

## 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

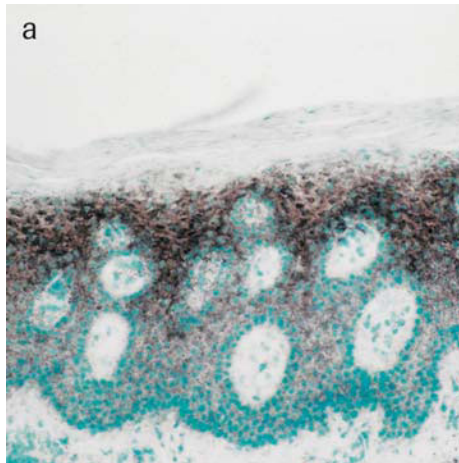
Choosing the type of probe (*i.e.*, DNA, RNA, or oligonucleotide) is essential for a good final result. For the optimization of the probe labeling, we have tested four methods of incorporating the DIG label:

- ▶ 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:

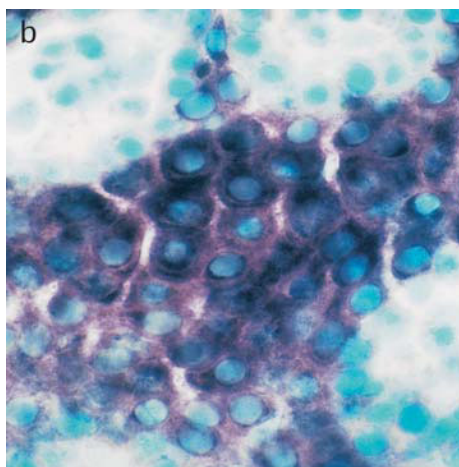
- ▶ For cRNA probe synthesis, subclone a cDNA molecule in an appropriate vector. The vector should have sequences flanking the insert that allow the insert to be transcribed. Typically, commercially available vectors have SP6 and T7 RNA polymerase sites flanking the multiple cloning sites.
- ▶ Regulate 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).



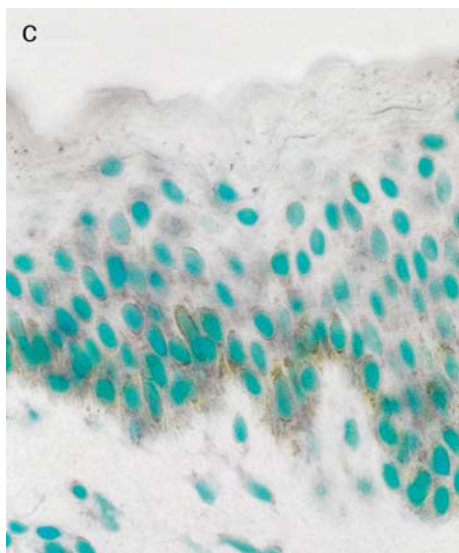


**Figure 1: RNA *in situ* hybridization on a frozen section of human psoriatic epidermis with DIG-labeled cRNA probes coding for human SKALP.**

Panel a: An antisense cRNA probe (150 bp) was used on a 10  $\mu\text{m}$  thick section (magnification  $\times 125$ ).




Panel b: An antisense cRNA probe (150 bp) was used on a 20  $\mu\text{m}$  thick section (magnification  $\times 500$ ).





Panel c: A sense cRNA probe (150 bp) was used on a 10  $\mu\text{m}$  thick section (magnification  $\times 270$ ). 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.*

