



III. Nick-translation labeling of ds DNA with Nick Translation Mixes for *in situ* Probes

The nick translation procedure was originally described by Rigby et al. (1977) and used for incorporating nucleotide analogs by Langer et al. (1981). The procedure described here incorporates one modified nucleotide (DIG-, biotin-, fluorescein-, AMCA-, or tetramethylrhodamine-dUTP) at approximately every 20–25th position in the newly synthesized DNA. This labeling density allows optimal enzymatic incorporation of the modified nucleotide and produces the most sensitive targets for indirect (immunological) detection.

For *in situ* hybridization procedures, the length of the labeled fragments obtained from this procedure should be about 200–500 bases.

 DNA does not need to be denatured before it is labeled by nick translation.

A. Labeling reaction with DIG-dUTP or Biotin-dUTP

- 1 Place a 1.5 ml microcentrifuge tube on ice and add to the tube:
 - ▶ 16 μ l sterile double dist. water containing 1 μ g template DNA (either linear or supercoiled).
 - ▶ 4 μ l of *either* DIG-Nick Translation Mix for *in situ* Probes or Biotin-Nick Translation Mix for *in situ* Probe.
 -  Each Nick Translation Mix contains 5 \times concentrated reaction buffer; 50% glycerol; DNA Polymerase I; DNase I; 0.25 mM each of dATP, dCTP, and dGTP; 0.17 mM dTTP; and 0.08 mM X-dUTP (X = DIG or biotin).
- 2 Mix ingredients and centrifuge tube briefly.
- 3 Incubate at 15°C for 90 min.
- 4 Chill the reaction tube to 0°C.
- 5 Take a 3 μ l aliquot from the tube and analyze it as follows:
 - ▶ Mix the aliquot with enough gel loading buffer to make a sample which will fit in one well of an agarose minigel.
 - ▶ Denature the sample (DNA aliquot + gel loading buffer) for 3 min at 95°C.
 - ▶ Place the denatured sample on ice for 3 min.
 - ▶ Run the sample on an agarose minigel with a DNA molecular weight marker.
- 6 Depending on the average size of the probe, do one of the following:
 - ▶ If the probe is between 200 and 500 nucleotides long, go to Step 7.
 - ▶ If the probe is longer than 500 nucleotides, incubate the reaction tube further at 15°C until the fragments are the proper size.
 -  If the fragment is too long, the labeled probe can also be sonicated to the proper size.
- 7 Stop the reaction as follows:
 - ▶ Add 1 μ l 0.5 M EDTA (pH 8.0) to the tube.
 - ▶ Heat the tube to 65°C for 10 min.


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B. Labeling reaction with Fluorescein-dUTP, or Tetramethylrhodamine-dUTP

- 1 Prepare 50 μ l of a 5x fluorophore labeling mixture (enough for about 12 labeling reactions) by mixing the following in a 1.5 ml microcentrifuge tube on ice:
 - ▶ 5 μ l 2.5 mM dATP.
 - ▶ 5 μ l 2.5 mM dCTP.
 - ▶ 5 μ l 2.5 mM dGTP.
 - ▶ 3.4 μ l 2.5 mM dTTP.
 - ▶ 4 μ l of either 1 mM Fluorescein-dUTP or 1 mM Tetramethylrhodamine-dUTP.
 - ▶ 27.6 μ l sterile double dist. water.


- 2 Place a 1.5 ml microcentrifuge tube on ice and add to the tube:
 - ▶ 12 μ l sterile double dist. water containing 1 μ g template DNA (either linear or supercoiled).
 - ▶ 4 μ l 5x concentrated fluorophore labeling mixture (from Step 1).
 - ▶ 4 μ l of Nick Translation Mix for *in situ* Probes.

 *Nick Translation Mix contains 5x concentrated reaction buffer; 50% glycerol; DNA Polymerase I; and DNase I.*


- 3 Mix, incubate and stop the reaction as in Steps 2–7 for DIG- and biotin-labeling above.

