

Production of High-Activity Digoxigenin-Labeled Riboprobes for *In Situ* Hybridization Using the AmpliScribe™ T7 High Yield Transcription Kit

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Introduction

Our laboratory uses non-radioactive *in situ* hybridization (ISH) to study switching of collagen gene expression in skeletal tissues during normal and abnormal growth and development.^{1,2} Digoxigenin-labeled, antisense riboprobes are well-suited for this purpose because they can be stored frozen for long periods before use, they bind tightly to their cognate mRNA, and they give superior histological resolution compared to radioactive labels. Despite the fact, however, that collagen genes are generally highly expressed, we found some commercial *in vitro* transcription systems were yielding low levels of probe activity with too much background. We therefore tested EPICENTRE's AmpliScribe™ T7 High Yield Transcription Kit to prepare digoxigenin-labeled riboprobes according to a modified procedure described by Kaplan *et al.*³

Methods and Results

Preparation of the T7 transcription template

Transcription templates were generated by PCR amplification of regions of rat collagen cDNA clones (Figure 1). By using PCR-generated transcription templates we effectively eliminate backgrounds caused by uncut plasmid or other DNA. Briefly, a reverse PCR primer (5' TTTC-CATTGCCATTTCAG 3') was designed to bind 169 bases "downstream" from the T7 transcription promoter contained on the cloning vector. This 169-base "leader" sequence helps ensure that the T7 RNA polymerase can initiate at its promoter site efficiently. We then designed a different forward PCR primer for each of the different collagen cDNAs we wanted to detect. The forward primers were selected so that the final PCR product would be about 700 to 1000 bp long—a good length for ISH probes.

Typically 1 ng of cDNA clone DNA per 500- μ l PCR reaction volume was used as PCR template. We performed 5 X 100- μ l PCR reactions under standard conditions for 38 cycles. Following PCR, the reaction product was cleaned up on a spin column, the OD measured, and then sodium acetate/ethanol precipitated and washed. The PCR product was then resuspended to approximately 1 μ g/ μ l and used as template for the AmpliScribe T7 *in vitro* transcription reaction.

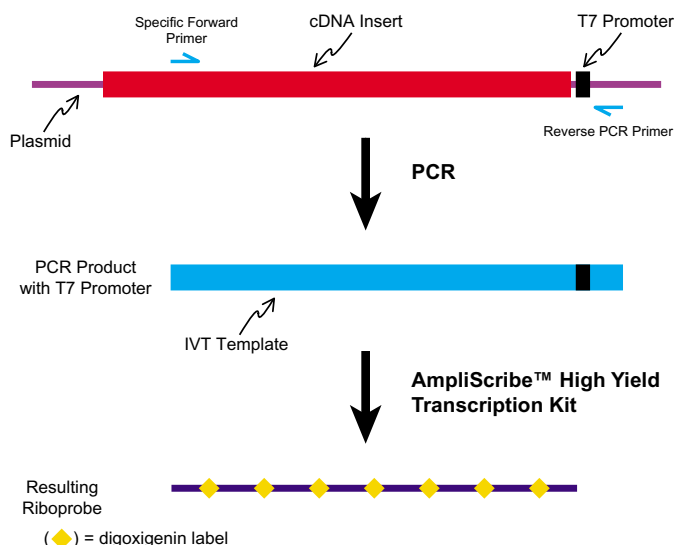


Figure 1. Schematic overview of how digoxigenin-labeled riboprobes were prepared. PCR was carried out on plasmid containing a collagen cDNA. This yields a dsDNA template for *in vitro* transcription (IVT) that includes 700-1000 bases of cDNA plus 169 bases of vector that includes the T7 transcription initiation site. Having the short downstream vector sequence increased the efficiency of the IVT reaction.

Transcription of digoxigenin-labeled riboprobes using the AmpliScribe T7 High Yield Transcription Kit

The AmpliScribe T7 *in vitro* transcription reaction was set up at room temperature in the following order:

PCR product template.	1 μ g (about 1 μ l)
AmpliScribe™	
10X Reaction Buffer.	2 μ l
ATP, CTP, GTP (100 mM each).	1.5 μ l each
100 mM UTP	1 μ l
10 mM Digoxigenin-11UTP*	6 μ l
100 mM DTT	2 μ l
Water	to 18 μ l
AmpliScribe™ T7 Enzyme Solution	2 μ l

TOTAL VOLUME 20 μ l

*Roche Applied Science, Cat. No. 1209256

The reaction was mixed and incubated 2 hours at 37°C and then treated with RNase-Free DNase to remove the template DNA. The digoxigenin-labeled riboprobe was precipitated with ammonium acetate, washed with ethanol, dried, and resuspended in 52 μ l of water. Yields measured by A_{260} were up to 200 times the amount of input DNA template.

