3. PCR can be used to prepare DIG-labeled DNA probes. DNA probes are efficiently labeled with DIG-11-dUTP using PCR, because small amounts of DNA (100 ng–1 μg) can be directly amplified and labeled in a single reaction. Although the PCR could be done directly on intact plasmid DNA, Boehringer have recommended that insert be restriction-digested from vector sequences, followed by phenol:chloroform extraction and ethanol precipitation before labeling. We have found this improves the probe sensitivity, compared to using undigested plasmid.

   The optimal PCR conditions have to be adapted and optimized to each template–primer combination. The concentrations of $MgCl_2$, enzyme, template, and primers, and the thermocycler reaction times we have given may not suit all situations. It is possible and convenient to use reduced PCR volumes (typically 50 μL) when determining optimal conditions for making a new probe.
4. Probes in regular use can be stored at 4°C, or at –20°C, if intended for long-term storage.
5. Random priming is a fast and efficient method for preparation of probes and is well-suited for labeling relatively long probes. However, there may be only limited introduction of labeled nucleotide into the probe. Using a Boehringer DIG-High Prime kit, DIG-labeled probes can be generated within an hour, but, for increased yield, an overnight reaction is preferable. It is important to digest the vector to remove the target insert prior to labeling; labeled vector sequences can lead to nonspecific hybridization.
6. Typically, we use a DNA probe concentration of 5–25 ng/mL to detect RNA. However, the optimum concentration of a new probe should be determined, taking background signal into account.
7. The samples should include a lane containing a conventional RNA marker, which can be cut off before transfer, and ethidium bromide stained. An alternative, and preferable, method is to use DIG-labeled RNA mol-wt marker supplied by Boehringer; there are three size ranges available. This has the advantage that it will be transferred during blotting and will be visible on the X-ray film alongside the sample bands. DIG-labeled mol-wt marker should be heated at 65°C for 10 min before being placed on ice; other details for using mol-wt markers can be found in the manufacturer's technical leaflet.
8. It is not necessary to prewet nylon membrane, but, if required, it wets faster in 10X SSC than in 20X SSC.
9. Estimates of transcript RNA concentration can be made by comparing the intensities of hybridized nucleic acid spots with those given by dilutions of a known concentration of purified RNA. Estimates of relative amounts of RNA can also be determined by comparing the intensities given by different samples. The range of dilutions that is suitable for previously untested RNA extracts, should be determined empirically. It is very important to include RNA samples from nontransgenic plants to act as controls for nonspecific hybridization.
10. There are various buffers that can be used for hybridization. Dig Easy Hyb is nontoxic and does not contain formamide. It is ready to use and is both DNase- and RNase-free. The optimum hybridization temperature should be estimated