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).

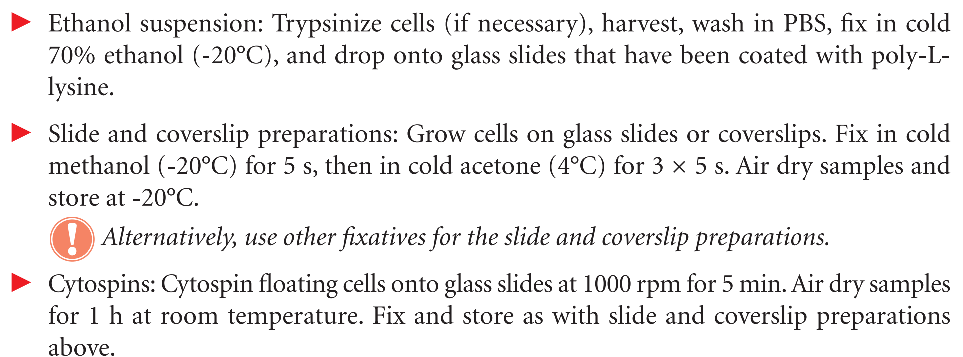
We show examples of single- and multiple-target ISH experiments on standard human lymphocyte metaphase spreads, as well as on interphase cell preparations. These results demonstrate the potential of this detection methodology for metaphase and interphase cytogenetics, *e.g.*, for studying chromosome 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).

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.

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-E1		
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

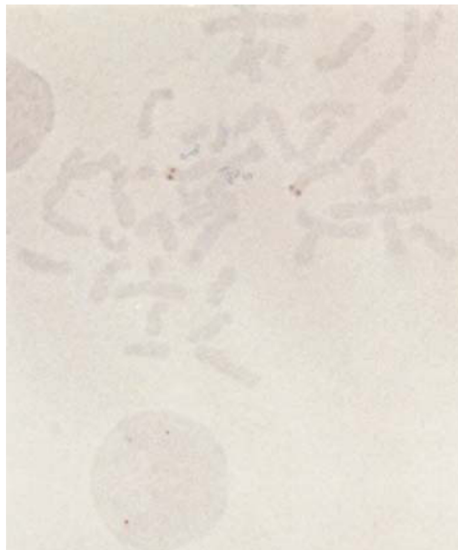


Figure 1: Single-target ISH on a normal human lymphocyte metaphase spread with a peroxidase detection system. The probe was a biotinylated cosmid that recognizes 40 kb of chromosome 11q23. The detection system included monoclonal anti-biotin Ab (DAKO), rabbit anti-mouse Ab-PO, and the PO-DAB reaction.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

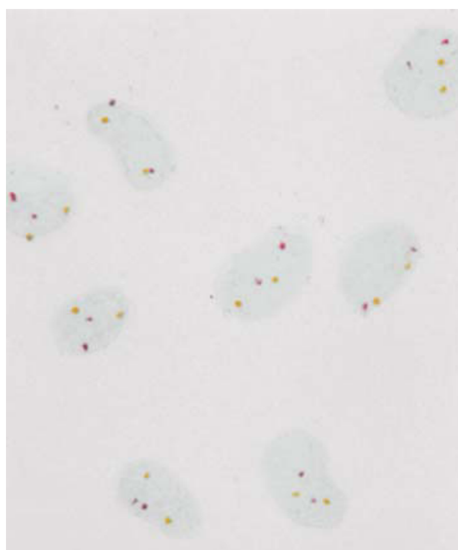


Figure 2: Double-target ISH on normal human umbilical vein endothelial cells with brightfield viewing. The centromere of chromosome 1 (brown) was detected with a biotinylated probe; that of chromosome 7 (red), with a digoxigenin-labeled probe. The detection steps included (1) avidin-PO, (2) monoclonal 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.

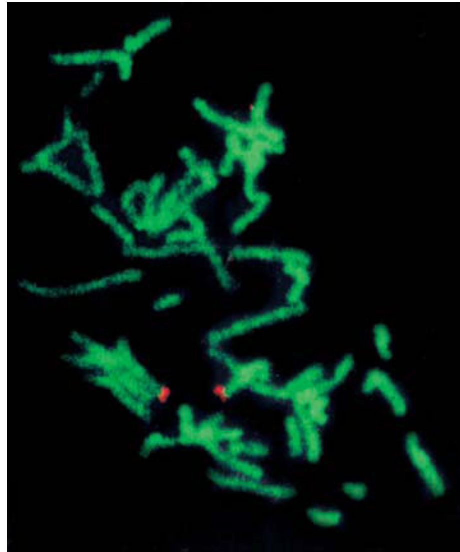


Figure 3: Same ISH experiment as in Figure 1, but with a fluorescent alkaline phosphatase detection system. The detection system for the biotinylated cosmid probe included monoclonal anti-biotin, horse anti-mouse Ab-biotin, avidin-biotinylated APase complex, and the APase-Fast Red reaction (Speel et 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.

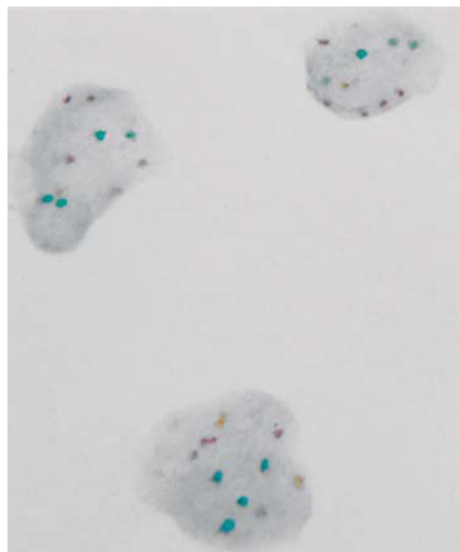


Figure 5: Triple-target ISH on human bladder tumor cell line T24. The probes and experimental procedures were the same as in Figure 4.

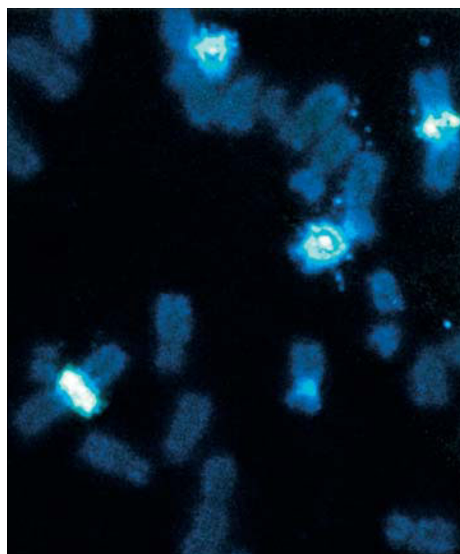


Figure 6: Double-target ISH on a normal human lymphocyte metaphase spread with reflection contrast viewing. The centromere of chromosome 1 (yellow) was detected with a biotinylated probe; that of chromosome 17 (white), with a digoxigenin-labeled probe. The detection steps included (1) monoclonal anti-biotin 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.

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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.