptor tyrosine kinase in brain. *Neuroscience* **63**, 163–178.

Vass, K.; Berger, M. L.; Nowak, T. S. Jr.; Welch, W. J.; Lassmann, H. (1989) Induction of stress protein HSP 70 in nerve cells after status epilepticus in the rat. *Neurosci. Lett.* **100**, 259–264.

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Reagents available from Roche Applied Science for this procedure

Reagent	Description	Cat. No.	Pack size
DIG RNA Labeling Kit (SP6/T7)*	For RNA labeling with digoxigenin-UTP by *in vitro* transcription with SP6 and T7 RNA polymerase.	11 175 025 910	1 Kit (2 × 10 labeling reactions)
Proteinase K, rec., PCR Grade	Lyophilizate	03 115 836 001	25 mg
		03 115 879 001	100 mg
		03 115 801 001	2 × 250 mg
		03 115 852 001	4 × 250 mg
Tris	Powder	10 708 976 001	1 kg
		11 814 273 001	5 kg
Blocking Reagent	Powder	11 096 176 001	50 g
Anti-Digoxigenin-AP*	Fab Fragments from sheep	11 093 274 910	150 U (200 µl)
NBT/BCIP	Stock solution	11 681 451 001	8 ml

* The labeling of nucleic acids with DIG is covered by EP patents 0 324 474 and 0 371 262 as well as the following US patents 5.344.757, 5.354.657 and 5.702.888 owned by Roche Diagnostics GmbH.

RNA-RNA *in situ* hybridization using DIG-labeled probes: the effect of high molecular weight polyvinyl alcohol on the alkaline phosphatase indoxyl-nitroblue tetrazolium reaction

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The indoxyl-nitroblue tetrazolium (BCIP-NBT) reaction is relatively slow. During the reaction, intermediates (indoxyls) diffuse away into the medium, making it difficult to localize the site of hybridization (Van Noorden and Jonges, 1987) and reducing the hybridization signal.

In this paper, an improved nonradioactive RNA-RNA *in situ* hybridization protocol using alkaline phosphatase-conjugated digoxigenin- (DIG-) labeled probes is presented. The addition of polyvinyl alcohol (PVA) of high molecular weight (40–100 kD) to the BCIP-NBT detection system enhances the alkaline phosphatase reaction and prevents diffusion of reaction intermediates, resulting in a twentyfold increase in sensitivity without increasing the background. Due to the more localized precipitation of the formazan, the site of hybridization can be determined more precisely.

I. Fixation, dehydration, and embedding

Follow the protocol described by Jackson (1992) to fix, dehydrate, and embed the tissue in paraffin, with the following modifications:

1 For fixation, prepare either of the following solutions:
- 100 mM phosphate buffer, pH 7, containing 0.25% gluteraldehyde and 4% freshly depolymerized paraformaldehyde.
- Formalin-acetic acid (50% ethanol; 10% formalin, containing 37% formaldehyde; 5% acetic acid).

2 Using either of the solutions from Step 1, fix the tissue for 4 h at room temperature. During the fixation:
- Vacuum infiltrate the tissue (with a water aspirator) for 10 min once 1 h.
- After each vacuum infiltration, renew the fixative solution.

3 Depending on the fixative used in Step 2, wash the fixed tissue as follows:
- *If* gluteraldehyde-paraformaldehyde was the fixative, wash the fixed tissue 2 × 30 min with 100 mM phosphate buffer, pH 7.
- *If* formalin-acetic acid was the fixative, wash the fixed tissue 2 × 30 min with 50% ethanol.

4 Dehydrate the tissue by incubating in the following series of ethanol solutions:
- *Either* 90 min (after gluteraldehyde-paraformaldehyde fixation) *or* 30 min (after formalin-acetic acid fixation) at room temperature in 50% ethanol.
- 90 min at room temperature in 70% ethanol.
- Overnight at 4°C in 85% ethanol.
- 3 × 90 min at room temperature in 100% ethanol.

II. Sectioning

1. Cut the paraffin-embedded tissues in 10 µm sections.
2. Attach the sections at 75°C for 1 h to slides that have been treated with Vectabond (Vector Laboratories, Burlingame, CA, U.S.A.).

III. Prehybridization treatments

1. Dewax and hydrate the sections as previously described (Jackson, 1992).
2. Incubate the sections with a Proteinase K solution (100 mM Tris, pH 7.5; 50 mM EDTA; 2 µg/ml Proteinase K) for 30 min at 37°C.
3. After the Proteinase K treatment, wash the slides 2× in phosphate buffered saline (PBS).
4. Dehydrate the sections in ascending concentrations of ethanol as previously described (Jackson, 1991).

IV. Hybridization

1. Label the probe RNA with DIG-UTP according to the procedures given in Chapter 4 of this manual.
2. Reduce the length of the probe to approximately 200 bases as follows:
   - To 50 µl of labeled probe RNA in a microcentrifuge tube, add 30 µl 200 mM Na_2CO_3 and 20 µl 200 mM $NaHCO_3$.
   - Hydrolyze the probe at 60°C for t min, where:

     $t = (L_0 - L_f) / (K \cdot L_0 \cdot L_f)$

     L_0 = starting length of probe RNA (in kb)

     L_f = length of probe RNA (in kb) (In this case, $L_f = 0.2$ kb.)

     K = rate constant (In this case,

     K = 0.11 kb/min.)

     t = hydrolysis time in min
3. After hydrolysis, purify the probe RNA as follows:
   - Add the following to the hydrolyzed probe solution:
     - 5 µl 10% acetic acid
     - 11 µl 3 M sodium acetate (pH 6.0)
     - 1 µl of a 10 mg/ml tRNA stock
     - 1.2 µl 1 M $MgCl_2$
     - 300 µl (about 2.5 volumes) cold ethanol
   - Incubate 4–16 h at -20°C.
   - Centrifuge in a microcentrifuge for 15 min at 4°C to pellet the RNA.
   - Discard the supernatant and dry the RNA pellet in a vacuum desiccator.
   - Resuspend labeled probe RNA in DEPC-treated water at 10–50 µg/ml.

4 Prepare a hybridization mixture containing the following:

- 50% deionized formamide.
- 2.25× SSPE (300 mM NaCl; 20 mM NaH_2PO_4; 2 mM EDTA; pH 7.4).
- 10% dextran sulfate.
- 2.5× Denhardt's solution.
- 100 µg/ml sheared and denatured herring sperm DNA.
- 100 µg/ml tRNA.
- 5 mM DTT.
- 40 units/ml RNase inhibitor.
- 0.2–1.0 µg/ml hydrolyzed and denatured probe (200 bases long).

5 Cover each section with 250–500 µl of hybridization mixture (depending on the size of the section) and incubate in a humidified box at 42°C overnight.

Do not use a coverslip during the hybridization incubation.

6 After hybridization, wash the slides as follows:

- 5 min at room temperature with 3× SSC.

  1× SSC contains 150 mM NaCl, 15 mM Na-citrate; pH 7.

- 5 min at room temperature with NTE (500 mM NaCl, 10 mM Tris-HCl, 1 mM EDTA; pH 7.5).

7 To remove unhybridized single-stranded RNA probe, put slides into a humidified box and cover each section with 500 µl of NTE buffer containing 50 µg/ml RNase A. Incubate for 30 min at 37°C.

8 After RNase treatment, wash the slides 3 × 5 min at room temperature with NTE.

9 To remove nonspecifically hybridized probe, wash the slides as follows:

- 30 min at room temperature with 2× SSC.
- 1 h at 57°C with 0.1× SSC.

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V. Detection of DIG-labeled hybrids

1. Incubate the slides first with blocking solution, then with blocking solution containing 1.25 units/ml of alkaline phosphatase-conjugated anti-DIG Fab fragments as recommended in the pack insert of the DIG Nucleic Acid Detection Kit.
2. After the antibody incubation, wash the slides to remove unbound antibody as recommended in the Roche Applied Science DIG Application Manual for Filter Hybridization.
3. Prepare the BCIP-NBT-PVA color development solution as follows:
   - Prepare a Tris-NaCl-PVA stock solution by dissolving 10% (w/v) polyvinyl alcohol [PVA, *either* 40 kD *or* 70 –100 kD (Sigma)] at 90°C in 100 mM Tris-HCl (pH 9) containing 100 mM NaCl.
   - Cool the Tris-NaCl-PVA stock solution to room temperature.
   - Add $MgCl_2$, BCIP, and NBT to the Tris-NaCl-PVA stock to produce a final color development solution that contains: – 5 mM MgCl – 0.2 mM 5-bromo-4-chloro-3-indolyl phosphate (BCIP) – 0.2 mM nitroblue tetrazolium salt (NBT).
4. After the washes in Step 2, perform the visualization step as follows:
   - Place the slides in 30 ml of BCIP-NBT-PVA color development solution in a vertical staining dish suited for eight slides.
   - Incubate the slides in the color development solution for 2–16 h at 30°C.
   - Monitor color formation visually.
5. When the color on each slide is optimal, stop the color reaction by washing the slide 3 × 5 min in distilled water.
6. Dehydrate the sections by incubating the slides without shaking in the following ethanol solutions:
   - 15 s in 70% ethanol.
   - 2 × 15 s in 100% ethanol.
7. Air dry the slides and mount them with Eukitt (O. Kindler GmbH, FRG).
8. Examine the sections with a Dialux 22 microscope (Leitz, Wetzlar, FRG) equipped with Normaski differential interference contrast.

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Results and discussion

The direct influence of the polymers on the BCIP-NBT alkaline phosphatase reaction in a test tube is shown in Table 1. From these results it is clear that polyvinyl alcohol was the only polymer that enhanced formazan formation in the alkaline phosphatase reaction.

This enhancement was even more pronounced in *in situ* hybridization experiments. Figure 1 shows the results of an *in situ* hybridization in which sections of tobacco pistils were hybridized with an antisense RNA probe from a pistil-specific cDNA clone (de S. Goldman et al., 1992). After three hours development no hybridization could be detected if no PVA had been added to the reaction buffer. A clear but still weak signal was visible after three h if 20% PVA (molecular weight, 10 kD) was used (Figure 1a). However, with 10% PVA (molecular weight, 70 –100 kD), a strong hybridization signal appeared in the transmitting tissue of the pistil after a few h (Figure 1b). In the control with the sense RNA probe no signal could be detected, even after 24 h of development (Figure 1c).

Polymer	Molecular weight	Concentration[b]	Effect on the alkaline phosphatase BCIP-NBT reaction[e]
PVP	15 kD	10, 20, 30%	Autoreduction of NBT
	25 kD	10, 20, 30%	
	44 kD	10, 20, 30%	
PEG	6 kD	10, 20, 30%	No enhancement (0.01 mM formazan)
	20 kD	10, 20, 30%	
PVA	10 kD	10, 20%[c]	Approx. 4-fold enhancement (0.04 mM formazan)
	40 kD	10%[d]	Approximately 6- to 8-fold enhancement (0.06 to 0.08 mM formazan)
	70 – 100 kD	10%[d]	

Table 1: Influence of polymers on the alkaline phosphatase BCIP-NBT reaction[a].

[a] Each reaction was done with a total volume of 2 ml BCIP-NBT reaction mixture in a test tube. The BCIP-NBT reaction mixture was as described in the text, except that it contained 7.5×10^{-3} units alkaline phosphatase/ml and the polymer indicated in the table. Incubation was for 30 min at 24°C.

[b] The polymers were dissolved in the alkaline phosphatase reaction buffer in concentrations between 10% and 30%. The concentrations are given as a percentage of weight to volume.

[c] A solution of 30% 10 kD PVA was too viscous.

[d] Solutions of 20 or 30% 40 kD PVA and 20 or 30% 70 – 100 kD PVA were too viscous.

[e] The amount of formazan formed was measured at 605 nm (Eadie et al., 1970). The enhancement factor is expressed with respect to the controls (no polymer added). If no polymer was added, about 0.01 mM formazan was formed.

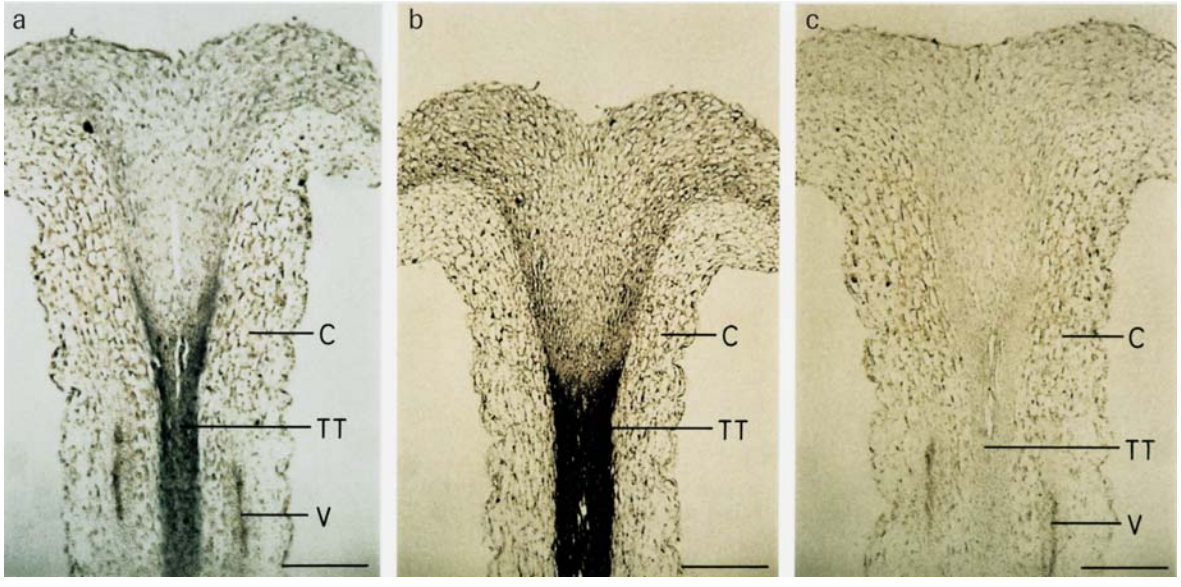

Figure 1: The influence of PVA on the alkaline phosphatase BCIP-NBT reaction in *in situ* hybridizations. The hybridizations were carried out on 10 µm paraffin sections of stigmas and styles of tobacco with antisense and sense DIG-labeled RNA probes of pMG07 (de S. Goldman et al., 1992).

C, cortex tissue; TT, transmitting tissue;

V, vascular tissue. Bars = 200 µm.

Panel a: Hybridization with antisense probe. Twenty percent 10 kD PVA was added to the alkaline phosphatase reaction buffer. Development was done for 4 h.

Panel b: Hybridization with antisense probe. Ten percent 70 – 100 kD PVA was added to the alkaline phosphatase reaction buffer. Development was done for 2 h.

Panel c: Hybridization with sense RNA probe. Ten percent 70 – 100 kD PVA was added to the alkaline phosphatase reaction buffer. Development was done for 20 h.

References

DeBlock, M.; Debrouwer, D. (1993) RNA-RNA *in situ* hybridization using digoxigenin-labeled probes: the use of high molecular weight polyvinyl alcohol in the alkaline phosphatase indoxyl-nitroblue tetrazolium reaction. *Anal. Biochem.* **215**, 86–89.

de S. Goldman, M. H.; Pezzotti, M.; Seurinck, J.; Mariani, C. (1992) Developmental expression of tobacco pistil-specific genes encoding novel extensin-like proteins. *Plant Cell* **4**, 1041–1051.

Eadie, M. J.; Tyrer, J. H.; Kukums, J. R.; Hooper, W. D. (1970) *Histochemie* **21**, 170–180.

Jackson, D. (1991) *in situ* hybridization in plants. In: Gurr, S. J.; McPherson; Bowles, D. J. (Eds.) Molecular Plant Pathology, A Practical Approach. Oxford, England: Oxford University Press, Vol. 1, pp. 163–174.

Van Noorden, C. J. F.; Jonges, G. N. (1987) Quantification of the histochemical reaction for alkaline phosphatase activity using the indoxyl-tetranitro BT method. *Histochem. J.* **19**, 94–102.

Reagents available from Roche Applied Science for this procedure

Reagent	Description	Cat. No.	Pack size
DIG RNA Labeling Kit (SP6/T7)*	For RNA labeling with digoxigenin-UTP by *in vitro* transcription with SP6 and T7 RNA polymerase.	11 175 025 910	1 Kit (2× 10 labeling reactions)
Proteinase K, rec., PCR Grade	Lyophilizate	03 115 836 001	25 mg
		03 115 879 001	100 mg
		03 115 801 001	2 × 250 mg
		03 115 852 001	4 × 250 mg
tRNA	From baker's yeast, lyophilizate	10 109 495 001	100 mg
		10 109 509 001	500 mg
DTT	Purity: >97%	10 197 777 001	2 g
		10 708 984 001	10 g
		11 583 786 001	25 g
		10 708 992 001	50 g
		10 709 000 001	100 g
RNase A	Powder	10 109 142 001	25 mg
		10 109 169 001	100 mg
DIG Nucleic Acid Detection Kit*	For detection of digoxigenin-labeled nucleic acids by an enzyme-linked immunoassay with a highly specific anti-DIG-AP antibody conjugate and the color substrates NBT and BCIP.	11 175 041 910	1 Kit

* The labeling of nucleic acids with DIG is covered by EP patents 0 324 474 and 0 371 262 as well as the following US patents 5.344.757, 5.354.657 and 5.702.888 owned by Roche Diagnostics GmbH.

Detection of neuropeptide mRNAs in tissue sections using oligo-nucleotides tailed with fluorescein-12-dUTP or DIG-dUTP

Department of Cytochemistry and Cytometry, University of Leiden, The Netherlands.

The protocol given below has been developed with the neuropeptidergic system of the pond snail *Lymnaea stagnalis*. More information concerning the application and *in situ* hybridization methodology can be found in Van Minnen et al. (1989), Dirks et al. (1988), Dirks et al. (1990), and Dirks et al. (1991).

I. Tissue preparation

1. Dissect the tissue, embed in O.C.T. compound (Miles Scientific, USA), and freeze in liquid nitrogen.
2. Prepare sections as follows:
   - Cut cryostat sections of 8 µm.
   - Mount on poly-L-lysine-coated slides.
   - Air dry.
3. After mounting, do the following:
   - Fix the sections for 30 min at 4°C with modified Carnoy's [2% formalin, (from 37% stock), 75% ethanol, 23% acetic acid].
   - Rinse the slides with water.
   - Dehydrate the sections.

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II. Probe preparation

Synthesize and purify the oligonucleotides according to routine procedures. Tail the oligonucleotide with DIG-dUTP or fluorescein-dUTP according to the procedures given in Chapter 4, but without using dATP.

If you use fluorescein-dUTP, add to the labeling mixture equal amounts of unmodified dTTP and fluorescein-dUTP, then carry out the labeling procedure in the dark.

III. *In situ* hybridization

18-mers are used in this study. For oligonucleotides with other lengths and/or composition, you may have to alter the stringency of hybridization by changing the formamide concentration or the hybridization temperature listed below.

1. Perform the hybridization as follows:
   - Prepare hybridization mixture [25% formamide; 3× SSC; 0.1% polyvinylpyrrolidone; 0.1% ficoll, 1% bovine serum albumin; 500 µg/ml sheared salmon sperm DNA; 500 µg/ml yeast RNA].
     1× SSC contains 150 mM sodium chloride, 15 mM sodium citrate; pH 7.0.
   - To the cryostat sections, add hybridization mixture containing 1 ng/µl labeled probe.
   - Incubate for 2 h at room temperature.
     If the probe contains a fluorochrome label, perform the hybridization incubation in the dark.
2. After hybridization, do the following:
   - Rinse the section 3 times in 4× SSC at room temperature.
   - Dehydrate the sections.
     If the probe contains a fluorochrome label, perform the washes in the dark.

IV. Hybrid detection

Depending on the label used on the probe, follow one of these procedures:

- *If* sections are hybridized with fluorescein-labeled oligonucleotides:
  - Mount sections in PBS/glycerol (1:9; v/v) containing 2.3% 1,4-diazabicyclo-(2,2,2)-octane (DABCO, from Sigma) and 0.1 µg/µl 4',6'-diamidino-2-phenylindole (DAPI).
  - Evaluate under a fluorescence microscope.
- *If* sections are hybridized with DIG-labeled oligonucleotides:
  - Make a 1:250 dilution of fluorescein-conjugated anti-DIG antibody (from sheep) in incubation buffer [100 mM Tris-HCl, pH 7.4; 150 mM NaCl; 1% blocking reagent].
  - Overlay the sections with the diluted antibody in incubation buffer.
  - Incubate sections at room temperature for 30 min.
  - Rinse sections 3 × 5 min in Tris-NaCl [100 mM Tris, pH 7.4; 150 mM NaCl].
  - Mount and evaluate as for fluorescein-labeled oligonucleotides.

