Part I
RNA Synthesis and Detection

Handbook of RNA Biochemistry, Second Edition.
Edited by R.K. Hartmann, A. Bindereif, A. Schön, and E. Westhof.

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Enzymatic RNA Synthesis Using Bacteriophage T7 RNA Polymerase

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1.1 Introduction

Bacteriophage T7 RNA polymerase (T7 RNAP) was first cloned and overexpressed from bacteriophage T7-infected *Escherichia coli* cells in the early 1980s [1]. In contrast to multisubunit DNA-dependent RNAPs from eukaryotes and prokaryotes, T7 RNAP consists of a single subunit of about 100 kDa [2]. The subdomains adopt a hand-like shape with palm, thumb, and fingers around a central cleft where the active site containing the functionally essential amino acid residues is located, creating a binding cavity for magnesium ions and ribonucleotide substrates. For RNA synthesis, the unwound template strand is positioned such that the template base −1 is anchored in a hydrophobic pocket in direct vicinity of the active site [3].

T7 RNAP is highly specific to its own promoters and exhibits no affinity even to closely related phage T3 promoters, although the 23 bp consensus sequences are very similar (Figure 1.1a). During the initiation process, the polymerase goes through several elongation attempts, generating short abortive oligoribonucleotides. Only when the nascent RNA transcript exceeds 9–12 nt do initiation complexes convert to stable elongation complexes. Transcription proceeds with an average rate of 200–260 nt s^{-1} until the elongation complex encounters a termination signal or falls off the template end during *in vitro* run-off transcription [4, 5]. The error frequency in transcripts of wild-type (wt) T7 RNAP is about 6×10^{-5} [6].

In the following sections, we describe protocols that have been used routinely for T7 transcriptions. Further, a robust and simple protocol for the partial purification of T7 RNAP is included, which yields an enzyme preparation that fully satisfies all *in vitro* transcription demands. The given transcription protocols suffice for most purposes. However, in special cases, such as the synthesis of milligram quantities, modified RNAs, or very A + U-rich RNAs, it may be worthwhile to further optimize the transcription conditions. We would also like to draw the reader's attention to the paper by Milligan and Uhlenbeck [10], which briefly discusses many fundamental aspects of T7 transcription.

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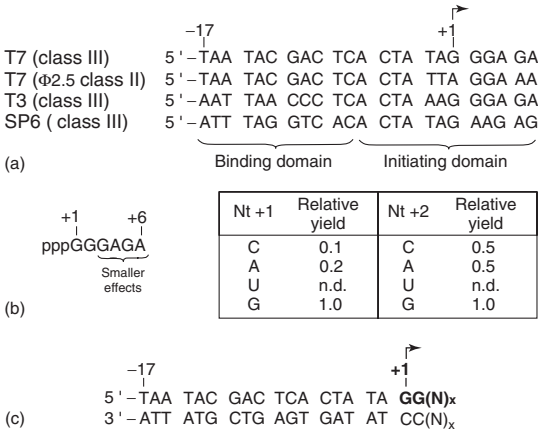

Figure 1.1 (a) Consensus sequences of class III promoters of bacteriophages T7, T3, and SP6, and sequence of the T7ϕ2.5 class II promoter; [5, 7–9]. Phage-polymerase-initiating domains also include the first 5–6 nt of the transcribed template strand. The transcription start (position +1) is indicated by the arrow. The phage T7 genome encodes a total of 17 promoters, including five class III promoters and one replication promoter (ϕOR), which are all completely conserved in the region from nt −17 to +6. In addition, there are ten T7 class II promoters plus one more replication promoter (ϕOL); among these eleven promoters, which display some sequence variation within the −17 to +6 region, only the ϕ2.5 and ϕOL promoters initiate transcription with an A instead of a G residue [8]. (b) Effect of sequence variations in the +1 to +6 regions of the T7 class III promoter on transcription efficiency; n.d., not determined (adapted from Milligan and Uhlenbeck [10]). (c) T7 class III promoter region with the recommended G identities at positions +1 and +2 of the RNA transcript shown in gray.

1.2
Description of Method – T7 Transcription *In vitro*

T7 RNAP can be used *in vitro* to produce milligram amounts of RNA polymers ranging from less than 100 to 30 000 nt [10, 11]. Since the commonly used T7 class III promoter, usually referred to as *the T7 promoter*, is also strictly conserved in the transcribed region of nt +1 to +6, sequence variations especially at nt +1 and +2 influence transcription yields significantly (Figure 1.1b,c; [10]).

1.2.1
Templates

Templates can be generated in three different ways: (i) by insertion into a plasmid (double-stranded DNA (dsDNA)), (ii) by polymerase chain reaction (PCR) (dsDNA), or (iii) by annealing a T7 promoter DNA oligonucleotide to a single-stranded template DNA oligonucleotide.

1.2.1.1 **Strategy (i): Insertion into a Plasmid**

We prefer to work with plasmid dsDNA templates because once the correct sequence of a plasmid clone has been confirmed, the DNA can be conveniently

amplified by *in vivo* plasmid replication exploiting the high fidelity of bacterial DNA polymerases. The RNA expression cassette (either with or without the T7 promoter sequence) is usually obtained by PCR and cloned into a bacterial plasmid. Since PCR amplification is error-prone, plasmid inserts ought to be sequenced. When the T7 RNAP promoter region from −17 to−1 is not encoded in the PCR fragment, one can use commercially available T7 transcription vectors (e.g., pGEM® 3Z and derivatives from Promega, or the pPCR-Script series from Agilent Technologies/Stratagene) containing the T7 promoter and a multiple cloning site for insertion of the RNA expression cassette. If there are no sequence constraints at the transcript 5′ end, we routinely design templates encoding 5′−GGA at positions +1 to +3 of the RNA transcript, which usually results in high transcription yields. Whenever possible, at least the nucleotide preferences at positions +1 and +2 should be taken into account (Figure 1.1b,c). Directly downstream of the expression cassette, a restriction site is required for template linearization to terminate RNA synthesis (run-off transcription); restriction enzymes producing 5′ overhangs are preferred over those producing blunt ends or 3′ overhangs [10]. Beyond common type II restriction enzymes generating 5′ overhangs (e.g., *Bam* HI, *Eco* RI), ''type IIS'' enzymes (e.g., *Fok*I) are of interest because they cleave sequence-independently outside of their recognition sequence and thus permit to design RNA transcript 3′ ends of complete identity to natural counterparts. Individual steps of template preparation are (i) ligation of (PCR) insert into plasmid, (ii) cloning in *E. coli*, purification and sequencing of plasmid, (iii) linearization of plasmid DNA for run-off transcription, (iv) phenol/chloroform extraction and ethanol precipitation of template DNA before (v) use in T7 transcription assays.

1.2.1.2 Strategy (ii): Direct Use of Templates Generated by PCR

Direct use of PCR fragments as templates is faster than insertion into a plasmid and preferred if only minor amounts of RNA are required. In this case, the T7 promoter sequence is encoded by the 5′ primer used in the PCR reaction. A downstream restriction site producing 5′ overhangs may be conveniently included in the 3′ primer.

