


## In situ hybridization to mRNA in *in vitro* cultured cells with DNA probes

Department of Cytochemistry and Cytometry, University of Leiden, The Netherlands.

The protocol given below has been developed with a cell line (rat 9G) which has integrated into its genome the major immediate early transcription unit 1 of human cytomegalovirus (Boom et al., 1986). The protocol is written in general terms and is applicable to abundantly expressed mRNA species. More information concerning this specific *in situ* hybridization application can be found in Raap et al. (1991).


### I. Cell preparation, fixation and permeabilization

 All solutions must be treated with RNase inhibitors.

- 1** Culture cells at 37°C in a 5% CO<sub>2</sub> atmosphere on poly-L-lysine coated microscopic slides. As culture medium, use Dulbecco's minimal essential medium without phenol red.
- 2** Wash the cells with PBS at 37°C, then fix at room temperature for 30 min in a solution of 4% (w/v) formaldehyde, 5% (v/v) acetic acid, and 0.9% (w/v) NaCl.
- 3** Wash the fixed cells with PBS at room temperature and store them in 70% ethanol at 4°C.
- 4** Before *in situ* hybridization, treat the fixed cells as follows:
  - ▶ Dehydrate by incubating successively in 70%, 90%, and 100% ethanol.
  - ▶ Wash in 100% xylene to remove residual lipids.
  - ▶ Rehydrate by incubating successively in 100%, 90%, and 70% ethanol.
  - ▶ Finally, incubate in PBS.
- 5** Treat the fixed cells at 37°C with 0.1% (w/v) pepsin in 0.1 N HCl, to increase permeability to macromolecular reagents.
- 6** Finally, treat the fixed cells as follows:
  - ▶ Wash with PBS for 5 min.
  - ▶ Post-fix with 1% formaldehyde for 10 min.
  - ▶ Wash again with PBS.

### II. *In situ* hybridization


- 1** Label a suitable probe DNA with digoxigenin (DIG), using any of the protocols given elsewhere in this manual.
- 2** Prepare hybridization solution which contains 60% deionized formamide, 300 mM NaCl, 30 mM sodium citrate, 10 mM EDTA, 25 mM NaH<sub>2</sub>PO<sub>4</sub> (pH 7.4), 5% dextran sulfate, and 250 ng/μl sheared salmon sperm DNA.
- 3** Denature the DIG-labeled probe DNA at 80°C shortly before use and add it to the hybridization solution at a concentration of 5 ng/μl.
- 4** Add 10 μl of the hybridization mixture (hybridization solution plus denatured probe) to the fixed, permeabilized cells and cover with an 18 × 18 mm coverslip.
 

 *An in situ denaturation step is optional. Inclusion of such a step may intensify the RNA signal since it makes the sample more accessible to the probe.*
- 5** Hybridize at 37°C for 16 h.

### III. Washes

- 1 After hybridization, remove coverslips by shaking the slides at room temperature in a solution of 60% formamide, 300 mM NaCl, and 30 mM sodium citrate .
- 2 Using the same formamide-salt solution as in Step 1, wash the slides as follows:
  - ▶ Wash 3× at room temperature.
  - ▶ Wash 1× at 37°C.
- 3 Finally, wash the slides 1 × 5 min in PBS.

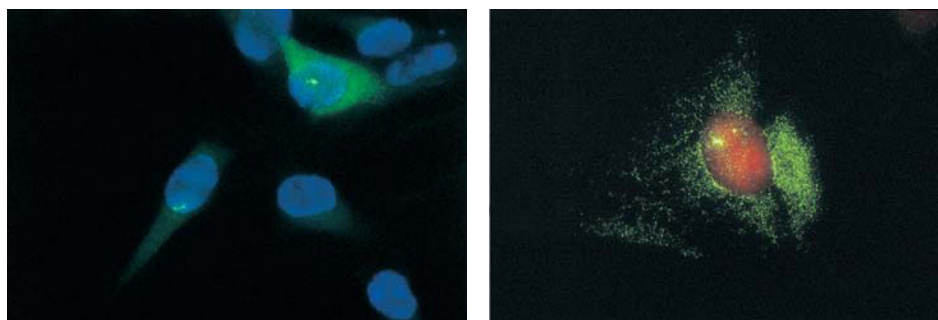
### IV. Immunofluorescent detection

 Other protocols for amplifying the signal may also be used. See the protocols under “Single color fluorescent detection with immunological amplification” on page 72.

