

## RNA Labeling using *In Vitro* Transcription

Labeled RNA probes permit very sensitive detection. DIG-labeled RNA probes produce higher sensitivity (10 to 100-fold) than DIG-labeled DNA probes for detecting RNA. This is because RNA-RNA hybrids are stronger than RNA-DNA and DNA-DNA hybrids. RNA probes are ideal for detecting rare mRNAs on northern blots and for localizing nucleic acids using *in situ* hybridization. RNA probes are also used for Southern blots, library screening, and dot/slot blots. RNA probes however require strict RNase-free handling during all experimental steps.

Labeled RNA probes are produced by *in vitro* transcription from linearized DNA templates prepared from plasmid clones by restriction enzyme digestion. Plasmids contain promoters either for SP6, T3 or T7 RNA polymerase on both sides of the multiple cloning site. For *in situ* hybridization, it is important to be able to prepare a sense strand as negative control, and target-specific antisense RNA strand. During *in vitro* transcription, many DIG-labeled RNA copies of the plasmid DNA product are generated. From 1  $\mu\text{g}$  of DNA template, up to 20  $\mu\text{g}$  labeled RNA are synthesized.

A straightforward method for generating templates with DIG suitable for *in vitro* transcription is to prepare templates by PCR using specially designed primers that include the RNA polymerase promoter sequence used. This approach makes cloning, plasmid isolation, and restriction enzyme digestion unnecessary.

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Not for use in diagnostic procedures.



## Probe Preparation and Labeling

For RNA labeling by *in vitro* transcription with DIG, Roche recommends individual primer sets that specifically amplify the required sequence, so that the reverse primers of the antisense RNAs contained 5'-AATACGACTCACTATAGG-3' for T7 RNA transcription, and 5'-ATTAACCCTCAC-TAAAGG-3' as T3 minimal promoter sequence for T3 RNA transcription, as described in Roche's **DIG Northern Starter Kit**.

Specific PCR products containing the RNA polymerase promoter binding site are used without prior purification. RNA is labeled by *in vitro* transcription using digoxigenin-11-UTP inserted at approximately every third uracil position of the newly synthesized RNA.

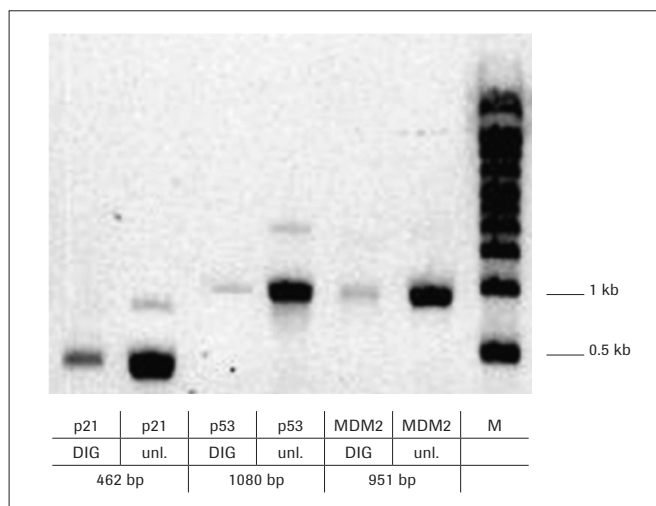
### Technical Tip:

The detailed protocol is found

- in the Instructions for Use provided in Roche's **DIG Northern Starter Kit**.
- For non-labeled RNA, use Roche's **SP6/T7 Transcription Kit**.

## Analyzing Probe Labeling and Sensitivity

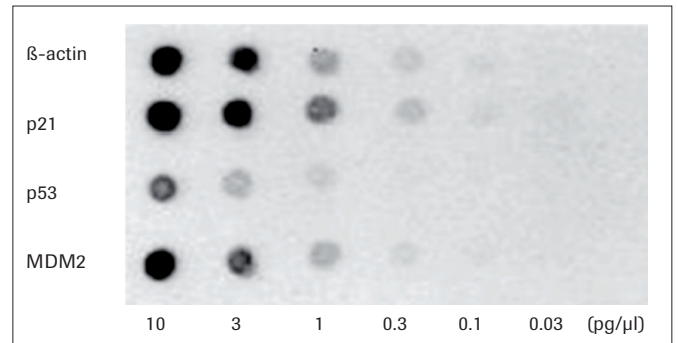
DIG-labeled RNA, hybridization probes, and unlabeled control RNAs can be analyzed for correct size and purity using an agarose gel. Labeled RNAs show a slight shift in molecular weight due to the incorporation of DIG, illustrating the effectiveness of the *in vitro* transcription (**see Figure 1**).