1.2.1.3 Strategy (iii): Annealing of a T7 Promoter DNA Oligonucleotide to a Single-Stranded Template

This strategy is the fastest, and we have used it to synthesize small amounts of an RNA 30-mer for 5′−labeling purposes (Protocol 4). Here, the shorter T7 promoter DNA oligonucleotide is annealed to the complementary single-stranded DNA template oligonucleotide. The complementary double-stranded region is sufficient to initiate transcription by T7 RNAP.

1.2.2 Special Demands on the RNA Product

1.2.2.1 Homogeneous 5′ and 3′ Ends, Small RNAs, Functional Groups at the 5′ End

While T7 RNAP usually initiates transcription at a defined position, it tends to append one or occasionally even a few more non-templated nucleotides to the

product 3′ terminus [10, 12]. 5′ End heterogeneity may become a problem when the template encodes unusual 5′-terminal sequences, such as 5′-CACUGU, 5′-CAGAGA, or 5′-GAAAAA [13], or when transcripts are initiated with multiple guanosines [14]. For example, in the case of transcripts starting with 5′-GGGGG, 75% had canonical 5′ ends, relative to >99% for 5′ -GCGGA, 87% for 5′-GGGCC, 97% for 5′-GGGAG, and only 66% for 5′-GGGGC [14]. Thus, it is recommended that more than two consecutive G residues at the 5′ end be avoided. 5′ End heterogeneity seems to be a problem associated with T7 class III promoters (Figure 1.1a) because almost complete 5′ end homogeneity of T7 transcripts has been achieved with templates directing transcription from the more rarely used T7ϕ2.5 class II promoter (Figure 1.1a), at which T7 RNAP initiates synthesis with an A instead of a G residue. Transcription yields from this promoter were reported to equal those of the commonly used T7 class III promoter [15].

For the production of RNAs with 100% 5′ and 3′ end homogeneity, several methods are available. In one approach (Chapter 3), the downstream PCR primer introduces two 2′-OCH_3-modified RNA nucleotides at the 5′-terminal positions of the template strand, which suppresses the addition of 3′-terminal non-templated residues during transcription. Alternatively, hammerhead or hepatitis delta virus (HDV) ribozymes may be tethered to the RNA of interest on one or both sides (Chapter 2). The ribozyme(s) will release the RNA product by self-cleavage during transcription. Such a *cis*-acting ribozyme placed upstream releases the RNA of interest with a 5′-OH terminus directly accessible to 5′ endlabeling (Chapter 9), and simultaneously eliminates the problem of 5′ end heterogeneity as well as constraints on the identity of the 5′ terminal nucleotide of the RNA of interest (Chapter 2). The same strategy may also be considered for the synthesis of large amounts of smaller RNAs. Chemical synthesis and purification of 10 mg of, for example, an RNA 15-mer by a commercial supplier can be quite expensive. In such a case, a cheaper alternative would be to transcribe the 15-mer sandwiched between two *cis*-cleaving ribozymes, resulting in posttranscriptional release of the 15-mer with uniform 5′ and 3′ ends. Purification of the 15-mer (and separation from the released ribozyme fragments) can then be achieved by preparative denaturing polyacrylamide gel electrophoresis (PAGE; Section 1.3.4). If T7 RNAP is self-prepared according to the protocol described in this chapter, synthesis of 10 mg of a 15-mer will become quite affordable.

Normally, transcription by T7 RNAP is initiated with GTP, resulting in 5′-triphosphate ends. If, however, 5′-OH ends or 5′-monophosphate termini are preferred, T7 RNAP can be prompted to initiate transcripts with guanosine or 5′-ApG (to generate 5′-OH ends for direct endlabeling with ^{32}P), or 5′-GMP (to generate 5′-monophosphates), when these components are added to reaction mixtures in excess of GTP [16]. RNA transcripts with 5′-GMP ends are preferred when the RNA is used for ligation with other RNA molecules.

1.2.2.2 **Modified Substrates**

There are a number of modified nucleoside-5′-triphosphates known to be substrates for T7 RNAP. Table 1.1 has been adopted from Milligan and Uhlenbeck [10] and expanded by addition of more recent information.

Table 1.1 Nucleotide analogs for internal or 5′-terminal incorporation into T7 transcripts.

NTP	wt T7 RNAP	Y639F T7 RNAP	Y639F/H784A T7 RNAP	References
NTPαS (*S*p)	+			[17]
NTPαS (*R*p)	–			[17]
5-Br-UTP	+			[10]
5-F-UTP	+			[10]
5-Hexamethyleneamino-UTP	+			[10]
6-Aza-UTP	+			[10]
4-Thio-UTP	+			[10]
Pseudo-UTP	+			[10]
8-Br-ATP	+			[10]
7-Me-GTP	–			[10]
ITP (with initiator)[a]	+			[18]
2′-dNTP	+/–,–[b]	+		[10, 19, 20]
2′-dNTPαS	+/–	+		[21–23]
2′-O-Me-NTP or -NTPαS	+/–		+	[21, 24]
2′-O-Me-NTP		+		[20]
2′-N_3-NTP	–[c]		+	[19, 24]
2′-F-(A,C,U)TP	(+), +/–[d]	+		[19, 20]
2′-Amino-UTP	(+), +/–[d]			[19]
2′-Amino-(A,C,U)TP		+		[20]
LNA-ATP	+			[24]
LNA-UTP	+			[25]
tCTP[e]	+			[26]
GTPγS	+			[27]
5′-Biotin-GMP		+		[28]
6-Thio-GMP	+			[29]
GMPS[f]	+			[30]

+/–: Low incorporation efficiency.
(+): Reduced incorporation efficiency.
[a] Inosine triphosphate (ITP) cannot be used to start transcription, but can substitute for GTP during elongation if a primer, such as 5′-ApG or 5′-GMP, is present as initiator of transcription.
[b] Tested for 2′-dCTP fully replacing CTP during transcription [19].
[c] Tested for 2′-N_3-CTP fully replacing CTP during transcription [19].
[d] Transcription efficiency strongly dependent on the specific RNA transcript [19].
[e] 1,3-Diaza-2-oxophenothiazine-ribose-5′-triphosphate (fluorescent ribonucleotide).
[f] Guanosine 5′-monophosphorothioate.

