V. RNA labeling by *in vitro* transcription of DNA with DIG, Biotin or Fluorescein RNA Labeling Mix

The DNA to be transcribed should be cloned into the polylinker site of a transcription vector which contains a promoter for SP6, T7, or T3 RNA Polymerase (Dunn and Studier, 1983; Kassavetis, 1982). To synthesize "run-off" transcripts, use a restriction enzyme that creates a 5'-overhang to linearize the template before transcription. Alternatively, use the circular vector DNA as template to create "run-around" transcripts.

A PCR fragment that has the appropriate promoter ligated to its 5'-ends can also serve as a transcription template.

The procedure described here incorporates one modified nucleotide (DIG-, Biotin-, or Fluorescein-UTP) at approximately every 20 – 25th position in the transcripts. Since the nucleotide concentration does not become limiting in the following reaction, 1 µg linear plasmid DNA (with a 1 kb insert) can produce approximately 10 µg of full-length labeled RNA transcript in a 2 h incubation. Larger amounts of labeled RNA can be synthesized by scaling up the reaction components.

! *The amount of newly synthesized labeled RNA depends on the amount, size, site of linearization, and purity of the template DNA.*

1 Purify template DNA in one of the following ways:
- Use phenol/chloroform extraction and ethanol precipitation to purify *linearized plasmid* DNA after the linearizing restriction digestion. Resuspend the pellet in 10 mM Tris-HCl, pH 8.0.
- Ethanol precipitate *circular plasmid* DNA and resuspend it in 10 mM Tris-HCl, pH 8.0.
- Use acrylamide gel electrophoresis and elution (in 10 mM Tris-HCl, pH 8.0) to purify a PCR *fragment* which has an RNA polymerase promoter ligated to its 5'-ends.

2 Add the following to a 1.5 ml microcentrifuge tube on ice:
- 1 µg purified, linearized plasmid DNA *or* 1 µg purified, circular plasmid DNA *or* 100 – 200 ng purified PCR fragment.
- 2 µl of *either* 10× concentrated DIG RNA Labeling Mix *or* 10× concentrated Biotin RNA Labeling Mix *or* 10× concentrated Fluorescein RNA Labeling Mix.

  ! *Concentrated RNA Labeling Mix contains 10 mM each of ATP, CTP, and GTP; 6.5 mM UTP; 3.5 mM X-UTP (X = DIG, Biotin or Fluorescein); pH 7.5 (20°C)].*
- 2 µl 10× concentrated Transcription Buffer [400 mM Tris-HCl (pH 8.0, 20°C), 60 mM $MgCl_2$, 100 mM Dithiothreitol (DTT), 20 mM spermidine].

  ! *10x concentrated Transcription Buffer is supplied with SP6, T3, or T7 RNA Polymerase.*
- 2 µl RNA Polymerase (SP6, T7, or T3).
- Enough sterile, redistilled water to make a total reaction volume of 20 µl.

3 Mix the components and centrifuge the tube briefly.

4 Incubate the tube for 2 h at 37°C.

! *Longer incubations do not increase the yield of labeled RNA. To produce larger amounts of RNA, scale up the reaction components.*

5 Do one of the following:

- **Optional:** *If* you want to remove the template DNA, add 2 U DNase I, RNase-free to the tube and incubate for 15 min at 37°C. Then, go to Step 6.
- *If* you do not want to remove the template DNA, go to Step 6.

  Since the amount of labeled RNA transcript is far in excess of the template DNA (by a factor of approx. 10), it is usually not necessary to remove the template DNA by DNase treatment before an in situ hybridization experiment.

6 Add 2 µl 0.2 M EDTA (pH 8.0) to the tube to stop the polymerase reaction.

7 **Optional:** Precipitate the labeled RNA transcript by performing the following steps.

As an alternative to the ethanol procedure below you may purify labeled probes which are 100 bp or longer with the High Pure PCR Purification Kit. See the procedure on page 47 in this chapter.

- To the reaction tube, add 2.5 µl 4 M LiCl and 75 µl prechilled (-15 to -25°C) 100% ethanol. Mix well.
- Let the precipitate form for at least 30 min at -70°C or 2 h at -15 to -25°C.
- Centrifuge the tube (at 13,000 × *g*) for 15 min at 2–8°C.
- Discard the supernatant.
- Wash the pellet with 50 µl ice-cold 70% (v/v) ethanol.
- Centrifuge the tube (at 13,000 × *g*) for 5 min at 2–8°C.
- Discard the supernatant.
- Dry the pellet under vacuum.
- Dissolve the RNA pellet for 30 min at 37°C in 100 µl DEPC (diethylpyrocarbonate)-treated (or sterile) double dist. water.

8 To estimate the yield of the transcript, do the following:

- Run an aliquot of the transcript on an agarose or acrylamide gel beside an RNA standard of known concentration.
- Stain with ethidium bromide.
- Compare the relative intensity of staining between the labeled transcripts and the known standard.

9 Do one of the following:

- *If* you are not going to use the labeled probe immediately, store the probe solution at -70°C.

  Avoid repeated freezing and thawing of the probe.

- *If* you are going to use the probe immediately, dilute an aliquot of the probe solution to its working concentration (*e.g.*, 0.2 – 10 ng/µl) in the hybridization buffer to be used for the *in situ* experiment (as described in Chapter 5 of this manual).