Quantifying the activity of the digoxigenin-labeled riboprobe

One microliter dilutions of the riboprobe were used to titrate the probe to measure incorporation of digoxigenin by chemiluminescent immuno-dot blots probed with alkaline phosphatase-conjugated anti-digoxigenin (Roche Applied Science) and developed with CSPD chemiluminescent substrate. A parallel dilution series was made with a positive control digoxigenin-labeled RNA that is supplied with Roche Applied Science's digoxigenin labeling kit (which represents "maximum" labeling, according to the manufacturer) and digoxigenin-labeled riboprobe produced by a competitor's *in vitro* transcription kit. As shown in Figure 2, the AmpliScribe T7 Kit yielded 3-fold higher incorporation than Roche's "maximum" control and 10-

to 30-fold higher probe activity than the competitor's kit. Following quantitation, the riboprobes were divided into 5- μ l aliquots, flash frozen in liquid nitrogen, and stored at -70°C .

In situ hybridization

Optimum dilutions for probing tissue samples were determined empirically. Preparation of tissues and hybridization conditions have been described in detail.² Briefly, tissues are dissected and immediately placed in cold, neutral 4% para-formaldehyde, fixed overnight, then decalcified by storing in 12.5% EDTA.

Tissues were embedded in paraffin, sectioned (5-7 μm), and deparaffinized by standard techniques. After prehybridization for one hour, probe is incubated with the tissue overnight at 42°C , and any non-hybridized probe was removed by RNase A digestion. After washes of increasing stringency, anti-digoxigenin-alkaline phosphatase conjugate was incubated with the sections for an hour, followed by PBS washes. Finally, NBT-BCIP chromogenic substrate was added and incubated at room temperature until signal develops, the reaction was stopped with water, and the slides dried and cover slipped (Figure 3).

Conclusion

The AmpliScribe T7 High Yield Transcription Kit proved to be the most effective product for preparing the high-activity, digoxigenin-labeled riboprobes needed for our projects. With typically a 30-fold higher yield, we spend substantially less time, effort, and expense in reagent preparation and optimization. This provides us a long-lasting supply of consistent probe to process samples in large experimental series.

Acknowledgment

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References

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2. Marks, Jr., S.C. *et al.* (2000) *Int. J. Dev. Biol.* 44 (3), 309.
3. Kaplan, E.D. *et al.* (1996) *EPICENTRE Forum* 3 (2), 1.

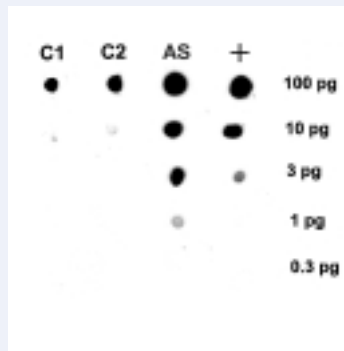


Figure 2. An AmpliScribe™ T7 High Yield Transcription reaction yielded 10- to 30-fold higher digoxigenin-labeled riboprobe activity than the competitor's reaction. Each column is a dilution series of one sample. C1, digoxigenin-labeled rat collagen type II (α 1) riboprobe produced in a competitor's *in vitro* transcription reaction; C2, a repeat of the competitor's reaction; AS, digoxigenin-labeled rat collagen type II (α 1) riboprobe produced in an AmpliScribe T7 transcription reaction; +, digoxigenin-labeled positive control RNA (Roche Applied Science).

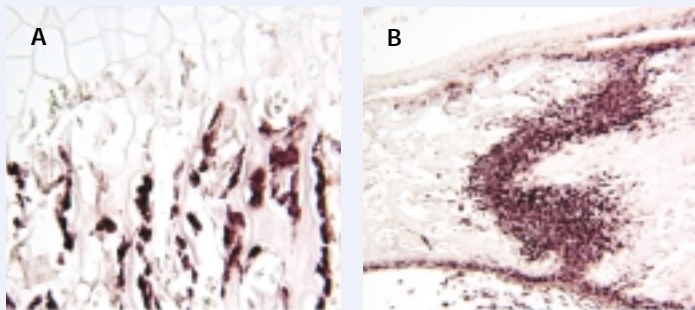


Figure 3. *In situ* hybridization results using digoxigenin-labeled riboprobes produced using the AmpliScribe™ T7 High Yield Transcription Kit. Panel A, type I collagen in the rat tibia. Type I-positive osteoblasts are intensely stained along the bone trabeculae of the metaphysis in the lower portion of the panel, while the cartilage at the top is completely negative, as expected. Panel B, type III collagen in the rat premaxillary-maxillary suture. The sigmoidal shape of the suture is seen clearly in the right panel, with type III-expressing, undifferentiated precursor cells filling the space between the bony surfaces. Original magnifications, Panel A 160X and Panel B 43X.

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AmpliScribe™ T7 High Yield Transcription Kit

AS2607	25 Reactions
AS3107	50 Reactions

AmpliScribe™ T3 High Yield Transcription Kit

AS2603	25 Reactions
AS3103	50 Reactions

AmpliScribe™ SP6 High Yield Transcription Kit

AS2606	25 Reactions
AS3106	50 Reactions

Contents:

AmpliScribe™ T7, T3, or SP6 Enzyme Solution (with added RNase inhibitor), 100 mM ATP, CTP, GTP & UTP Solutions, AmpliScribe™ 10X Reaction Buffer, RNase-Free Water, RNase-Free DNase I, DTT, and Control Template DNA (linearized).

AmpliScribe™ T7-Flash™ Transcription Kit

Now Available

- Fast 30-minute *in vitro* transcription reaction
- Higher yields of RNA – up to 180 μg from a 20- μl reaction

See page 8 for more information.