Owing to discrimination of NTPs and dNTPs by wt T7 RNAP, the polymerase incorporates NTPs 70- to 80-fold more efficiently than dNTPs in the presence of Mg^{2+} as the metal ion cofactor. However, a T7 RNAP mutant (Y639F) carrying a tyrosine to phenylalanine exchange at position 639 [31] was shown to have only about fourfold higher preference for NTPs than dNTPs [31, 32], and thus permits more efficient incorporation of substrates lacking the ribose 2′-hydroxyl, such as 2′-fluoro or 2′-amino nucleotides [32]. Incorporation of substrate analogs with 2′-ribose modifications can also be stimulated to some extent in reactions catalyzed by wt T7

RNAP upon addition of Mn^{2+} [21]. Similarly, dNTPαS analogs were partially incorporated into RNase P RNA in a sequence-specific manner under mixed metal ion conditions (Mg^{2+}/Mn^{2+}; [23]). Despite these achievements, the Y639F mutant T7 RNAP is nowadays the enzyme of choice for the incorporation of all nucleotides with 2′-ribose modifications. For detailed protocols tailored to the specific nucleotide analog, the reader is referred to [20, 32, 33]. The Y639F T7 RNAP can, of course, also be used for the synthesis of RNA transcripts with standard nucleotides. This mutant T7 RNAP can be purchased from Epicentre Biotechnologies (Madison, WI, USA) under the product name T7 R&DNA™ Polymerase, and a strain overexpressing the Y639F enzyme can be obtained from the Sousa Laboratory (Section 1.5.1).

A double mutant of T7 RNAP (Y639F/H784A) was shown to increase the efficiency of full-length RNA synthesis using modified NTPs with bulky 2′-substituents, such as 2′-OCH_3 and 2′-N_3 ([34]; 2′-OCH_3– and 2′-N_3–modified 5′-NTPs are available from TriLink BioTechnologies, San Diego, CA, USA). Analog incorporation was efficient during the elongation phase of transcription, but still inefficient during the poorly processive initiation phase (<9 nt), particularly for the 2′-OCH_3 analog [34].

Further modifications can be introduced into transcripts by initiator (oligo)nucleotides. Di- to hexanucleotides with a 3′ terminal guanine base, including di- to tetranucleotides with internal or terminal 2′-deoxy- or 2′-O-methylated residues, were tested as initiators of transcription by wt T7 RNAP [35]. 5′-Terminal incorporation varied between 20% (hexamer) and 80–95% in the case of 5′-ApG or a 5′-biotinylated ApG. ApG, 5′-biotin-ApG, 5′-fluorescein-ApG, and 5′-TAMRA-ApG are available from IBA, Göttingen, Germany (furthermore, a series of other ApG derivatives on request). For 5′-terminal labeling with biotin, 5′-biotin-GMP (TriLink BioTechnologies) can be added as starter nucleotide to transcription reactions in excess of GTP (e.g., 2 mM GTP, 3 mM 5′-biotin-GMP [28]). Similarly, 6-thio-GMP and guanosine 5′-monophosphorothioate (GMPS) were used as starter nucleotides to directly introduce a photoagent for site-specific crosslinking (6-thio-GMP [29]) or for derivatization with a photoagent (GMPS [30]) (Chapter 11). Also, transcription by T7 RNAP, in this case from the T7 ϕ2.5 class II promoter, was initiated with coenzymes containing an adenosine moiety, such as CoA (3′-dephospho-coenzyme A), NAD, or FAD. Reduced NADH and oxidized FAD are highly fluorescent, which opens up the perspective to employ coenzyme-linked RNAs for the study of RNA–RNA or RNA–protein interactions by fluorescence techniques [36].

1.3 Transcription Protocols

Generally, all solutions are prepared using double-distilled RNase-free water (dd H_2O) (exception: deionized H_2O to prepare and dilute 5 x TBE (Tris/Borate/EDTA) to 1 x TBE electrophoresis buffer) and analytical grade reagents. All stock solutions, except for electrophoresis buffers at their final dilution, are subjected to sterile filtration (Filtropur S 0.2; Sarstedt) and stored at −20 °C if not stated otherwise.

1.3.1
Transcription with Unmodified Nucleotides

The protocols given below have been applied to template DNAs directing transcription from the T7 class III promoter. Transcription yields can differ substantially, depending on the individual DNA template and the origin of T7 RNAP. In Protocols 1–5 (Hartmann Laboratory), T7 RNAP from Fermentas or home-made T7 RNAP (Section 1.5.2) has been used. These protocols may be suboptimal for some RNA transcripts and when using T7 RNAP from other sources. This has been accounted for by including protocols from the Kirsebom (Protocols 6 and 7) and Schön (Protocol 8) Laboratories. Independent of the type of protocol, it is advisable to put some effort into the optimization of the transcription protocol if large amounts of RNA are to be produced or if the transcript represents a ''standard RNA'' in the laboratory, used over longer periods and requiring repeated synthesis. In addition to commercially available T7 RNAP, protocols for the partial purification of T7 RNAP from bacterial overexpression strains have been reported [1, 37–39]. We have used and refined the protocol detailed in Section 1.5.

When a new DNA template is used for the first time, one approach is to perform small test transcriptions on a 50 µl scale according to a basic protocol (e.g., Protocol 1). Reaction mixtures should be prepared at room temperature, since DNA may precipitate in the presence of spermidine at low temperatures. In the case of plasmid DNA, template amounts of 40–80 $\mu g\,ml^{-1}$ (final assay concentration) are used as a rule of thumb, whereas a PCR template of 400 bp is adjusted to about 5 $\mu g\,ml^{-1}$ final assay concentration. In the Hartmann Laboratory, we usually incubate transcription mixes for 4–6 h at 37 °C, although overnight incubations have been used as well. A variation is to add another aliquot (e.g., 2 U μl^{-1} of reaction mix) of T7 RNAP after 2 h at 37 °C, followed by a further 2 h incubation period at 37 °C. In transcriptions according to, for example, Protocol 6, a white precipitate may appear because of pyrophosphate accumulation. This is avoided, for example, in Protocol 1, where pyrophosphate is hydrolyzed because of the presence of pyrophosphatase. Extension of incubation periods beyond 4–6 h did not prove to be advantageous in our hands and may be associated with some product degradation since T7 RNAP has a DNase and RNase function, which is normally inhibited by NTP substrates added in excess to *in vitro* transcription assays. However, after extended incubation periods, the NTP concentration may drop below a critical limit, thereby favoring RNA degradation [40]. Protocol 2 represents an inexpensive strategy to incorporate a 5′-terminal guanosine. Since guanosine has a low solubility, a 30 mM solution is prepared and kept at 75 °C; the reaction mixture – except for guanosine and T7 RNAP – is prepared at room temperature and prewarmed to 37 °C before addition of guanosine and T7 RNAP.

We would like to note here that transcription from the T7 ϕ2.5 class II promoter is initiated with A instead of G (Figure 1.1a), opening up the perspective to incorporate an adenosine at the 5′ end. Adenosine would fulfill the same purpose as the aforementioned guanosine used in the case of the class III promoter, but is advantageous because of its better water solubility [15, 36].