Results

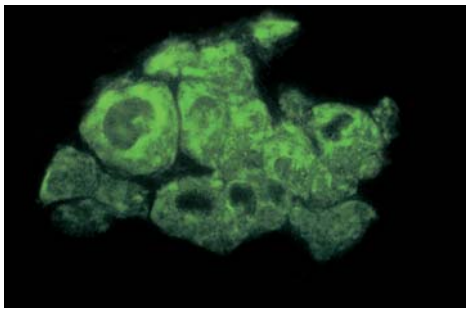

Figure 1: Direct detection of caudodorsal cells with fluorescein-labeled CDCH-I.

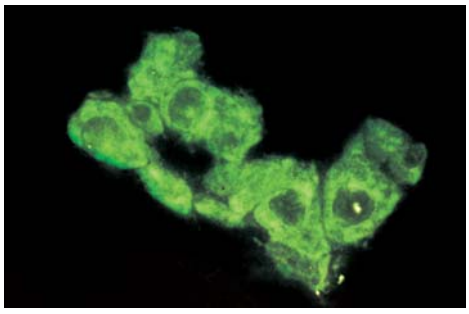

Figure 2: Indirect detection of caudodorsal cells with DIG-labeled CDCH-I and sheep anti-DIG-fluorescein conjugate. The same cells are positive, but the hybridization signal is clearly more intense than that obtained with the direct technique (Figure 1).

References

Dirks, R. W.; van Gijlswijk, R. P. M.; Vooijs, M. A.; Smit, A. B.; Bogerd, J.; Van Minnen, J.; Raap, A. K.; Van der Ploeg, M. (1991) 3'-end fluorochromized and haptenized oligonucleotides as *in situ* hybridization probes for multiple, simultaneous RNA detection. *Exp. Cell Res.* **194**, 310–315.

Dirks, R. W.; van Gijlswijk, R. P. M.; Tullis, R. H.; Smit, A. B.; Van Minnen, J.; Van der Ploeg, M.; Raap, A. K. (1990) Simultaneous detection of different mRNA sequences coding for neuropeptide hormones by double *in situ* hybridization using FITC- and biotin-labeled oligonucleotides. *J. Histochem. Cytochem.* **38**, 467–473.

Dirks, R. W.; Raap, A. K.; Van Minnen, J.; Vreugdenhil, E.; Smit, A. B.; Van der Ploeg, M. (1989) Detection of mRNA molecules coding for neuropeptide hormones of the pond snail Lymnaeae stagnalis by radioactive and nonradioactive *in situ* hybridization: a model study for mRNA detection. *J. Histochem. Cytochem.* **37**, 7–14.

Van Minnen, J.; Van de Haar, Ch.; Raap, A. K.; Vreugdenhil, E. (1988) Localization of ovulation hormone-like neuropeptide in the central nervous system of the snail Lymnaeae stagnalis by means of immunocytochemistry and *in situ* hybridization. *Cell Tissue Res.* **251**, 477–484.

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Reagents available from Roche Applied Science for this procedure

Reagent	Description	Cat. No.	Pack size
DIG Oligonucleotide Tailing Kit, 2nd generation*	For tailing oligonucleotides with digoxigenin-dUTP.	03 353 583 910	1 Kit (25 tailing reactions)
Fluorescein-12-dUTP	Tetralithium salt, solution	11 373 242 910	25 nmol (25 µl)
tRNA	From baker's yeast, lyophilizate	10 109 495 001 10 109 509 001	100 mg 500 mg
Anti-Digoxigenin-Fluorescein*	Fab Fragments from sheep	11 207 741 910	200 µg
Anti-Digoxigenin-Rhodamine*	Fab Fragments from sheep	11 207 750 910	200 µg
DAPI	Fluorescence dye for staining of chromosomes	10 236 276 001	10 mg
Blocking Reagent	Blocking reagent for nucleic acid hybridization	11 096 176 001	50 g

* The labeling of nucleic acids with DIG is covered by EP patents 0 324 474 and 0 371 262 as well as the following US patents 5.344.757, 5.354.657 and 5.702.888 owned by Roche Diagnostics GmbH.

RNA *in situ* hybridization using DIG-labeled cRNA probes

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In recent years, the *in situ* hybridization (ISH) technique has found widespread application in both basic science and diagnostic clinical research. The ISH technique has frequently been used to localize specific genes on metaphase chromosomes and to detect viral and bacterial genomes in infected tissues. The RNA *in situ* hybridization (RISH) technique for the examination of mRNA expression has gained less attention due to the many technical problems associated with this technique.

In this report, we present a step-by-step protocol for a nonradioactive RISH technique on frozen sections using digoxigenin-labeled copy RNA (cRNA) probes. We demonstrate this technique on frozen sections of mouse kidney using DIG-labeled cRNA probes for the ectoenzyme aminopeptidase A, a low-copy RNA (Assmann et al., 1992). For a high-copy RNA, we studied human psoriatic epidermis using a DIG-labeled cRNA probe for elafin/SKALP, an inhibitor of leukocyte elastase and proteinase 3 (Alkemade et al., 1994). This protocol has been optimized to give strong hybridization signals, even with low-copy mRNA molecules.

For a full discussion of each step of this protocol, along with more hints on enhancing the result of this often laborious and troublesome technique, see the previously published version of this report (Dijkman et al., 1995a; 1995b).

I. Probe selection

Choosing the type of probe (*i.e.*, DNA, RNA, or oligonucleotide) is essential for a good final result. For the optimization of the probe labeling, we have tested four methods of incorporating the DIG label:

- Direct labeling of cDNA molecules using the DIG DNA Labeling Kit.
- DIG labeling of oligonucleotides using the DIG Oligonucleotide Tailing Kit.
- DIG labeling of PCR products according to the method of Hannon et al. (1993).
- DIG labeling of cRNA molecules using the DIG RNA Labeling Kit.

The RNA labeling method gave by far the best results. Therefore, we provide some hints on how to transcribe DIG-labeled cRNA molecules:

- For cRNA probe synthesis, subclone a cDNA molecule in an appropriate vector. The vector should have sequences flanking the insert that allow the insert to be transcribed. Typically, commercially available vectors have SP6 and T7 RNA polymerase sites flanking the multiple cloning sites.
- Regulate the length of the cDNA molecule since this greatly affects the hybridization efficiency. Usually, a cDNA length between 200 and 500 nucleotides gives the best results, allowing efficient hybridization and good penetration of the tissue.
- When a molecule of interest is expressed as a high-copy RNA but the target RNA is masked by proteins, make probes between 100 and 200 nucleotides long for better penetration (Figure 1).

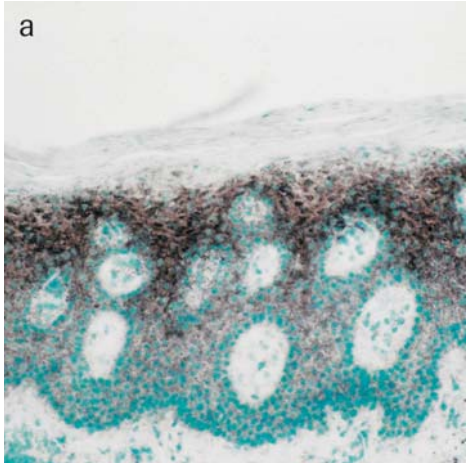
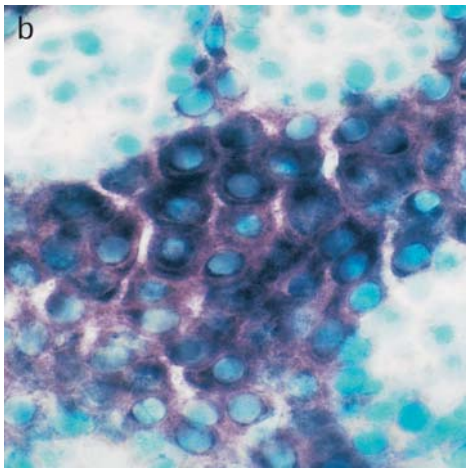
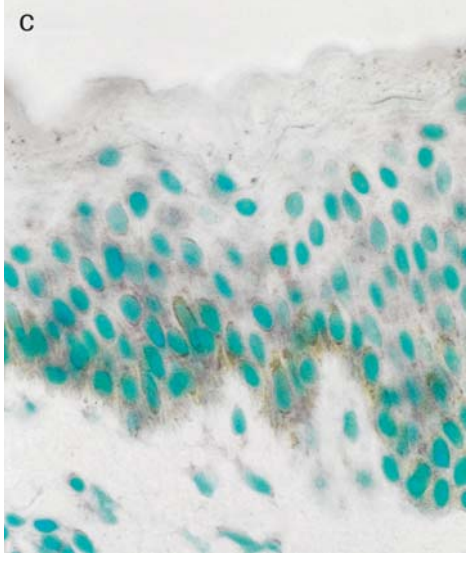


Figure 1: RNA *in situ* hybridization on a frozen section of human psoriatic epidermis with DIG-labeled cRNA probes coding for human SKALP.

Panel a: An antisense cRNA probe (150 bp) was used on a 10 µm thick section (magnification × 125).



Panel b: An antisense cRNA probe (150 bp) was used on a 20 µm thick section (magnification × 500).



Panel c: A sense cRNA probe (150 bp) was used on a 10 µm thick section (magnification × 270). Intense staining of both the stratum spinosum and stratum granulosum is observed with the antisense probe.

II. Probe labeling

1. After subcloning the cDNA molecule, linearize the circular vector with restriction enzymes that cut the multiple cloning site in 2 orientations, allowing sense and antisense synthesis.

   ⚠ *Control this linearization step carefully by monitoring the digestion with agarose gel electrophoresis. Circular molecules that are left in the digestion mixture affect the transcription efficiency.*

2. Extract the linearized molecules from the digestion mixture by phenol extraction.

3. Label the transcripts from the linearized molecules with DIG-labeled nucleotides and the DIG RNA Labeling Kit according to the instructions given in the kit, but with the following modifications:
   - Perform the SP6 polymerase incubation at 40°C instead of 37°C.
   - Precipitate the labeled molecules overnight at -20°C, rather than 30 min at -70°C.
   - Monitor the transcription reaction by agarose gel electrophoresis to check for correct cRNA probe length.

4. Monitor probe labeling by spotting diluted aliquots of the labeled cRNA probes on nylon membranes and analyzing with the DIG Luminescent Detection Kit. See also Chapter 4, page 63.

   ! *The sense and antisense cRNA probes should be labeled equally as efficiently as the control DNA included in the DIG RNA Labeling Kit.*

5. If necessary, adjust the concentration of the labeled sense and antisense cRNA probes so they contain equal amounts of label.

6. Aliquot the cRNA probes in polypropylene tubes at -70°C.

   ! *Repeated freezing and thawing affects the labeled cRNA probe.*

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III. Preparation of tissue sections

! *For detection of low-copy RNA molecules, always prepare frozen sections, since paraffin embedding causes a loss of approximately 30% of the RNA. The method described here has been optimized for frozen sections.*

1. For the processing of tissue, clean all knives and other materials with RNase ZAP (AMBION) and work as aseptically as possible.

2. Remove the tissue from the animal, immediately snap-freeze the tissue, and store it in liquid nitrogen. Work quickly to avoid degradation of RNA (Barton et al., 1993).

3. Cut 10 µm thick frozen sections.

   ! *To get a higher signal, cut sections thicker than 10 µm. For better signal localization, cut sections thinner than 10 µm.*

4. Mount tissue directly on Superfrost Plus slides (Menzel Gläser, Omnilabo, Breda, The Netherlands) to prevent detachment of the section during the RISH procedure.

■

IV. Pretreatment of slides

Prepare all solutions for Procedures IV with water that has been treated with 0.1% DEPC.

1. Directly after mounting, heat the sections on a stove for 2 min at 50°C to fix the RNA in the tissue.

   Vary the time (from 10–120 s) and temperature (from 50°–90°C) of the fixation step to accommodate different types of tissue and RNA.

2. Dry the sections for 30 min.

3. Circle the sections with a silicone pen (DAKO A/S, Glostrup, Denmark) to prevent smudging of the substrate.

4. Use the following criteria to decide the next step of the procedure:
   - *If* the target tissue has lipid vesicles (Figure 2) that interfere with the RISH detection, go to Step 5.
   - *If* the target tissue does not have lipid vesicles that interfere with the RISH detection, go to Step 6.

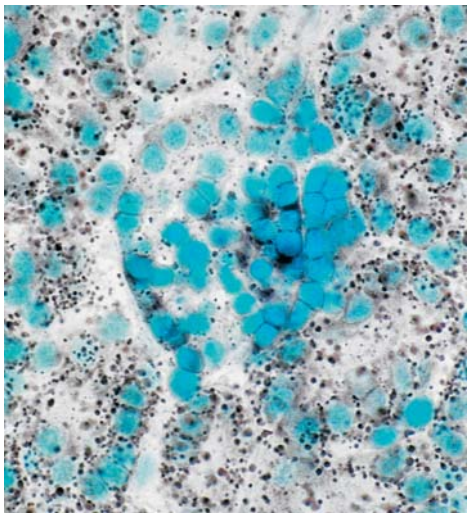


Figure 2: RNA *in situ* hybridization on a frozen kidney section with a DIG-labeled cRNA probe for mouse aminopeptidase A (without delipidization). The section was 10 µM thick and was taken from a male BALB/c mouse. Note the many nonspecific lipid vesicles in the section. The specific hybridization signal appears in cells of the glomerulus (arrows). Magnification, 600×.

5. **Optional:** To minimize nonspecific background caused by lipid vesicles, do the following (all at room temperature):
   - Delipidize the sections by extracting them for 5 min in chloroform.
   - Dry the section to evaporate the chloroform.

   This delipidation step may be omitted in pilot studies on a new tissue.

6. Fix the tissue sections as follows (all at room temperature):
   - Incubate tissue in PBS containing 4% paraformaldehyde for 7 min.
   - Wash 1 × 3 min with PBS.
   - Wash 2 × 5 min with 2× SSC.

   1× SSC contains 150 mM NaCl, 15 mM sodium citrate; pH 7.2.

V. Prehybridization, hybridization, and post-hybridization

Prepare all solutions for Procedures V with water that has been treated with 0.1% DEPC.

1. Prehybridize each section for 60 min at 37°C in 100 µl hybridization buffer [4× SSC; 10% dextran sulfate; 1× Denhardt's solution (0.02% Ficoll 400, 0.02% polyvinyl pyrolidone, 0.02% bovine serum albumin); 2 mM EDTA; 50% deionized formamide; 500 µg/ml herring sperm DNA].

   You may vary the temperature of the prehybridization and hybridization steps between 37°C and 50°C.

2. Perform hybridization as follows:
   - Remove and discard the buffer from the prehybridization step.
   - Cover each section with 100 µl hybridization buffer containing 200 ng/ml of DIG-labeled cRNA probe.

     Do not use coverslips, since they decrease the signal up to fourfold.

     A probe concentration of 200 ng/ml holds only if the cRNA was labeled efficiently. If, in Procedure II, the cRNA probe was not labeled as efficiently as the controls from the DIG RNA Labeling Kit, adjust the concentration of the cRNA probe in the hybridization mixture.
   - Incubate for 16 h at 37°C.

   You may vary the temperature of the prehybridization and hybridization steps between 37°C and 50°C.

3. After the hybridization, wash unbound cRNA probe from the section as follows:
   - 1 × 5 min with 2× SSC at 37°C.

     To make the wash more stringent and wash away nonspecifically bound cRNA probe, lower the salt concentration or increase the formamide concentration in the washing buffer and perform the washing step at a temperature 5°C beneath the melting temperature of the probe.
   - 3 × 5 min with 60% formamide in 0.2× SSC at 37°C.
   - 2 × 5 min with 2× SSC at room temperature.

VI. Immunological detection

1. Wash the sections for 5 min at room temperature with 100 mM Tris-HCl (pH 7.5), 150 mM NaCl.
2. Incubate the sections for 30 min at room temperature with blocking buffer [100 mM Tris-HCl (pH 7.5), 150 mM NaCl; saturated with blocking reagent].
3. Prepare a 1:200 dilution of alkaline phosphatase-conjugated anti-DIG antibody (polyclonal, Fab fragments, from sheep) in blocking buffer (from Step 2).
4. Incubate the sections for 120 min at room temperature with the diluted anti-DIG antibody conjugate prepared in Step 3.
5. Wash the sections as follows:
   - 2 × 5 min at room temperature with 100 mM Tris-HCl (pH 7.5), 150 mM NaCl.
   - 1 × 10 min at room temperature with 100 mM detection buffer [Tris-HCl (pH 9.5), 100 mM NaCl, 50 mM $MgCl_2$].
6. Cover the sections with detection buffer containing 0.18 mg/ml BCIP, 0.34 mg/ml NBT, and 240 µg/ml levamisole. Incubate for 16 h at room temperature (for detection of low abundance RNA).

   This development should be carried out with the slides standing up in a small container to prevent nonspecifically converted substrate from falling onto the section.
7. Stop the color reaction by washing the sections for 5 min in 10 mM Tris (pH 8), 1 mM EDTA.

VII. Counterstain

1. Wash the sections for 5 min with distilled H_2O at room temperature.
2. Counterstain the sections for 5–10 min with 1% methylene green at 37°C.
3. Repeat wash (from Step 1).
4. Mount coverslips in Kaiser's solution (available from Merck).

Results and discussion

Figure 3 shows a typical RISH signal obtained with this protocol and a labeled antisense cRNA probe coding for mouse aminopeptidase A. The protocol included the optional delipidation step with chloroform (Procedure IV, Step 5). Figure 4 shows the control reaction with the labeled sense cRNA probe.

We have provided a standard protocol for RISH on low- and high-copy RNA molecules. Using this protocol, one should be able to perform RISH on each RNA molecule of interest, with only minor modifications depending on the abundance of the RNA of interest.

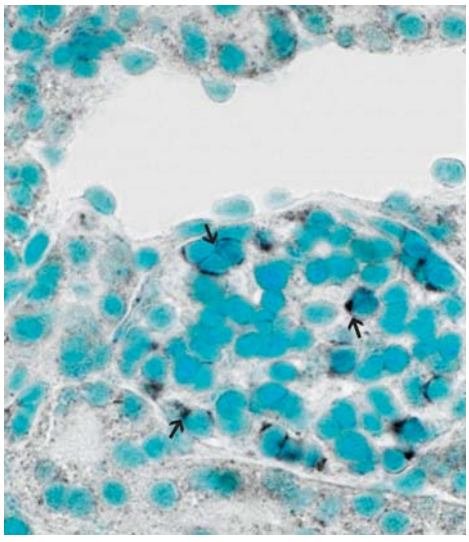


Figure 3: RNA *in situ* hybridization on a frozen kidney section with a DIG-labeled antisense cRNA probe for mouse aminopeptidase A (after delipidization). The section was 10 µm thick and was taken from a male BALB/c mouse. Pretreatment of the section with chloroform prior to the hybridization delipidized the section and reduced a nonspecific signal previously observed in lipid vesicles (Figure 2). The specific hybridization signal (arrows) was undiminished by the chloroform treatment (magnification × 600).

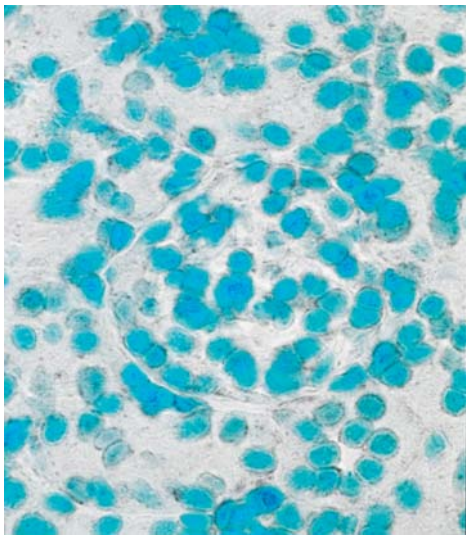


Figure 4: RNA *in situ* hybridization on a frozen kidney section from the same mouse used in Figure 3 with a DIG-labeled sense cRNA probe for mouse aminopeptidase A. As in Figure 3, the section was 10 µm thick and was taken from a male BALB/c mouse. The section was pretreated with chloroform prior to the hybridization. No signal could be seen, indicating the specificity of the antisense hybridization signal seen in Figure 3 (magnification, 600×).

Acknowledgments

The cDNA clone coding for the mouse aminopeptidase A cDNA sequence was kindly provided by Dr. Max D. Cooper from the University of Alabama at Birmingham, USA (Wu et al., 1990). The cRNA probe for elafin/SKALP was kindly provided by Dr. J. Schalkwijk, department of Dermatology, University Hospital Nijmegen, The Netherlands.

References

Alkemade, J. A. C.; Molhuizen, H. O. F.; Ponec, M.; Kempenaar, J. A.; Zeeuwen, P. L. J. M.; de Jongh, G. J.; van Vlijmen-Willems, I. M. J. J.; van Erp, P. E. J.; van de Kerkhof, P. C. M.; Schalkwijk, J. (1994) SKALP/elafin is an inducible proteinase inhibitor in human epidermal keratinocytes. *J. Cell Sci.* **107**, 2335–2342.

Assmann, K. J. M.; van Son, J. P. H. F.; Dijkman, H. B. P. M.; Koene, R. A. P. (1992) A nephritogenic rat monoclonal antibody to mouse aminopeptidase A. Induction of massive albuminuria after a single intravenous injection. *J. Exp. Med.* **175**, 623–636.