Regulation of RNA Probe Length by Alkaline Hydrolysis

Some applications require shorter RNA probes than other techniques. When performing *in situ* hybridizations, probes must be short enough to allow diffusion into and out of the tissue. Alkaline hydrolysis allows you to regulate the size of RNA probes.

Procedure

The following procedure is a modification of the protocol regulating the size of RNA probes by alkaline hydrolysis described by Cox, et al. (1984, Develop. Biol. 101, 485 – 502). This protocol was adapted for use with DIG-UTP-labeled RNA probes.

1. Hydolyze 1 µg RNA by adding an equal volume of DMPC-treated H_2O and two volumes of carbonate buffer. Incubate for 10 – 60 min at +60°C.
   Calculate the incubation time of according to the formula below, however the optimal incubation time must be determined empirically. We have found that hydrolysis starts as early as 30 s after the addition of the carbonate buffer.
2. Add an equal volume of hydolysis-neutralization buffer to stop the hydolysis.
3. Add 3 volumes of chilled ethanol to precipitate the RNA. Mix well and incubate at -70°C for 30 min.
4. Centrifuge at 13,000 × *g* for 15 min at +4°C in a microcentrifuge.
5. Decant the ethanol, and wash the pellet with 100 µl of cold 70% ethanol. Centrifuge at 13,000 × *g* for 5 min at +4°C in the microcentrifuge, then remove the 70% ethanol.
6. Dry the pellet and resuspend in 100 µl DMPC-treated H_2O. If not used immediatly, store the probe at -70°C.
7. Check the resulting probe length by electrophoresis of 10 µl hydrolyzed RNA on a 1% ethidium bromide-stained agarose gel.

Additionally required solution	Description
DMPC-treated H_2O	sterile, distilled water treated with 0.1% dimethylpyrocarbonate
Carbonate buffer	60 mM Na_2CO_3; 40 mM $NaHCO_3$; pH 10.2
Hydrolysis-neutralization buffer	3 M sodium acetate; 1% (v/v) acetic acid; pH 6.0
Ethanol	Absolute ethanol, chilled at -20°C; when 70% ethanol is indicated, dilute the ethanol with DMPC-treated water.

Limited alkaline hydrolysis (Optional)

Calculate the incubation time as follows:

$$t = \frac{L_O - L_f}{k \times L_O \times L_f}$$

L_o = initial lenght of transcript (in kb)

L_f = desired probe lenght (in kb)

k = constant = 0.11 kb/min

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Reagents available from Roche Applied Science for this procedure

Reagent	Description	Available as
DIG RNA Labeling Kit (SP6, T7)*	Kit for 2 × 10 labeling reactions	Cat. No. 11 175 025 910
DIG RNA Labeling Mix*	10 mM ATP, 10 mM CTP, 10 mM GTP, 6.5 mM UTP, 3.5 mM DIG-UTP; in Tris-HCl, pH 7.5 (+20°C)	▶ Vial 7, DIG RNA Labeling Kit (SP6/T7) (Cat. No. 11 175 025 910) ▶ DIG RNA Labeling Mix (Cat. No. 11 277 073 910)
or One of the following		
Biotin RNA Labeling Mix	10 mM ATP, 10 mM CTP, 10 mM GTP, 6.5 mM UTP, 3.5 mM biotin-16-UTP; in Tris-HCl, pH 7.5 (+20°C)	Cat. No. 11 685 597 910
Fluorescein RNA Labeling Mix	10 mM ATP, 10 mM CTP, 10 mM GTP, 6.5 mM UTP, 3.5 mM fluorescein-12-UTP	Cat. No. 11 685 619 910
10× Transcription buffer	400 mM Tris-HCl, pH 8.0; 60 mM $MgCl_2$, 100 mM dithioerythritol (DTE), 20 mM spermidine, 100 mM NaCl, 1 U/ml RNase inhibitor	▶ Vial 8, DIG RNA Labeling Kit (SP6/T7) (Cat. No. 11 175 025 910) ▶ Supplied with the RNA Polymerase
DNase I, RNase-free	10 U/µl DNase I, RNase-free	▶ Vial 9, DIG RNA Labeling Kit (SP6/T7) (Cat. No. 11 175 025 910)
Protector RNase Inhibitor	2.000 U 10.000 U	Cat. No. 03 335 399 001 Cat. No. 03 335 402 001
One of the following		
SP6 RNA Polymerase	20 U/µl SP6 RNA Polymerase	▶ Vial 11, DIG RNA Labeling Kit (SP6/T7) (Cat. No. 11 175 025 910) ▶ SP6 RNA Polymerase (Cat. Nos. 10 810 274 001, 11 487 671 001)
T7 RNA Polymerase	20 U/µl T7 RNA Polymerase	▶ Vial 12, DIG RNA Labeling Kit (SP6/T7) (Cat. No. 11 175 025 910) ▶ T7 RNA Polymerase (Cat. Nos. 10 881 767 001, 10 881 775 001)
T3 RNA	20 U/µl T3 RNA Polymerase	▶ T3 RNA Polymerase (Cat. Nos. 11 031 163 001, 11 031 171 001)

* 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.