

### **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:

Step	Action
1	For fixation, prepare either of the following solutions: <ul style="list-style-type: none"> <li>▶ 100 mM phosphate buffer, pH 7, containing 0.25% gluteraldehyde and 4% freshly depolymerized paraformaldehyde.</li> <li>▶ Formalin-acetic acid (50% ethanol; 10% formalin, containing 37% formaldehyde; 5% acetic acid).</li> </ul>
2	Using either of the solutions from Step 1, fix the tissue for 4 h at room temperature. During the fixation: <ul style="list-style-type: none"> <li>▶ Vacuum infiltrate the tissue (with a water aspirator) for 10 min once 1 h.</li> <li>▶ After each vacuum infiltration, renew the fixative solution.</li> </ul>
3	Depending on the fixative used in Step 2, wash the fixed tissue as follows: <ul style="list-style-type: none"> <li>▶ If gluteraldehyde-paraformaldehyde was the fixative, wash the fixed tissue 2 x 30 min with 100 mM phosphate buffer, pH 7.</li> <li>▶ If formalin-acetic acid was the fixative, wash the fixed tissue 2 x 30 min with 50% ethanol.</li> </ul>
4	Dehydrate the tissue by incubating in the following series of ethanol solutions: <ul style="list-style-type: none"> <li>▶ Either 90 min (after gluteraldehyde-paraformaldehyde fixation) or 30 min (after formalin-acetic acid fixation) at room temperature in 50% ethanol.</li> <li>▶ 90 min at room temperature in 70% ethanol.</li> <li>▶ Overnight at 4°C in 85% ethanol.</li> <li>▶ 3 x 90 min at room temperature in 100% ethanol.</li> </ul>

## II. Sectioning

Step	Action
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

Step	Action
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 2x in phosphate buffered saline (PBS).
4	Dehydrate the sections in ascending concentrations of ethanol as previously described (Jackson, 1991).

## IV. Hybridization

Step	Action
1	Label the probe RNA with DIG-UTP according to the procedures given in Chapter 4 of this manual.
2	<p>Reduce the length of the probe to approximately 200 bases as follows:</p> <ul style="list-style-type: none"> <li>▶ To 50 <math>\mu</math>l of labeled probe RNA in a microcentrifuge tube, add 30 <math>\mu</math>l 200 mM <math>\text{Na}_2\text{CO}_3</math> and 20 <math>\mu</math>l 200 mM <math>\text{NaHCO}_3</math>.</li> <li>▶ Hydrolyze the probe at 60°C for t min, where:  <math display="block">t = (L_0 - L_f) / (K \cdot L_0 \cdot L_f)</math> <p><math>L_0</math> = starting length of probe RNA (in kb)  <math>L_f</math> = length of probe RNA (in kb) (In this case, <math>L_f = 0.2</math> kb.)  <math>K</math> = rate constant (In this case,  <math>K = 0.11</math> kb/min.)  <math>t</math> = hydrolysis time in min</p></li> </ul>
3	<p>After hydrolysis, purify the probe RNA as follows:</p> <ul style="list-style-type: none"> <li>▶ Add the following to the hydrolyzed probe solution: <ul style="list-style-type: none"> <li>- 5 <math>\mu</math>l 10% acetic acid</li> <li>- 11 <math>\mu</math>l 3 M sodium acetate (pH 6.0)</li> <li>- 1 <math>\mu</math>l of a 10 mg/ml tRNA stock</li> <li>- 1.2 <math>\mu</math>l 1 M <math>\text{MgCl}_2</math></li> <li>- 300 <math>\mu</math>l (about 2.5 volumes) cold ethanol</li> </ul> </li> <li>▶ Incubate 4 - 16 h at -20°C.</li> <li>▶ Centrifuge in a microcentrifuge for 15 min at 4°C to pellet the RNA.</li> <li>▶ Discard the supernatant and dry the RNA pellet in a vacuum desiccator.</li> <li>▶ Resuspend labeled probe RNA in DEPC-treated water at 10 - 50 <math>\mu</math>g/ml.</li> </ul>
4	<p>Prepare a hybridization mixture containing the following:</p> <ul style="list-style-type: none"> <li>▶ 50% deionized formamide.</li> <li>▶ 2.25x SSPE (300 mM NaCl; 20 mM <math>\text{NaH}_2\text{PO}_4</math>; 2 mM EDTA; pH 7.4).</li> <li>▶ 10% dextran sulfate.</li> <li>▶ 2.5x Denhardt's solution.</li> <li>▶ 100 <math>\mu</math>g/ml sheared and denatured herring sperm DNA.</li> <li>▶ 100 <math>\mu</math>g/ml tRNA.</li> <li>▶ 5 mM DTT.</li> <li>▶ 40 units/ml RNase inhibitor.</li> <li>▶ 0.2 - 1.0 <math>\mu</math>g/ml hydrolyzed and denatured probe (200 bases long).</li> </ul>
5	<p>Cover each section with 250 - 500 <math>\mu</math>l of hybridization mixture (depending on the size of the section) and incubate in a humidified box at 42°C overnight.</p> <p><b>Note:</b> Do not use a coverslip during the hybridization incubation.</p>
6	<p>After hybridization, wash the slides as follows:</p> <ul style="list-style-type: none"> <li>▶ 5 min at room temperature with 3x SSC.</li> </ul> <p><b>Note:</b> 1x SSC contains 150 mM NaCl, 15 mM Na-citrate; pH 7.</p> <ul style="list-style-type: none"> <li>▶ 5 min at room temperature with NTE (500 mM NaCl, 10 mM Tris-HCl, 1 mM EDTA; pH 7.5).</li> </ul>
7	<p>To remove unhybridized single-stranded RNA probe, put slides into a humidified box and cover each section with 500 <math>\mu</math>l of NTE buffer containing 50 <math>\mu</math>g/ml RNase A. Incubate for 30 min at 37°C.</p>
8	<p>After RNase treatment, wash the slides 3 x 5 min at room temperature with NTE.</p>
9	<p>To remove nonspecifically hybridized probe, wash the slides as follows:</p> <ul style="list-style-type: none"> <li>▶ 30 min at room temperature with 2x SSC.</li> <li>▶ 1 h at 57°C with 0.1x SSC.</li> </ul>

