

## Combination of Chemical and Enzymatic RNA Synthesis

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### Summary

The potential of standard in vitro transcription reactions can be dramatically expanded, if chemically synthesized low-mol-wt compounds are used as building blocks in combination with standard nucleotide 5' triphosphates (NTPs). Short oligonucleotides that terminate in guanosine effectively compete with guanosine 5' triphosphate (GTP) as starter building blocks, and they are incorporated at the 5'-end of transcripts. Applications include production of RNAs with “unfriendly 5'-ends” (they do not begin with G), variations of the 5'-sequence are possible with the same DNA template, site-specific insertion of nucleotide modifications, and addition of 5'-labels, such as fluorescein for detection or biotin for capture. Clearly, chemically synthesized, modified NTPs are inserted at internal sites. The combination with phosphorothioate linkages for detection has been developed into a powerful high-throughput method to study site-specific interference of modifications with RNA function.

**Key Words:** Biotin; digoxigenin; FAM; fluorescence; initiator oligonucleotide; 5'-label; modification; mutation; NAIM; nonradioactive; 5'-<sup>32</sup>P-label; phosphorothioate.

### 1. Introduction

In vitro transcription reactions with bacteriophage RNA polymerases (SP6, T3, and now, most used T7) have been developed into a very powerful technique to produce large quantities of long RNA molecules. Although all effective DNA templates include the homologous double-stranded promoter, the template types vary from the standard transcription plasmid to specifically designed PCR products and to mostly single-stranded templates, containing only the promoter in double stranded form (*1*). The power of this technology can be dramatically expanded by combining chemical synthesis of low-mol-wt compounds with standard NTPs as RNA building blocks.

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The discovery that short, synthetic oligonucleotides, terminating with guanosine, effectively compete with GTP as starter building blocks enables the convenient and precise manipulation of the 5'-proximal section of RNA transcripts. Otherwise, this is only possible in the complete chemical RNA synthesis that is limited to short lengths. Applications of these so-called initiator oligonucleotides (2) include: i) overcoming the limitation that in vitro transcripts must begin with G, ii) variations of the 5'-sequence without the need for a series of different templates, iii) site-specific insertion of nucleotide modifications, iv) direct 5'-labeling during the transcription reaction with fluorescein for detection or with biotin for capture, and v) the direct production of transcripts with 5'-OH, for simplified and very effective 5'-<sup>32</sup>P-labeling, avoiding removal of the recalcitrant 5'-triphosphate.

Chemically synthesized, modified NTPs offer a wide range, and are clearly inserted at many internal sites. The combination with phosphorothioate linkages for detection has been developed into a powerful high-throughput method to study site-specific interference of modifications with RNA function (3,4).

## 2. Materials

1. Template DNA.
2. 10X transcription buffer: 400 mM Tris-HCl, pH 8.0, 200 mM MgCl<sub>2</sub>, 20 mM spermidine.
3. Ribonucleoside triphosphates (NTP): a solution containing each NTP (adenosine 5' triphosphate [ATP], cyndine 5' triphosphate [CTP], guanosine 5' triphosphate [GTP], uridine 5' triphosphate [UTP]) at 10 mM.
4. 100 mM dithiothreitol (DTT); do not autoclave.
5. RNase-inhibitor RNasin from human placenta (e.g., Fermentas, Roche, Promega).
6. 50% (w/v) Polyethylene glycol (PEG), M<sub>r</sub> 6000; can be autoclaved.
7. 0.1% Triton X-100 (Roche); do not autoclave.
8. T7 RNA polymerase or other appropriate phage RNA polymerase (Fermentas, NE-Biolabs, Roche).
9. Optional: [ $\alpha$ -<sup>32</sup>P]-UTP (Amersham, ICN, Hartmann-Analytic).
10. 4 M ammonium acetate, 20 mM ethylenediaminetetraacetic acid (EDTA). Adjust to pH 7.0, autoclave.
11. Cold ethanol, p.a. (stored at -20°C).
12. Equipment for polyacrylamide gel electrophoresis and elution.
13. If appropriate: Replace **items 2–8** by High-yield transcription kit, e.g., AmpliScribe (Epicentre), MEGAscript, or MEGAshortscript (Ambion).
14. Initiator oligonucleotides—a wide range is commercially available (e-mail: krupp@artus-biotech.com). Fluorescent-labeled materials should be stored in the dark (wrapped in aluminum foil). Purity is a very important issue, since these short oligos are difficult to separate from work-up products from the chemical synthesis.
15. Modified NTP $\alpha$ S: a wide range is commercially available (e-mail: krupp@artus-biotech.com).