As an alternative to starting class III-promoter-directed transcripts with guanosine, the dinucleotide 5′-ApG (see above) may be employed. 5′-Terminal incorporation of the dinucleotide leads to a −1 adenosine extension of the transcript (Protocol 3). ApG, which is more convenient to use than guanosine, is available from TriLink BioTechnologies and IBA, but much more expensive than guanosine. Transcripts can further be initiated with 5′-GMP (Protocol 3) if present in excess of 5′-GTP (15 mM *vs* 3.75 mM). Although the majority of RNA products should possess a 5′-terminal monophosphate, a dephosphorylation/phosphorylation strategy (Chapter 3) may be preferred to obtain RNA products with 100% 5′-monophosphate ends.

Protocol 4 is a quick protocol for the synthesis of small amounts of shorter RNAs for 5′-endlabeling purposes. The promoter and template DNA oligonucleotides are simply added to the reaction mixture, which is then preincubated for 1 h at 37 °C before starting transcription by adding T7 RNAP.

Protocol 5 is tailored to the enzymatic synthesis of large amounts of RNA (e.g. for structural studies).

Protocol 6 (from the Kirsebom Laboratory) has the characteristics of high T7 RNAP concentrations and the presence of RNase inhibitor, suitable for large-scale transcriptions. Protocol 7 is used in the Kirsebom Laboratory for the production of internally labeled RNA.

Protocol 8 (from the Schön Laboratory) has been used for standard transcriptions as well as for the synthesis of extremely A+U-rich RNAs, with the ratio of individual NTPs adapted to their proportion in the final transcript.

Protocol 1: Standard Protocol (Hartmann Laboratory)

	Final concentration	1000 µl
HEPES pH 7.5, 1 M	80 mM	80 µl
DTT 100 mM	5 mM	50 µl
$MgCl_2$ 3 M	20 mM	6.7 µl
Spermidine 100 mM	1 mM	10 µl
NTP mix (25 mM each)	4 mM (each)	160 µl
Template (linearized plasmid 3.2 kb) 1 µg µl^{-1}	40 µg ml^{-1}	40 µl
Pyrophosphatase[a] 200 U ml^{-1}	1 U ml^{-1}	5 µl
T7 RNAP 200 U µl^{-1}	1000–2000 U ml^{-1}	5–10 µl
RNase-free water	—	to 1000 µl

For small scale transcriptions (50 µl final volume), reaction mixes are incubated for 4–6 h at 37 °C. For preparative transcription, usually 1 ml reaction mixtures are prepared and then incubated in 200 µl aliquots (for better thermal equilibration) for 2 h at 37 °C; then a second aliquot of T7 RNAP is added (400 U per 200 µl reaction mix), followed by another 2 h of incubation at 37 °C. Efficient transcription reactions in the 1 ml scale result in a product yield of about 3 nmol.

[a] Pyrophosphatase from yeast (Roche Cat.No. 10108987001, EC 3.6.1.1, 200 U mg^{-1}, < 0.01% ATPase and phosphatases each).

Transcription yields depend on the particular template DNA and RNA transcript. If the yield and quality (fraction of full-length transcript) of the transcription is unsatisfactory, we vary the dithiothreitol (DTT) concentration between 5 and

15 mM, and that of Mg^{2+} between 10 and 40 mM (in 50 µl test reactions). Furthermore, the DNA template, pyrophosphatase, and T7 RNAP concentrations may be increased to 80 µg ml^{-1}, 2–5 U ml^{-1}, and 3000 U ml^{-1}, respectively. We further observed that addition of 3% dimethyl sulfoxide (DMSO) can increase transcription yields severalfold (test the range between 0.5 and 10% DMSO). Another additive worth testing is betaine (Chapter 51).

Protocol 2: Transcripts Initiated with Guanosine for 5′ Endlabeling (Hartmann Lab)

	Final concentration	1000 µl
HEPES pH 7.5, 1 M	80 mM	80 µl
DTT 100 mM	5 mM[a]	50 µl
$MgCl_2$ 3 M	20 mM[a]	6.7 µl
Spermidine 100 mM	1 mM	10 µl
NTP mix (25 mM each)	3.75 mM (each)	150 µl
Template (linearized plasmid 3.2 kb) 1 µg $µl^{-1}$	40 µg ml^{-1}	40 µl
Pyrophosphatase[b] 200 U ml^{-1}	1 U ml^{-1}	5 µl
RNase-free water	—	348.3 µl

[a] May require optimization, as described for Protocol 1.
[b] See Protocol 1.

1) Prewarm mixture to 37 °C.
2) Then add 300 µl 30 mM guanosine (kept at 75 °C) to a final concentration of 9 mM.
3) Finally add 10 µl T7 RNAP (200 U $µl^{-1}$) to a final concentration of 2000 U ml^{-1}.
4) For incubation, see Protocol 1.

Protocol 3: Initiation with 5′-GMP or 5′-ApG, to Provide Transcripts with 5′-Monophosphates (5′-GMP) or with 5′-Hydroxyls for 5′-Endlabeling Purposes (5′-ApG) (Hartmann Lab)

	Final concentration	1000 µl
HEPES pH 7.5, 1 M	80 mM	80 µl
DTT 100 mM	5 mM[a]	50 µl
$MgCl_2$ 3 M	20 mM[a]	6.7 µl
Spermidine 100 mM	1 mM	10 µl
NTP mix (25 mM each)	3.75 mM (each)[b]	150 µl
5′-GMP 100 mM (initiator)[b]	15 mM	150 µl
Template (linearized plasmid 3.2 kb) 1 µg $µl^{-1}$	40 µg ml^{-1}	40 µl
Pyrophosphatase[c] 200 U ml^{-1}	5 U ml^{-1}	25 µl
T7 RNAP 200 U $µl^{-1}$	2000 U ml^{-1}	10 µl
RNase-free water	—	ad 1000 µl

[a] May require optimization, as described for Protocol 1.
[b] When 5′-GMP is replaced with the dinucleotide 5′-ApG for transcription initiation, adjust 5′-ApG to 7.5 mM and NTPs to 2.5 mM each (final concentrations). For incubation, see Protocol 1.
[c] See Protocol 1.

Protocol 4: DNA Template Assembled from Oligonucleotides, Transcripts Initiated with Guanosine (Hartmann Lab)

	Final concentration	500 µl
HEPES pH 8.0, 1 M	160 mM	80 µl
DTT 100 mM	15 mM	75 µl
$MgCl_2$ 3 M	33 mM	5.5 µl
Spermidine 100 mM	1 mM	5 µl
Promoter DNA oligonucleotide 3.3 µg µl^{-1}[a]	132 µg ml^{-1}	20 µl
Template DNA oligonucleotide 2.1 µg µl^{-1}[b]	84 µg ml^{-1}	20 µl
BSA (bovine serum albumin) 20 mg ml^{-1}	0.12 mg ml^{-1}	3 µl
RNase-free water	—	51.5 µl

[a] Promoter DNA oligonucleotide: 5′-TAA TAC GAC TCA CTA TAG.
[b] In this example, the template DNA oligonucleotide had the sequence 5′-GGT CAT AGG TAT TCC CCC TCT CTC CAT TCC TAT AGT GAG TCG TAT TAA, resulting in an RNA product with the sequence 5′-GGA AUG GAG AGA GGG GGA AUA CCU AUG ACC; the design of DNA oligonucleotides was adapted from [12, 41].

