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 non-isotopic in situ hybridization with digoxigenin-labeled probes (Komminoth, 1992; Komminoth et al., 1992). In those reports we demonstrated:

- Digoxigenin-labeled probes are as sensitive as 35S-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 Boehringer Mannheim DIG/Genius™ System User’s Guide for Membrane Hybridization.

I. Preparation of slides

Note: 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 x 10 min in distilled water.
3. Dry slides overnight at 60°C.

Note: 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.

Caution: Prepare all solutions for procedures IIA and IIB 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.

Note: Perform all procedures below at room temperature unless a different temperature is stated.

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)).
   Note: 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.
   Note: Slides with cryostat sections may be stored at −80°C for several weeks.

IIIA. ISH protocol for detection of mRNA with DIG-labeled RNA probes

Caution: 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.

Note: Perform all procedures below at room temperature unless a different temperature is stated.

Probes labeling

Note: 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).

Note: As an alternative to linearized plasmids, use PCR-generated templates containing RNA polymerase promoter sequences for in vitro transcription (Young et al., 1991).
Procedures for In Situ Hybridization to Chromosomes, Cells, and Tissue Sections
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Caution: 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.

Post-fix sections for 5 min at 4°C with DEPC-treated PBS containing 4% paraformaldehyde.

Wash sections 2 x 5 min with DEPC-treated PBS.

To acetylate sections, place slide containers on a rocking platform and incubate slides 2 x 5 min with 0.1 M triethanolamine (TEA) buffer, pH 8.0, containing 0.25% (v/v) acetic anhydride (Sigma). Caution: Acetic anhydride is highly unstable. Add acetic anhydride to each change of TEA-acetic anhydride solution immediately before incubation.

Incubate sections at 37°C for at least 10 min with prehybridization buffer [4 x SSC containing 50% (v/v) deionized formamide]. Note: Deionize formamide with Dowex® MR 3 (Sigma) according to protocols described in Sambrook et al. (1989).

1 x SSC = 150 mM NaCl, 15 mM sodium citrate, pH 7.2.

In situ hybridization

Prepare hybridization buffer containing:
- 40% deionized formamide.
- 10% dextran sulfate.
- 1 x D enhardt's solution [0.02% Ficoll®, 0.02% polyvinylpyrrolidone, 10 mg/ml RNase-free bovine serum albumin].
- 4 x SSC.
- 10 mM DTT.
- 1 mg/ml yeast t-RNA.
- 1 mg/ml denatured and sheared salmon sperm DNA.

Caution: Denature and add salmon sperm DNA to buffer shortly before hybridization.

Note: Hybridization buffer (minus salmon sperm DNA) can be stored at -20°C for several months.

Purify linearized plasmid by phenol-chloroform extraction and ethanol precipitation.

Caution: 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.

To avoid RNA polymerase inhibition, resuspend the plasmid in EDTA-free buffer or DEPC-treated ddH₂O.

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.

Purify labeled probes according to procedures in the DIG/Genius™ System User’s Guide for Membrane Hybridization or in Chapter 4 of this manual.

Caution: Probes longer than 500 bases may not penetrate tissue. If probes are longer than 500 bases, shorten them by alkaline hydrolysis according to procedures in the DIG/Genius™ System User’s Guide for Membrane Hybridization.

Estimate the yield of labeled probes by direct blotting procedures as described in Chapter 4 of this manual.

Aliquot labeled probes and store them at -80°C.

Note: Probes are stable for up to one year.

Prehybridization

Incubate sections as follows:
- 2 x 5 min with DEPC-treated PBS (pH 7.4) (Sambrook et al., 1989).
- Treat sections for 15 min with DEPC-treated PBS containing 0.3% Triton® X-100.
- Wash 2 x 5 min with DEPC-treated PBS.
- 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).

Note: Hybridization buffer (minus salmon sperm DNA) can be stored at -20°C for several months.

*Sold under the trade name of Genius in the US.
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.

Cover samples with a 24 x 30 mm hydrophobic plastic coverslip (e.g., cut from Gel Bond Film, FM BioProducts, Rockland, ME, USA).

Incubate sections at 42°C overnight in a humid chamber.

Posthybridization

Caution: Use a separate set of glassware for posthybridization and prehybridization procedures to avoid RNase contamination in prehybridization steps.

Remove coverslips from sections by immersing slides for 5–10 min in 2 x SSC.

Caution: Do not place samples which are hybridized with different probes in the same container.

In a shaking water bath at 37°C, wash sections as follows:
- 2 x 15 min with 2 x SSC.
- 2 x 15 min with 1 x SSC.

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.

Caution: 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.

In a shaking water bath at 37°C, wash sections 2 x 30 min with 0.1 x SSC.

Note: If this procedure gives high background or nonspecific signals, try posthybridization washings at 52°C with 2 x SSC containing 50% formamide.

Immunological detection

Note: 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.

Using a shaking platform, wash sections 2 x 10 min with buffer 1 [100 mM Tris-HCl (pH 7.5), 150 mM NaCl].

