Reagents available from Roche Applied Science for this procedure

Reagent	Description	Cat. No.	Pack size
DIG-High Prime*,‡	5× solution with: 1 mM dATP, dCTP, dGTP (each); 0.65 mM dTTP; 0.35 mM DIG-11-dUTP, alkali-labile; random primer mixture; 1 U/µl Klenow enzyme, labeling grade, in reaction buffer, 50% glycerol (v/v).	11 585 606 910	160 µl (40 reactions)
Biotin-High Prime‡	5× solution with: 1 mM dATP, dCTP, dGTP (each); 0.65 mM dTTP; 0.35 mM biotin-16-dUTP; random primer mixture; 1 U/µl Klenow enzyme, labeling grade, in reaction buffer, 50% glycerol (v/v).	11 585 649 910	100 µl (25 reactions)
Fluorescein-High Prime‡	5× solution with: 1 mM dATP, dCTP, dGTP (each); 0.65 mM dTTP; 0.35 mM fluorescein-12-dUTP; random primer mixture; 1 U/µl Klenow enzyme, labeling grade, in reaction buffer, 50% glycerol (v/v).	11 585 622 910	100 µl (25 reactions)

‡ This product or the use of this product may be covered by one or more patents owned by Roche Diagnostics GmbH, including the following: US patent 5.814.502.
* 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.

4

II. PCR labeling of ds DNA with the PCR DIG Probe Synthesis Kit or PCR Labeling Mixes

The procedures given here are necessarily generalized. Optimal PCR reaction conditions are very dependent on the sequence of the template DNA and the primer. The optimal concentrations of template, primer, Mg^{2+} ion, and polymerase, as well as the optimal incubation times and temperatures should be determined empirically for each new primer/template combination (Innis et al., 1990, Rolfs, A. et al., 1992).

The three procedures in this section use PCR to produce probes that are directly labeled with digoxigenin (DIG) or fluorescein. The procedures will be especially useful for producing highly labeled probes from limited amounts of template DNA. Each procedure takes advantage of a premixed labeling solution or kit that has been optimized for the production of certain types of probes. They are:

- **PCR DIG Probe Synthesis Kit**, which contains a 2 + 1 ratio of dTTP:DIG-dUTP and is ideal for generating highly labeled hybridization probes containing unique sequences. Such probes can detect target sequences present at a low copy number in complex genomes.
- **PCR DIG Labeling Mix**, which contains a 19 + 1 ratio of dTTP:DIG-dUTP and is ideal for generating moderately labeled hybridization probes containing repetitive elements. Such probes can detect target sequences (*e.g.*, human alphoid sequences) which are present at a high copy number in complex genomes.
- **PCR Fluorescein Labeling Mix**, which contains a 3 + 1 ratio of dTTP:fluorescein-dUTP and produces optimally labeled hybridization probes suitable for direct *in situ* detection experiments.

A. PCR DIG labeling reaction for highly labeled probes containing unique sequences

The following labeling reaction produces 50 µl of DIG-labeled probe solution. In a control experiment, 50 µl of DIG-labeled probe was enough to perform 25 hybridization reactions.

To produce less probe, scale the reaction volume and components down proportionally.

1 Place a sterile microcentrifuge tube on ice and, for each PCR, add to the tube:

- 5 µl 10× concentrated PCR buffer with 15 mM $MgCl_2$ (vial 3).
  - Numbered vials are included in the PCR DIG Probe Synthesis Kit.
- 5 µl 10× concentrated PCR DIG probe synthesis mix, containing a 2:1 ratio of dTTP:DIG-dUTP (2 mM each of dATP, dCTP, and dGTP; 1.3 mM dTTP; 0.7 mM DIG-dUTP, alkali-labile; pH 7.0) (vial 2).
- Upstream primer 1–10 µM solution (0.1–1 µM final conc.).
- Downstraeam primer 1–10 µM solution (0.1–1 µM final conc.).
  - *The concentration of PCR primers must be determined empirically (Innis et al.; 1990). Initially, try a 0.3 mM concentration of each primer in the reaction mixture.*
- Template DNA:
  Plasmid DNA 10–100 pg (optimal amount, 10 pg).
  Genomic DNA 1–50 ng (optimal amount, 10 ng).
- 0.75 µl (2.6 U) Enzyme mix,Expand High Fidelity (vial 1).
- Add sterile double dist. water to a final reaction volume of 50 µl.

2 Mix reagents and centrifuge briefly to collect the sample at the bottom of the tube.

3 Overlay with 100 µl mineral oil to reduce evaporation of the mix during amplification.

If your thermal cycler has a top heater, the oil overlay is not necessary.

4 Place samples in a thermal cycler and start PCR.

Cycling conditions depend on the combination of template, primers and thermal cycler. The conditions below may not be optimal for your template/primer combination, but are a good starting point for initial experiments.