1) Mix and preincubate for 1 h at 37 °C.
2) Add 75 µl NTP mix (25 mM each) to a final concentration of 3.75 mM (each)[c]
3) Add 5 µl Pyrophosphatase (200 U ml^{-1})[d] to a final concentration of 2 U ml^{-1}.
4) Prewarm mixture to 37 °C.
5) Then add 150 µl 30 mM guanosine (kept at 75 °C) to a final concentration of 9 mM.
6) Add 10 µl T7 RNAP (200 U µl^{-1}) to a final concentration of 4000 U ml^{-1}.
7) Incubate at 37 °C for 4 h.

[c] To increase the percentage of transcripts initiated with guanosine, the ratio of guanosine to NTPs may be increased; for example, by reducing the NTP concentration to 1.5 mM each.
[d] See Protocol 1.

Protocol 5: T7 Synthesis of Large Amounts of RNA for Structural Studies (Hartmann Lab)

For such large-scale transcriptions, we first determine the optimal Mg^{2+} concentration (variation between 10 and 40 mM) in 50 µl test reactions.

	Final concentration	~15 ml
Tris-HCl pH 7.9, 1 M	200 mM	3 ml
DTT 1 M	20 mM	0.3 ml
$MgCl_2$ 150 mM	15 mM	1.5 ml
Spermidine 100 mM	2 mM	0.3 ml
Template (linearized plasmid 3.2 kb) 200 µg ml^{-1}	100 µg ml^{-1}	7.5 ml
NTP mix (25 mM each)	4 mM (each)	2.4 ml
T7 RNAP (in-house preparation)	—	37.5 µl
RNase-free water	—	—

1) Incubate at 37 °C overnight.
2) *Optional*: add 0.5 M Na_2EDTA (pH 7.5 or 8.0) until all insoluble Mg^{2+}-pyrophosphates (Section 1.3.4) have been dissolved.
3) Precipitate the RNA with ethanol (or isopropanol).

4) After ethanol precipitation, centrifuge, wash the RNA pellet with 70% ethanol, briefly centrifuge again, air-dry the pellet, dissolve it in 4–6 ml ddH_2O, and add an equal volume of denaturing loading buffer (for more details, see Section 1.3.4).
5) Load the sample (e.g., 12 ml) onto five preparative (e.g., 10%) polyacrylamide (PAA) 8 M urea gels (30 cm (width) × 20 cm (height) × 1 mm, (gel thickness), gel pocket 23 cm wide). For each gel, one may load 2 µg of a reference RNA into a small pocket (0.7 cm wide) next to the preparative sample, which helps to locate the RNA of interest during UV shadowing. Run the gels at 40 mA for 4–5 h in 1 x TBE at room temperature.
6) Localize the RNA of interest by UV shadowing (Chapter 3) and excise the corresponding gel strip. Combine the gel strips from all five gels, chop into smaller gel slices and transfer into a 50 ml Falcon tube containing 20 ml 0.3 M NaOAc pH 5; shake in an Orbital Shaker overnight at 4–10 °C, withdraw the solution using a syringe and filter over a sterile filter to get rid of residual gel pieces; precipitate with ethanol and redissolve in ~100 µl ddH_2O. Perform a second elution in the same manner as the first.

Using this protocol (without Na_2EDTA addition), we were able to purify 7.5 mg of the catalytic domain of *E. coli* RNase P RNA (~220 nt) involving two consecutive gel elutions.

Protocols 6 and 7: Kirsebom Laboratory

• 10 x Transcription buffer (TRX), for transcription Protocols 6 and 7:

	Final concentration	1000 µl
Tris-HCl pH 7.7, 1 M	400 mM	400 µl
$MgCl_2$ 3 M	240 mM	80 µl
Spermidine 100 mM	20 mM	200 µl
RNase-free water	—	320 µl

Protocol 6: Nonradioactive Transcription, Volume Sufficient for 4 Reactions (Mix Prepared for 4.5 Reactions)

	Final concentration[a]	432 µl
10 x TRX	1 x	45 µl
DTT 0.5 M	10 mM	9 µl
0.2% Triton X100	0.01%	22.5 µl
ATP (100 mM)	2 mM	9 µl
GTP (100 mM)	2 mM	9 µl
CTP (100 mM)	2 mM	9 µl
UTP (100 mM)	2 mM	9 µl
RNase inhibitor 40 $U\mu l^{-1}$ (Fermentas)	32 U ml^{-1}	0.6 µl
T7 RNAP 200 $U\mu l^{-1}$ (Ambion or prepared in-house)	10 000 U ml^{-1}	22.5 µl
RNase-free water	—	296 µl

[a] Final concentrations after addition of template.

1) To 96 µl of this mix, add 4 µl template DNA (linearized plasmid ≈ 3.2 kb; 1 µg µl^{-1}; final concentration 40 µg ml^{-1}).
2) Incubate at 37 °C for ≤ 10 h.

Protocol 7: Internal Radioactive Labeling Mix, Volume Sufficient for 9 Reactions (Mix Prepared for 10 Reactions)

	Final concentration[a]	230 µl
10 x TRX	1 x	25 µl
DTT 0.5 M	10 mM	5 µl
0.2% Triton X100	0.01%	12.5 µl
ATP (100 mM)	2 mM	5 µl
GTP (100 mM)	2 mM	5 µl
CTP (100 mM)	2 mM	5 µl
UTP 1 mM	0.2 mM	50 µl
[α−^{32}P]UTP 800 Ci mmol^{-1} (20 mCi ml^{-1})[b]	2 mCi ml^{-1}	25 µl
RNase inhibitor 24 U µl^{-1}	31.7 U ml^{-1}	0.33 µl
T7 RNAP 200 U µl^{-1}	10 000 U ml^{-1}	12.5 µl
RNase-free water	—	84.7 µl

[a] Final concentrations after addition of template.
[b] Other ^{32}P-labeled nucleotides may be used as well, also in combinations and with different specific activities.

1) To 23 µl of this mix, add 2 µl template DNA (linearized plasmid ≈ 3.2 kb; 1 µg µl^{-1}; final concentration 80 µg ml^{-1}).
2) Incubate at 37 °C for ≤ 10 h.

Protocol 8: Preparative Transcription of RNAs with Biased Nucleotide Composition (Schön Lab)

To account for unbalanced nucleotide composition, such as in RNase P RNAs from the *Cyanophora paradoxa* cyanelle [42] or from a plant-pathogenic phytoplasma [43], the relative concentrations of NTPs are adjusted accordingly. For phytoplasma RNase P RNA (about 73% A+U), the composition of the nucleotide mix was calculated as follows:

	Calculated mol% of each nucleotide in the transcript	Concentration of each NTP in the nucleotide mix (mM)	Final concentration of each NTP in the reaction mix (mM)
ATP	41.08	33	3.3
CTP	11.06	9	0.9
GTP	16.03	12.5	1.25
UTP	31.83	25.5	2.55
Total	100	80	8.0

The following sample protocol is routinely used for the preparation of large amounts of RNA and can be easily adjusted to the transcription of templates with a biased nucleotide composition. In such cases, the "standard" NTP mix (20 mM each NTP) is replaced with the template-specific NTP mix with adjusted nucleotide concentrations.