Dijkman, H. B. P. M.; Mentzel, S.; de Jong, A. S.; Assmann; K. J. M. (1995a) RNA *in situ* hybridization using digoxigenin-labeled cRNA probes. Biochemica (U.S. version) No. 2 [1995], 23–27.

Dijkman, H. B. P. M.; Mentzel, S.; de Jong, A. S.; Assmann; K. J. M. (1995b) RNA *in situ* hybridization using digoxigenin-labeled cRNA probes. Biochemica (worldwide version) No. 2 [1995], 21–25.

Barton, A. J. L.; Pearson, R. C. A.; Najlerahim, A.; Harrison, P. J. (1993) Pre- and postmortem influences on brain RNA. *J. Neurochem.* **61**, 1–11.

Hannon, K.; Johnstone, E.; Craft, L. S.; Little, S. P.; Smith, C. K.; Heiman, M. L.; Santerre, R. F. (1993) Synthesis of PCR-derived, single-stranded DNA probes suitable for *in situ* hybridization. *Anal. Biochem.* **212**, 421–427.

Wu, Q.; Lahti, J. M.; Air, G. M.; Burrows, P. D.; Cooper, M. D. (1990) Molecular cloning of the murine BP-1/6C3 antigen: a member of the zinc-dependent metallopeptidase family. *Proc. Natl. Acad. Sci. USA* **87**, 993–997.

Reagents available from Roche Applied Science for this procedure

Reagent	Description	Cat. No.	Pack size
DIG RNA Labeling Kit (SP6/T7)*	For RNA labeling with digoxigenin-UTP by *in vitro* transcription with SP6 and T7 RNA polymerases.	11 175 025 910	1 Kit (2 × 10 labeling reactions)
Blocking Reagent	For nucleic acid hybridization	11 096 176 001	50 g
Anti-Digoxigenin-AP*	Anti-Digoxigenin, Fab fragments conjugated with alkaline phosphatase	11 093 274 910	150 U (200 µl)
Tris	For preparation of buffer solutions	10 708 976 001 11 814 273 001	1 kg 5 kg
NBT solution	100 mg/ml nitroblue tetrazolium salt in 70% (v/v) dimethylformamide	11 383 213 001	3 ml (300 mg) (dilute prior to use)
BCIP solution	50 mg/ml 5-bromo-4-chloro-3-indolyl phosphate (BCIP), toluidinium salt in 100% dimethylformamide	11 383 221 001	3 ml (150 mg)

* The labeling of nucleic acids with DIG is covered by EP patents 0 324 474 and 0 371 262 as well as the following US patents 5.344.757, 5.354.657 and 5.702.888 owned by Roche Diagnostics GmbH.

Detection of mRNAs on cryosections of the cardiovascular system using DIG-labeled RNA probes

G. Plenz, B. Weissen, and I. Steffen, Institute for Arteriosclerosis Research, Department of Thoracic and Cardiovascular Surgery, and Department of Cardiology and Angiology, University of Münster, Germany

The following protocol was optimized from a protocol using 35S-labeled RNA probes (Plenz et al., 1993 and 1994). It allows to detect the expression of low abundant mRNAs in the cardiovascular system, *e.g.* of the proinflammatory cytokine GM-CSF in normal human coronary arteries, and of IL6 and gp130 in human failing hearts (Plenz et al., 2001). The protocol can be combined with immunohistochemistry (Plenz et al., 1997 and 1999).

All solutions used for in situ hybridization have to be treated with 0.1% DEPC or to be prepared with DEPC treated distilled water. The protocol can also be used for paraffin and methacrylate embedded sections.

I. Preparation of DIG-labeled RNA probes

1. Amplify the desired cDNA by RT-PCR and clone the cDNA into an *in vitro* transcription vector.
2. Prepare the linearized template plasmide for *in vitro* transcription according to standard molecular techniques.
3. *In vitro* transcribe DIG-labeled RNA probes from the plasmide according to the method described in chapter 4, page 49 of this manual or according to the instructions in the DIG RNA Labeling Kit.

   Best results will be obtained with digoxigenin-labeled RNA probes <600 bp. For larger probes adjusting of the Proteinase K digest is preferred to denaturing of the probes.
4. Determine the amount of DIG-labeling by dot blot according to the method in chapter 4, page 59 of this manual.

   Thoroughly evaluate the concentration of the RNA probes. If the concentration is underestimated background will rise after in situ hybridization.

II. Preparation of silane coated slides

1. Incubate glass slides for 60 min in silane solution.
2. Rinse slides for 10 min in distilled water.
3. Dry slides overnight at 50°C.

   Store slides dust free and dry. The slides may be used for several month.

III. Tissue preparation

Important: *Freeze tissue as soon as possible after excision to prevent degradation of mRNA.*

1. Cut tissue to appropriate size.
   Remove as much of the fatty tissue as possible.
2. Cool down 2-methylbutan in liquid nitrogen.
3. Immerse tissue in cryoprotective and freeze on cork disks in nitrogen cooled 2-methylbutan.
   Freezing in precooled 2-methylbutan results in optimal conservation of the tissue structure.
4. Store tissue at -80°C or in liquid nitrogen.
   The tissue can be stored for years.

IV. Preparation of cryosections

1. Prewarm the tissue samples to -22°C.
2. Cut sections (4 µm–12 µm).
   Thicker sections may be preferred for confocal microscopy.
3. Place the sections (2–4) on silane coated slides.
   The slides may be immediately used or stored at -80°C for several months.

V. Prehybridization procedure

1. Dry the slides for 1 h at room temperature or in an oven for 10 min at 50°C.
   If tissue is rich in lipids delipidize the sections for 10 min in chloroform.
2. Fix the sections for 10 min with phosphate buffered 4% paraformaldehyde.
3. Rinse three-times in 5× TE (50 mM Tris-HCl, pH 8.0, 5 mM EDTA).
4. If necessary treat the sections with Proteinase K (to increase the efficiency of the Proteinase K treatment we recommend to preincubate the stock solution for 1 hr at 37°C) for 10 min at room temperature.
   Whether Proteinase K treatment is required and which concentration of Proteinase K is used strongly depends on the kind of tissue and fixation. For blood vessels and myocardial tissue we used:
   Cryosections: up to 2 µg/ml
   Paraffin embedded sections: up to 20 µg/ml
   Methacrylate embedded sections: up to 50 µg/ml
5. Rinse the sections in Tris-Glycine (100 mM Tris-HCl pH7.0, 100 mM glycine from a 10× stock solution).
6. Postfix for 10 min in 4% phosphate buffered paraformaldehyde.
7. Rinse three-times in TBS (50 mM Tris-HCl pH 7.5, 150 mM NaCl) for 5 min.

8. Rinse once in distilled water (5 min).

9. Dehydrate the sections in increasing concentrations of ethanol and dry in a dust free area.

   After the prehybridization procedure the sections may be stored for a few days in a refrigerator. However, optimal results will be achieved by immediately continuing with in situ hybridization.

VI. Hybridization procedure

Homologous probes are hybridized at 50–52°C. For heterologous probes we recommend lower temperatures.

1. Denature hybridization solution for 10 min at 80°C.
   (Hybridization solution: 50% formamide, 2× SSPE buffer, 10 mM DTT, 1 mg/ml herring sperm DNA, 500 µg/ml yeast t-RNA, 1 mg/ml BSA)

2. Preincubate the sections in a humidified chamber for 2 h in hybridization solution.

   The sections must be completely covered with the hybridization solution.

3. Remove prehybridization solution and add hybridization solution.
   Concentration of the DIG-labeled RNA probe: 0.3–1µg/ml.

   Smaller volumes of hybridization solution can be used if the sections are covered with hydrophobic plastic coverslips or sheets of parafilm cut to the appropriate size. However, to our experience the best results are obtained without covering the sections and using slightly larger volumes of hybridization solution.

4. Incubate the tightly sealed chambers overnight in a shaking water bath at 50°C.

VII. Posthybridization procedure

1. Remove the hybridization solution by thoroughly rinsing the slides in 4× SSC.

2. Wash twice in 2× SSC for 15 min at 50°C.

3. Wash twice in 1× SSC for 15 min at 50°C.

4. To remove not specifically bound DIG-labeled RNA probes incubate with RNase A (10 µg/ml NTE: 500 mM NaCl, 10 mM Tris-HCl pH 8.0, 1 mM EDTA) for 10 min at 37°C.

   RNase is extremely stable!!!! Avoid contamination. We strongly recommend the use of separate glassware and of a water bath in an area separate from the in situ hybridization unit.

5. Wash 2× in 0.1× SSC for 10 min at 50°C.

VIII. Detection procedure

For this procedure the compounds of the DIG Nucleic Acid Detection Kit are used. All washing steps are performed on a shaking platform.

1. Wash sections for 5 min in buffer 1.
2. Block unspecific background by incubating the sections for 1 hr in buffer 2 (buffer 1 with 0.5% Blocking Reagent).
3. Remove blocking solution.

A: Alkaline phosphatase conjugated antibodies

1. Incubate with anti-DIG antibody conjugated with alkaline phosphatase (FAB-fragments) (dilute 1:500–1:1000 in buffer 2) for 1 h at room temperature.
2. Rinse thoroughly in buffer 1 containing 0.05% Tween and wash twice for 15 min.
3. Incubate for 15 min in buffer 3.
4. Incubate with an appropriate amount of staining solution and incubate the slides in a humidified chamber for 30 min to 24 h.

   To obtain optimal staining results the development of the precipitate should be performed under microscopic control.

   Staining solution: Ad 335 µg NBT (Stock solution: 75 mg/ml in 70% dimethylformamide), 174 µg X-phosphate (Stock solution: 50 mg/ml in 100% dimethylformamide) and 240 µg Levamisole per ml buffer 3.
5. Remove the staining solution by rinsing in 5× TE.
6. Stop staining procedure by incubating the slides for 15 min in 5× TE.
7. Rinse briefly in distilled water.
8. Counterstain the sections with methylen green.
9. Mount the sections with Kaiser´s glycerin gelatin.

   Other substrates may be used. If immunofluorescence protocols are used in combination with in situ hybridization we recommend development with the ELF substrate (ELF Kit, Molecular Probes).

B: Fluorochrome conjugated antibodies

If the mRNA of interest is abundantly expressed anti-DIG antibodies conjugated with FITC or other fluorochromes can be used.

1. Incubate for 2 h with FITC-conjugated anti-DIG antibody (1:20–1:200 in buffer 1).
2. Wash twice for 5 min with buffer 2 (Buffer 1 containing 0.05% Tween).
3. Counterstain the sections with Hoechst Dye 33342.
4. Mount with fluorescence mounting medium (DAKO).

IX. Immunohistochemistry

Usually immunohistochemistry was performed immediately after *in situ* hybridization. However, with both detection procedures excellent results were also obtained several weeks up to months after *in situ* hybridization.

All washing steps are performed on a shaking platform.

A. The peroxidase staining procedure was performed according to the manufacturers recommendations (Vectastain elite kit, Vektor).

B. Immunofluorescence

1. Incubate slides with PBS/1–2% BSA for 1–2 h in a humidified chamber.
2. Remove the blocking solution.
3. Incubate with an appropriate dilution of the respective primary antibody (in PBS/1–2% BSA) for 1–4 h at room temperature or overnight at 4°C in a humidified chamber.
4. Remove excess antibody by washing three-times for 5 min in PBS containing 0.05% Tween.
5. Incubate with an appropriate dilution (1:500 –1:1000 in PBS/1–2% BSA) of the secondary antibody (for immunoconfocal detection we recommend Cy-conjugated antibodies such as provided by Chemicon).
6. Wash three-times for 5 min in PBS.
7. Counterstain with Hoechst Dye 33342.
8. Mount with fluorescence mounting medium (DAKO).

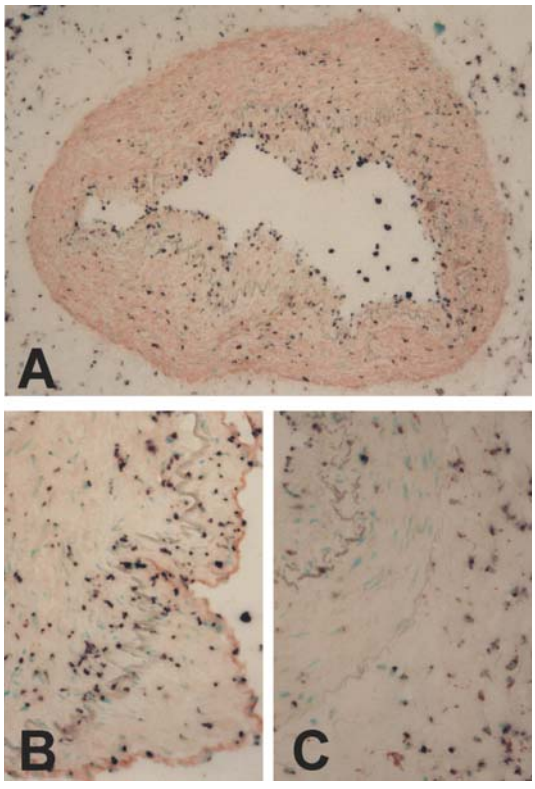


Figure 1: Detection of GM-CSF mRNA (purple) with a DIG-labeled antisense cRNA probe in an early stage of plaque development and immunohistochemical characterization of the GM-CSF expressing vascular cell types (red).

GM-CSF mRNA is expressed by smooth muscle cells (panel A), by endothelial cells (panel B) and macrophages (panel C). For detection of the *in situ* hybridization signal the alkaline phosphatase protocol was used. The cell type specific antibodies were visualized by using a peroxidase staining procedure (Vectastain elite kit, Vektor).

Results and discussion

Combined *in situ* hybridization and immunohistochemical staining has been used to identify vascular cells expressing GM-CSF and type VIII collagen in human coronary arteries. This non-radioactive procedure combined

1. *In situ* hybridization with DIG-labeled cRNA pobes (GM-CSF, type VIII collagen) and
2. immunohistochemical characterization of vascular cells by using cell type specific antibodies (smooth muscle cells: anti smooth muscle actin, Enzo; endothelial cells: von Willebrand factor, Sigma; macrophages: CD68, DAKO) and a peroxidase staining procedure (Vectastain elite kit, Vektor). About 70% of the antibodies tested worked in this protocol.

The method enabled us to identify the intimal and medial smooth muscle cells as the major cell type expressing GM-CSF in the development of atherosclerotic plaques, particularly in advanced lesions (Plenz et al., 1997). Other GM-CSF expressing cell types found in advanced lesions are endothelial cells and macrophages. In early lesions GM-CSF mRNA (*in situ* hybridization, purple stain) was expressed mainly by medial smooth muscle cells, some smooth muscle cells of the tunica intima (Figure 1A), about 50% of the endothelial cells (Figure 1B), and only a few macrophages located in the tunica adventitia (Figure 1C). In all stages of plaque development GM-CSF was coexpressed with type VIII collagen (Plenz et al., 1999) (Figure 2). Using the protocol described above, we easily characterized the GM-CSF and type VIII collagen expressing cell types in cryosections and paraffin embedded samples of various arteries, in the myocardium and in cultured vascular cells.

As shown in figures 1 and 2, the protocol described represents an easy to use and excellent means to evaluate the spatial and temporal expression pattern of mRNAs in various tissues and cultured cells. The protocol allows to simultaneously characterize the expressing cell types as well as the localization and distribution of other proteins. The protocol is as sensitive as radioactive *in situ* hybridization.

However it is less time consuming (Plenz et al., 1993 and 1994) and locates the expressing cells in a far more precise way.

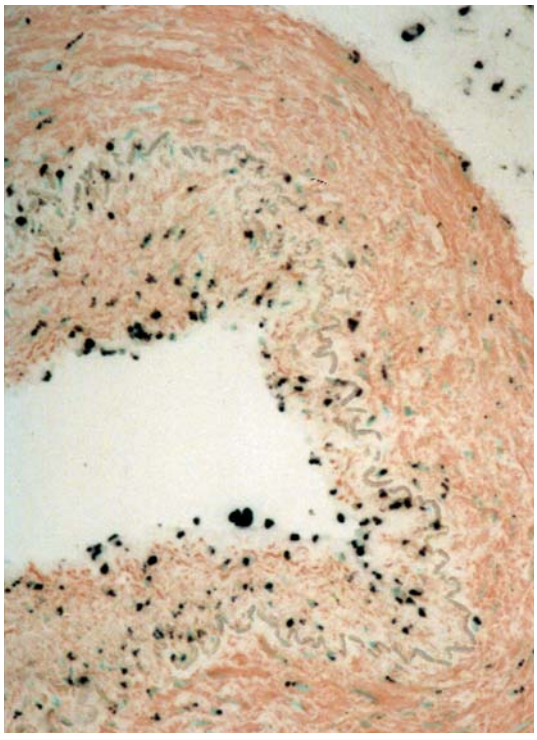


Figure 2: Detection of procollagen alpha1 type VIII mRNA (purple) with a DIG-labeled antisense cRNA probe in an early stage of plaque development.
Double staining to demonstrate type VIII collagen mRNA (purple) expressing smooth muscle cells (red) in early atherogenesis.

5

References

G Plenz, Y Gan, HM Raabe, PK Müller. Expression of vigilin in chicken cartilage and bone. *Cell Tissue Res 1993;* **273**, 381-389

G Plenz, S Kuegler, S Schnittger, H Rieder, C Fonatsch, PK Müller. The human vigilin gene: identification, chromosomal localization and expression pattern. *Hum Genet 1994,* **93**, 575-582

G Plenz, C Koenig, N Severs, H Robenek. Smooth muscle cells express granulocyte macrophage colony stimulating factor (GM-CSF) in the undiseased and atherosclerotic human coronary artery. *Atheroscler Thromb Vasc Biol 1997*, **17**, 2489-2499

G Plenz, S Reichenberg, C Koenig, J Rauterberg, M Deng, HA Baba, H Robenek. Granulocyte macrophage colony stimulating factor (GM-CSF) and typ VIII collagen are codistributed during atherogenesis and GM-CSF transiently stimulated the expression of type VIII collagen mRNA by smooth muscle cells. *Arterioscler Thromb Vasc Biol 1999,* **9**, 1658-1668

Plenz G, Song ZF, Tjan TDT, Koenig C, Baba H, Erren M, Flesch M, Wichter T, Scheld HH, Deng MC. Activation of the cardiac interleukin-6 system in advanced heart failure. *Eur J Heart Failure 2001,* **3**, 415-421

Reagents available from Roche Applied Science for this procedure

Reagent	Description	Cat. No.	Pack size
DIG RNA Labeling Kit (SP6/T7)*	For RNA labeling with Digoxigenin-UTP by *in vitro* transcription with SP6 and T7 RNA polymerases.	11 175 025 910	1 Kit (2 × 10 labeling reactions)
Proteinase K, rec., PCR Grade	Lyophilizate	03 115 836 001 03 115 879 001 03 115 801 001 03 115 852 001	25 mg 100 mg 2 × 250 mg 4 × 250 mg
tRNA from baker's yeast	Lyophilizate	10 109 495 001 10 109 509 001	100 mg 500 mg
BSA Bovine Serum Albumine	Special Quality for Molecular Biology	10 711 454 001	20 mg (1 ml)
RNase, DNase-free	from bovine pancreas, dry powder	11 119 915 001	500 µg (1ml)
DIG Nucleic Acid Detection Kit*	Kit for color detection of 40 blots of 100 cm^2	11 175 041 910	1 kit (40 blots)
Anti-Digoxigenin*	Fab fragments from sheep	11 207 741 910	200 µg

* The labeling of nucleic acids with DIG is covered by EP patents 0 324 474 and 0 371 262 as well as the following US patents 5.344.757, 5.354.657 and 5.702.888 owned by Roche Diagnostics GmbH.

Molecular and Biochemical Analysis of *Arabidopsis*

Rüdiger Simon, Department of Developmental Biology, University of Cologne, Germany. The protocol given below was part of a EMBO Course (Nonradioactive in situ hybridization, Cologne, 1998).

The standard protocol for *in situ* hybridizations in plants still involves fixing fresh tissue, embedding the tissue in wax, sectioning with a microtome and detection of the transcripts of interest using labeled RNA-probes. The RNA probes are synthesized by *in vitro* transcription, and either radioactive or nonradioactive labels can be used. This protocol concentrates only on nonradioactive methods, as they are easy to perform, very sensitive and even faster than techniques involving ra dioisotope labels.

I. Tissue fixation and embedding

Tissue to be fixed should be small (the smaller the better). Tissue should be placed into ice cold fixative immediately after dissection. If you are fixing stem tissue, cut it into short pieces about 5 mm in length. Root tissue is most easily harvested form plantlets that were grown on hard agar or in liquid culture. This avoids any problems with soil sticking to the roots.

Most plant tissues have a cuticle and will thus simply float on the surface of the fixative. The detergent Tween 20 (alternative: Triton X 100) will aid the penetration of the fixative. The open glass vials with tissue in fixative are placed into a exiccator that is connected to a vacuum pump. Vacuum is applied for about 10 minutes, until the tissue sinks down in the fixative solution at normal air pressure. Leave to fix overnight at 4°C.