Use the following thermal profile:
Initial denaturation 2 min at 95°C, 30 s

1–10 cycles of:

Denaturation,	95°C, 30 s
Annealing,	60°C, 30 s
Elongation,	72°C, 40 s

11–33 cycles of:

Denaturation,	95°C, 30 s
Annealing,	60°C, 30 s
Elongation,	72°C, 40 s + additional 20 s for each successive cycle

The increased elongation time is only required for long (3 kb) fragments. For amplification of shorter fragments, continue to use the 40 s elongation time for all 30 cycles.

Final elongation step, 72°C, 7 min

Hold, 4°C

5 Check PCR labeleed probe via agarose gel (load an aliquot, *e.g.*, 5 µl) and compare to PCR product without DIG label.

6 The PCR labeled probe should be stored:

- Short term, at 2–8°C until the PCR product is used for hybridization
- Long term, at -15 to -25°C, stable for at least one year.

7 **Optional:** Precipitate the labeled probe by performing the following steps:

As an 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 65 in this chapter.

- To the labeled DNA, add 5 µl 4 M LiCl and 150 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 100 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.

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.

- Dissolve the pellet in 50 µl TE (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) buffer.

If the hybridization buffer (used in Step 7 below) contains a high percentage formamide, dissolve the probe pellet in a smaller amount of TE buffer to form a more concentrated probe stock solution.

8 Do one of the following:

- *If* you are not going to use the probe immediately, 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, dilute an aliquot of the probe solution in the hybridization buffer to be used for the *in situ* experiment (as described in Chapters 2 and 5 of this manual).

The amount of probe solution to use in the hybridization reaction must be determined empirically. Initially, try using 2 µl of probe solution (out of the original 50 µl total) in 20 µl hybridization solution for each hybridization reaction (under a 24 × 24 mm coverslip). If the probe was dissolved in <50 µl TE (in Step 6 above), add correspondingly less of the concentrated probe stock to the hybridization buffer.

The DIG-dUTP used in this labeling reaction is alkali-labile. Avoid exposing the probe to strong alkali (e.g., 0.2 M NaOH). Standard in situ procedures should not detach the DIG label from the probe.

B. PCR DIG labeling reaction for moderately labeled probes

This labeling reaction produces 100 µl of DIG-labeled probe solution. In a control experiment, 100 µl of DIG-labeled probe was enough to perform 20 – 50 indirect *in situ* detections of repetitive (human alphoid) sequences in metaphase chromosomes. To produce less probe, scale the reaction volume and components down proportionally.

1 Briefly centrifuge all reagents before starting.

2 Prepare two mixes of reagents in sterile microcentrifuge tubes (on ice):

MIX 1 (final volume 25 µl)

- PCR DIG Labeling Mix 10×, containing a 19:1 ratio of dTTP:DIG-dUTP (2 mM each)
- Upstream primer 0.1 – 0.6 µM
- Dowmstream Primer 0.1 – 0.6 µM
- Template DNA:
  Plasmid DNA 0.1 ng – 15 ng.
  Human genomic DNA 10 – 250 ng.

  The optional buffer for template DNA is either sterile double dist. water or 5 – 10 mM Tris (ph 7 – 8). Avoid TE -buffer, because EDTA chelates Mg^{2+}.

MIX 2 (final volume 25 µl)

- 19.75 µl sterile double dist.water
- 5 µl PCR reaction buffer 10×, with 15 mM $MgCl_2$ (delivered with the Taq DNA Polymerase)
- 0.25 µl Taq DNA Polymerase

3 Combine Mix 1 and Mix 2 in a thin-walled PCR tube (on ice).

Gently vortex the mixture to produce a homogeneous reaction, then centrifuge briefly to collect the sample at the bottom of the tube.

Continue to thermal cycling immediatly.

Carefull overlay the reaction with mineral oil if required by your type of thermal cycler.

4 Thermal cycling

Place samples in the thermal cycler and start cycling using the thermal profiles mentioned in the Taq DNA Polymerase pack insert (Cat. No. 1 146 165 001) or also available at http://www.roche-applied-science.com/pack-insert/1146165a.pdf .

5 **Optional:** Precipitate the labeled probe by performing the following steps:

As an 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 10 µl 4 M LiCl and 300 µl prechilled (-15 to -25°C) 1 00% 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 100 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.

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.

- Dissolve the pellet in 100 µl TE (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) buffer.

If the hybridization buffer (used in Step 6 below) contains a high percentage formamide, dissolve the probe pellet in a smaller amount of TE buffer to form a more concentrated probe stock solution.

6 Do one of the following:

- *If* you are not going to use the probe immediately, store the probe solution at -15 to -25°C for at least 1 year.

Avoid repeated freezing and thawing of the probe.

- *If* you are going to use the probe immediately, dilute an aliquot of the probe solution in the hybridization buffer to be used for the *in situ* experiment (as described in Chapters 2 and 5 of this manual).