## V. Detection of DIG-labeled hybrids

Step	Action
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: <ul style="list-style-type: none"> <li>▶ 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.</li> <li>▶ Cool the Tris-NaCl-PVA stock solution to room temperature.</li> <li>▶ Add MgCl<sub>2</sub>, 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).</li> </ul>
4	After the washes in Step 2, perform the visualization step as follows: <ul style="list-style-type: none"> <li>▶ Place the slides in 30 ml of BCIP-NBT-PVA color development solution in a vertical staining dish suited for eight slides.</li> <li>▶ Incubate the slides in the color development solution for 2 – 16 h at 30°C.</li> <li>▶ Monitor color formation visually.</li> </ul>
5	When the color on each slide is optimal, stop the color reaction by washing the slide 3 x 5 min in distilled water.
6	Dehydrate the sections by incubating the slides without shaking in the following ethanol solutions: <ul style="list-style-type: none"> <li>▶ 15 s in 70% ethanol.</li> <li>▶ 2 x 15 s in 100% ethanol.</li> </ul>
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.

## 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.

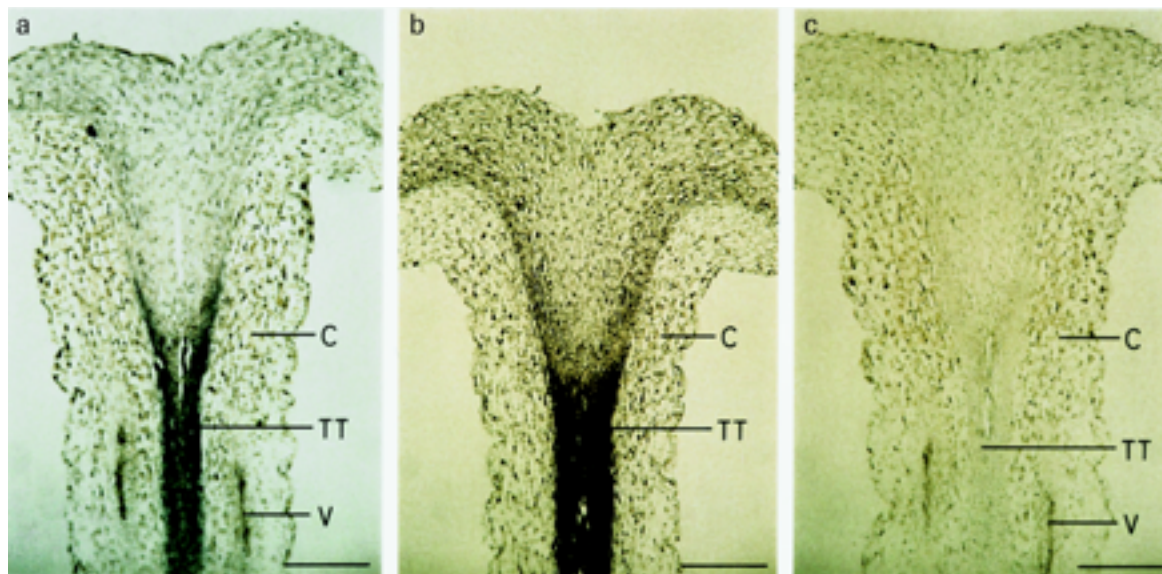
This enhancement was even more pronounced in *in situ* hybridization experiments. Figure 1 shows the results of an *in situ* hybridization in which sections of tobacco pistils were hybridized with an antisense RNA probe from a pistil-specific cDNA clone (de S. Goldman et al., 1992). After three hours 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 <sup>b</sup>	Effect on the alkaline phosphatase BCIP-NBT reaction <sup>e</sup>
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% <sup>c</sup>	Approx. 4-fold enhancement (0.04 mM formazan) Approximately 6- to 8-fold enhancement (0.06 to 0.08 mM formazan)
	40 kD	10% <sup>d</sup>	
	70 – 100 kD	10% <sup>d</sup>	

**Table 1: Influence of polymers on the alkaline phosphatase BCIP-NBT reaction<sup>a</sup>.**

- 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  $7.5 \times 10^{-3}$  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  $\mu$ m 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  $\mu$ m.

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

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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 polymerase.	1 175 025	1 Kit (2 x 10 labeling reactions)
<b>Proteinase K</b>	Lyophilizate	161 519 745 723 1 000 144 1 092 766	25 mg 100 mg 500 mg 1 g
<b>tRNA</b>	From baker's yeast, lyophilizate	109 495 109 509	100 mg 500 mg
<b>DNA</b> from fish sperm	Lyophilized, sodium salt	223 646	1 g
<b>DTT</b>	Purity: >97%	197 777 708 984 1 583 786 708 992 709 000	2 g 10 g 25 g 50 g 100 g
<b>RNase A</b>	Powder	109 142 109 169	25 mg 100 mg
<b>RNase Inhibitor</b>	From human placenta	799 017 799 025	2,000 units 10,000 units
<b>DIG Nucleic Acid Detection Kit</b>	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.	1 175 041	1 Kit