- 1 Block non-specific binding by the following steps:
  - ▶ Add 100 µl blocking solution [100 mM Tris-HCl; pH 7.5, 150 mM NaCl, 0.5% (w/v) blocking reagent] to each slide.
  - ▶ Cover with a 24 × 50 mm coverslip.
  - ▶ Place slide in a moist chamber.
- 2 To loosen the coverslips, wash the slides briefly with the buffer used for immunological detection.
- 3 Detect the hybridized DIG-labeled probe by incubating the slides in a moist chamber for 45 min with a 1:500 dilution of anti-DIG-fluorescein in blocking solution (from Step 1) .
- 4 Wash slides with a solution of 100 mM Tris-HCl, pH 7.5; 150 mM NaCl; 0.05% Tween 20.
- 5 To dehydrate the cell samples, incubate the slides for 5 min in each of a series of ethanol solutions: 70%, 90%, then 100% ethanol.
- 6 Air dry the slides.
- 7 Embed the cell samples in an anti-fading solution which contains:
  - ▶ 9 parts glycerol
  - plus*
  - ▶ 1 part staining mixture: 1 M Tris-HCl (pH 7.5), 2% 1,4-diaza-bicyclo-[2,2,2]-octane (DABCO), and a DNA counterstain [either propidium iodide (500 ng/ml) or DAPI (75 ng/µl)].

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## Results



Panel a: PI counterstain.

Panel b: V-30b

**Figure 1: Nuclear staining of integrated IE viral DNA after induction with cycloheximide.** The signal throughout the cytoplasm represents viral mRNA. The two panels show the same signal with different counterstains.

## References

Boom, R.; Geelen, J. L.; Sol, C. J.; Raap, A. K.; Minnaar, R. P.; Klaver, B. P.; Noordaa, J. v. d. (1986) Establishment of a rat cell line inducible for the expression of human cytomegalovirus immediate - early gene products by protein synthesis inhibition. *J. Virol.* **58**, 851-859.

Raap, A. K.; Van de Rijke, F. M.; Dirks, R. W.; Sol, C. J.; Boom, R.; Van der Ploeg, M. (1991) Bicolor fluorescence *in situ* hybridization to intron- and exon mRNA sequences. *Exp. Cell Res.* **197**, 319-322.

## Reagents available from Roche Applied Science for this procedure

Reagent	Description	Cat. No.	Pack size
<b>Pepsin</b>	Aspartic endopeptidase with broad specificity	10 108 057 001	1 g
<b>Tween 20</b>		11 332 465 001	5 × 10 ml
<b>Blocking Reagent</b>	Powder	11 096 176 001	50 g
<b>Anti-Digoxigenin Fluorescein*</b>	Fab Fragments from sheep	11 207 741 910	200 µg
<b>DAPI</b>	Fluorescence dye for staining of chromosomes	10 236 276 001	10 mg
<b>Digoxigenin-11-dUTP, alkali-stable<sup>∇</sup></b>		11 093 088 910	25 nmol (25 µl)
		11 558 706 910	125 nmol (125 µl)
		11 570 013 910	5 × 125 nmol (5 × 125 µl)
<b>Fluorescein-12-dUTP</b>	Tetralithium salt, 1 mM solution	11 373 242 910	25 nmol (25 µl)
<b>Tetramethyl-rhodamine-5-dUTP</b>	Tetralithium salt, 1 mM solution	11 534 378 910	25 nmol (25 µl)
<b>Biotin-16-dUTP</b>	Tetralithium salt, 1 mM solution	11 093 070 910	50 nmol (50 µl)
<b>DNase I</b>	Lyophilizate	10 104 159 001	100 mg
<b>DNA Polymerase I</b>	Nick Translation Grade	10 104 485 001	500 units
		10 104 493 001	1000 units

\* 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.

<sup>∇</sup> EP Patent 0371262 and US 5,198,537 owned by Roche Diagnostics GmbH.