5

1 Preparation of fixative

All solutions should be freshly prepared just before use.

⚠ *Paraformaldehyde and Formaldehyde solutions and vapour are toxic, so all steps involving these chemicals should be handled in a chemical fume hood. During the vacuum treatments, Formaldehyde vapours are released, so check that the outlet of your vacuum system will not pump toxic vapours into the lab.*

- Add a small pellet of NaOH to100 ml PBS, pH 6.5–7.

  ! *The pH will increase to about pH 11.*

- Heat to 70°C in the microwave.
- Add 4 g Paraformaldehyde and shake vigorously until the Paraformaldehyde is dissolved.
- Cool on ice.
- Add H_2SO_4 to adjust the pH to 7.
- Finally add 30 ml Tween 20.

The fixative is now ready for use. Aliquot the fixative into small glass scintillation vials.

2 Embedding

The tissue is dehydrated through a graded alcohol series, stained with a dye (Eosin Y) to facilitate sectioning and embedded into wax. This procedure will take at least 5 days.

Perform all steps in glass scintillation vials.

Day 1

- Remove the fixative, replace with ice-cold 50% Ethanol, incubate 90 min on ice.
- Ice-cold 70% Ethanol, incubate 90 min on ice.
- 85% Ethanol, incubate 90 min at 4°C.
- 95% Ethanol with 0.1% Eosin Y, incubate 90 min at 4°C.
- 100% Ethanol with 0.1% Eosin Y, incubate *overnight* at 4°C.

Day 2

- 100% Ethanol with 0.1% Eosin Y, incubate 90 min at 4°C.
- 100% Ethanol, incubate 60 min at RT.
- 50% Ethanol:50% Histoclear, incubate 60 min at RT.
- 100% Histoclear, incubate 60 min at RT.
- 100% Histoclear, incubate 60 min at RT.
- 100% Histoclear, incubate 60 min at RT.
- Pour off the last Histoclear, fill the glass vials up to the middle with Histoclear, then add wax pellets to the top. Incubate at 40–50°C *overnight*.

Day 3

- Melt wax pellets at 60°C, replace Histoclear/wax mixture with freshly molten wax and incubate at 60°C.
- Change the wax again in the evening.

The wax (Paraplast, or other brand names) to be used also contains plastic polymers and DMSO that shall facilitate the infiltration and sectioning. These additives are unstable at temperatures higher than 62°C. The Paraplast will solidify at 56–58°C. Be very careful to keep the temperature of the wax always at 60°C. All changes of wax and handling of the embedded materials has to be done quickly.

Day 4 and 5

- For additional 2 days keep changing the wax every morning and evening.

Day 6

Now the tissue blocks are made.

- Place the mould on a heating block at 60°C, pour some wax into the mould and empty a glass scintillation vial with the tissues into the mould.

Use plastic balance trays as moulds. The tissue can be quickly oriented in the mould using forceps that are preheated in the flame of a gas burner.

- When the tissue is in position, float the mould on cool water, thereby solidifying the wax.
- Store the embedded tissues in the fridge.

II. Sectioning

Sectioning requires a lot of patience and some practice. Use a standard microtome that allows to produce sections 5 to 10 mm thick. Check whether the blade is still unmarked. If possible, use a microtome with cheap, disposable blades that can be exchanged whenever it seems necessary.

Cut the wax in the moulds into small blocks with tissue.

1. Heat a spatula in the flame of a gas burner, press it quickly to the wax block and fix it with some molten wax onto a holder.
   These holders can be bought from companies or made yourself by cutting hard wood into small cubes (2 × 2 × 2 cm).
2. After fixing a number of tissue blocks to the holders, cool the blocks for 20 min.
3. Cut the wax blocks to a rectangular shape, leaving 1 to 2 mm of wax around the tissue.
   Such small wax blocks will allow you to fit more sections on each slide. Mount the holder to the microtome such that the longer side of the block is parallel to the blade.
4. Cut ribbons of sections at 5 to 10 mm thickness.
5. Place ribbons on coated glass slides (Superfrost Plus), using a fine paint brush.
6. Add sterile water so that the wax ribbons float freely.
7. Place the slide onto a hotplate at 42°C until the sections are fully flattened.
8. Drain off the water with a tissue paper (Kleenex).
9. Gently blot the sections dry with a very fine tissue paper.
   You can now check the section under a microscope.
10. Leave the slides on the hotplate overnight, and then store at 4°C until you are ready for the hybridization.

Common problems with sectioning:

The sections break up or appear brittle: material was not properly embedded or the wax was destroyed by overheating.

Sections split along the ribbon: the blade is chipped or dirty – clean or replace the blade.

Ribbons are not straight: the wax block is not rectangular or the long side of the block is not parallel to the blade.

Sections roll up, no ribbon is formed: change the angle of the blade.

Ribbon forms, but the whole ribbon rolls up or sticks to the blade: the blade is electrostatically charged, wipe it with a wet Kleenex.

III. Preparation of probes

The DNA fragment has to be subcloned into a transcription vector, *e.g.*, pBluescript or pGEM. The plasmid DNA to be used for the *in vitro* transcription reaction should be purified using a commercial purification system.

Linearise the plasmid with a restriction enzyme, and check the digest on a minigel.

It is important that the plasmid is cut to completion. Avoid using a restriction enzyme that leaves a 3´ overhang (e.g. Kpn I).
Purify the plasmid after the restriction digest by Phenol/Chloroform extraction and Ethanol precipitation. Be aware that the DNA has to be completely free of RNase!
Resuspend the cut plasmid DNA in autoclaved water at a concentration of 0.5 µg/µl.

In vitro transcription

Use any commercially available T7, T3 or SP6 RNA-Polymerase. The choice of polymerase depends of course on the plasmid vector used and on the orientation of your sequence of interest (Select the appropriate polymerase so that the antisense-transcript is made).

1. Set up the transcription reaction on the bench (not on ice):
   - 5 µl dH_2O
   - 5 µl 5× Transcription buffer
   - 1 µl RNAse Inhibitor (40 U/µl)
   - 2.5 µl 5mM ATP
   - 2.5 µl 5mM GTP
   - 2.5 µl 5mM CTP
   - 1 µl 5mM UTP
   - 2.5 µl 1mM DIG-UTP
   - 2 µl linearized template DNA (= 1 µg)
   - 1 µl RNA-Polymerase (20 U/µl)
2. Incubate at 37°C for 60 to 120 min.
3. Check the transcript on a gel. Therefore take 1 µl from each labeling reaction, add 9 µl TE.
   After electrophoresis a smear of synthesized RNA and a faint plasmid band should be visible.
4. Stop the reaction by adding
   - 75 µl TMS-buffer
   - 2 µl tRNA (100 mg/ml)
   - 1 µl DNase (RNase free!)
5. Incubate 10 min. at 37°C
6. Take 1 µl from each sample, add to 9 µl TE and check on gel whether template DNA has been removed
   - Add
     100 µl 3.8 M NH_4Ac
     600 µl EtOH
   - Incubate at -20°C for 60 min.
   - Spin down for 10 min. at maximum speed in a microcentrifuge.
7. Wash the pellet with ice cold 70% EtOH/0.15 M NaCl.
8. Centrifuge again, take supernatant off and air dry briefly.
9. Resuspend the air dryed pellet in 50 µl dH_2O.

IV. Hydrolysis

The optimum length for *in situ* probes is about 150 bp. The *in vitro* synthesized RNA probes can be hydrolysed under mild alkaline condition to achieve a mixture of RNA probes whose sizes average around 150 bp.

The following formula is used to calculate the time of hydrolysis:

$$t = \frac{L_i - L_f}{k \times L_i \times L_f}$$

t = time (minutes)
K = rate constant (= 0.11 kb/min)
L_i = initial length (kb)
L_f = final length (kb)

Example: If your cloned DNA fragment to be transcribed is 1.5 kb, the hydrolysis time will be:

$$t = \frac{1.5 - 0.15}{0.11 \times 1.5 \times 0.15} = 54.5$$

Procedure

1. Hydrolyse your *in vitro* transcripts by adding 50 µl 200 mM Carbonate buffer pH 10.2.
2. Incubate at 60°C for the calculated time.
3. Put your sample on ice and add
   10 µl 10% acetic acid
   12 µl 3M NaAcetate
   - Mix briefly, gas bubbles should appear, then add 312 µl Ethanol.
   - Incubate at -20°C, 60 min.
4. Spin down in a microcentrifuge for 10 min,
5. Wash the pellet with ice cold 70% Ethanol/0.15 M NaCl.
6. Centrifuge again, take supernatant off and air dry briefly.
7. Resuspend the air dryed pellet in 50 µl dH_2O and store at -20°C.

Checking the probe with an anti-DIG antibody

1. Spot 1 µl of your probe solution on a small piece of filter.
2. Fix by baking or UV-cross linking.
3. Wet briefly in 100 mM Tris-HCl pH 7.5, 150 mM NaCl.
4. Incubate 30 min. in 0.5% Blocking Reagent.
5. Wash briefly in 100 mM Tris-HCl pH 7.5, 150 mM NaCl.
6. Incubate 30 min. in 5 ml 100 mM Tris-HCl pH 7.5, 150 mM NaCl with 1 µl anti-DIG AP
   - Wash 2 × 15 min in 100 mM Tris-HCl pH 7.5, 150 mM NaCl
   - Wash briefly in 100 mM Tris-HCl pH 9.5, 100 mM NaCl, 50 mM $MgCl_2$
   - Develop for 10 min in 5 ml buffer 5 with 5 µl NBT and 5 µl BCIP
   - Stop the reaction by washing the filter in water.

   A dark blue spot should be visible where your probe solution was spotted on the filter.

V. *In situ* Hybridization

1 Preparation of cover slips

- Coverslips of the appropriate size (*e.g.* 24 × 50 mm) have to be cleaned and baked before they can be used.
- Wash coverslips in acetone for 15 min
- Drain the slides in a steel test tube rack
- Wrap in aluminum foil and bake at 180°C for 2 h.

2 Tissue pretreatments

On the day that you want to set up the hybridization reactions, the tissue has to be pretreated. These treatments consist of a number of steps in order to make the tissue more accessible for the RNA-probes and to reduce any unspecific binding of the probe to the slide. The slides are put into stainless steel racks (or plastic racks: check first whether they withstand the Histoclear) and passed through the following solutions:

- Dunk the slide rack several times and then leave it for the specified time in each solution.

Solution	Time	Remarks
100% Histoclear (or Rotihistol)	10 min	
100% Histoclear	10 min	
100% Ethanol	1 min	
100% Ethanol	1 min	
95% Ethanol	1 min	
85% Ethanol	1 min	
50% Ethanol	1 min	
30% Ethanol	1 min	
dH_2O	1 min	
0.2 M HCl	10 min	HCl treatment makes the tissue more accessible for the probe.
dH_2O	5 min	
PBS 1	2 min	PBS 1 and 2 are two separate boxes with 1 × PBS buffer.
Pronase (0.125 mg/ml in Pronase buffer)	10 min	Pronase treatment makes the tissue more accessible for the probe.
Formaldehyde (4% in PBS)	10 min	⚠ *Treat in a fume hood!* Formaldehyde has to be prepared freshly from Paraformaldehyde.
Glycine (0.2% in PBS)	2 min	Glycine will stop the Pronase activity.
PBS 1	2 min	
PBS 2	2 min	
Acetic anhydride (1ml in 100ml 0.1 M Triethanolamine pH 8.0)	10 min	⚠ *Treat in a fume hood!* Acetic anhydride is very unstable in water. The slides are incubated in Triethanolamine solution, and acetic anhydride is added while the slides are dunked repeatedly during the 10 min incubation. Acetylation of the tissue (the acetic anhydride step) shall reduce unspecific binding of the probe to the tissue.
PBS 2	2 min	

3

- Dehydrate through the Ethanol series up to the second 100% Ethanol
- Wash again in fresh 100% Ethanol.
- Store the slides in a box with a bit of Ethanol at 4°C while you are preparing the hybridization mix.

VI. Hybridization

In general, about 2 µl of the hydrolysed probe solution should be used per slide. However, it is advisable to try hybridizations with larger or smaller amounts of probe to find an optimum probe concentration. The final hybridization mix consists of 1 part "probe mix" and 4 parts of "Hybridization buffer" .

1 Preparation of required solutions

- Probe mix (per slide)
  - 2 µl hydrolysed DIG-labeled RNA probe
  - 2 µl dH_2O
  - 4 µl deionised Formamide
  - Mix, incubate for 2 min. at 80°C
  - Cool on ice.
- Hybridization buffer (for 25 slides):
  - 100 µl 3 M NaCl, 0.1 M Tris-HCl pH 6.8, 0.1 M $NaPO_4$-buffer, 50 mM EDTA
  - 400 µl Formamide (deionised)
  - 200 µl 50% Dextransulphate
  - 10 µl 100 mg/ml tRNA
  - 20 µl 50 × Denhardt´s solution
  - 70 µl dH_2O

2 Procedure

- Add 8 µl probe mix to 32 µl Hybridization buffer, this results in 40 µl Hybridization mix.
- Distribute the 40 µl on the tissue and cover with a 24 × 50 mm cleaned cover slip.

  Avoid any air bubbles.
- Place the slides on tissue paper soaked in 2× SSC, 50% Formamide in a small box.
- Seal the box with adhesive tape to avoid evaporation, and incubate it overnight in an oven or a waterbath at 50°C.

VII. Washing

1 Put the slides back into slide racks and immerse them in Wash buffer (2× SSC; 50% Formamide, no need to deionise) at 50°C.

The cover slips should have slided off the slides after a few minutes. You can also try to lift them off very gently, but be careful not to destroy your sectioned tissue.

2 Place the slides into fresh Wash buffer (2× SSC, 50% Formamide) and incubate at 50°C, 2 times for 60 min each.

3 Wash in NTE (500 mM NaCl; 10 mM Tris-HCl, pH7.5; 1 mM EDTA) at 37°C, 2 times for 5 min each.

4 Incubate in NTE with 20 mg/ml RNase A at 37°C for 30 min.

The RNase A will digest any unspecifically bound single stranded RNA, but will not affect the specifically bound (hybridized) and therefore double stranded probe-RNA.

5 Wash in NTE at RT, 2 times for 5 min each.

6 Wash in wash buffer at 50°C for 60 min.

7 Wash in PBS at RT for 5 min.

VIII. Detection

The hybridized probe-RNA will now be detected with an anti-DIG antibody that is coupled to alkaline phosphatase.

Handling instructions:

The following steps are performed either in slide racks, or in small trays to save solutions. This is recommended for the antibody incubation. Trays should be placed on a shaking platform. Trays should be changed and washed rather than just changing the solutions. All incubations are at Room Temperature.

Procedure

1 Incubate the slides for the specified time in each solution.

Step	Solution/Composition	Time
1	Buffer1: 100 mM Tris-HCl, pH 7.5; 150 mM NaCl	5 min
2	Buffer1 with 0.5% Blocking Reagent	60 min
3	Buffer1 with 1% BSA, 0.3% Triton	60 min
4	Anti-DIG-AP antibody diluted 1:3000 in Buffer 1 with 1% BSA, 0.3% Triton	60 min
5	Buffer 1 with 0.3% Triton	20 min, 4×
6	Buffer 1	5 min
7	Buffer 2: 100 mM Tris-HCl, pH 9.5; 100 mM NaCl; 50 mM $MgCl_2$	5 min
8	Buffer 2 with 10% Polyvinylalcohol (MW 70.000-100.000, *e.g.* Sigma P 1763); dissolve the polyvinylalcohol by boiling the solution on a heated stirrer. Let it cool down, then add 1.5 µl NBT and 1.5 µl BCIP per ml.	up to 3 days in the dark

Prepare shortly before use Incubate the slides in buffer 2 in trays with a transparent cover to avoid evaporation. You can then easily check the reaction under a microscope after 12 hours. Incubations for more than three days will result in increased background.

2 Put slides back into slide racks

- Wash in dH_2O, 5 min
- Wash in 70% Ethanol, 5 min.
- Wash in 95% Ethanol, 5 min.
- Wash in fresh 70% Ethanol, 5 min
- Wash in fresh dH_2O, 5 min.

3 The tissue can now be stained with a fluorescent dye (Calcofluor).

- Incubate in 0.1% Calcofluor (in dH_2O), 5 min.
- Wash briefly in dH_2O

The intensity of the staining can be easily checked by holding the slide over a normal UV-transilluminator. The tissue on the slides should show a bright blue intensive fluorescent color.

IX. Mounting

1. Dry the slides by air on the bench.

2. Add 2–3 drops of Entellan (or Euparal), cover with a cover slip of suitable size and leave to dry in the fume hood for 2 h.

   The slides are now ready to view with a light mircoscope. Use a microscope that is equipped with a UV source. Very faint signals can be more easily detected under dark field.

Reagents available from Roche Applied Science for this procedure

Reagent	Cat. No.	Pack size
Anti-Digoxigenin, Fab fragments*	11 093 274 910	150 U (200 µl)
Nylon membrane, positively charged	11 209 272 001 11 209 299 001 11 417 240 001	10 sheets (20 × 30 cm) 20 sheets (10 × 15 cm) 1 roll (0.3 × 3 m)
Blocking Reagent	11 096 176 001	50 g
NBT	11 383 213 001	3 ml (300 mg) dilute prior to use
BCIP	11 383 221 001	3 ml (150 mg)
tRNA, from baker´s yeast	10 109 495 001	100 mg
Set of ATP, CTP, GTP, UTP	11 277 057 001	1 set (4 × 20 µmol)
DIG-11-UTP	11 209 256 910	250 nmol (25 µl)
RNA Polymerase T7	10 881 767 001 10 881 775 001	1000 U 5000 U
RNA Polymerase Sp6	10 810 274 001 11 487 671 001	1000 U 5000 U
RNA Polymerase T3	11 031 163 001 11 031 171 001	1000 U 5000 U
DNase, RNase free	10 776 785 001	10 000 U
DIG RNA Labeling Kit (SP6/T7)*	11 175 025 910	1 kit
DIG RNA Labeling Mix*	11 277 073 910	40 µl (20 reactions)

* The labeling of nucleic acids with DIG is covered by EP patents 0 324 474 and 0 371 262 as well as the following US patents 5.344.757, 5.354.657 and 5.702.888 owned by Roche Diagnostics GmbH.

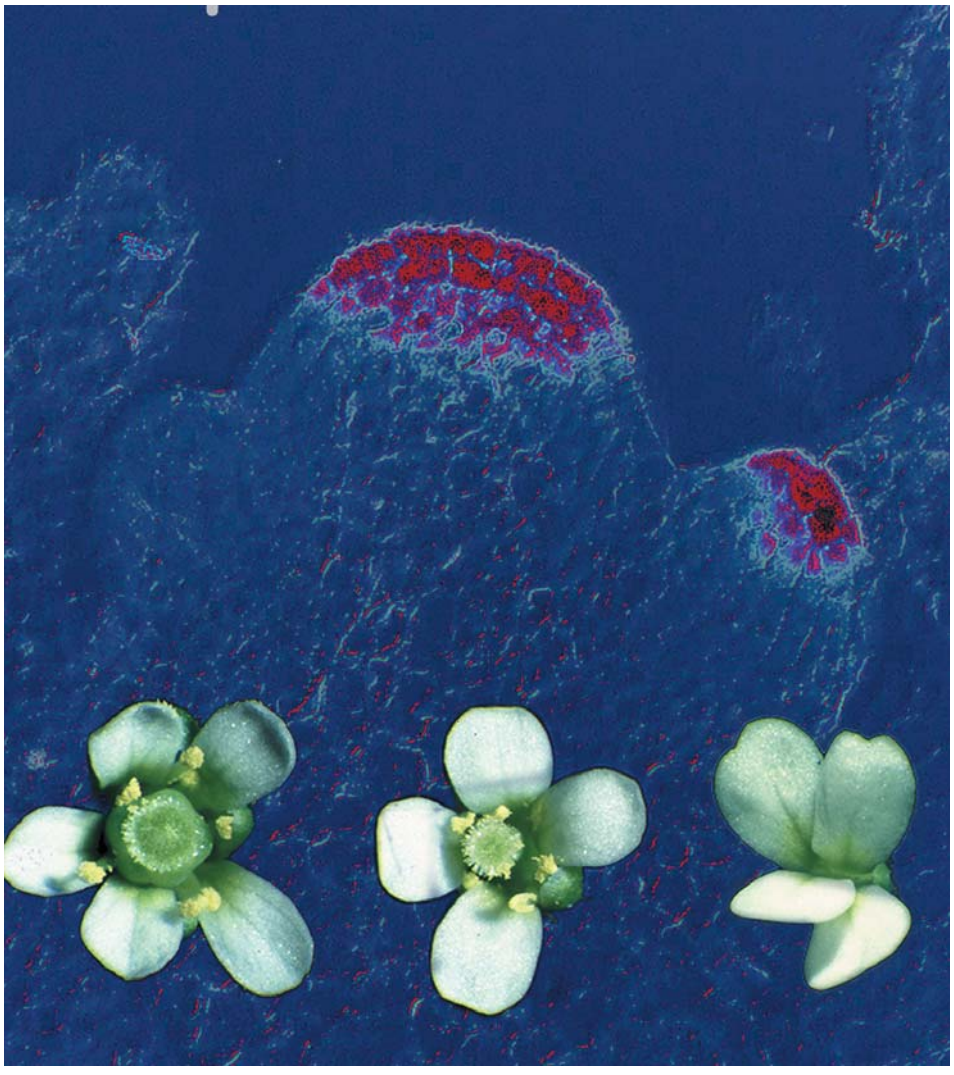
Acknowledgements and Literature

This protocol is a modification of several protocols that were tested out and written up by David Jackson, Enrico Coen, Sabine Hantke and other former colleagues at the John Innes Center, Norwich, U.K. Only a few steps were slightly altered to adapt this method for *Arabidopsis*.

