

## Detection of even-skipped transcripts in *Drosophila* embryos with PCR/DIG-labeled DNA probes

Dr. N. Patel and Dr. C. Goodman, Carnegie Institute of Washington, Embryology Department, Baltimore, Maryland, USA.

This protocol has been used to detect the transcript distribution of a number of genes by *in situ* hybridization, including even-skipped and seven-up, in whole mount *Drosophila* embryos, and engrailed Antennapedia in whole mount grasshopper embryos. The *in situ* hybridization and detection was performed essentially according to the protocol of Tautz and Pfeiffle (1989).

This laboratory uses PCR rather than random primed labeling to prepare DIG-labeled probes because:

- ▶ A much larger quantity of probe can be made with the same amount of starting nucleotide.
- ▶ The ratio of labeled DNA to unlabeled starting material is much higher (especially important when transcripts to be detected are not very abundant).
- ▶ PCR can produce strand-specific copies.

### Disadvantage:

- ▶ It is more difficult to control the probe size.

This laboratory has also found that biotin-16-dUTP incorporated in the same way and detected with streptavidin-alkaline phosphatase is about three- to fivefold less sensitive than DIG-labeled probes in *in situ* hybridization experiments.

## I. Probe labeling

- 1 Prepare the following stock solutions:
  - ▶ 10× concentrated reaction mix: 500 mM KCl; 100 mM Tris-HCl, pH 8.3; 15 mM MgCl<sub>2</sub>; 0.01% (w/v) gelatin.
  - ▶ 5× concentrated dNTP mix: 1 mM dATP, 1 mM dCTP, 1 mM dGTP, 0.65 mM dTTP, 0.35 mM digoxigenin-11-dUTP.
  - ▶ A primer stock solution containing
    - either
    - 30 ng/μl (approx. 5.3 mmol) primer 1 (e.g. generated by SP6 RNA polymerase)
    - or
    - 30 ng/μl (approx. 5.3 mmol) primer 2 (e.g. generated by T7 RNA polymerase).
- 2 With a restriction enzyme, linearize “dual promotor vector DNA” containing the insert, as one would to make run-off RNA transcripts.
  - ! The two different primers can be used to create an antisense strand and a sense strand (as control).
- 3 Heat inactivates the restriction enzyme.
- 4 Dilute the linearized DNA with water to a final concentration of about 100–200 ng/μl.
  - ! If the insert is much over 3 kb, the probe produced will probably not represent the entire insert.



- 
- 5** Set up the following reaction mix:
- ▶ 9.25  $\mu$ l water.
  - ▶ 2.5  $\mu$ l 10 $\times$  concentrated reaction mixture.
  - ▶ 5.0  $\mu$ l 5 $\times$  concentrated dNTP mix.
  - ▶ 5.0  $\mu$ l primer 1 or 2 (from 30 ng/ $\mu$ l stock).
  - ▶ 2.0  $\mu$ l linearized DNA (100–200 ng/ $\mu$ l).
- 
- 6** Add 40  $\mu$ l mineral oil, centrifuge, then boil the mix for 5 min.
- 
- 7** To the mix, add 1.25  $\mu$ l of 1 unit/ $\mu$ l Taq DNA Polymerase (1.25 units of Taq Polymerase). [Final volume of reaction mix with Taq is 25  $\mu$ l.]
- 
- 8** Mix the contents of the reaction tube and then centrifuge for 2 min.
- 
- 9** Incubate for 30 cycles in the PCR thermal cycler under the following conditions:
- ▶ Denaturation at 95°C for 45 s.
  - ▶ Annealing at 50°–55°C for 30 s.
    - ! *Annealing temperature depends on length of primer. Use 55°C for a 21-mer.*
  - ▶ Elongation at 72°C for 1.0–1.5 min.
    - ! *Elongation time depends on length of insert. Use 1 min for 1.0 kb or less, 1.5 min for 2.5 kb or more.*
- 
- 10** After the PCR run, add 75  $\mu$ l distilled H<sub>2</sub>O to the reaction tube, then centrifuge it.
- 
- 11** Remove 90–95  $\mu$ l of the reaction mix from beneath the oil.
- 
- 12** To recover the DNA, do an ethanol precipitation as follows:
- ▶ Add NaCl to a final concentration of 0.1 M.
  - ▶ Add 10  $\mu$ g of glycogen or tRNA as a carrier (0.5  $\mu$ l of a 20 mg/ml stock).
  - ▶ Add 3 volumes of 100% EtOH.
  - ▶ Mix well and leave at -70°C for 30 min.
  - ▶ Centrifuge.
  - ▶ Wash pellet with 70% ethanol.
  - ▶ Dry under vacuum.
- Optional:** Perform a second ethanol precipitation as above.
- 
- 13** Resuspend DNA pellet in 300  $\mu$ l of hybridization buffer [50% formamide; 5 $\times$  SSC (where 1 $\times$  SSC contains 150 mM NaCl, 15 mM sodium citrate); 50  $\mu$ g/ml heparin, 0.1% Tween 20; and 100  $\mu$ g/ml sonicated and denatured salmon sperm DNA] (according to Tautz and Pfeifle, 1989).
- 
- 14** To reduce the size of the single-stranded DNA, boil the probe for 40–60 min.
- ! *For efficient penetration of and hybridization to the embryos, the average probe length should be about 50–200 bp.*
- 
- 15** Dilute the probe as much as tenfold before use.
- ! *Optimal dilution varies depending on the abundance of the transcript and background staining. We recommend using the probe either undiluted (original 300  $\mu$ l) or diluted up to threefold for the initial experiment.*
- 





## II. Evaluation of labeling reaction

- 
- 1** Prepare an aliquot of the probe as follows:
    - ▶ Remove 1  $\mu$ l of probe from the reaction mix.
    - ▶ Add 5  $\mu$ l of 5 $\times$  SSC.
    - ▶ Boil 5 min.
    - ▶ Quick cool on ice.
    - ▶ Centrifuge.