**Figure 1.** Labeled DIG probes were analyzed using a 1.2 % agarose gel to estimate the effectiveness of the labeling reaction. One hundred nanograms of labeled and unlabeled RNA were loaded (data from Roche Cancer Research Application Note No. 10, Weil et al., June 2011).

## Analyzing Probe Labeling and Sensitivity

To determine the sensitivity of DIG-labeled probes, a dot blot analysis is performed (see **Figure 2**). Using the protocol of the DIG Northern Starter Kit, a dilution series of the DIG-labeled probes is applied to a small strip of positively charged nylon membrane. As a control, labeled  $\beta$ -actin RNA provided in the **DIG Northern Starter Kit** can be used. After crosslinking, the immunological chemiluminescent detection with anti-digoxigenin-AP-antibody and CDP-*Star* are carried out. Ideally, DIG-probes should exhibit a detectable spot as low as the 0.1 pg dilution. Probes with at least detectability to 1 pg are required or the probe will not be sensitive enough for hybridization studies.

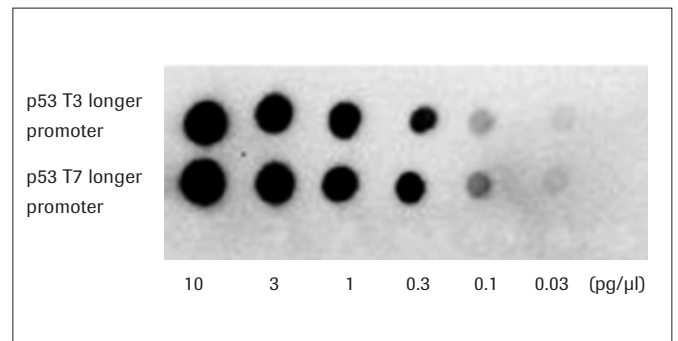


**Figure 2.** Sensitivity testing of labeled DIG-probes. For all templates, the appropriate minimal promoter sequence was used. For direct detection, all probes, except the p53 probe, showed a spot down to 0.1 pg concentration. Exposure time was 5 minutes after 17 hours incubation with Roche's chemiluminescent reagent, CDP-*Star* (data from Cancer Research Application Note No. 10, Weil et al. June 2011).

## Influence of Promoter Sequence on labeling efficiency

Longer promoter sequences may influence labeling efficiency. Such a longer T3 promoter is 5'-AATTAACCCT-CACTAAAGGGAGA-3'; a longer T7 promoter is 5'-TAATACGACTCACTATAGGGAGA-3'.

In the following figure, dot blot results are shown, documenting the influence of promoter sequence length. For best results, add four additional bases to the promoter sequence to optimize results (see **Figure 3**).



**Figure 3.** Influence of longer T3/T7 promoter sequences on the probe labeling efficiency. Four additional bases were attached to the minimal promoter sequences. In this case, sequences with longer promoters show a clear advantage over the minimal promoter sequences (data from Cancer Research Application Note No. 10, Weil et al. June 2011).

## Summary

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DIG-labeled RNA probes are sensitive and ideal for use in northern blots and *in situ* hybridization. Using RNA probes requires RNase-free handling procedures.

A straightforward way to generate DIG-labeled RNA is *in vitro* transcription of PCR products containing specially designed primers that include the appropriate RNA polymerase promoter sequence.

## Further Reading

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For best results, use the information provided in the package insert of Roche's **DIG Northern Starter Kit**.

Additional information and data are found in Roche Cancer Research Application Note No. 10, Weil et al., June 2011: **Cancer Research Workflow: Analysis of Nutlin-Induced Changes in mRNA Synthesis and Protein Expression using the xCELLigence System, qRT-PCR, Northern Blots, and Western Blots**.

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