The basic protocols can be found here:

Jackson, D.P. (1991): *in situ* hybridisation in plants. In: Molecular Plant Pathology: A Practical Approach, D.J. Bowles, S.J.Gurr, and M.McPherson, eds. Oxford University Press, England.

Coen, E.S., Romero, J.M., Doyle, S., Elliott, R., Murphy, G., and Carpenter, R. (1990). *floricaula*: A homeotic gene required for flower development in *Antirrhinum majus.* Cell **63**, 1311-1322.



Meristem size in Arabidopsis is controlled by a secreted signalling molecule, *CLV3*. This RNA *in situ* hybridization on a tissue section shows presence of *CLV3* RNA (in red, false colours) in the stem cells at the meristem tip. The tissue used here originated from a *clv1-4* mutant *Arabidopsis* plant that accumulates stem cells in the meristem. The flowers shown are from a *clv3-2* mutant (left), wildtype (middle) and a *CLV3* overexpressing *Arabidopsis* plant (right) (Reference: Brand, U., Fletcher, J.C., Hobe, M., Meyerowitz, E.M. und Simon, R. (2000): Dependence of stem cell fate in *Arabidopsis* on a feedback loop regulated by *CLV3* activity. *Science* **289**, 617-619)

Whole mount *in situ* hybridization for the detection of mRNA in *Drosophila* embryos*

Diethard Tautz, Institut für Genetik, University Cologne

In situ hybridization for the detection of mRNA in tissues has traditionally been performed on sectioned material. This was necessary since the probes were usually labeled with ^{3}H or ^{35}S, which required the use of a photographic film emulsion covering the sections for the detection of the signal. The development of highly sensitive nonradio-actively labeled probes allows now *in situ* hybridizations to be performed directly in tissues, such as whole *Drosophila* embryos (Tautz D, Pfeifle C, 1989). This "whole mount" *in situ* hybridization procedure is highly sensitive and the resolution of details is unparalleled. Complex expression patterns in particular, can only be analyzed in whole embryos, since the reconstruction of a three-dimensional pattern from sections can be very cumbersome. The method has therefore by now been adapted to other types of embryos and tissues as well, both from invertebrates and vertebrates and has become a general procedure in research laboratories studying questions of embryology and developmental biology.

In the original method DNA fragments were used that were labeled by random priming with DIG-dUTP. While this is still the method of choice if one wants to have a quick overview over the expression profile of a newly cloned gene, it has by now become clear that RNA probes can provide much better results (Lehmann R, Tautz D, 1994). Higher sensitivity can be obtained, because labeling efficiency is high and because they are single stranded. Moreover, since RNA-RNA hybrids are more stable than RNA-DNA hybrids, elevated hybridization temperatures can be employed, which result in a higher specificity and less background.

The detection of the hybridization signal is usually done with chromogenic substrates which develop a color at the place where the probe has bound. Different substrates are available, which allow to use different colors for differently labeled probes, *e.g.* biotin or fluorescein as haptens. A triple-labeling and detection procedure has been described for *Drosophila* (Hauptman G, Gerster T, 1996). Signal detection has also been done with fluorescently labeled antibodies against the respective haptene. While this results in a reduced sensitivity of detection, it is particularly useful in conjunction with the use of laser scanning microscopy, which allows a much better resolution of internal hybridization signals (Hughes et al.1996).

The experimental procedures given below are optimized for *Drosophila*. However, they may also be applied to other types of embryos. Only the procedures for the collection of the embryos and for the removal of the extra-embryonic membrane (if one exists) will have to be modified.

* Reprinted from Kessler, C. [ED]: Nonradioactive Analysis of Biomolecules, 2nd. ed. 2000; 573-580, Springer Lab. Manual, ISBN 3-540-64901-9, with permission of Springer Verlag GmbH.

I. Embryo collection and fixation

1. Collect the embryos on an apple juice agar plate and transfer them into a little basket made from polyethylene tubing (or an reaction vial) and stainless steel mesh (Wieschaus et al., 1986).
2. Wash embryos with double dist. water and dechorionate in a solution of 50% commercial bleach (Klorix) for about 2–3 min.

   Control this step under the binocular microscope. Dechorionated embryos float to the surface of the solution because the vitellin membrane is hydrophobic.
3. Wash with 0.1% Triton X-100 and transfer the embryos into a glass scintillation vial containing 4 ml Fixation solution [0.1 M Hepes, pH 6.9; 2mM $MgS0_4$; 1 mM EGTA].
4. Add 0.5 ml 37% Formaldehyde solution and 8 ml heptane.
5. Shake the vial for 15–20 min.
6. Remove the lower phase as far as possible (the embryos should be at the interphase).
7. Add 10 ml methanol and shake vigorously for 10 s.

   This step causes the vitellin membranes to burst and the devitellinized embryos will sink to the bottom.
8. Transfer the embryos into an reaction vial and wash them with methanol.

   The embryos may be stored at this stage for several weeks or even longer in the refrigerator (at -15 to -25°C).

II. Prehybridization treatment

Unless otherwise indicated, all the following steps are done in reaction vials in a volume of 1 ml at room temperature and on a rotating wheel.

Potential sources of RNAse contamination should be avoided.

1. Wash embryos 3 × 5 min in PBT [130 mM NaCl; 10 mM sodium phosphate, pH 7.2; 0.1% (v/v) Tween 20].
2. Postfix the embryos in 1 ml PBT, 4% Formaldehyde for 15 min.

   This postfixation may not be necessary when working with DNA probes, but is advisable for RNA probes, as these are hybridized under more harsh conditions.
3. Wash embryos 5 × 5 min in PBT.
4. Incubate the embryos for 2–5 min in a solution of 15-30 mg/ml Proteinase K in PBT.

   The exact length of this incubation step should be optimized for each new batch of Proteinase K. Too short digestion times result in a loss of signal intensity, too long digestion times may cause the embryos to burst during the subsequent steps.
5. Stop the Proteinase K digestion by incubating for 2 min in 2 mg/ml glycine in PBT

   This step may be dispensible if proteinase activity is low.
6. Wash 2 × 5 min with PBT.
7. Refix the embryos in 1 ml PBT, 4% Formaldehyde for 20 min.
8. Wash 5 × 5 min in PBT.

III. Hybridization

The hybridization procedure is similar for both DNA and RNA probes, both DIG labeled. Only the incubation temperatures are higher for RNA probes and the Hybridization buffer is more acidic, since this stabilizes the embryos during the high temperature incubation steps.

DNA probes should not be used under such acidic conditions, since they would become depurinated.

1. Wash the embryos in Hybridization solution [Hybridization solution for DNA probes:
   750 mM NaCl; 75 mM Na-citrate (= 5× SSC) pH 7.0; 50% (v/v) formamide; 0.1% (v/v) Tween 20; 50 µg/ml heparin; 50 µg/ml sonicated Salmon Sperm DNA.
   Hybridization solution for RNA probes: same as for DNA probes, but pH 5.0] diluted 1:1 with PBT for 10 min.

2. Wash 10 min in Hybridization solution.
   This stepwise transfer into the Hybridization solution is not strictly required, but embryos which are slightly overdigested with Proteinase K would burst, if they were brought directly into the formamide containing solution. The same considerations apply for the washing after the hybridization (see below).

3. Prehybridization
   - Prehybridize in Hybridization solution for 20–60 min in a waterbath at 45°C (55–65°C for RNA probes).
   - Remove most of the liquid, leaving about 2 mm of solution above the surface of the settled embryos.
     This corresponds usually to a hybridization volume of about 100 ml.

4. Probe preparation
   - Add 2 µl of the probe to 5 µl of a solution of 2 mg/ml sonicated salmon sperm DNA (this has to be scaled up appropriately if more then one hybridization is carried out).
   - Denature at 100°C for 3 min, cool shortly on ice and add directly to the embryos in Hybridization solution.

5. Hybridization
   - Mix thoroughly and incubate at 45°C (55-65°C for RNA probes) overnight.
     Slight agitation may be advantageous to avoid clumping of the embryos, but is not strictly necessary.

IV. Washing and detection

The following washing protocol is very extensive and is necessary if high background is encountered. However, fewer and shorter steps may be sufficient for many applications.

1. Wash steps
   - Wash 2 × 30 min in 500 µl Hybridization solution at the hybridization temperature.
   - Proceed with washes in serial dilutions (4:1, 3:2, 2:3, 1:4) of Hybridization solution in PBT for l0 min each at room temperature.
   - Wash 2 × 10 min in PBT.
2. ***Optional:*** Block embryos with 1% serum of the species the antibody is derived.
3. Preparation of anti-DIG antibody conjugate

   The anti-DIG antibody conjugate should be freshly preabsorbed for 1 h against fixed embryos in order to remove any unspecifically binding material. The final working dilution of the antibody conjugate is 1:2000 in PBT. The preabsorption step should be adjusted accordingly.

   Example: *If ten reactions are processed in parallel, use about 200 µl embryos in 1 ml PBT with an antibody conjugate dilution of 1:200. This solution is then further diluted 1:10 in the next step.*

   The diluted antibody solution may be reused for two or more stainings within a few days.
4. Antibody incubation

   Incubate the embryos for l h in 500 µl diluted and preabsorbed anti-DIG antibody complex.
5. Washing steps
   - Wash 3 × 20 min in PBT.
   - Wash 3 × 5 min in Staining buffer [100 mM NaCl; 50 mM $MgC1_2$; l00mM Tris-HCl, pH 9.5].
6. Staining of embryos

   Transfer embryos in a small dish with 1 ml Staining buffer containing 4.5 µl NBT solution and 3.5 µl BCIP solution.

   Let the color develop in the dark with occasional inspection under the binocular microscope. Color develops usually within 1 h, but the reaction may also be left overnight.
7. Stop the staining reaction by washing in PBT.
8. Transfer embryos into 70% glycerol, equilibrate for several hours and transfer them onto a microscope slide for inspection and photography.

   For permanent mounting dehydrate the embryos in an alcohol series (70%, 90%, and 100%) and mount in Euparal.

V. Results

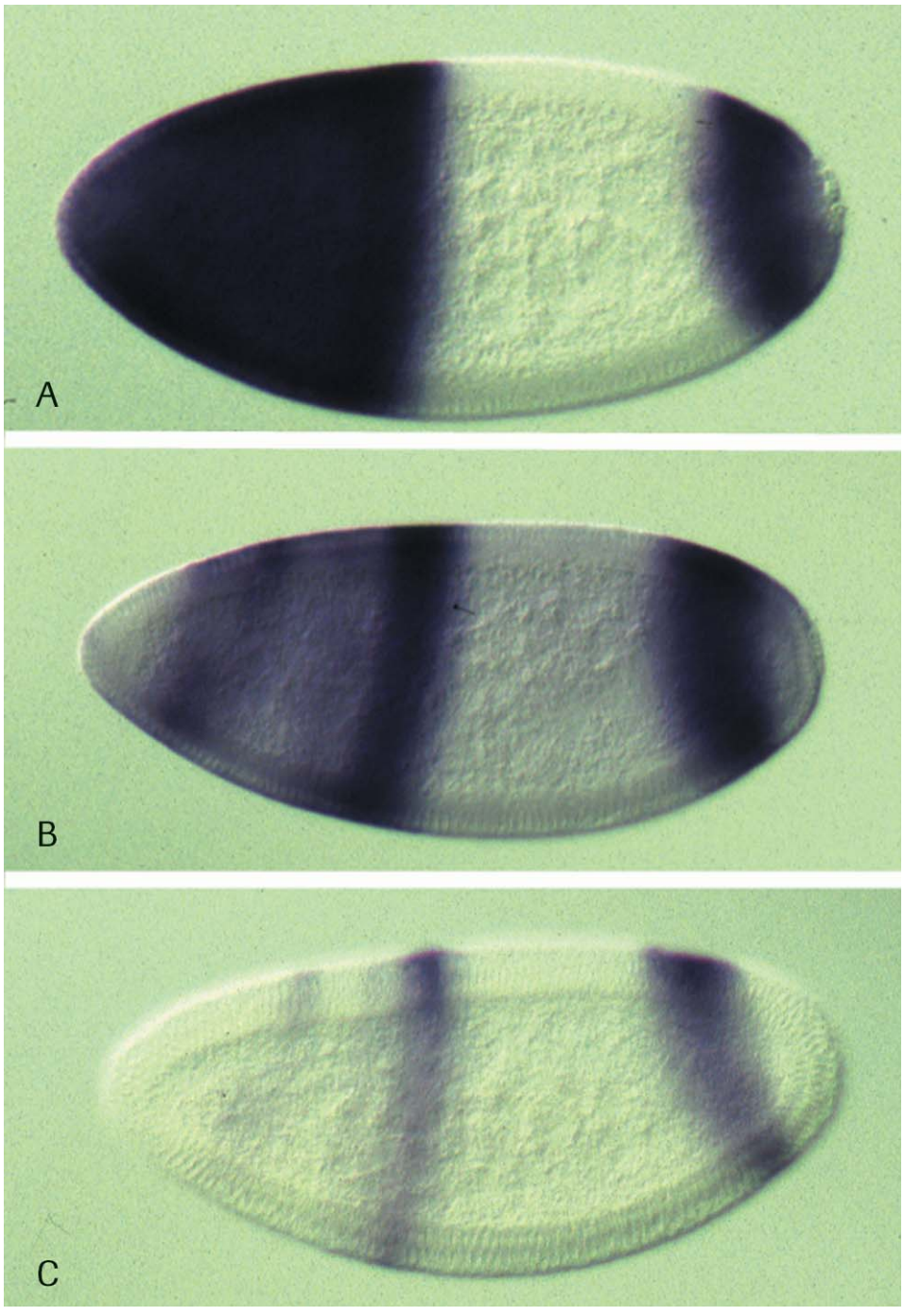


Figure 1: Expression of the segmentation gene hunchback in *Drosophila* in different stages of development. Whole mount *in situ* hybridization is particularly useful to follow dynamically developing spatially expressed patterns. The stages shown here are approximately 20 min part from each other in developmental time. For further information on hunchback expression see Tautz, Nature 332, 281.

VI. Troubleshooting

A. *If* a poor signal is observed, then:

1. Check whether the probe is correctly labeled (see Sect. 3.1).
2. Check Proteinase K digestion. Depending on the tissue and the Proteinase activity, it may be necessary to perform rather extensive Proteinase K digestions. Set up a series of digestion conditions and test these.
3. Check under the binocular microscope whether the vitellin membranes are fully removed after the methanol step.
4. Check for RNAse contamination of the solutions. Treat the solutions with diethylpyrocarbonate (Sigma) before use, in particular the PBT solution before addition of Tween 20.

B. *If* there is too much background, then:

1. Prolong the washing step, because of insufficient washing each step may be done for longer times.
2. Perform longer preabsorption of the anti-digoxigenin complex.
3. Include levamisole in the staining solution. Levamisole acts as a potent inhibitor for endogeneous lysosomal phosphatases. These are, however, usually not a problem in early *Drosophila* embryos.
4. Increase detergent concentration in the PBT. Tween 20 may also be replaced by SDS.
5. Include a xylene treatment step after the fixation.

5

References

Hauptman G, Gerster T (1996) Multicolour whole-mount *in situ* hybridization to *Drosophila* embryos. *Dev Gen Evol* **206**:292-295

Hughes SC, Saulier-Le Drean B, Livne-Bar I, Krause HM (1996) Fluorescence *in situ* hybridization in whole-mount *Drosophila* embryos. *BioTechniques* **20**:748-750

Lehmann R, Tautz D (1994) *in situ* hybridization to RNA. *Methods in Cell Biology* **44**: 575-598

Tautz D, Pfeifle C (1989) A nonradioactive *in situ* hybridization method for the localization of specific RNAs in *Drosophila* embryos reveals translational control of the segmentation gene *hunchback*. *Chromosoma* **98**:81-85

Wieschaus E, Nüsslein-Vollhard C (1986) Looking at embryos. In: Roberts DB (ed). *Drosophila* – A Practical Approach. IRL Press, Oxford, pp 199-228

Reagents available from Roche Applied Science for this procedure

Reagent	Description	Cat. No.	Pack size
Triton X-100	Vicous, liquid	10 789 704 001	100 ml
Hepes	Purity: 98% (from N)	10 737 151 001 10 242 608 001	500 g 1 kg
DIG RNA Labeling Kit*	2× 10 labeling reaction	11 175 025 910	1 kit
DIG RNA Labeling Mix*	10× solution (20 reaction)	11 277 073 910	40 µl
DIG DNA Labeling and Detection Kit*	25 labeling reaction and 50 blots	11 093 657 910	1 kit
DIG-High Prime*,‡	Premixed solution for 40 random-primed DNA labeling reactions with DIG-11-dUTP	11 585 606 910	160 µl (40 labeling reactions)
Anti-Digoxigenin-AP*	750 units/ml Anti-Digoxigenin, Fab fragments conjugated to alkaline phosphatase	11 093 274 910	150 U (200 µl)
NBT solution	100 mg/ml nitroblue tetrazolium salt in 70% (v/v) dimethylformamide	11 383 213 001	3 ml (300 mg) (dilute priorto use)
BCIP solution	50 mg/ml 5-bromo-4-chloro-3-indolyl phosphate (BCIP), toluidinium salt in 100% dimethylfo rmanide	11 383 221 001	3 ml (150 mg)
NBT/BCIP solution	Stock Solution	11 681 451 001	8 ml
Proteinase K, rec., PCR Grade	Lyophilizate	03 115 836 001 03 115 879 001 03 115 801 001 03 115 852 001	25 mg 100 mg 2 × 250 mg 4 × 250 mg
Tween 20		11 332 465 001	5 × 10 ml

* The labeling of nucleic acids with DIG is covered by EP patents 0 324 474 and 0 371 262 as well as the following US patents 5.344.757, 5.354.657 and 5.702.888 owned by Roche Diagnostics GmbH.

‡ This product or the use of this product may be covered by one or more patents owned by Roche Diagnostics GmbH, including the following: US patent 5.814.502.

Detection of even-skipped transcripts in *Drosophila* embryos with PCR/DIG-labeled DNA probes

Dr. N. Patel and Dr. C. Goodman, Carnegie Institute of Washington, Embryology Department, Baltimore, Maryland, USA.

This protocol has been used to detect the transcript distribution of a number of genes by *in situ* hybridization, including even-skipped and seven-up, in whole mount *Drosophila* embryos, and engrailed Antennapedia in whole mount grasshopper embryos. The *in situ* hybridization and detection was performed essentially according to the protocol of Tautz and Pfeiffle (1989).

This laboratory uses PCR rather than random primed labeling to prepare DIG-labeled probes because:

- A much larger quantity of probe can be made with the same amount of starting nucleotide.
- The ratio of labeled DNA to unlabeled starting material is much higher (especially important when transcripts to be detected are not very abundant).
- PCR can produce strand-specific copies.

Disadvantage:

- It is more difficult to control the probe size.

This laboratory has also found that biotin-16-dUTP incorporated in the same way and detected with streptavidin-alkaline phosphatase is about three- to fivefold less sensitive than DIG-labeled probes in *in situ* hybridization experiments.

5

I. Probe labeling

1. Prepare the following stock solutions:
   - 10× concentrated reaction mix: 500 mM KCl; 100 mM Tris-HCl, pH 8.3; 15 mM $MgCl_2$; 0.01% (w/v) gelatin.
   - 5× concentrated dNTP mix: 1 mM dATP, 1 mM dCTP, 1 mM dGTP, 0.65 mM dTTP, 0.35 mM digoxigenin-11-dUTP.
   - A primer stock solution containing
     either
     30 ng/µl (approx. 5.3 mmol) primer 1 (*e.g.* generated by SP6 RNA polymerase)
     or
     30 ng/µl (approx. 5.3 mmol) primer 2 (*e.g.* generated by T7 RNA polymerase).
2. With a restriction enzyme, linearize "dual promotor vector DNA" containing the insert, as one would to make run-off RNA transcripts.

   ! *The two different primers can be used to create an antisense strand and a sense strand (as control).*
3. Heat inactivates the restriction enzyme.
4. Dilute the linearized DNA with water to a final concentration of about 100–200 ng/µl.

   ! *If the insert is much over 3 kb, the probe produced will probably not represent the entire insert.*

▶▶▶

5

5 Set up the following reaction mix:
- 9.25 µl water.
- 2.5 µl 10× concentrated reaction mixture.
- 5.0 µl 5× concentrated dNTP mix.
- 5.0 µl primer 1 or 2 (from 30 ng/µl stock).
- 2.0 µl linearized DNA (100–200 ng/µl).