### 3. Methods

#### 3.1. Overcoming the Limitation of Standard Protocols: *In Vitro* Transcription of RNAs That Have No Guanosine as Their 5'-End

Commercially available transcription systems with bacteriophage RNA polymerases (T7, T3, or SP6) all require guanosine as the 5'-terminal first nucleotide in the transcript. Frequently, functional RNA molecules do not start with G—for example, many tRNAs.

One approach to overcome this limitation is the introduction of a ribozyme structure that cleaves the primary transcript and liberates the desired 5'-end (5,6). Based on our previously published observations (2), we present a simple protocol if the desired RNAs have a G at least near the required 5'-end, at the second, third, or fourth nucleotide.

For this purpose, the template DNA codes for a transcript beginning with the first G in your RNA. The *in vitro* transcription reactions are performed as usual, but in addition, a short “initiator oligonucleotide” is added. This oligonucleotide contains your desired 5'-sequence ending at the first G. If preferred, the oligonucleotide may already contain a 5'-phosphate, and a schematic example would be:

5'-terminal sequence of desired RNA	5'-CAGGCCAGUAAA.....
template-encoded transcript	5'-pppGGCCAGUAAA.....
<i>in vitro</i> transcript with the trinucleotide (p)CAG	5'-(p) CAGGCCAGUAAA.....

The incorporation efficiencies listed in **Table 1** were obtained using a twofold molar excess of the initiator oligonucleotide over GTP that competes as an initiator in the transcription reaction. Example results are shown in **Fig. 1**. Reactions can be performed with all four NTPs at the same concentration—e.g., all in the standard range of 0.5–2 mM. The “high-yield transcription kits,” such as Ampliscribe (from Epicentre) or Megascript (from Ambion) contain much higher NTPs (4–7 mM), and in this case, a lower GTP concentration of approx 1 mM can be used to reduce the required amount of the more expensive oligonucleotide.

##### 3.1.1. Protocol With Standard Transcription Method

1. For a 100-μL reaction: Use approx 1–10 pmoles of DNA template (e.g., standard transcription plasmid, PCR product, or a combination of synthetic oligos (7, *see Note 1* and 2).
2. Set up the reaction with final concentrations of 40 mM Tris-HCl, pH 8.0, 20 mM MgCl<sub>2</sub>, 2 mM spermidine, 10 mM DTT, 1 mM NTPs each (up to 2 mM). Optional additions: 50 U of RNasin; the enhancing additives 8% polyethylene glycol 6000 and 0.01% Triton X-100.  
If desired, a tracer amount of [ $\alpha$ -<sup>32</sup>P]-UTP can be added, for visualization by autoradiography and quantification by scintillation counter.

**Table 1**  
**Incorporation Efficiency of Initiator Oligonucleotides**

All sequences NxG are possible, but oligo(G) homopolymers should be avoided

Dinucleotides ( <i>one</i> extra nucleoside at 5'-end) unmodified, or with label—e.g., <b>biotin</b> or <b>fluorescein</b>	>95%
Trinucleotides ( <i>two</i> extra nucleosides at 5'-end)	>85%
Tetranucleotides ( <i>three</i> extra nucleosides at 5'-end)	>80%
Pentanucleotides ( <i>four</i> extra nucleosides at 5'-end)	>60%
Hexanucleotides ( <i>five</i> extra nucleosides at 5'-end)	about 40%