- 10 x Transcription buffer: 400 mM Tris-HCl (pH 7.9 at 25 °C), 120 mM $MgCl_2$, 50 mM DTT, 50 mM NaCl and 10 mM spermidine.

	Final concentration	**Volume added**
10 x Transcription buffer	1 x buffer	25 µl
NTP mix (here: 33 mM ATP/9 mM CTP/12.5 mM GTP/25.5 mM UTP)	0.1 x NTP mix	25 µl
Template (linearized plasmid 3.2 kb) 0.1 µg μl^{-1}	50 µg ml^{-1}	125 µl
T7 RNAP (own preparation, 5–10 µg μl^{-1} total protein)[a]	10–50 µg ml^{-1}[a]	2–5 µl[a]
RNase-free water	—	ad 250 µl

[a] Depending on the specific activity of the individual T7 RNAP preparation; see Section 1.5.2.2.
Note that because of the high concentrations of NTPs and Mg^{2+} in the reaction, insoluble precipitates may form if the complete mix is kept on ice. It is thus advisable to start with water before adding the other components, and to prewarm the mix to 37 °C before adding the template and polymerase. We let the reactions proceed for at least 2 h (up to overnight) at 37 °C and quench the excess Mg^{2+} by adding Na_2EDTA to a final concentration of 25 mM before phenol extraction and EtOH precipitation (Section 1.3.4, step 1).

After transcription, product yield and quality (5–10 µl aliquot plus an equal volume of gel loading buffer) are checked by PAGE in the presence of 8 M urea and staining with toluidine blue or ethidium bromide. Load at least one reference RNA on the gel to identify the genuine product, since sometimes a complex mixture of bands is observed. A small number of bands in addition to the product band points to good transcription performance, and high yields of transcription correlate with the observation that the RNA product appears as a prominent band, while the DNA template is faintly visible. However, with some templates one has to be satisfied with product amounts exceeding that of the template less than tenfold. Aberrant transcripts of similar size and abundance as the desired product, sometimes even appearing as a smear, can make identification and gel purification of the RNA product of interest impossible. In view of such potential problems, the best transcription protocol will be the one generating the highest amount of specific product at the lowest cost of incorrect products. If RNA yields are not satisfactory, vary concentrations of template, $MgCl_2$, T7 RNAP, or DTT for further optimization.

1.3.2 Transcription with 2′-Fluoro-Modified Nucleotides

To produce nuclease-resistant 2′-fluoro-modified RNAs with the Y639F mutant T7 RNAP, we replaced UTP and CTP with the corresponding 2′-fluoro analogs (Protocol 9: 2′-fluoro analogs supplied by IBA, Protocol 10: 2′-fluoro analogs supplied by Epicentre Biotechnologies, or TriLink BioTechnologies). Protocol 9 has been employed to transcribe an internally ^{32}P-labeled RNA library for *in vitro* selection using a 117 bp double-stranded PCR template including an internal segment of 60 randomized positions. Transcription assays were incubated for 3–4 h at 37 °C. Protocol 10 was used to enzymatically synthesize one individual aptamer (~100 nt; without ^{32}P-labeling) isolated in the above *in vitro* selection study. In this particular case, the optimal Mg^{2+} concentration was determined as 6 mM.

2′-F-ATP and 2′-F-GTP analogs have become available as well (TriLink BioTechnologies), opening the perspective to also replace purines with 2′-fluoro analogs in T7 transcripts.

Epicentre Biotechnologies also offers the DuraScribe® T7 and SP6 Transcription Kits for the synthesis of transcripts with 2′-F-pyrimidine substitutions. Such transcripts, additionally started with 5′-biotin-GMP, have been synthesized in an aptamer study [28].

Protocol 9: Transcription of a 2′-Fluoro- Modified and ^{32}P-Labeled RNA Library (Hartmann Lab)

- 10 x Transcription buffer: 400 mM Tris-HCl (pH 8.0), 200 mM $MgCl_2$, 10 mM spermidine, 0.1% Triton X100.

	Final concentration	150 µl
10 x Transcription buffer	1 x buffer	15 µl
DTT 100 mM	5 mM	7.5 µl
2′-F-CTP, 2′-F-UTP (10 mM each)	1.25 mM (each)	18.75 µl
ATP, GTP (10 mM each)	1.25 mM (each)	18.75 µl
[α–^{32}P]ATP 800 Ci mmol^{-1} (10 mCi ml^{-1})	0.2 mCi ml^{-1}	3 µl
PCR template	1–3.33 nmol ml^{-1}	0.15–0.5 nmol
Y639F mutant T7 RNAP (~ 4 µg µl^{-1})	100 µg ml^{-1}	3.8 µl
RNase-free water	—	ad 150 µl

Protocol 10: Transcription of an Individual 2′-Fluoro-Modified RNA Aptamer (Hartmann Lab)

- 2 x Transcription buffer: 160 mM HEPES (pH 7.5), 4 mM spermidine, 20 mM DTT, 0.24 mg ml^{-1} BSA (Sigma–Aldrich A7906).

	Final concentration	50 µl
2 x Transcription buffer	1 x buffer	25 µl
$MgCl_2$ 0.5 M	6 mM	0.6 µl
Pyrophosphatase 0.1 mg/ml[a]	5 µg ml^{-1}	2.5 µl
Template (BamH1-linearized plasmid 3.1 kb) 1 µg $µl^{-1}$	80 µg ml^{-1}	4.0 µl
2′-F-CTP, 2′-F-UTP (25 mM each)	1.88 mM (each)	3.75 µl
ATP, GTP (25 mM each)	1.88 mM (each)	3.75 µl
Y639F mutant T7 RNAP (prepared according to Section 1.5.2.2)	—	1 µl
RNase-free water	—	ad 50 µl

[a] Pyrophosphatase (Roche) diluted from stock (1 mg ml^{-1}) with 50% glycerol.

