thermocycler. The following reaction cycles were found to be good using a Perkin-Elmer 9600, but should be altered to suit respective template, primers, and thermocycler.

Hold at	95°C for 1 min
5 cycles at	94°C for 1 min
	55°C for 30 s
	72°C for 30 s
25 cycles at	94°C for 30 s
	55°C for 30 s
	72°C for 30 s
Hold at	72°C for 5 min

5. If an aliquot of the reaction mixture is analyzed by agarose gel electrophoresis after amplification, the mol wt of the PCR product will be found to have increased significantly because of incorporation of DIG-11-dUTP, compared to the unlabeled product. The concentration of the probe should be determined as described in **Subheading 3.3.** and then stored at 4°C or –20°C (*see* **Note 4**).

3.2. Labeling Probes by Random Priming

The use of a Boehringer DIG-High Prime (random priming) kit is recommended. *See* **Note 5** for discussion some theoretical aspects.

1. Resuspend 1 µg template DNA in 16 µL of sterile water.
2. Denature the DNA thoroughly by boiling the template for 10 min and chilling on an ice–salt-water mix until cool.
3. Add 4 µL of DIG High Prime, mix thoroughly, then centrifuge briefly.
4. Incubate overnight at 37°C.
5. Stop the reaction by adding 2 µL 0.2*M* EDTA, pH 8.0.
6. Determine the concentration of the probe as described in **Subheading 3.3.** and store at 4°C or –20°C (*see* **Note 4**).

3.3. Estimating Concentration of Labeled Probe

1. Make 10-fold serial dilutions of DIG-labeled control DNA in TE buffer from 1 ng/µL to 0.01 pg/µL (equivalent to dilutions of 1:20:1:2,000,000 of the original DNA). Spot 1 µL of each dilution onto a positively charged nylon membrane, approx 60 × 30 mm.
2. Prepare a similar dilution series of the labeled probe, direct from the PCR product, and spot 1 µL of each dilution onto the membrane.
3. Bind the DNA to the membrane by exposing to UV or by heating (*see* **Note 2**).
4. Equilibrate the membrane for 1 min in wash buffer.
5. Incubate membrane at 37°C for 30 min in 50 mL of buffer 2 in a dish.
6. Dilute anti–DIG-AP, Fab fragments to 75 mU/mL (1:10,000) in buffer 2. Diluted antibody solutions are stable for 12 h at 4°C. A precipitate can form in the antibody solution during storage, this should be removed by brief centrifugation before use. Failure to do this can result in background spots on the membrane.