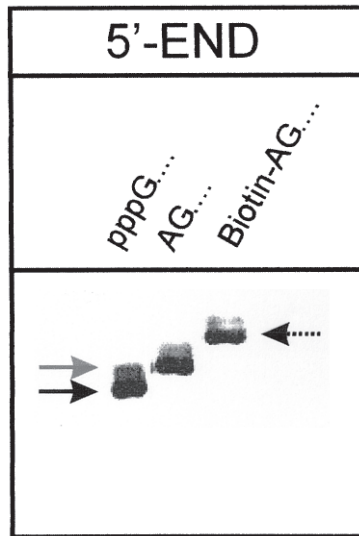


Fig. 1. Incorporation of initiator oligonucleotides in transcripts. Transcriptions were performed as described in **Subheading 3.1.1.**, including a tracer amount of [ $\alpha$ - $^{32}\text{P}$ ]-UTP. The plasmid template encodes mature tRNA<sup>Phe</sup> from yeast (2), T7 RNA polymerase was used. Analysis of transcripts was performed by 8% denaturing PAGE, followed by autoradiography. The 5'-terminal sequence is indicated above the lanes. pppG: normal triphosphate end in standard transcription reaction. ApG: addition of dinucleotide AG results in extra adenosine with 5'-OH end. Biotin-AG: addition of biotinylated dinucleotide results in extra adenosine with 5'-biotin end. As usual, transcripts terminate with the last template-encoded nucleotide (black arrow at left side), and about 30% are extended by one extra nucleotide (gray arrow at left side). The product with one extra 5'-terminal adenosine migrates slightly above the gray arrow, because of the missing negative charges (5'-OH instead of triphosphate). Addition of the bulky group biotin results in further shift (dotted arrow at right side). Please note: the initiator oligonucleotides (twofold molar excess over GTP) effectively outcompete formation of standard transcript; further, all products display a similar 3'-heterogeneity.

3. Add the appropriate initiator oligonucleotide at twofold excess—e.g., at 2 mM (or at 4 mM).
4. Add 100 U (or up to 10-fold higher amount, but not exceeding 10% of the total reaction volume to avoid excessive glycerol addition) of T7 RNA polymerase.
5. Incubate at 37°C for 1–4 h.
6. If desired, remove DNA template by adding 20 U of RNase-free DNase, incubate an additional 30 min at 37°C.
7. Transcripts are recovered by ethanol precipitation: add 100  $\mu$ L of 4 M ammonium acetate/20 mM EDTA, mix, add 500  $\mu$ L cold ethanol, and mix again. Chill for 15 min on dry ice (or 30 min at –70°C, or >60 min at –20°C), microfuge for 15 min, and discard supernatant. Dry briefly.
8. Dissolve pellet in 10–20  $\mu$ L gel loading solution, denature by heating for 2 min at 96°C, and load on denaturing polyacrylamide gel.
9. After electrophoresis, RNA can be visualized by autoradiography or for unlabeled RNA, by UV-shadowing or by staining—e.g., with ethidium bromide.

### 3.1.2. Protocol for Using a High-Yield Transcription Kit

1. Kits are available—for example, from Epicentre (Ampliscribe) or from Ambion (MEGAscript or MEGAsortscript).
2. For a 20- $\mu$ L reaction, 1–10 pmols of DNA template.
3. Set up the reaction as specified in the kit. The NTPs are used at high concentrations, about 5–7 mM each. Although this will compromise the transcript yields, reduce GTP concentration to 2 mM, thus reducing the required amount of initiator oligonucleotide. If desired, a tracer amount of [ $\alpha$ -<sup>32</sup>P]-UTP can be added, for visualization by autoradiography and quantification by scintillation counter.
4. Add the appropriate initiator oligonucleotide at twofold excess—e.g., at 4 mM.
5. Add the RNA polymerase from the kit.
6. Incubate at 37°C for 1–4 h.
7. If desired, remove DNA template by adding 20 U of RNase-free DNase, incubate an additional 30 min at 37°C.
8. Transcripts are recovered by ethanol-precipitation: add 20  $\mu$ L of 4 M ammonium acetate/20 mM EDTA, mix, add 100  $\mu$ L cold ethanol, and mix again. Chill for 15 min on dry ice (or 30 min at –70°C, or >60 min at –20°C), microfuge for 15 min, and discard supernatant. Dry briefly.
9. Dissolve pellet in 10–20  $\mu$ L gel-loading solution, denature by heating for 2 min at 96°C, and load on denaturing polyacrylamide gel.
10. After electrophoresis, RNA can be visualized by autoradiography or for unlabeled RNA, by UV-shadowing or by staining—e.g., with ethidium bromide.

### 3.1.3. Introducing Defined Sequence Changes in the 5'-Terminal Sequence, Without Using Different Templates

An example of this approach is the generation of tRNAs with different extra 5'-terminal sequences as 5'-flanks, suitable for studies of pre-tRNA processing by RNase P (3,8).