Cover sections for 30 min with blocking solution [buffer 1 containing 0.1% Triton® X-100 and 2% normal sheep serum (Sigma)].

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].

Note: For optimal detection, incubate several sections (from the same sample) with different dilutions of the antibody (1:100; 1:500, and 1:1000).

Using a shaking platform, wash sections 2 x 10 min with buffer 1.

Incubate sections for 10 min with buffer 2 [100 mM Tris-HCl (pH 9.5), 100 mM NaCl, 50 mM MgCl₂].

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-indolylphosphate (BCIP or X-phosphate) solution (50 mg X-phosphate/ml in 100% dimethylformamide).
- 1 mM (2.4 mg/10 ml) levamisole (Sigma).

Note: For convenience, prepare a 1 M stock solution of levamisole in ddH₂O (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.

Note: 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.

Cover each section with approximately 200 µl color solution and incubate slides in a humid chamber for 2–24 h in the dark.

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].

Dip slides briefly in distilled water.

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Q 1. Counterstain sections for 1–2 min with 0.02% fast green FCF or 0.1% nuclear Fast Red (Aldrich Chemical Corp., Milwaukee, WI, U SA) in distilled H₂O.
Q 2. Wash sections 2 x 10 min in tap water.
Q 3. Mount sections using an aqueous mounting solution (e.g. Aqua-Mount from Lerner Laboratories, Pittsburgh, PA, U SA).
Caution: 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
Q 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.
Q 2. Purify labeled probes according to procedures in the DIG/Genius™ System User’s Guide for Membrane Hybridization or in Chapter 4 of this manual.
Q 3. Estimate the yield of labeled probes by direct blotting procedures as described in Chapter 4 of this manual.
Q 4. Aliquot the labeled probes and store them at −20°C.
Note: Probes are stable for up to one year.

Note: 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
Q 1. Follow Steps 1–7 from the prehybridization section of Procedure II A (for RNA probes).
Q 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).

A 3. Add a 24 x 30 mm coverslip to each section and incubate slides in a humid chamber at 37°C for 2 h.
A 4. Remove coverslips by immersing slides for 5 min in 2x SSC.

In situ hybridization
Note: To increase the sensitivity of in situ hybridization procedures, use mixtures of oligonucleotide probes that are complementary to different regions of the target RNA.

Q 1. Prepare hybridization buffer containing:
   1. 2 x SSC.
   2. 1 x Denhardt’s solution (0.02% Ficoll®, 0.02% polyvinylpyrrolidone, 10 mg/ml RNase-free bovine serum albumin).
   3. 10% dextran sulfate.
   4. 50 mM phosphate buffer (pH 7.0).
   5. 50 mM DTT.
   6. 250 µg/ml yeast t-RNA.
   7. 5 µg/ml polydeoxyadenylic acid.
   8. 100 µg/ml polyadenylic acid.
   9. 0.05 pM/ml Randomer Oligonucleotide Hybridization Probe (DuPont).
   10. 500 µg/ml denatured and sheared salmon sperm DNA.
Caution: Denature and add salmon sperm DNA to buffer shortly before hybridization.

Q 2. Enough deionized formamide (%dF, as calculated by the formula below) to produce stringent hybridization conditions at 37°C (that is, to make 37°C = Tm (oligonucleotide probe) − 10°C) (Long et al., 1992).
Caution: % dF should not exceed 47% of the total volume of the hybridization buffer. If dF is less than 47%, add ddH₂O so that ddH₂O plus dF equals 47% of the volume.

Note: Use the following formula to calculate the formamide concentration (%dF) in the hybridization buffer for each oligonucleotide probe:

% dF = \((-7.9) + 81.5 + 0.41(\% G C) - 675/L - \% mismatch - (47)\) / 0.65

where, in this case:
- 7.9 = 16.6 log10 [Na⁺] (for 2 x SSC)
- % G C = [% 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)

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Note: Hybridization buffer (minus dF, ddH₂O, and salmon sperm DNA) can be stored at -20°C for several months.

Drain 2 x 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.

Cover sections with a 24 x 30 mm hydrophobic plastic coverslip (e.g. cut from Gel Bond Film, FMC BioProducts, Rockland, ME, USA).

Incubate samples at 37°C overnight in a humid chamber.

Posthybridization

Remove coverslips from sections by immersing slides for 5–10 min in 2 x SSC.

In a shaking water bath at 37°C, wash sections as follows:

- 2 x 15 min with 2 x SSC.
- 2 x 15 min with 1 x SSC.
- 2 x 15 min with 0.25 x 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

Positive sample: Tissue or cell line known to contain mRNA of interest.

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

Negative sample: Tissue or cell line known to lack the sequence of interest.

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 35S- 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 Boehringer Mannheim for this procedure

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<td>For RNA labeling with digoxigenin-UTP by in vitro transcription with SP6 and T7 RNA polymerase.</td>
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**EP Patent 0 324 474 granted to Boehringer Mannheim GmbH.
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****EP Patents 0 124 657/0 324 474 granted to Boehringer Mannheim GmbH.
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References