6 Add 40 µl mineral oil, centrifuge, then boil the mix for 5 min.

7 To the mix, add 1.25 µl of 1 unit/µl Taq DNA Polymerase (1.25 units of Taq Polymerase). [Final volume of reaction mix with Taq is 25 ml.]

8 Mix the contents of the reaction tube and then centrifuge for 2 min.

9 Incubate for 30 cycles in the PCR thermal cycler under the following conditions:
- Denaturation at 95°C for 45 s.
- Annealing at 50°–55°C for 30 s.

  Annealing temperature depends on length of primer. Use 55°C for a 21-mer.

- Elongation at 72°C for 1.0–1.5 min.

  Elongation time depends on length of insert. Use 1 min for 1.0 kb or less, 1.5 min for 2.5 kb or more.

10 After the PCR run, add 75 µl distilled H_2O to the reaction tube, then centrifuge it.

11 Remove 90–95 µl of the reaction mix from beneath the oil.

12 To recover the DNA, do an ethanol precipitation as follows:
- Add NaCl to a final concentration of 0.1 M.
- Add 10 µg of glycogen or tRNA as a carrier (0.5 µl of a 20 mg/ml stock).
- Add 3 volumes of 100% EtOH.
- Mix well and leave at -70°C for 30 min.
- Centrifuge.
- Wash pellet with 70% ethanol.
- Dry under vacuum.

Optional: Perform a second ethanol precipitation as above.

13 Resuspend DNA pellet in 300 µl of hybridization buffer [50% formamide; 5× SSC *(where 1× SSC contains 150 mM NaCl, 15 mM sodium citrate)*; 50 µg/ml heparin, 0.1% Tween 20; and 100 µg/ml sonicated and denatured salmon sperm DNA] (according to Tautz and Pfeifle, 1989).

14 To reduce the size of the single-stranded DNA, boil the probe for 40–60 min.

For efficient penetration of and hybridization to the embryos, the average probe length should be about 50–200 bp.

15 Dilute the probe as much as tenfold before use.

Optimal dilution varies depending on the abundance of the transcript and background staining. We recommend using the probe either undiluted (original 300 µl) or diluted up to threefold for the initial experiment.

II. Evaluation of labeling reaction

1. Prepare an aliquot of the probe as follows:
   - Remove 1 µl of probe from the reaction mix.
   - Add 5 µl of 5× SSC.
   - Boil 5 min.
   - Quick cool on ice.
   - Centrifuge.
2. Spot 1–2 µl of the probe aliquot onto a small nitrocellulose strip cut to fit into a 1.5 ml microcentrifuge tube or a 5 ml snap cap tube.
3. Bake the filter between two sheets of filter paper in an 80°C vacuum oven for 30 min.

   ⚠ *The residual formamide may cause the nitrocellulose to warp. If this is a problem, reduce the time in the baking oven or do this spot test before the second precipitation (Procedure I, Step 12).*

   ! *Unincorporated nucleotide binds only slightly to the nitrocellulose.*
4. Treat the baked filter as follows:
   - Wet the filter with 2× SSC.
   - Wash filter 2 × 5 min in PBT (1× PBS, 0.2% BSA, 0.1% Triton X-100).
   - Place filter into a 1.5 ml microcentrifuge tube or 5 ml snap cap tube.
5. Detect the labeled probe as follows:
   - Block the filter by incubating it 30 min in PBT.
   - Incubate in PBT for 30–60 min with a 1:2000 dilution (in PBT) of alkaline phosphatase-conjugated anti-DIG antibody.
   - Wash 4 × 15 min in PBT.
   - Wash 2 × 5 min in a solution containing 100 mM NaCl; 50 mM $MgCl_2$; 100 mM Tris, pH 9.5; 0.1% Tween 20.

   ! *Levamisole is not needed.*
6. Develop color with NBT and BCIP as described in the appropriate Roche Applied Science pack insert.
7. Stop the reaction when the spots are visible.

   ! *Spots should be visible within a few minutes and dark after 10–15 min.*

■

5

III. Preparation of embryos, hybridization and detection

Perform the preparation of the embryos, subsequent hybridization of the DIG-PCR probe, and immunological detection as described in the Tautz article, "Localization of the expression of the segmentation gene hunchback in *Drosophila* embryos with digoxigenin-labeled DNA probes," page 158??? in this manual.

Results

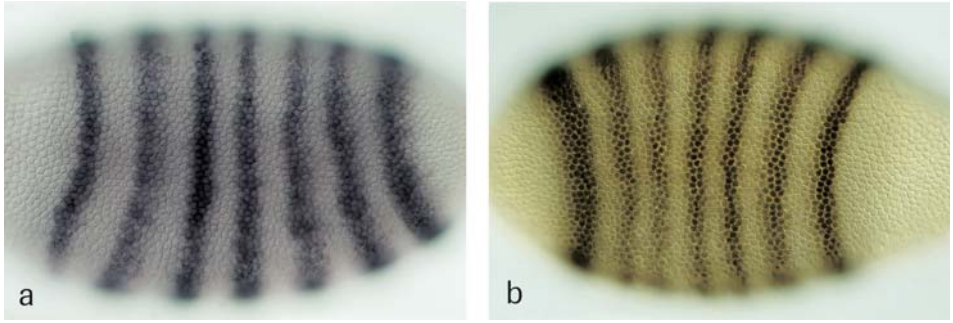


Figure 1: Detection of the even-skipped phenotype on blastoderm stage *Drosophila* embryos. Panel a shows an *in situ* hybridization using the ***Drosophila*** even-skipped gene as probe. Panel b demonstrates the even-skipped product using the specific antibody as probe. The seven stripe pattern is associated with the even-skipped phenotype.

Reference

Tautz, D.; Pfeiffle, C. (1989) A nonradioactive *in situ* hybridization method for the localization of specific RNAs in *Drosophila* embryos reveals a translational control of the segmentation gene hunchback. *Chromosoma (Berl.)* **98**, 81–85.

5

Reagents available from Roche Applied Science for this procedure

Reagent	Description	Cat. No.	Pack size
PCR DIG Probe Synthesis Kit*	For generating highly sensitive probes labeled with DIG-dUTP (alkali-labile) in the polymerase chain reaction (PCR) using a ratio of 1 + 2 (DIG-dUTP:dTTP)	11 636 090 910	1 Kit (25 reactions)
Tween 20		11 332 465 001	5 × 10 ml
Triton X-100	Vicous, liquid	10 789 704 001	100 ml
Anti-Digoxigenin-AP*	750 units/ml Anti-Digoxigenin, Fab fragments conjugated to alkaline phosphatase	11 093 274 910	150 U (200 µl)
NBT solution	100 mg/ml nitroblue tetrazolium salt in 70% (v/v) dimethylformamide	11 383 213 001	3 ml (300 mg) (dilute prior to use)
BCIP solution	50 mg/ml 5-bromo-4-chloro-3-indolyl phosphate (BCIP), toluidinium salt in 100% dimethylformamide	11 383 221 001	3 ml (150 mg)

* The labeling of nucleic acids with DIG is covered by EP patents 0 324 474 and 0 371 262 as well as the following US patents 5.344.757, 5.354.657 and 5.702.888 owned by Roche Diagnostics GmbH.

Whole mount fluorescence *in situ* hybridization (FISH) of repetitive DNA sequences on interphase nuclei of the small cruciferous plant *Arabidopsis thaliana*

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[1] Laboratory for Genetics, Gent University, Gent, Belgium
[2] Laboratory for Biochemistry and Molecular Cytology, Gent University, Gent, Belgium

Hybridizing fluorescently labeled DNA probes *in situ* to the chromatin of interphase nuclei in whole mounts allows the study of nuclear architecture in morphologically well preserved specimens. Fluorescent labeling of the DNA probes (either directly or indirectly) allows the simultaneous, but differential, detection of several sequences (*e.g.* Lengauer et al., 1993; Nederlof et al., 1990). Also, fluorescent signals can be imaged by confocal microscopy so that stacks of optical section images can be recorded through the whole mounts. Multiple labeling FISH on whole mounts therefore allows the study of the relative positions of different chromosomes or chromosome segments in individual interphase nuclei as well as between nuclei of related cells.

In the experiments presented here, two tandemly repeated sequences, rDNA and a 500 bp repeat sequence, were hybridized to interphase nuclei of seedlings and flowers (inflorescences) of the small cruciferous plant *Arabidopsis thaliana*. The procedure used in the experiments, based on the protocols published by Ludevid et al. (1992) and Tautz and Pfeifle (1989), was described earlier by Bauwens et al. (1994).

I. Seed sterilization and germination

Procedure I is based on the protocol of Valvekens et al. (1988).

1. Surface sterilize seeds of *Arabidopsis thaliana* (C24) by immersing them as follows:
   - 2 min in 70% (v/v) ethanol.
   - 15 min in a solution of 5% (v/v) NaOCl and 0.05% (v/v) Tween 20.
2. Wash seeds 5 times in sterile, distilled water.
3. Pipette onto germination medium (1× Murashige and Skoog salt mixture (Flow Laboratories, USA); 0.5 g/L 2-(N-morpholino)ethane sulphonic acid (MES), pH 5.7 (adjusted with 1 M KOH); 0.8% (w/v) Bacto-agar (Difco Laboratories, USA).
4. Allow the seeds to germinate at room conditions for 4 days.
5. Transfer part of the seedlings to soil.
6. Grow plants under continuous light conditions at desk temperature.
7. Harvest flowers and inflorescences after 3–4 weeks.

II. Tissue fixation

1. Place approximately 30–40 seedlings or a few flowers or inflorescences in a glass vial containing:
   4.365 ml fixation buffer [1.1× PBS; 0.067 M EGTA, pH 7.5 (adjusted with NaOH)].
   10× PBS contains 1.3 M NaCl, 0.0027 M KCl, 0.07 M Na_2HPO_4, 0.03 M NaH_2PO_4; pH 7.2.
   - 0.135 ml 37% formaldehyde (Sigma, USA).
   - 0.5 ml DMSO.
   Final concentrations in vial are 1% formaldehyde and 10% DMSO.
2. Rock the glass vial for 25 min at room temperature.
3. Remove the fixative and rinse as follows:
   - 2× with 5 ml methanol.
   - 4× with 5 ml ethanol.
4. Discard the last ethanol wash, then cover sample with a final 5 ml ethanol.
5. Leave sample at -20°C for 2–4 days.
   Keeping the material for longer periods of time in ethanol makes it brittle.

III. Labeling of the probe DNA

1. Use the following as hybridization probes:
   - A mixture of three ribosomal DNA (rDNA) inserts, each cloned into pBS (I)KS$^+$ (Stratagene, USA) from *A. thaliana*. The inserts contain the 5.8S, 18S and 25S rRNA genes, as well as the intergenic region (IGR) (Unfried and Gruendler, 1990; Unfried et al., 1989).
   - A 500 bp repeat DNA sequence, cloned into pGem-2 (Promega, USA).
     The repeat is one of three classes of highly repetitive, tandemly arranged DNA sequences in *A. thaliana* (Simoens et al., 1988).
2. Label undigested samples of both probes according to the nick translation procedures in Chapter IV of this manual. Use the following labels:
   - Label the rDNA with either DIG-dUTP or fluorescein-dUTP.
     The concentration of substituted nucleotide in the nick translation labeling mixture should be the same for either fluorescein-dUTP or DIG-dUTP.
   - Label the 500 bp repeat DNA with DIG-dUTP.
3. After nick translation, treat each labeled probe as follows:
   - Co-precipitate 1 µg of labeled DNA with 55 µg of sonicated salmon sperm DNA (Sigma, USA).
   - Redissolve probe in 25 µl H_2O to a concentration of 40 ng labeled DNA per µl.

IV. Pretreatment

1. Remove ethanol from seedlings or flowers (or inflorescences) and transfer material to microcentrifuge tubes.

2. Fix each sample as follows:
   - Rinse 2× with 1 ml ethanol.
   - Replace ethanol with 1 ml ethanol/xylene (1:1) and incubate for 30 min.
   - Rinse 2× with 1 ml ethanol.
   - Rinse 2× with 1 ml methanol.
   - Replace methanol with 1 ml of a 1:1 mixture of methanol and [PBT containing 1% (v/v) formaldehyde]. Rock for 5 min.

     PBT contains 1× PBS and 0.1% (v/v) Tween 20.

3. Post-fix sample for 25 min in 1 ml PBT containing 1% formaldehyde.

4. Remove fixative and rinse sample with 5 × 1 ml PBT.

5. Wash sample 3 × 1 ml of 2× SSC (each wash, 5 min).

   1× SSC contains 150 mM NaCl and 15 mM sodium citrate, pH 7.0.

6. Digest with RNase A (100 µg/ml in 2× SSC) for 1 h at 37°C.

7. Wash 3 × 5 min with 1 ml PBT.

8. Digest with Proteinase K (40 µg/ml in PBT) for 8 min at 37°C.

9. After the Proteinase K digestion, do the following:
   - Rinse 2× with 1 ml PBT.
   - Wash 2× 2 min with 1 ml PBT.
   - Rinse 2× with 1 ml PBT.

10. Postfix a second time for 25 min with 1 ml PBT containing 1% formaldehyde.

11. Remove fixative and rinse 5× with 1 ml PBT.

V. *In situ* hybridization

1. Wash each sample 10 min with 1 ml of a 1:1 mixture of PBT and hybridization solution [hybridization solution contains 50% formamide (Ultra Pure from USB, USA) in 2× SSC].

2. Rinse sample with 2 × 1 ml hybridization solution.

3. Remove the hybridization solution and add the following to each sample (to produce a final volume of 500 µl of hybridization solution):
   - 250 µl formamide.
   - 50 µl 20× SSC.
   - Enough H_2O to make a total volume, including probes, of 500 µl.
   - 25 µl of each labeled rDNA probe (for single or double labeling experiments).
   - 25 µl labeled 500 bp repeat probe (for double labeling experiments only).

   Each labeled probe has a final concentration of 2 ng/µl.

   This incubation mixture can be used for either a single label hybridization (fluorescein-labeled probe) with direct detection; a single label hybridization (DIG-labeled probe) with indirect detection; or a double label hybridization (both fluorescein- and digoxigenin-labeled probes). See Procedure VIII below for details on these different types of experiments.

4. Treat the sample as follows:
   - Denature target and probe in hybridization solution for 4 min at 100°C.
   - Place immediately on ice for 3 min.
   - Centrifuge very briefly.
   - Incubate overnight at 37°C to hybridize probe and target.

   If using a directly labeled (i.e., fluorescent) probe, perform the hybridization incubation and the rest of the procedure in the dark.

VI. Pre-absorption of antibodies (for indirect detection only)

1. Prepare powdered *A. thaliana* root or seedling extract as follows:
   - Grind the root or seedling under liquid nitrogen.
   - Extract the ground powder with acetone under liquid nitrogen.
   - Decant the acetone supernatant.
   - Let the residual acetone evaporate from the precipitate.
   - Use the dry, powdered precipitate in the pre-absorption procedure below.

2. Dilute each detection antibody, in 4× SSC containing 1% (w/v) BSA, to the working dilution suggested by the manufacturers and a final volume of 500 µl.

3. Add approximately 2 mg of powdered *A. thaliana* root or seedling extract (from Step 1 above) to the diluted antibody.

4. Pre-absorb the antibodies overnight at 15°C, in the dark.

5. Centrifuge the pre-absorption mixture.

6. Use the supernatant in the immunocytochemical detection reaction.

VII. Posthybridization washes

1. After overnight hybridization at 37°C (Procedure V, Step 4), treat the hybridization sample as follows:
   - Remove the hybridization solution.
   - Wash the sample for 1 h in fresh hybridization solution at 37°C.
   - Wash the sample 4 × 30 min in hybridization solution at 37°C.
   - *If* performing an *in situ* hybridization experiment with directly labeled probe DNA (rDNA) in a single labeling experiment, proceed to procedure VIII a.
   - *If* performing an *in situ* hybridization experiment requiring indirect detection with antibodies, proceed to step 2.
2. Bring the seedlings or flowers (or inflorescences) gradually to 4× SSC through the following washes (all at room temperature):
   - 20 min with a 3:1 mix of hybridization solution and 4× SSC.
   - 20 min with a 1:1 mix of hybridization solution and 4× SSC.
   - 20 min with a 1:3 mix of hybridization solution and 4× SSC.
   - 4 × 5 min with 4× SSC.

VIII. Immunocytochemical detection

Use the immunocytochemical detection schemes detailed in Table 1 to analyze the single and double labeling *in situ* hybridization experiments.

Type of Experiment	Probe	Label	Antibody[a]		
			1st (detecting)	2nd (amplifying)	Procedure to Follow
Single label, direct detection	rDNA	Fluorescein-dUTP	–	–	VIIIa
Single label, indirect detection	rDNA	DIG-dUTP	Fluorescein-conjugated anti-DIG antibody (from sheep)[b]	Fluorescein-conjugated anti-sheep antibody (from donkey)[c]	VIIIb
Double label	rDNA 500 bp	Fluorescein-dUTP DIG-dUTP	– Tetramethylrhodamine-conjugated anti-DIG antibody (from sheep)[d]	– Tetramethylrhodamine-conjugated anti-sheep antibody body (from rabbit)[e]	VIIIb

Table 1: Immunocytochemical detection schemes for single and double labeling experiments.
[a] All antibodies should be pre-absorbed, according to Procedure VI above.
[b] Fab fragments.
[c] Fab fragments, from Sigma, USA.
[d] Fab fragments.
[e] Whole molecule, from Chemicon, USA.

VIIIa. Direct detection

1. Bring the samples to 1× PBS gradually through the following washes (all at room temperature):
   - ▶ 20 min with a 3:1 mix of hybridization solution and 1× PBS.
   - ▶ 20 min with a 1:1 mix of hybridization solution and 1× PBS.
   - ▶ 20 min with a 1:3 mix of hybridization solution and 1× PBS.
   - ▶ 4 × 15 min in 1 ml 1× PBS.
2. Proceed to the staining and mounting procedure (Procedure IX).

■

VIIIb. Indirect detection

1. After the posthybridization washes (Procedure VII), remove the 4× SSC solution from the samples.
2. Add the first pre-absorbed antibody (from Table 1) to the sample and incubate overnight at 15°C in the dark.
3. Remove the first antibody and wash the material 4 × 15 min in 1 ml 4× SSC containing 0.05% (v/v) Tween 20.
4. Depending on whether you are using an amplifying (second antibody), do either of the following:
   - ▶ *If* you use an amplifying antibody, proceed to Step 5.
   - ▶ *If* you do not use an amplifying antibody, proceed to Step 7.
5. If amplifying the signal, wash the sample 4 × 15 min with 4× SSC containing 1% bovine serum albumin.
6. Remove the wash solution; add the second pre-absorbed antibody (from Table 1) to the sample, and incubate overnight at 15°C in the dark.
7. After the last (first or second) antibody incubation, remove the antibody and wash the sample 4 × 15 min in 1 ml 1× PBS.
8. Proceed to the staining and mounting procedure (Procedure IX).

■

IX. Staining and mounting

1. Counterstain the sample as follows:
   - For single labeling experiments with fluorescein-conjugated antibodies or nucleotides: Counterstain the chromatin of the interphase nuclei with 0.5 µg/ml propidium iodide (in 1× PBS) for 1 h in the dark.
   - For double labeling experiments with fluorescein- and tetramethylrhodamine-conjugated nucleotides and antibodies: Counterstain the chromatin of the interphase nuclei with 0.2 µg/ml DAPI (in 1× PBS) for 1 h in the dark.

     DAPI is 4,6'-diamidino-2-phenylindole.
2. Place a few seedlings or flowers (or part of an inflorescence) on a slide.
3. Apply some tape to the slides to create a support for the cover slip, so as not to crush the seedlings or flowers.
4. Apply a drop of antifade reagent (Vectashield, Vector Laboratories, USA) and cover with a cover slip.

X. Fluorescence microscopy

Xa. Conventional fluorescence microscopy

1. Visually inspect the slides on a DIAPHOT 300 inverted microscope (Nikon, Japan), fitted with *either* a 60×, NA 1.40 oil immersion lens (Olympus, Japan) *or* an NPL FLUOTAR, 40×, NA 1.30 oil immersion lens (Leitz, FRG).
2. Use the following filter combinations (Chroma Technology Corp., USA):
   - To localize propidium iodide-stained nuclei and tetramethylrhodamine-labeled hybrids: filter block 31014 404.
   - To localize DAPI-stained nuclei: filter block 31000 404.
   - To localize fluorescein-labeled hybrids: filter block 31001404.
3. As light source, use a mercury arc lamp (100 W).

Xb. Confocal fluorescence microscopy

1. Record images with the MRC-600 Confocal Scanning Laser Microscope (CSLM) System (Bio-Rad, USA). Attach the CSLM to the DIAPHOT 300 inverted microscope fitted with appropriate lenses (same microscope and lenses as described in Procedure Xa, Step 1 above).
2. Use the K1/K2 filter block combinations (Bio-Rad, USA) and either:
   - The 568 nm line from a Krypton-Argon laser (Ion Laser Technology, Utah, USA), to image the propidium iodide-stained interphase nuclei and the tetramethylrhodamine-labeled hybrids.
   - The 488 nm line from the same Krypton-Argon laser, to image the fluorescein-labeled hybrids.