The approach is very similar. In this case, the provided template DNA codes for a transcript beginning with 5'-terminal G of the mature tRNA. The in vitro transcription reactions are performed as usual, but in addition, a short “initiator oligonucleotide” is added. This oligonucleotide contains the desired extra 5'-sequence, including the 5'-terminal G of the mature tRNA.

5'-terminal sequence mature tRNA <sup>Phe</sup> from yeast	5'-GCGGAUUUAGC.....
template-encoded transcript	5'-pppGCGGAUUUAGC.....
in vitro transcript with the trinucleotide AAG	5'-AAGCGGAUUUAGC.....

Protocols are exactly as described in **Subheading 3.1**.

#### 3.1.4. Producing RNAs With Modified Nucleotides in the 5'-Terminal Sequence

Another example is the generation of RNAs that contain 5'-proximal, well-defined nucleotide modifications, suitable for studies of RNA processing. Already, this 5'-modified RNA can be the desired final product (9), or the modifications can be internalized by combining two RNA molecules (10,11).

Again, the approach is very similar, and the provided template DNA codes for a transcript beginning with 5'-terminal G. The in vitro transcription reactions are performed as usual, but in addition, the “initiator oligonucleotide” contains a well-defined modification, and includes the 5'-terminal G of the normal transcript. An example is the site-specific introduction of a 2'-deoxyribose:

5'-terminal sequence of normal transcript	5'-pppGCGGAUUUAGC.....
in vitro transcript with the trinucleotide dAAG	5'-dAAGCGGAUUUAGC.....

Another example is the introduction of a fully characterized stereoisomer of a phosphorothioate (R or S isomer; as a reminder: at internal sites, only the R isomer can be introduced by in vitro transcription):

5'-terminal sequence of normal transcript	5'-pppGCGGAUUUAGC.....
in vitro transcript with the dinucleotide A(pS)G	5'-A(pS)GCGGAUUUAGC.....

A further example is the introduction of a modified base in long RNA transcripts, such as 7-deazaadenine (c<sup>7</sup>A):

5'-terminal sequence of normal transcript	5'-pppGCGGAUUUAGC.....
in vitro transcript with the dinucleotide c <sup>7</sup> AG	5'-c <sup>7</sup> AGCGGAUUUAGC.....

Protocols are exactly as described in **Subheading 3.1.**, using the proper modified initiator oligonucleotide.

