


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 ($4\ \mu\text{m}$ – $12\ \mu\text{m}$).
! 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 $5\times$ 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 $2\ \mu\text{g}/\text{ml}$
Paraffin embedded sections: up to $20\ \mu\text{g}/\text{ml}$
Methacrylate embedded sections: up to $50\ \mu\text{g}/\text{ml}$
- 5 Rinse the sections in Tris-Glycine (100 mM Tris-HCl pH7.0, 100 mM glycine from a $10\times$ 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.



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- 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.*
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VI. Hybridization procedure


 *Homologous probes are hybridized at 50–52°C. For heterologous probes we recommend lower temperatures.*

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- 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: 0.3–1 µg/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.
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VII. Posthybridization procedure

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- 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.
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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).

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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 immunofluorescence 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).



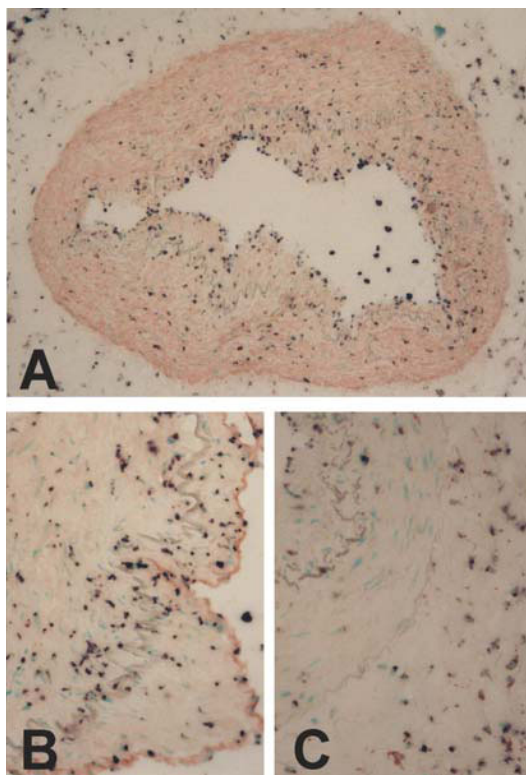


Figure 1: Detection of GM-CSF mRNA (purple) with a DIG-labeled antisense cRNA probe in an early stage of plaque development and immunohistochemical characterization of the GM-CSF expressing vascular cell types (red).

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 probes (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.

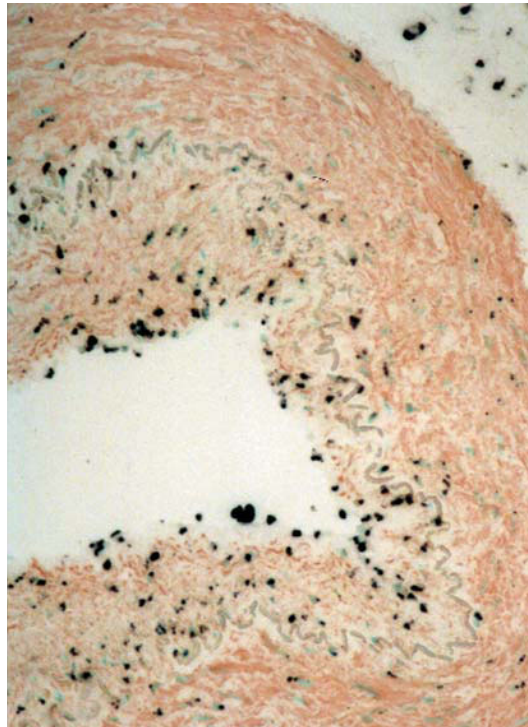


Figure 2: Detection of procollagen alpha1 type VIII mRNA (purple) with a DIG-labeled antisense cRNA probe in an early stage of plaque development.

Double staining to demonstrate type VIII collagen mRNA (purple) expressing smooth muscle cells (red) in early atherogenesis.

References

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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 <i>in vitro</i> 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	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
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.

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