Results and discussion

Figures 1 and 2 show some of the results obtained by FISH of rDNA and the 500 bp repeat on whole mounts of flowers and seedlings from *A. thaliana*.

The rDNA from *A. thaliana* covers about 5.7 Mbp per haploid genome (Meyerowitz and Pruitt, 1985), and is distributed over two large tandem repeats on chromosomes 2 and 4 (Maluszynska and Heslop-Harrison, 1991; Murata et al. 1990). Diploid interphase nuclei from *A. thaliana* exhibit, however, a number of rDNA-loci ranging from two to more than four (Bauwens et al. 1991) as can clearly be seen from the merged image in Figure 1.

The 500 bp repeat sequence covers about 0.3–0.6 Mbp per haploid genome (Bauwens et al., 1991; Simoens et al., 1988) and exhibits a chromosome-specific large cluster (Bauwens and Van Oostveldt, 1991; Bauwens et al., 1991), resulting in two distinct signals in diploid interphase nuclei as can be seen from the merged image in Figure 2.

Some helpful remarks should be made about confocal observation of fluorescent signals in whole mounts.

Sequential excitation with the 568 nm and the 488 nm lines of the Kr-Ar-laser and the K1/K2 filter combinations from the Bio-Rad MRC-600 CSLM, allowed a clear separation of the red (tetramethylrhodamine) and the green (fluorescein) signal. [See the signals from the 500 bp repeat (red) and the rDNA (green) probes in Figure 2.]. Especially, the yellow 568 nm line allows specific excitation of the red fluorescing dyes such as tetramethylrhodamine or, preferably, Texas Red with almost no excitation of fluorescein.

Confocal observation of the preparations appeared to be absolutely necessary to obtain clear images of the signals, especially in the very dense meristematic tissues of the seedling roottips and the developing flowers in the inflorescences. This was especially true for the weaker signal of the 500 bp repeat that, in many cases, could not be observed through the autofluorescence haze by conventional fluorescence microscopy. Acquiring digital images through confocal microscopy has the added advantage that images recorded at different wavelengths can be easily and accurately merged and compared.

Finally, the development of FISH protocols on whole mounts for localizing mRNA sequences, as already suggested by de Almeida Engler et al. (1994), will make it possible to follow the expression of different genes simultaneously at the cellular level, in three dimensions, through confocal observation.

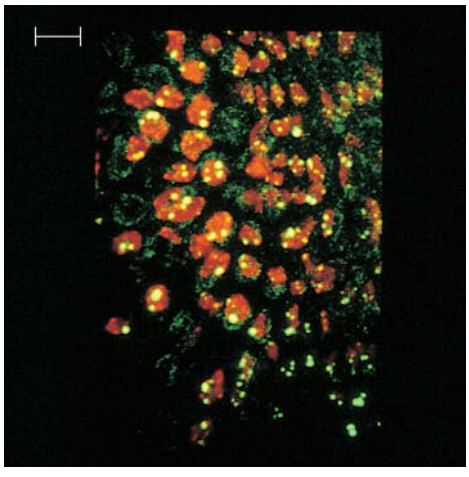

Figure 1: Whole mount FISH with rDNA on a developing flower from *A. thaliana*. The image is a merged image representing part of the pistil of a developing flower, showing fluorescein-labeled rDNA loci (yellow-green) and propidium iodide-stained interphase nuclei (red). An extended focus image of 15 optical sections through the pistil was created by maximum brightness projection before merging the 'green' (rDNA) and 'red' (nuclei) images. Bar 25 µm ≙ 17 mm.

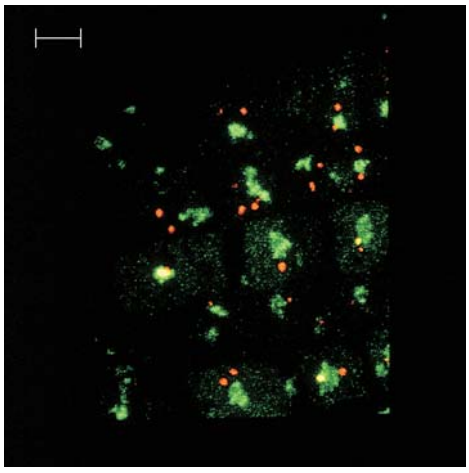

Figure 2: Whole mount double labeling FISH with rDNA and the 500 bp repeat on a seedling from *A. thaliana*. This merged image represents part of the meristematic zone of a seedling roottip. It shows fluorescein-labeled rDNA loci (green) and tetramethylrhodamine-labeled 500 bp repeat loci (red). An extended focus image of 20 optical sections through the meristematic zone of the roottip was created by maximum brightness projection before merging the 'green' (rDNA) and 'red' (500 bp repeat) images. Bar 25 µm ≙15 mm.

References

Bauwens, S.; Katsanis, K.; Van Montagu, M.; Van Oostveldt, P.; Engler, G. (1994) Procedure for whole mount fluorescence *in situ* hybridization of interphase nuclei on *Arabidopsis thaliana*. *Plant J.* **6**, 123–131.

Bauwens, S.; Van Oostveldt, P. (1991) Cytogenetic analysis on interphase nuclei with non-isotopic *in situ* hybridization and confocal microscopy. *Med. Fac. Landbouww. Rijksuniv. Gent.* **56/3a**, 753–758.

Bauwens, S.; Van Oostveldt, P.; Engler, G.; Van Montagu, M. (1991) Distribution of the rDNA and three classes of highly repetitive DNA in the chromatin of interphase nuclei of *Arabidopsis thaliana*. *Chromosoma* **101**, 41–48.

de Almeida Engler, J.; Van Montagu, M.; Engler, G. (1994) Hybridization *in situ* of whole-mount messenger RNA in plants. *Plant Mol. Biol. Reporter* **12**, 321–331.

Lengauer, C.; Speicher, M. R.; Popp, S.; Jauch, A.; Taniwaki, M.; Nagaraja, R.; Riethman, H. C.; Donis-Keller, H.; D'Urso, M.; Schlessinger, D.; Cremer, T. (1993) Chromosomal bar codes produced by multicolor fluorescence *in situ* hybridization with multiple YAC clones and whole chromosome painting probes. *Hum. Mol. Gen.* **2**, 505–512.

Ludevid, D.; Höfte, H.; Himelblau, E.; Chrispeels, M. J. (1992) The expression pattern of the tonoplast intrinsic protein g-TIP in *Arabidopsis thaliana* is correlated with cell enlargement. *Plant Physiol.* **100**, 1633–1639.

Maluszynska, J.; Heslop-Harrison, J. S. (1991) Localization of tandemly repeated DNA sequences in *Arabidopsis thaliana*. *Plant J.* **1**, 159–166.

Meyerowitz, E. M.; Pruitt, R. E. (1985) *Arabidopsis thaliana* and plant molecular genetics. *Science* **229**, 1214–1218.

Murata, M.; Varga, F.; Maluszynska, J.; Gruendler, P.; Schweitzer, D. (1990) Chromosomal localization of the ribosomal RNA genes in *Arabidopsis thaliana* by *in situ* hybridization. In: Schweitzer, D.; Peuker, K.; Loidl, J. (Eds) Fourth International Conference on *Arabidopsis* Research, Vienna, Abstracts, 5.

Nederlof, P. M.; van der Ploeg, S.; Wiegant, J.; Raap, A. K.; Tanke, H. J.; Ploem, J. S.; van der Ploeg, M. (1990) Multiple fluorescence *in situ* hybridization. *Cytometry* **11**, 126–131.

Simoens, C. R.; Gielen, J.; Van Montagu, M.; Inzé, D. (1988) Characterization of highly repetitive sequences of *Arabidopsis thaliana*. *Nucleic Acids Res.* **16**, 6753–6766.

Tautz, D.; Pfeifle, C. (1989) A nonradioactive *in situ* hybridization method for the localization of specific RNAs in *Drosophila* embryos reveals translational control of the segmentation gene hunchback. *Chromosoma* **98**, 81–85.

Unfried, I.; Gruendler, P. (1990) Nucleotide sequence of the 5.8S and 25S rRNA genes and of the internal transcribed spacers from *Arabidopsis thaliana*. *Nucleic Acids Res.* **18**, 4011.

Unfried, I.; Stocker, U.; Gruendler, P. (1989) Nucleotide sequence of the 18S rRNA gene from *Arabidopsis thaliana* Co 10. *Nucleic Acids Res* **7**, 7513.

Valvekens, D.; Van Lijsebettens, M.; Van Montagu, M. (1988) Agrobacterium *tumefaciens-mediated* transformation of *Arabidopsis thaliana* root explants by using kanamycin selection. P*roc. Natl. Acad. Sci. USA* **85**, 5536–5540.

Reagents available from Roche Applied Science for this procedure

Reagent	Description	Cat. No.	Pack size
DIG-Nick Translation Mix*	For generation of highly sensitive probes for *in situ* hybridization with digoxigenin-11-dUTP. Premixed solution for 40 labeling reactions	11 745 816 910	160 µl
Nick Translation Mix*	For generation of highly sensitive probes for fluorescence *in situ* hybridization. The Nick Translation Mix for *in situ* probes is designed for direct fluorophore-labeling of *in situ* probes.	11 745 808 910	200 µl
Fluorescein-12-dUTP	Tetralithium salt, solution, 1 mmol/l	11 373 242 910	25 nmol (25 µl)
dNTP Set	Set of dATP, dCTP, dGTP, dTTP, lithium salts, solutions	11 277 049 001	1 set, 4 × 10 µmol (100 µl)
Anti-Digoxigenin-Rhodamine*	Fab Fragments from sheep	11 207 750 910	200 µg
Anti-Digoxigenin-Fluorescein*	Fab Fragments from sheep	11 207 741 910	200 µg
Tween 20		11 332 465 001	5 × 10 ml
Proteinase K, rec., PCR Grade	Lyophilizate	03 115 836 001 03 115 879 001 03 115 801 001 03 115 852 001	25 mg 100 mg 2 × 250 mg 4 × 250 mg
RNase A	Dry powder	10 109 142 001 10 109 169 001	25 mg 100 mg

* The labeling of nucleic acids with DIG is covered by EP patents 0 324 474 and 0 371 262 as well as the following US patents 5.344.757, 5.354.657 and 5.702.888 owned by Roche Diagnostics GmbH.

6

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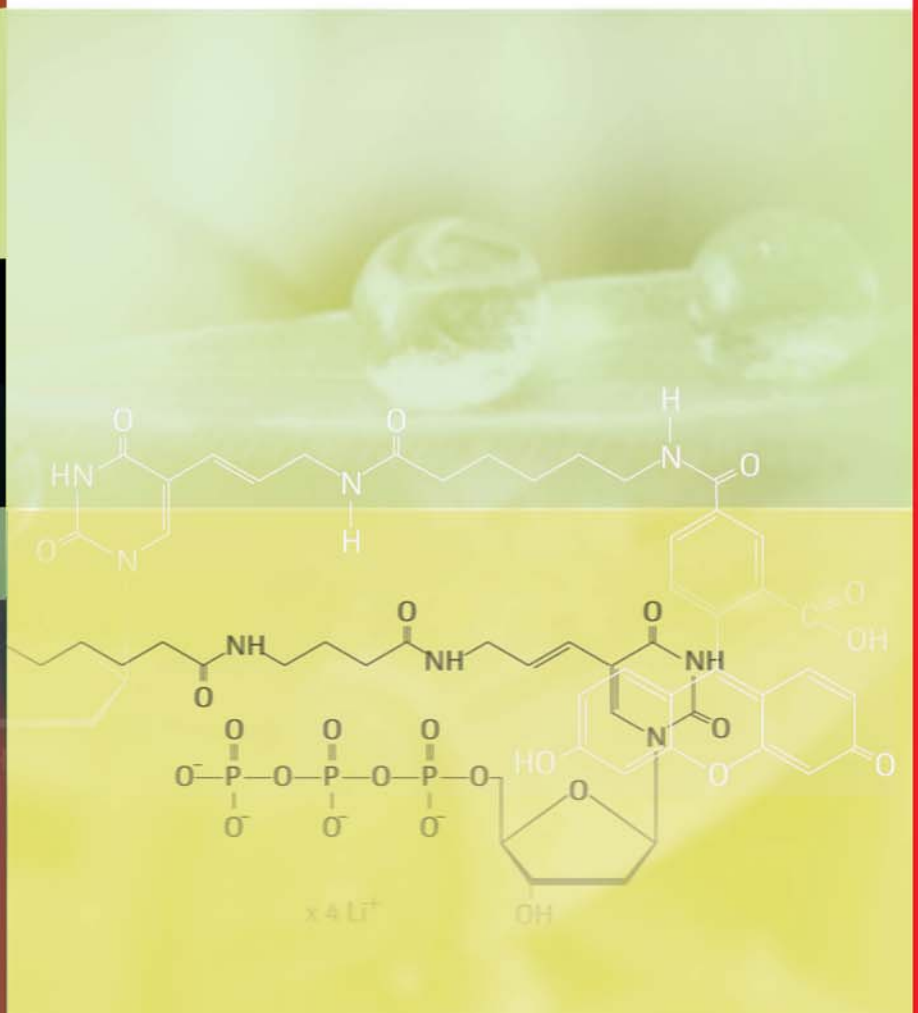

Product Selection Guide and Ordering Information

DNA Labeling Guide

Labeling Methods		DIG - Labeling Reagents	Other Labeling Reagents
By PCR ssDNA Template 3' 5' + Specific Primer + Label dUTP + Taq DNA Polymerase Labeled Probe 5' 3' / 3' 5'	Kits for Labeling	■ **PCR DIG Probe Synthesis Kit*** 11 636 090 910 - 25 reactions ■ **PCR ELISA, DIG-Labeling** 11 636 120 910 - 50 reactions	
	Mixes for Labeling with Enzyme	■ **PCR DIG Labeling Mix*** 11 585 550 910 - 2 × 250 µl ■ **PCR DIG Labeling Mix^{PLUS}*** 11 835 289 910 - 2 × 250 µl	■ **PCR Fluorescein Labeling Mix*** 11 636 154 910 - 100 µl
	Nucleotides for Labeling	■ **Digoxigenin-11-dUTP, alkali-stable‡** 11 093 088 910 - 25 nmol (25 µl) 11 558 706 910 - 125 nmol (125 µl) 11 570 013 910 - 5 × 125 nmol (5 × 125 µl) ■ **Digoxigenin-11-dUTP, alkali-labile*** 11 573 152 910 - 25 nmol (25 µl) 11 573 179 910 - 125 nmol (125 µl) ■ **Deoxynucleoside Triphosphate Set** 11 969 064 001 - 4 × 250 µl 03 622 614 001 - 4 × 1,250 µl	■ **Biotin-16-dUTP** 11 093 070 910 - 50 nmol (50 µl) ■ **Fluorescein-12-dUTP** 11 373 242 910 - 25 nmol (25 µl) ■ **Tetramethyl-Rhodamine-5-dUTP** 11 534 378 910 - 25 nmol (25 µl)
	Enzymes	■ **Expand High Fidelity^{PLUS} PCR System¥** 03 300 242 001 - 125 U 03 300 226 001 - 500 U (2 × 250 U) 03 300 234 001 - 2,500 U (10 × 250 U)	■ **Expand High Fidelity^{PLUS} PCR System¥** 03 300 242 001 125 U 03 300 226 001 500 U (2 × 250 U) 03 300 234 001 2,500 U (10 × 250 U)
By Nick Translation dsDNA Template 5' 3' / 3' 5' + DNase + Label dUTP + DNA Polymerase Labeled Probe 5' 3' / 3' 5'	Kits for Labeling		■ **Nick Translation Kit** 10 976 776 001 - 50 reactions
	Mixes for Labeling with Enzyme	■ **DIG-Nick Translation Mix*** 11 745 816 910 - 160 µl	■ **Nick Translation Mix*** 11 745 808 910 - 200 µl ■ **Biotin-Nick Translation Mix*** 11 745 824 910 - 160 µl
	Nucleotides for Labeling	■ **Digoxigenin-11-dUTP, alkali-stable‡** 11 093 088 910 - 25 nmol (25 µl) 11 558 706 910 - 125 nmol (125 µl) 11 570 013 910 - 5 × 125 nmol (5 × 125 µl) ■ **Digoxigenin-11-dUTP, alkali-labile‡** 11 573 152 910 - 25 nmol (25 µl) 11 573 179 910 - 125 nmol (125 µl) ■ **Deoxynucleoside Triphosphate Set** 11 969 064 001 - 4 × 250 µl 03 622 614 001 - 4 × 1,250 µl	■ **Biotin-16-dUTP** 11 093 070 910 - 50 nmol (50 µl) ■ **Fluorescein-12-dUTP** 11 373 242 910 - 25 nmol (25 µl) ■ **Tetramethyl-Rhodamine-5-dUTP** 11 534 378 910 - 25 nmol (25 µl)
	Enzymes	■ **DNA Polymerase I, endonuclease-free** 10 642 711 001 - 250 U 10 642 720 001 - 1,000 U ■ **DNase I recombinant, RNase-free** 04 716 728 001 - 10,000 U	■ **DNA Polymerase I, endonuclease-free** 10 642 711 001 - 250 U 10 642 720 001 - 1,000 U ■ **DNase I recombinant, RNase-free** 04 716 728 001 - 10,000 U

▶▶▶

Labeling Methods		DIG - Labeling Reagents	Other Labeling Reagents
By Random Priming ssDNA Template 3' 5' + Random Primer (Hexamer) 5' 3' + Label dUTP + Klenow Enzyme Labeled Probe 5' 3' / 3' 5'	**Kits for Labeling and Detection**	■ **DIG High Prime DNA Labeling and Detection Starter Kit I*** 11 745 832 910 - 12 labeling and 24 detection reactions ■ **DIG High Prime DNA Labeling and Detection Starter Kit II*** 11 585 614 910 - 12 labeling and 24 detection reactions ■ **DIG DNA Labeling and Detection Kit*** 11 093 657 910 - 25 labeling reactions and detection of 50 blots	
	Kits for Labeling	■ **DIG DNA Labeling Kit*** 11 175 033 910 - 40 reactions	■ **Random Primed DNA Labeling Kit** 11 004 760 001 - 50 reactions
	Mixes for Labeling with Enzyme	■ **DIG-High Prime*,◆** 11 585 606 910 - 160 µl ■ **DIG DNA Labeling Mix*** 11 277 065 910 - 50 µl	■ **High Prime** 11 585 592 001 - 200 µl ■ **Biotin-High Prime◆** 11 585 649 910 - 100 µl ■ **Fluorescein-High Prime◆** 11 585 622 910 - 100 µl
	Nucleotides for Labeling	■ **Digoxigenin-11-dUTP, alkali-stable‡** 11 093 088 910 - 25 nmol (25 µl) 11 558 706 910 - 125 nmol (125 µl) 11 570 013 910 - 5 × 125 nmol (5 × 125 µl) ■ **Digoxigenin-11-dUTP, alkali-labile*** 11 573 152 910 - 25 nmol (25 µl) 11 573 179 910 - 125 nmol (125 µl)	■ **Hexanucleotide Mix** 11 277 081 001 - 100 µl ■ **Biotin-16-dUTP** 11 093 070 910 - 50 nmol (50 µl) ■ **Fluorescein-12-dUTP** 11 373 242 910 - 25 nmol (25 µl) ■ **Tetramethyl-Rhodamine-5-dUTP** 11 534 378 910 - 25 nmol (25 µl)
	Enzymes	■ **Klenow Enzyme, labeling grade** 11 008 404 001 - 100 U 11 008 412 001 - 500 U	■ **Klenow Enzyme, labeling grade** 11 008 404 001 - 100 U 11 008 412 001 - 500 U
	Additional Products	■ **Primer "random"** 11 034 731 001 - 2 mg	■ **Primer "random"** 11 034 731 001 - 2 mg