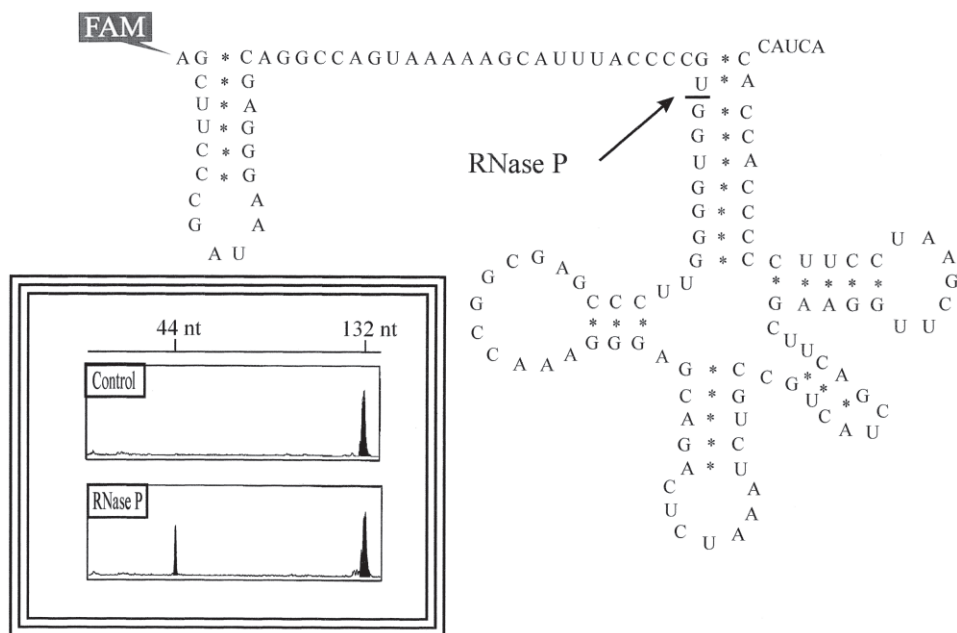


Fig. 2. Processing of fluorescently-labeled pre-tRNA, monitored by automated sequencer. Transcriptions were performed as described in **Subheadings 3.1.1.** and **3.1.3.** The plasmid template encodes pre-tRNA<sup>Tyr</sup> from *E. coli* (3), T7 RNA polymerase, and the initiator oligonucleotide FAM-AG was used. Transcripts were purified by 8% denaturing PAGE, transcript was directly visible in the gel as green band, or visualized by fluorescence scanning (Storm 860 from Amersham-Pharmacia). The transcript structure is shown, including the extra 5'-terminal A, linked to the fluorescent dye. The cleavage position of the pre-tRNA processing RNase P is indicated (3). Insert: two runs on the ABI Prism 310 capillary sequencer. Control: incubation without enzyme, only peak for full-size 132-nucleotide pre-tRNA is visible. RNase P: treatment with RNase P from yeast (8) results in additional peak for liberated 44-nucleotide 5'-flank.

### 3.1.5. Direct Nonradioactive 5'-Labeling of RNAs During In Vitro Transcription (e.g., With fluorescein or With biotin)

The 5'-fluorescent-labeled RNAs are convenient for analysis with polyacrylamide gel electrophoresis combined with a fluorescence scanner or for use in standard automated DNA sequencers. An example is shown in **Fig. 2** with a 5'-FAM-labeled pre-tRNA, processed by RNase P and analyzed in an ABI 310 capillary sequencer.

Furthermore, even real-time analysis of ribozyme reactions is possible by observing changes in fluorescence polarization (12; see also Chapter 4).

5'-biotinylated RNAs were previously used for nonradioactive detection in polyacrylamide gels (2), and an equivalent option would be digoxigenin. An attractive property of these site-specifically biotinylated RNAs is their highly efficient recovery with streptavidin-beads. Applications could be the isolation of high-affinity binding compounds after incubation with complex biological samples, or the immobilization of RNA aptamers without compromising their activity and without requiring a chemical synthesis of the full-size RNA.

Again, the template DNA codes for a transcript beginning with 5'-terminal G. The *in vitro* transcription reactions are performed as usual, but in addition, a biotin- or FAM-AG (indicated as X-AG in the following scheme) is used as “initiator oligonucleotide.”

5'-terminal sequence of normal transcript	5'-pppGCGGAUUUAGC.....
<i>in vitro</i> transcript with the dinucleotide X-AG	5'-X-AGCGGAUUUAGC.....

Protocols are exactly as described in **Subheading 3.1**. Illustrative results are shown in **Table 1**, and a biotinylated RNA is shown in **Fig. 1**.

### 3.2. Functional RNA Studies With Transcripts Containing Internal, “Partially Modified” Nucleotides

This approach is only briefly presented, to show another context in which chemically synthesized RNA building blocks are used (*see also* Chapter 6). Here, internal sites can be screened for functional importance of ribose or base moieties. The crucial step is a semi-quantitative, site-specific detection of modification levels in RNA transcripts. This can be achieved by combining a phosphorothioate linkage (specifically cleaved and thus semi-quantitatively detected by iodine/ethanol treatment) with the modification of interest (*see Note 3*). Initially, the only commercially available RNA modification type was deoxyribose, in the form of dNTP $\alpha$ S, and the technique was established in the identification of important ribose moieties in RNase P substrates (3). Subsequently, it was used to define chemical groups in base moieties that were essential for the function of other ribozymes (4,13) and the technique was known as nucleotide analog interference mapping (NAIM). This technique awaits further use, since the number of commercially available, modified NTP $\alpha$ S building blocks has dramatically increased (e-mail: krupp@artus-biotech.com).

## 4. Notes

1. Avoid using plasmids linearized with a restriction enzyme such as *Pst*I that generates 3'-protruding ends. If unavoidable, blunt ends can be generated by brief treatment with T4 DNA polymerase.



2. If synthetic oligos or PCR products are used as templates, DNA and transcript size are similar, and to ensure DNA removal, a DNase treatment is advisable.
3. Phosphorothioate and other modified RNAs are more sensitive to degradation, and elution buffers should be adjusted to pH 7.0 (measuring in the final mixture).

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