## 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.
 

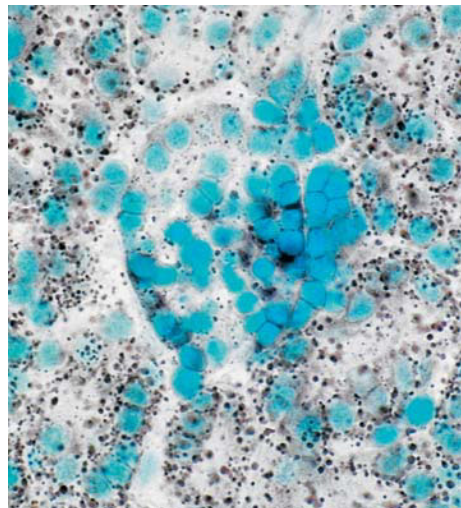
 *To get a higher signal, cut sections thicker than 10 µm. For better signal localization, cut sections thinner than 10 µm.*
- 4 Mount tissue directly on Superfrost Plus slides (Menzel Gläser, Omnilabo, Breda, The Netherlands) to prevent detachment of the section during the RISH procedure.

## 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.

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## 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 pyrrolidone, 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<sub>2</sub>].
- 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

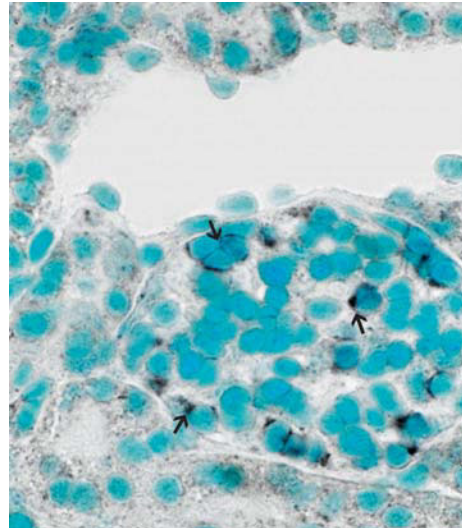
- 1 Wash the sections for 5 min with distilled H<sub>2</sub>O at room temperature.
- 2 Counterstain the sections for 5–10 min with 1% methylene green at 37°C.
- 3 Repeat wash (from Step 1).
- 4 Mount coverslips in Kaiser's solution (available from Merck).

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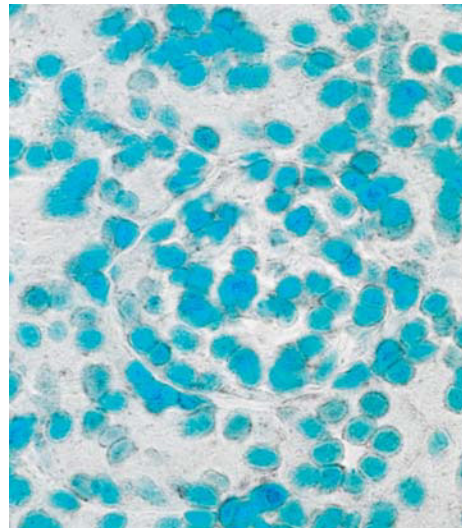
## Results and discussion

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  $\mu\text{m}$  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  $\times 600$ ).



**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  $\mu\text{m}$  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,  $600\times$ ).

## 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.

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## Reagents available from Roche Applied Science for this procedure

Reagent	Description	Cat. No.	Pack size
<b>DIG RNA Labeling Kit (SP6/T7)*</b>	For RNA labeling with digoxigenin-UTP by <i>in vitro</i> transcription with SP6 and T7 RNA polymerases.	11 175 025 910	1 Kit (2 × 10 labeling reactions)
<b>Blocking Reagent</b>	For nucleic acid hybridization	11 096 176 001	50 g
<b>Anti-Digoxigenin-AP*</b>	Anti-Digoxigenin, Fab fragments conjugated with alkaline phosphatase	11 093 274 910	150 U (200 µl)
<b>Tris</b>	For preparation of buffer solutions	10 708 976 001 11 814 273 001	1 kg 5 kg
<b>NBT solution</b>	100 mg/ml nitroblue tetrazolium salt in 70% (v/v) dimethylformamide	11 383 213 001	3 ml (300 mg) (dilute prior to use)
<b>BCIP solution</b>	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.

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