C. Purification of labeled probe (optional)

- 1 Precipitate the labeled probe (from either procedure above) by performing the following steps. *As alternative to the ethanol precipitation procedure below, you may purify labeled probes which are 100 bp or longer with the High Pure PCR Product Purification Kit. See the procedure on page 64 in this chapter.*
 - ▶ To the labeled DNA, add 2.5 μ l 4 M LiCl and 75 μ l 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 $\times g$) for 15 min at 2–8°C.
 - ▶ Discard the supernatant.
 - ▶ Wash the pellet with 50 μ l ice-cold 70% (v/v) ethanol.
 - ▶ Centrifuge the tube (at 13,000 $\times g$) for 5 min at 2–8°C.
 - ▶ Discard the supernatant.
 - ▶ Dry the pellet under vacuum.

 *Drying the pellet is important because small traces of residual ethanol will cause precipitation if the hybridization mixture contains dextran sulfate. Trace ethanol can also lead to serious background problems.*

- 2 Do one of the following:
 - ▶ *If you are not going to use the probe immediately, dissolve the pellet in a minimal amount of TE (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) buffer and store the probe solution at -15 to -25°C.*

 *Avoid repeated freezing and thawing of the probe.*
 - ▶ *If you are going to use the probe immediately, dissolve the pellet in a minimal amount of an appropriate buffer (TE, sodium phosphate, etc.), then dilute the probe solution to a convenient stock concentration (e.g., 10–40 ng/ μ l) in the hybridization buffer to be used for the *in situ* experiment (as described in Chapters 2 and 5 of this manual).*

Reagents available from Roche Applied Science for this procedure

Reagent	Description	Cat. No.	Pack size
DIG-Nick Translation Mix*	5× conc. stabilized reaction buffer in 50% glycerol (v/v) and DNA Polymerase I, DNase I, 0.25 mM dATP, 0.25 mM dCTP, 0.25 mM dGTP, 0.17 mM dTTP and 0.08 mM alkali-stable DIG-11-dUTP.	11 745 816 910	160 µl (40 labeling reactions)
Biotin-Nick Translation Mix*	5× conc. stabilized reaction buffer in 50% glycerol (v/v) and DNA Polymerase I, DNase I, 0.25 mM dATP, 0.25 mM dCTP, 0.25 mM dGTP, 0.17 mM dTTP and 0.08 mM biotin-16-dUTP.	11 745 824 910	160 µl (40 labeling reactions)
Nick Translation Mix*	5× conc. stabilized reaction buffer in 50% glycerol, DNA Polymerase I and DNase I.	11 745 808 910	200 µl (50 labeling reactions)
dNTP Set	Set of dATP, dCTP, dGTP, dTTP 100 mM solutions, lithium salts.	11 277 049 001	4 × 10 µmol (100 µl)
Fluorescein-12-dUTP	Tetralithium salt, 1 mM solution	11 373 242 910	25 nmol (25 µl)
Tetramethyl-rhodamine-5-dUTP	Tetralithium salt, 1 mM solution	11 534 378 910	25 nmol (25 µl)

* 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.

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IV. Nick-translation labeling of ds DNA with DIG-, Biotin-, or Fluorochrome-labeled dUTP

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For *in situ* hybridization procedures, the length of the labeled fragments obtained from this procedure should be about 200 – 400 bases.

- 1** Place a 1.5 ml microcentrifuge tube on ice and add to the tube:
 - ▶ 27 µl sterile, double dist. water.
 - ▶ 5 µl 10× concentrated nick translation buffer [500 mM Tris-HCl (pH 7.8), 50 mM MgCl₂, 0.5 mg/ml Bovine Serum Albumin (nuclease-free)].
 - ▶ 5 µl 100 mM Dithiothreitol.
 - ▶ 4 µl Nucleotide Mixture (0.5 mM each of dATP, dGTP, and dCTP; 0.1 mM dTTP).
 - ▶ 2 µl 1 mM DIG-dUTP or 1 mM Biotin-dUTP or 1 mM fluorochrome-labeled dUTP.
 - ▶ 1 µg template DNA.
 - ▶ 5 µl (5 ng) DNase I.
 - ▶ 1 µl (10 U) DNA Polymerase I.
- 2** Mix ingredients and centrifuge tube briefly.
- 3** Incubate at 15°C for 2 h.
- 4** Chill the reaction tube to 0°C.