1.3.3
T7 Transcripts with 5′-Cap Structures

Eukaryotic mRNAs, snRNAs, and many viral RNAs carry 5′-cap structures that are important for RNA stability, splicing, intracellular transport, initiation of protein synthesis, or infectivity (viral RNAs). 5′-Capped RNAs for biochemical studies can be synthesized by T7 RNAP, with four different cap structures available from Epicentre Biotechnologies CellScript: unmethylated (G[5′]ppp[5′]G), monomethylated (m^7G[5′]ppp[5′]G), trimethylated ($m_3^{2,2,7}$G[5′]ppp[5′]G), and antireverse cap analog (ARCA; $m_2^{7,3'\text{-}O}$G[5′]ppp[5′]G). With the m^7G[5′]ppp[5′]G analog, 50% of the cap is incorporated in the reverse orientation, which compromises translation efficiency. In contrast, ARCA is incorporated exclusively in the correct orientation, but the priming of transcripts with ARCA is less efficient than with the standard monomethylated cap analog [44]. Protocol 11 (see below) has been routinely applied to transcription of RNAs with unmethylated or monomethylated cap analogs. If the outcome with this protocol is unsatisfactory for ARCA-initiated T7 transcription, Epicentre offers the MessageMAX™ T7 ARCA-Capped Message Transcription Kit for high-yield transcription.

Protocol 11: Transcription with 5′-Cap Analogs (Bindereif Laboratory)

- 5 x Cap transcription buffer: 200 mM Tris-HCl (pH 7.9), 30 mM $MgCl_2$, 50 mM DTT, 50 mM NaCl, 10 mM spermidine
- T7 RNAP (Fermentas)
- RNaseOUT™ Recombinant Ribonuclease Inhibitor (Life Technologies Invitrogen).

	Final concentration	25 µl
5 x Cap transcription buffer	1 x	5 µl
ATP, CTP, UTP (5 mM each)	0.5 mM (each)	2.5 µl
GTP 1 mM	0.1 mM	2.5 µl
Cap analog (10 mM)	1 mM	2.5 µl
Template (linearized plasmid 3.2 kb) 1 µg µl^{-1}	40 µg ml^{-1}	1 µl
T7 RNAP 20 U µl^{-1}	800 U ml^{-1}	1 µl
RNaseOUT 40 U µl^{-1}	1600 U ml^{-1}	1 µl
RNase-free water	—	ad 25 µl

Incubate for 1–2 h at 37 °C.

1.3.4 Purification

Denaturing PAGE is most commonly used to purify RNA transcripts. However, the denaturing conditions may lead to aberrant folding or aggregation of the RNA sample, which may persist despite a refolding procedure applied before RNA use. For such cases, Chapter 5 describes the method of non-denaturing RNA purification by weak anion-exchange chromatography. Alternative methods are preparative high-performance liquid chromatography (HPLC) if available (see Chapters 7 and 23) or native PAGE to purify a homogeneously folded RNA conformer. If the full-length RNA of interest is the main product of the *in vitro* transcription, one may simply use Amicon® Ultra centrifugal filter devices provided with different molecular weight cutoffs (e.g., 50 kDa; Millipore) to concentrate the RNA, to remove salts and exchange buffer.

The purification steps outlined below involve denaturing PAGE. Some purification steps are optional and depend on transcription quality and the demands on product purity. Often, a purification procedure only including steps 5–8 is sufficient. Another protocol for purification of RNA by denaturing PAGE is described in Chapter 3, Section 3.4, Protocol 3.

1) *Insoluble pyrophosphate complexes*. In transcription reactions without pyrophosphatase or when pyrophosphatase activity is low, a white pyrophosphate precipitate may form. In such cases, remove the precipitate before steps 2 (see below) by centrifugation at 14000 *g* for about 5 min directly after transcription. Carefully remove the clear supernatant and transfer to a new Eppendorf tube for further sample processing. Also, Na_2EDTA (500 mM, pH 7.5) may be added immediately after transcription to give a final concentration of 50–100 mM. By chelating Mg^{2+}, the formation of insoluble precipitates is substantially reduced.
2) *DNase I digestion to remove template DNA*. Add 10 U DNase I (RNase-free, Roche) per 200 µl and incubate for 20 min at 37 °C.
3) *Phenol and chloroform extractions to remove the enzyme(s)*. For extraction with phenol (Roti®Aqua-Phenol, water-saturated, pH 4.5–5.0; Roth #A980.3), mix

the sample with 1 vol. phenol, vortex for 30 s, centrifuge 1– 5 min (until phases have cleared) at 12 000 *g*, withdraw the aqueous upper phase and mix it with 1 vol. chloroform, vortex for 30 s, centrifuge 3 min at 12 000 *g*, and transfer the aqueous upper phase to a new tube, avoiding to withdraw any chloroform.

4) *Removal of salt for better gel resolution*. For example, use illustra NAP-10 Columns (GE Healthcare); column material: Sephadex G-25.
5) *Ethanol precipitation*. Ethanol precipitation is performed to remove residual chloroform and salts, and to concentrate the RNA. Mix sample with 2.5 vol. ethanol, 0.1 vol. 3 M NaOAc (pH 4.7), and 1 µl glycogen (20 µg μl^{-1}); leave for 10–20 min at −70 °C or at least 2 h at −20 °C. Centrifuge for 30–45 min at 4 °C and 16 000 *g*. Wash the pellet with 70% ethanol and centrifuge again for 10 min. After ethanol precipitation and air drying of the pellet, redissolve it in a small volume of RNase-free water and add an equal volume of gel loading buffer (2 x TBE (see step 6), pH 8.0, 2.7 M urea, 67% formamide, 0.02% (w/v) each bromophenol blue (BPB) and xylene cyanol blue (XCB)).
6) *Preparative denaturing PAGE*. A gel well, 6–7 cm wide and 1 mm thick, is appropriate for loading the product RNA from an efficient 1 ml transcription reaction. The pocket size is of some importance, as an overloaded gel may cause separation problems; on the other hand, if the pocket is too large, RNA bands may be barely visible and elution efficiency may decrease. After electrophoresis, the desired RNA band is visualized at 254 nm by UV shadowing and marked for gel excision (for details, see Chapter 3); gel running buffer: 1 x TBE (89 mM Tris base, 89 mM boric acid, 2 mM EDTA).
7) *Elution of RNA product*.
   a. **Diffusion elution**. Cover the excised gel pieces with elution buffer and shake overnight at 4 °C; in the case of efficient transcriptions, a second gel elution step in fresh elution buffer may substantially increase the yield. Different elution buffers can be used: buffer A: 1 mM EDTA, 200 mM Tris-HCl (pH 7.0)/buffer B: 0.3–1.0 M NaOAc (pH 4.7–5.0)/buffer C (successfully used for the elution of phosphorothioate-modified RNAs): 1 M NH_4OAc (pH 7.0). Usually, buffer A is used; buffer B was found to be advantageous in cases where elution efficiency with buffer A was low. After elution, RNA is concentrated by ethanol precipitation. A lower NaOAc concentration of buffer B (e.g., 0.3 M) lowers the risk of precipitating NaOAc by ethanol precipitation, which may compromise RNA solubility.
   b. **Alternatively – electroelution**. Excised gel pieces containing the RNA are placed in an Elutrap® chamber (Schleicher & Schuell BioScience, VWR) following the manufacturer's protocol. The RNA is eluted in 0.5 x TBE buffer (see step 6). The final volume of RNA solution after elution is approximately 600 µl, depending on the extent of evaporation during the elution process. The elution is permitted to proceed overnight at 150 V/20 mA or for 4–6 h at 200–300 V/30 mA. During the elution process there is evaporation, resulting in condensation on the lid of the Elutrap chamber. This lid has to be closed when the Elutrap is running. To minimize evaporation/condensation, the Elutrap should not be run at a voltage higher than 150 V overnight. After

elution, the RNA is extracted once with phenol and twice with chloroform, followed by ethanol precipitation.