---

  - 2** Spot 1–2  $\mu$ l of the probe aliquot onto a small nitrocellulose strip cut to fit into a 1.5 ml microcentrifuge tube or a 5 ml snap cap tube.


---

  - 3** Bake the filter between two sheets of filter paper in an 80°C vacuum oven for 30 min.
    -  *The residual formamide may cause the nitrocellulose to warp. If this is a problem, reduce the time in the baking oven or do this spot test before the second precipitation (Procedure I, Step 12).*
    -  *Unincorporated nucleotide binds only slightly to the nitrocellulose.*

---

  - 4** Treat the baked filter as follows:
    - ▶ Wet the filter with 2 $\times$  SSC.
    - ▶ Wash filter 2  $\times$  5 min in PBT (1 $\times$  PBS, 0.2% BSA, 0.1% Triton X-100).
    - ▶ Place filter into a 1.5 ml microcentrifuge tube or 5 ml snap cap tube.



---

  - 5** Detect the labeled probe as follows:
    - ▶ Block the filter by incubating it 30 min in PBT.
    - ▶ Incubate in PBT for 30–60 min with a 1:2000 dilution (in PBT) of alkaline phosphatase-conjugated anti-DIG antibody.
    - ▶ Wash 4  $\times$  15 min in PBT.
    - ▶ Wash 2  $\times$  5 min in a solution containing 100 mM NaCl; 50 mM MgCl<sub>2</sub>; 100 mM Tris, pH 9.5; 0.1% Tween 20.
    -  *Levamisole is not needed.*

---

  - 6** Develop color with NBT and BCIP as described in the appropriate Roche Applied Science pack insert.

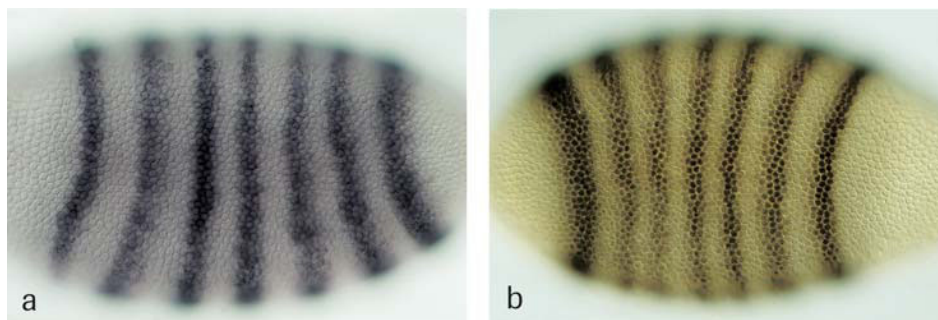
---

  - 7** Stop the reaction when the spots are visible.
    -  *Spots should be visible within a few minutes and dark after 10–15 min.*
- 
- 

### III. Preparation of embryos, hybridization and detection

Perform the preparation of the embryos, subsequent hybridization of the DIG-PCR probe, and immunological detection as described in the Tautz article, "Localization of the expression of the segmentation gene hunchback in *Drosophila* embryos with digoxigenin-labeled DNA probes," page 158?? in this manual.

#### Results



**Figure 1: Detection of the even-skipped phenotype on blastoderm stage *Drosophila* embryos.** Panel a shows an *in situ* hybridization using the *Drosophila* even-skipped gene as probe. Panel b demonstrates the even-skipped product using the specific antibody as probe. The seven stripe pattern is associated with the even-skipped phenotype.

#### Reference

Tautz, D.; Pfeiffle, C. (1989) A nonradioactive *in situ* hybridization method for the localization of specific RNAs in *Drosophila* embryos reveals a translational control of the segmentation gene hunchback. *Chromosoma (Berl.)* **98**, 81-85.

#### Reagents available from Roche Applied Science for this procedure

Reagent	Description	Cat. No.	Pack size
<b>PCR DIG Probe Synthesis Kit*</b>	For generating highly sensitive probes labeled with DIG-dUTP (alkali-labile) in the polymerase chain reaction (PCR) using a ratio of 1 + 2 (DIG-dUTP:dTTP)	11 636 090 910	1 Kit (25 reactions)
<b>Tween 20</b>		11 332 465 001	5 × 10 ml
<b>Triton X-100</b>	Vicous, liquid	10 789 704 001	100 ml
<b>Anti-Digoxigenin-AP*</b>	750 units/ml Anti-Digoxigenin, Fab fragments conjugated to alkaline phosphatase	11 093 274 910	150 U (200 µl)
<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.