The amount of probe solution to use in the hybridization reaction must be determined empirically. Initially, try using 2 – 5 µl of probe solution (out of the original 100 µl total) in 20 µl hybridization solution for each hybridization reaction (under a 24 × 24 mm coverslip). If the probe was dissolved in <100 µl TE (in Step 3 above), add correspondingly less of the concentrated probe stock to the hybridization buffer.

C. PCR fluorescein labeling reaction for direct *in situ* probes

This labeling reaction produces 100 µl of fluorescein-labeled probe solution. In a control experiment, 100 µl of fluorescein-labeled probe was enough to perform 20 – 50 direct *in situ* detections of repetitive (human alphoid) sequences in metaphase chromosomes. To produce less probe, scale the reaction volume and components down proportionally.

1 Add the following components to a sterile 1.5 ml microcentrifuge tube on ice:

- 10 µl 10× concentrated PCR buffer without $MgCl_2$ (100 mM Tris-HCl, 500 mM KCl, pH 8.3) (vial 2).
  Numbered vials are included with the PCR Fluorescein Labeling Mix.
- 12 – 20 µl 25 mM $MgCl_2$ (vial 3).
  The concentration of $MgCl_2$ must be determined empirically. We use 4 mM $MgCl_2$ (16 µl vial 3) as our standard concentration.
- 10 µl 10× concentrated PCR Fluorescein Labeling Mix (2 mM each of dATP, dCTP, and dGTP; 1.5 mM dTTP; 0.5 mM fluorescein-dUTP; pH 7.0) (vial 1).
- 0.1 – 1.0 µM PCR primer 1.
- 0.1 – 1.0 µM PCR primer 2.
  The concentration of PCR primers must be determined empirically (Innis et al., 1990). Initially, try a 0.3 mM concentration of each primer in the reaction mixture.
- Template DNA (1 – 100 ng human genomic DNA or 10 – 100 pg plasmid DNA).
  The amount of template DNA must be determined empirically (Innis et al., 1990). Initially, try 50 ng human genomic DNA or 50 pg plasmid DNA.
- 1 – 5 U Taq DNA Polymerase.
  The amount of polymerase must be determined empirically. As a starting amount, try 2.5 units.
- Add sterile, double dist. water to a total volume of 100 µl.

2 Mix the reagents, overlay with oil, and perform PCR exactly as in Steps 2 – 5 of Procedure IIA above (for labeling of probes containing unique sequences).

3 **Optional:** Precipitate the labeled probe by performing the following steps:

As an 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 65 in this chapter.

- To the labeled DNA, add 10 µl 4 M LiCl and 300 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 100 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.

  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.
- Dissolve the pellet in 100 µl TE (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) buffer.
  If the hybridization buffer (used in Step 4 below) contains a high percentage formamide, dissolve the probe pellet in a smaller amount of TE buffer to form a more concentrated probe stock solution.

4 Do one of the following:

- *If* you are not going to use the probe immediately, 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, dilute an aliquot of the probe solution in the hybridization buffer to be used for the *in situ* experiment (as described in Chapters 2 and 5 of this manual).

The amount of probe solution to use in the hybridization reaction must be determined empirically. Initially, try using 2–5 µl of probe solution (out of the original 100 µl total) in 20 µl hybridization solution for each hybridization reaction (under a 24 × 24 mm coverslip). If the probe was dissolved in <100 µl TE (in Step 3 above), add correspondingly less of the concentrated probe stock to the hybridization buffer.

Reagents available from Roche Applied Science for these procedures

Reagent	Description	Cat. No.	Pack size
PCR DIG Probe Synthesis Kit*	Kit for 25 labeling reactions which incorporate alkali-labile DIG-dUTP by PCR	11 636 090 910	1 kit (25 reactions)
PCR DIG Labeling Mix*	dATP, dCTP, dGTP, 2 mM each; dTTP 1.9 mM; alkali-stable DIG-dUTP, 0.1 mM; pH 7.0	11 585 550 910	500 µl (for 2 × 25 PCR reactions)
PCR Fluorescein Labeling Mix (for rapid and reliable synthesis of fluorescein-dUTP labeled probes that are particular useful for fluorescence in situ hybridization [FISH])	Contents: dATP, dCTP, dGTP 2 mM each; dTTP, 1.5 mM; fluorescein-dUTP, 0.5 mM; premixed in 100 µl water; ph 7.0 10× PCR buffer without $MgCl_2$ 25 mM $MgCl_2$ stock solution	11 636 154 910	for 10 PCR reactions
Taq DNA Polymerase, 5 U/µl#	PCR buffer included	11 146 165 001 11 146 173 001 11 418 432 001 11 596 594 001 11 435 094 001	100 units 500 units 4 × 250 units 10 × 250 units 20 × 250 units
Taq DNA Polymerase 1 U/µl#	PCR buffer included	11 647 679 001 11 647 687 001	250 units 4 × 250 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.

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