RNA Labeling Guide

Labeling Methods		DIG - Labeling Reagents	Other Labeling Reagents
By *In Vitro* Transcription **Expression Plasmid** **Linearization** **Label + T3/T7/SP6 RNA Polymerase** **Labeled Probe**	**Kits for Labeling**	■ **DIG Northern Starter Kit*** 12 039 672 910 - 10 labeling reactions and detection of 10 blots ■ **DIG RNA Labeling Kit (SP6/T7)*** 11 175 025 910 - 2 × 10 reactions	■ **SP6/T7 Transcription Kit** 10 999 644 001 - 2 × 20 reactions
	Mixes for Labeling with Enzyme	■ **DIG RNA Labeling Mix*** 11 277 073 910 - 40 µl	■ **Biotin RNA Labeling Mix** 11 685 597 910 - 40 µl ■ **Fluorescein RNA Labeling Mix** 11 685 619 910 - 40 µl
	Nucleotides for Labeling	■ **Digoxigenin-11-UTP*,‡** 11 209 256 910 - 250 nmol (10 mM, 25 µl) 03 359 247 910 - 200 nmol (3.5 mM, 57 µl)	■ **Biotin-16-UTP** 11 388 908 910 - 250 nmol (25 µl) ■ **Fluorescein-12-UTP** 11 427 857 910 - 250 nmol (25 µl) ■ **Biotin-11-CTP** 04 739 205 001 - 250 nmol (25 µl)
	Enzymes	■ **SP6 RNA Polymerase** 10 810 274 001 - 1,000 U 11 487 671 001 - 5,000 U ■ **T3 RNA Polymerase** 11 031 163 001 - 1,000 U 11 031 171 001 - 5,000 U ■ **T7 RNA Polymerase** 10 881 767 001 - 1,000 U 10 881 775 001 - 5,000 U	■ **SP6 RNA Polymerase** 10 810 274 001 - 1,000 U 11 487 671 001 - 5,000 U ■ **T3 RNA Polymerase** 11 031 163 001 - 1,000 U 11 031 171 001 - 5,000 U ■ **T7 RNA Polymerase** 10 881 767 001 - 1,000 U 10 881 775 001 - 5,000 U
	Additional Products	■ **Protector RNase Inhibitor** 03 335 399 001 - 2,000 U 03 335 402 001 - 5 × 2,000 U	■ **Protector RNase Inhibitor** 03 335 399 001 - 2,000 U 03 335 402 001 - 5 × 2,000 U

Oligonucleotide Labeling Guide

Labeling Methods		DIG - Labeling Reagents	Other Labeling Reagents
By Tailing Oligonucleotide 5' 3' + Label dUTP + Terminal Transferase Labeled Probe 5' 3'	**Kits for Labeling**	■ **DIG Oligonucleotide 5'-End Labeling Set*** 11 480 863 001 - 10 reactions	
	Nucleotides for Labeling	■ **Digoxigenin-11-dUTP, alkali-stable‡** 11 093 088 910 - 25 nmol (25 µl) 11 558 706 910 - 125 nmol (125 µl) 11 570 013 910 - 5 × 125 nmol (5 × 125 µl) ■ **Digoxigenin-11-dUTP, alkali-labile*** 11 573 152 910 - 25 nmol (25 µl) 11 573 179 910 - 125 nmol (125 µl)	■ **Biotin-16-dUTP** 11 093 070 910 - 50 nmol (50 µl) ■ **Fluorescein-12-dUTP** 11 373 242 910 - 25 nmol (25 µl) ■ **Tetramethyl-Rhodamine-5-dUTP** 11 534 378 910 - 25 nmol (25 µl)
	Enzymes	■ **Terminal Transferase** 03 333 566 001 - 8,000 U 03 333 574 001 - 24,000 U	■ **Terminal Transferase** 03 333 566 001 - 8,000 U 03 333 574 001 - 24,000 U
By 3' -End Labeling Oligonucleotide 5' 3' Label + Terminal Transferase ddUTP Labeled Probe 5' 3'	**Kits for Labeling**	■ **DIG Oligonucleotide 3'-End Labeling Kit, 2nd generation*** 03 353 575 910 - 25 reactions ■ **DIG Gel Shift Kit, 2nd generation** 03 353 591 910 - 1 kit	
	Nucleotides for Labeling	■ **Digoxigenin-11-ddUTP** 11 363 905 910 - 25 nmol (25 µl)	■ **Biotin-16-ddUTP** 11 427 598 910 - 25 nmol (25 µl)
	Enzymes	■ **Terminal Transferase** 03 333 566 001 - 8,000 U 03 333 574 001 - 24,000 U	■ **Terminal Transferase** 03 333 566 001 - 8,000 U 03 333 574 001 - 24,000 U
By 5'-End Labeling Oligonucleotide 5' 3' Chemical Synthesis + 5'-NH2 Group X-NHS ester Labeled Probe 5' 3'	**Kits for Labeling**	■ **DIG Oligonucleotide 5'-End Labeling Set*** 11 480 863 001 - 10 reactions	
	Additional Products	■ **Digoxigenin-3-O-methylcarbonyl-ε-aminocaproic acid-N-hydroxy-succinimide ester‡** 11 333 054 001 - 5 mg ■ **Alkaline Phosphatase** 10 713 023 001 - 1,000 U (1 U/µl) 11 097 075 001 - 1,000 U (20 U/µl)	■ **Alkaline Phosphatase** 10 713 023 001 - 1,000 U (1 U/µl) 11 097 075 001 - 1,000 U (20 U/µl)

Workflow of Detection

Purification of Labeled Probes	
■ **High Pure PCR Product Purification Kit**	11 732 668 001 - up to 50 purifications
	11 732 676 001 - up to 250 purifications
■ **High Pure PCR Cleanup Micro Kit**	04 983 955 001 - up to 50 purifications
	04 983 912 001 - up to 200 purifications
Quick Spin Columns	
■ **mini Quick Spin DNA Columns**	11 814 419 001 - 50 columns
■ **mini Quick Spin RNA Columns**	11 814 427 001 - 50 columns
■ **mini Quick Spin Oligo Columns**	11 814 397 001 - 50 columns
Immobilization of Target (Filter Bound or *In Situ*)	
Agaroses	
■ **Agarose LE**	11 685 660 001 – 100 g
	11 685 678 001 – 500 g
■ **Agarose MP**	11 388 983 001 – 100 g
	11 388 991 001 – 500 g
■ **Agarose MS**	11 816 586 001 – 100 g
	11 816 594 001 – 500 g
Buffers in a Box	
■ **Buffers in a Box, Premixed PBS Buffer, 10x**	11 666 789 001 - 4 l
■ **Buffers in a Box, Premixed SSC Buffer, 20x**	11 666 681 001 - 4 l
■ **Buffers in a Box, Premixed TAE Buffer,**	11 666 690 001 - 4 l
■ **Buffers in a Box, Premixed TBE Buffer, 10x**	11 666 703 001 - 4 l
Nucleic Acids and Probes, DIG-labeled	
■ **DIG-labeled Control DNA***	11 585 738 910 - 50 µl
■ **DIG-labeled Control RNA***	11 585 746 910 - 50 µl
■ **Actin RNA Probe, DIG-labeled**	11 498 045 001 - 2 µg
DNA Molecular Weight Markers, DIG-labeled	
■ **DNA Molecular Weight Marker II, DIG-labeled***	11 218 590 910 - 5 µg (500 µl)
■ **DNA Molecular Weight Marker III, DIG-labeled***	11 218 603 910 - 5 µg (500 µl)
■ **DNA Molecular Weight Marker VI, DIG-labeled***	11 218 611 910 - 5 µg (500 µl)
■ **DNA Molecular Weight Marker VII, DIG-labeled***	11 669 940 910 - 5 µg (500 µl)
■ **DNA Molecular Weight Marker VIII, DIG-labeled***	11 449 451 910 - 5 µg (500 µl)
RNA Molecular Weight Markers, DIG-labeled	
■ **RNA Molecular Weight Marker I, DIG-labeled***	11 526 529 910 - 4 µg (200 µl)
■ **RNA Molecular Weight Marker II, DIG-labeled***	11 526 537 910 - 2 µg (200 µl)
■ **RNA Molecular Weight Marker III, DIG-labeled***	11 373 099 910 - 2 µg (200 µl)

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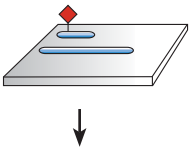
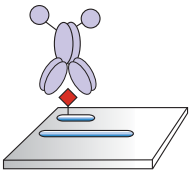


Hybridization of Labeled Probes	
■ **Blocking Reagent**	11 096 176 001 – 50 g
■ **DIG Easy Hyb**	11 603 558 001 – 500 ml
■ **DIG Wash and Block Buffer Set**	11 585 762 001 - 1 set (30 blots)
■ **Nylon Membranes, positively charged**	11 209 272 001 - 10 sheets (20 × 30 cm) 11 209 299 001 - 20 sheets (10 × 15 cm) 11 417 240 001 - 1 roll (0.3 × 3 m)
■ **Nylon Membranes for Colony and Plaque Hybridization**	11 699 075 001 - 50 discs (each 82 mm diameter) 11 699 083 001 - 50 discs (each 132 mm diameter)
■ **Lumi-Film Chemiluminescent Detection Film**	11 666 916 001 - 100 films (7.1 × 9.4 inches ≅ 18 × 24 cm)
■ **Hybridization Bags**	11 666 649 001 - 50 bags
■ **Buffers in a Box**	see page 6

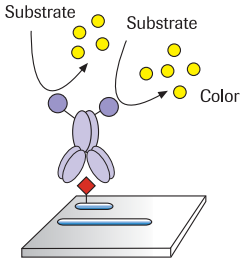


Detection – Binding of Antibody or Antibody Conjugate to Labeled Probe

Available Antibodies and their Recommended Use

Filter Hybridization	*In situ* Hybridization	Tube and Microplate Formats (ELISA)
With Conjugation		
Anti-Digoxigenin-AP, Fab fragments* 11 093 274 910 - 150 U (200 µl) **Anti-Digoxigenin-POD, Fab fragments*** 11 207 733 910 – 150 U **Anti-Fluorescein-AP, Fab fragments** 11 426 338 910 - 150 U (200 µl) **Anti-Fluorescein-POD, Fab fragments** 11 426 346 910 – 150 U	**Anti-Digoxigenin-AP, Fab fragments*** 11 093 274 910 - 150 U (200 µl) **Anti-Digoxigenin-Fluorescein, Fab fragments*** 11 207 741 910 - 200 µg **Anti-Digoxigenin-POD, Fab fragments*** 11 207 733 910 – 150 U **Anti-Digoxigenin-Rhodamine, Fab fragments*** 11 207 750 910 - 200 µg **Anti-Fluorescein-AP, Fab fragments** 11 426 338 910 - 150 U (200 µl) **Streptavidin-AP, for biotin** 11 093 266 910 -150 U **Streptavidin-POD, for biotin** 11 089 153 001 – 500 U	**Anti-Digoxigenin-AP, Fab fragments*** 11 093 274 910 - 150 U (200 µl) **Anti-Digoxigenin-POD, Fab fragments*** 11 207 733 910 – 150 U **Anti-Fluorescein-AP, Fab fragments** 11 426 338 910 - 150 U (200 µl) **Anti-Fluorescein-POD, Fab fragments** 11 426 346 910 – 150 U **Streptavidin-AP, for biotin** 11 093 266 910 -150 U **Streptavidin-POD, for biotin** 11 089 153 001 – 500 U
Without Conjugation		
Anti-Digoxigenin, polyclonal from sheep* 11 333 089 001 - 200 µg **Anti-Digoxigenin, monoclonal*** 11 333 062 910 - 100 µg	**Anti-Digoxigenin, polyclonal from sheep*** 11 333 089 001 - 200 µg **Anti-Digoxigenin, monoclonal*** 11 333 062 910 - 100 µg **Anti-Digoxigenin, Fab fragments*** 11 214 667 001 – 1 mg **Anti-Fluorescein, monoclonal** 11 426 320 001 - 100 µg	**Anti-Digoxigenin, polyclonal from sheep*** 11 333 089 001 - 200 µg **Anti-Digoxigenin, monoclonal*** 11 333 062 910 - 100 µg **Anti-Digoxigenin, Fab fragments*** 11 214 667 001 – 1 mg **Anti-Fluorescein, monoclonal** 11 426 320 001 - 100 µg

Detection using Alkaline Phosphatase (AP)

		Filter Hybridization	*In Situ* Hybridization	Tube and Microplate Formats (ELISA)
Colorimetric Detection – Alkaline Phosphatase (AP)				
	**Conjugated Antibodies**	■ **Anti-Fluorescein-AP, Fab fragments** 11 426 338 910 - 150 U (200 µl) ■ **Anti-Digoxigenin-AP, Fab fragments*** 11 093 274 910 - 150 U (200 µl)	■ **Streptavidin-AP, for biotin** 11 093 266 910 - 150 U ■ **Anti-Fluorescein-AP, Fab fragments** 11 426 338 910 - 150 U (200 µl) ■ **Anti-Digoxigenin-AP, Fab fragments*** 11 093 274 910 - 150 U (200 µl)	■ **Streptavidin-AP, for biotin** 11 093 266 910 - 150 U ■ **Anti-Fluorescein-AP, Fab fragments** 11 426 338 910 - 150 U (200 µl) ■ **Anti-Digoxigenin-AP, Fab fragments*** 11 093 274 910 - 150 U (200 µl)
	Single Reagents	■ **BM purple, AP substrate precipitating** 11 442 074 001 – 100 ml ■ **NBT/BCIP Ready-to-Use Tablets** 11 697 471 001 – 20 tablets ■ **NBT/BCIP Stock Solution** 11 681 451 001 – 8 ml	■ **BM purple, AP substrate precipitating** 11 442 074 001 – 100 ml ■ **NBT/BCIP Ready-to-Use Tablets** 11 697 471 001 – 20 tablets ■ **NBT/BCIP Stock Solution** 11 681 451 001 – 8 ml ■ **Fast Red Tablets** 11 496 549 001 – 20 tablets	
	Kits	■ **DIG-High Prime DNA Labeling and Detection Starter Kit I*** 11 745 832 910 - 12 labeling and 24 detection reactions ■ **DIG DNA Labeling and Detection Kit*** 11 093 657 910 - 25 labeling reactions and 50 blots		

▶▶▶

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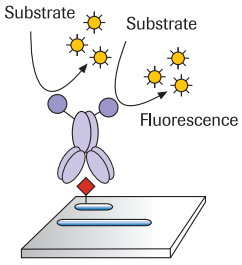
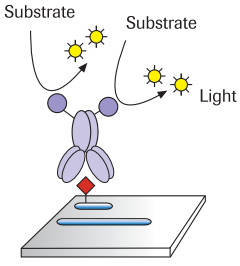
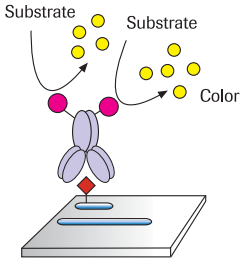
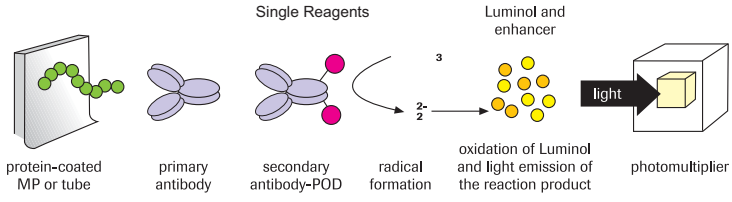
		Filter Hybridization	*In Situ* Hybridization	Tube and Microplate Formats (ELISA)
Fluorescent Detection – Alkaline Phosphatase (AP)				
	**Single Reagents**			■ **AttoPhos** 11 681 982 001 – for 1,800 wells or 720 tubes
	Kits	■ **HNPP Fluorescent Detection Set** 11 758 888 001 – 1 set	■ **HNPP Fluorescent Detection Set** 11 758 888 001 – 1 set	
	**Conjugated Antibodies**	■ **Anti-Fluorescein-AP, Fab fragments** 11 426 338 910 - 150 U (200 µl) ■ **Anti-Digoxigenin-AP, Fab fragments*** 11 093 274 910 - 150 U (200 µl)	■ **Streptavidin-AP, for biotin** 11 093 266 910 - 150 U ■ **Anti-Fluorescein-AP, Fab fragments** 11 426 338 910 - 150 U (200 µl) ■ **Anti-Digoxigenin-AP, Fab fragments*** 11 093 274 910 - 150 U (200 µl)	■ **Streptavidin-AP, for biotin** 11 093 266 910 - 150 U ■ **Anti-Fluorescein-AP, Fab fragments** 11 426 338 910 - 150 U (200 µl) ■ **Anti-Digoxigenin-AP, Fab fragments*** 11 093 274 910 - 150 U (200 µl)
	Single Reagents	■ **CSPD, ready-to-use** 11 755 633 001 – 2 × 50 ml ■ **CDP-*Star*, ready-to-use** 12 041 677 001 – 2 × 50 ml		
	Kits	■ **DIG Northern Starter Kit*** 12 039 672 910 - 10 labeling reactions and detection of 10 blots 10 × 10 cm^2) ■ **DIG-High Prime DNA Labeling and Detection Starter Kit II*** 11 585 614 910 - 12 labeling and 24 detection reactions ■ **DIG Luminescent Detection Kit*** 11 363 514 910 – 50 blots		■ **BM Chemiluminescence ELISA Substrate (AP)** 11 759 779 001 – 150 ml

Detection using Peroxidase (POD)

		Filter Hybridization	*In Situ* Hybridization	Tube and Microplate Formats (ELISA)
Colorimetric Detection – Peroxidase (POD)				
	**Antibodies**	■ **Anti-Fluorescein-POD, Fab fragments** 11 426 346 910 – 150 U ■ **Anti-Digoxigenin-POD, Fab fragments*** 11 207 733 910 – 150 U	■ **Streptavidin-POD, for biotin** 11 089 153 001 – 500 U ■ **Anti-Digoxigenin-POD, Fab fragments*** 11 207 733 910 – 150 U	■ **Streptavidin-POD, for biotin** 11 089 153 001 – 500 U ■ **Anti-Fluorescein-POD, Fab fragments** 11 426 346 910 – 150 U ■ **Anti-Digoxigenin-POD, Fab fragments*** 11 207 733 910 – 150 U
	Single Reagents	■ **BM Blue POD Substrate, precipitating, TMB ready-to-use solution** 11 442 066 001 – 100 ml ■ **DAB Substrate, metal enhanced, precipitating** 11 718 096 001 – 1 pack	■ **DAB Substrate, metal enhanced, precipitating** 11 718 096 001 – 1 pack	■ **ABTS Solution, ready-to-use** 11 684 302 001 – 3 × 100 ml ■ **ABTS Tablets** 11 204 521 001 – 20 tablets ■ **BM Blue POD Substrate, soluble** 11 484 281 001 – 100 ml
	Kits			■ **PCR ELISA, DIG Detection** 11 636 111 910 – 192 reactions ■ **PCR ELISA, DIG Detection, 5-pack** 11 965 409 910 – 1 kit
Chemiluminescent Detection –Peroxidase (POD)				
				■ **BM Chemiluminescence ELISA Substrate (POD)** 11 582 950 001 – 250 ml

Product Literature and Support Material

The DIG System is the nonradioactive technology of choice to label and detect nucleic acids for multiple applications such as filter hybridization or in situ hybridization. For an overview of our extensive DIG System product line, consult the **DIG Product Selection Guide**.

Explore detailed information about using DIG products for filter hybridization, view protocols for labeling and detection, find helpful hints and more in the **DIG Application Manual for Filter Hybridization**.
Access additional information on our **Labeling and Detection Special Interest Site** at **www.roche-applied-science.com/dig**

Use the **Tools for Amplification Brochure** to identify the right product for both routine and unique amplification reactions. Any amplification need and situation is addressed with this brochure.
Access detailed information about all our products related to PCR and RT-PCR by visiting our **PCR Special Interest Site** at **www.roche-applied-science.com/pcr**

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6

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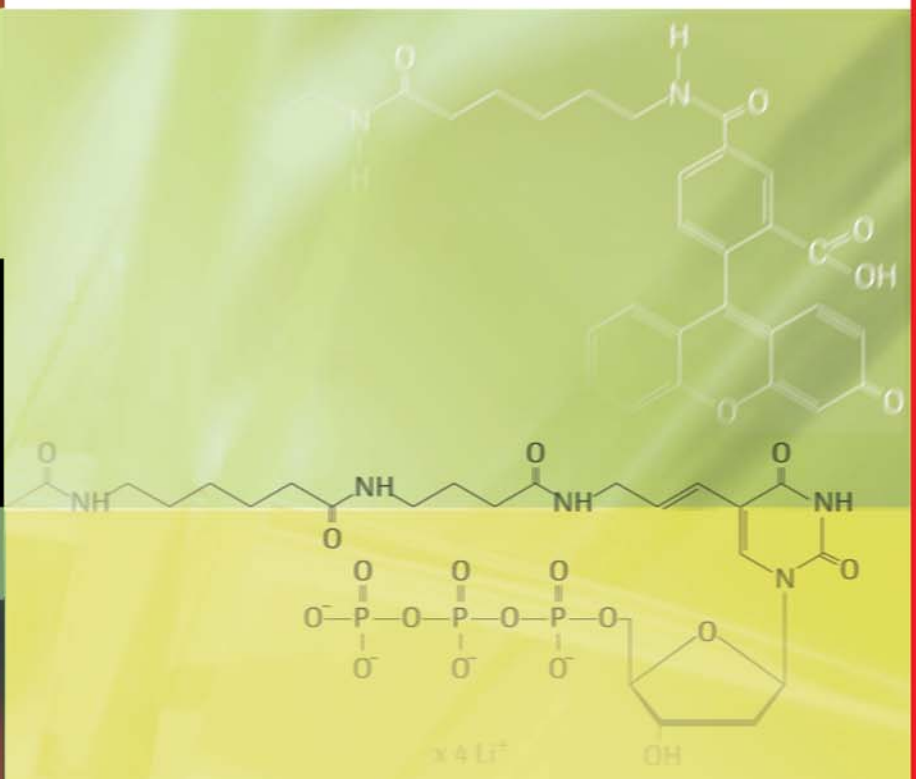

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