

## Detection of neuropeptide mRNAs in tissue sections using oligo-nucleotides tailed with fluorescein-12-dUTP or DIG-dUTP

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The protocol given below has been developed with the neuropeptidergic system of the pond snail *Lymnaea stagnalis*. More information concerning the application and *in situ* hybridization methodology can be found in Van Minnen et al. (1989), Dirks et al. (1988), Dirks et al. (1990), and Dirks et al. (1991).

### I. Tissue preparation

- 1 Dissect the tissue, embed in O.C.T. compound (Miles Scientific, USA), and freeze in liquid nitrogen.
- 2 Prepare sections as follows:
  - ▶ Cut cryostat sections of 8  $\mu\text{m}$ .
  - ▶ Mount on poly-L-lysine-coated slides.
  - ▶ Air dry.
- 3 After mounting, do the following:
  - ▶ Fix the sections for 30 min at 4°C with modified Carnoy's [2% formalin, (from 37% stock), 75% ethanol, 23% acetic acid].
  - ▶ Rinse the slides with water.
  - ▶ Dehydrate the sections.


### II. Probe preparation



Synthesize and purify the oligonucleotides according to routine procedures. Tail the oligonucleotide with DIG-dUTP or fluorescein-dUTP according to the procedures given in Chapter 4, but without using dATP.


-  If you use fluorescein-dUTP, add to the labeling mixture equal amounts of unmodified dTTP and fluorescein-dUTP, then carry out the labeling procedure in the dark.

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### III. *In situ* hybridization

 18-mers are used in this study. For oligonucleotides with other lengths and/or composition, you may have to alter the stringency of hybridization by changing the formamide concentration or the hybridization temperature listed below.

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- 1** Perform the hybridization as follows:
    - ▶ Prepare hybridization mixture [25% formamide; 3× SSC; 0.1% polyvinylpyrrolidone; 0.1% ficoll, 1% bovine serum albumin; 500 µg/ml sheared salmon sperm DNA; 500 µg/ml yeast RNA].
      -  1× SSC contains 150 mM sodium chloride, 15 mM sodium citrate; pH 7.0.
    - ▶ To the cryostat sections, add hybridization mixture containing 1 ng/µl labeled probe.
    - ▶ Incubate for 2 h at room temperature.
      -  If the probe contains a fluorochrome label, perform the hybridization incubation in the dark.

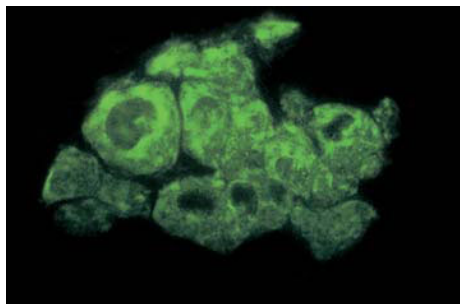
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- 2** After hybridization, do the following:
    - ▶ Rinse the section 3 times in 4× SSC at room temperature.
    - ▶ Dehydrate the sections.
      -  If the probe contains a fluorochrome label, perform the washes in the dark.
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### IV. Hybrid detection

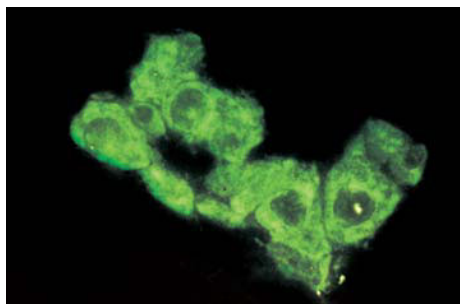
Depending on the label used on the probe, follow one of these procedures:

- ▶ If sections are hybridized with fluorescein-labeled oligonucleotides:
  - ▶ Mount sections in PBS/glycerol (1:9; v/v) containing 2.3% 1,4-diazabicyclo-(2,2,2)-octane (DABCO, from Sigma) and 0.1 µg/µl 4',6'-diamidino-2-phenylindole (DAPI).
  - ▶ Evaluate under a fluorescence microscope.
- ▶ If sections are hybridized with DIG-labeled oligonucleotides:
  - ▶ Make a 1:250 dilution of fluorescein-conjugated anti-DIG antibody (from sheep) in incubation buffer [100 mM Tris-HCl, pH 7.4; 150 mM NaCl; 1% blocking reagent].
  - ▶ Overlay the sections with the diluted antibody in incubation buffer.
  - ▶ Incubate sections at room temperature for 30 min.
  - ▶ Rinse sections 3 × 5 min in Tris-NaCl [100 mM Tris, pH 7.4; 150 mM NaCl].
  - ▶ Mount and evaluate as for fluorescein-labeled oligonucleotides.

## Results



**Figure 1: Direct detection of caudodorsal cells with fluorescein-labeled CDCH-I.**



**Figure 2: Indirect detection of caudodorsal cells with DIG-labeled CDCH-I and sheep anti-DIG-fluorescein conjugate.** The same cells are positive, but the hybridization signal is clearly more intense than that obtained with the direct technique (Figure 1).

## References

Dirks, R. W.; van Gijlswijk, R. P. M.; Vooijs, M. A.; Smit, A. B.; Bogerd, J.; Van Minnen, J.; Raap, A. K.; Van der Ploeg, M. (1991) 3'-end fluorochromized and haptenized oligonucleotides as *in situ* hybridization probes for multiple, simultaneous RNA detection. *Exp. Cell Res.* **194**, 310–315.

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Van Minnen, J.; Van de Haar, Ch.; Raap, A. K.; Vreugdenhil, E. (1988) Localization of ovulation hormone-like neuropeptide in the central nervous system of the snail *Lymnaea stagnalis* by means of immunocytochemistry and *in situ* hybridization. *Cell Tissue Res.* **251**, 477–484.

## Reagents available from Roche Applied Science for this procedure

Reagent	Description	Cat. No.	Pack size
<b>DIG Oligonucleotide Tailing Kit, 2<sup>nd</sup> generation*</b>	For tailing oligonucleotides with digoxigenin-dUTP.	03 353 583 910	1 Kit (25 tailing reactions)
<b>Fluorescein-12-dUTP</b>	Tetralithium salt, solution	11 373 242 910	25 nmol (25 µl)
<b>tRNA</b>	From baker's yeast, lyophilizate	10 109 495 001 10 109 509 001	100 mg 500 mg
<b>Anti-Digoxigenin-Fluorescein*</b>	Fab Fragments from sheep	11 207 741 910	200 µg
<b>Anti-Digoxigenin-Rhodamine*</b>	Fab Fragments from sheep	11 207 750 910	200 µg
<b>DAPI</b>	Fluorescence dye for staining of chromosomes	10 236 276 001	10 mg
<b>Blocking Reagent</b>	Blocking reagent for nucleic acid hybridization	11 096 176 001	50 g

\* 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.