8) *Quantification (UV spectroscopy, see* Chapter 3 *and Appendix) and quality check (analytical denaturing PAGE).*

1.4 Troubleshooting

1.4.1 Low or No Product Yield

- Evidence for RNase contamination: check electrophoresis equipment; the use of a single graduated cylinder for diluting 5 x TBE stocks to 1 x TBE buffer, irrespective of DNA or RNA work, is an obvious source of RNase contamination. Decontaminate electrophoresis equipment by soaking in 3% hydrogen peroxide for 10 min. Remove peroxide by extensively rinsing with RNase-free water (diethyl pyrocarbonate (DEPC)-treated or double-distilled). Also, freshly prepare 1 x TBE buffer. Check quality of deionized water used for preparing the TBE electrophoresis buffer, which may contain traces of transition metal ions. If necessary, switch to double-distilled or DEPC-treated water as used for all other aqueous solutions in RNA work. Several companies offer RNase inhibitors, but this is not a solution in case of general RNase degradation problems. For avoidance of RNase contamination, see also web pages of, for example, New England Biolabs (NEB) or Ambion (NEB web page, Technical Reference, General Molecular Biology Data ''Avoiding Ribonuclease contamination''; Ambion Life Technologies, RNA Technical Resources page, ''The Basics: RNase Control'').
- If product yields are low with a protocol that had already been successfully used for the same template, repeat transcription assay once without any alteration on a 50 µl scale; if unsuccessful, test different enzyme batches or enzymes from alternative suppliers as well. Differences between enzyme preparations can be considerable.
- Be sure that all components (except enzymes) have been warmed up to ambient temperature before preparation of reaction mixtures.
- Check that thawed stock solutions, particularly concentrated transcription buffers, do not contain precipitated ingredients. For nucleotide solutions, limit freeze/thawing cycles, store in aliquots at $-20\,^{\circ}C$, and adjust stock solutions (in H_2O) with NaOH to pH 7.0 or consider to buffer with 10–40 mM Tris-HCl adjusted to the pH used in transcription reactions; be aware that diluted working solutions may degrade rapidly.
- Check integrity of the DNA template (prepare new template if quality is equivocal), do not use linearized plasmids with 3′ overhangs and consider transcribing a control template for comparison. Take particular care to effectively remove traces of SDS, EDTA, RNases, salts (~50% inhibition of T7 RNAP by NaCl or KCl above 150 mM) as well as traces of phenol and chloroform.

- For templates with a highly biased nucleotide composition (e.g., coding for RNAs with extremely high A+U content [42, 43]), adjust the NTP solution according to the nucleotide composition of the transcript (Protocol 8). However, do not alter the total nucleotide concentration of the reaction mix. For G+C – rich template DNAs, increased GTP and CTP levels, an elevated assay temperature (42 °C), addition of *E. coli* single-stranded DNA-binding protein (SSB; [45]) or addition of 3% DMSO may be considered to increase transcript yields.
- In transcription reactions without pyrophosphatase, pyrophosphate precipitates may form. Such precipitates can be removed as described in step 1 of Section 1.3.4.
- Gel entry problems or smear on gel: consider to apply steps 1–5 of Section 1.3.4 before proceeding to gel purification (step 6).

1.5 Rapid Preparation of T7 RNA Polymerase

This protocol is based on the publications of Grodberg and Dunn [37] and Zawadzki and Gross [38] and provides a fast and efficient procedure for the preparation of a highly stable T7 RNAP, which is sufficiently pure for most purposes. The chromatography is described for fast-performance liquid chromatography (FPLC), but any standard low-pressure equipment will give satisfactory results if the procedure is adapted accordingly.

1.5.1 Required Material

- *E. coli* BL21 pAR1219 ([1, 46], provided by F.W. Studier, Biology Department, Brookhaven National Laboratory, Upton, NY 11973, USA) or *E. coli* BL21 pDPT7Y639F ([47], provided by R. Sousa, Department of Biochemistry, University of Texas Health Science Center, San Antonio, Texas 78229, USA).
- Branson Sonifier 250 with Tip 1/2'' (end diameter 13 mm) (Heinemann, Schwäbisch Gmünd, Germany, Cat. No. 101-063-674 and 101-148-013).

1.5.1.1 Medium

LB (Luria-Bertani) medium [48] supplemented with 50–100 μg ml^{-1} ampicillin.

1.5.1.2 Buffers and Solutions

- 1 M IPTG;
- TEN buffer (50 mM Tris HCl pH 8.1; 2 mM Na_2EDTA; 20 mM NaCl);
- Phenylmethyl sulfonyl fluoride (PMSF), 20 mg ml^{-1} in isopropanol;
- Leupeptin, 5 mg ml^{-1};
- 0.8% Na-deoxycholate solution;
- 2 M ammonium sulfate (enzyme grade);
- Polymin P: 10% solution, adjusted to pH 8 with HCl;
- Saturated ammonium sulfate solution (4.1 M; adjust pH to 7 with some drops of concentrated Tris base, keep at 4 °C where a precipitate will form);

- Buffer C-10, C-100, C-300, C-500, C-1000: buffer C (20 mM Na-phosphate pH 7.7; 1 mM Na_2EDTA; 1 mM DTT; 5% (w/v) glycerol) adjusted to 10, 100, 300, 500, or 1000 mM NaCl. The basis for all C buffers is a 1 M stock of phosphate buffer pH 7.7 (diluted from a mixture of 84.5 vol. of 1 M Na_2HPO_4 and 15.5 vol. of 1 M NaH_2PO_4; if crystals have formed, they need to be dissolved before this solution is used). Directly before use, supplement with NaCl (stock 5 M), PMSF, DTT (stock 1 M), and glycerol to the desired final concentrations; adjust the final volume by addition of double-distilled water precooled to 4 °C.
- 4 x Laemmli gel loading buffer: 100 mM Tris/HCl pH 6.8, 8% (w/v) SDS, 30% glycerol (w/v), 8% (v/v) β-mercaptoethanol, 0.04% (w/v) BPB; adjust pH before addition of BPB.
- sterile glycerol (autoclaved) 99.5%.

1.5.1.3 Electrophoresis and Chromatography

- Laemmli-type SDS gel for protein separation under denaturing conditions (10% PAA);
- Merck EMD Fractogel SO_3^-, equilibrated in buffer C-100 in a 2 × 10 cm column.

1.5.2 Procedure

1.5.2.1 Cell Growth, Induction, and Test for Expression of T7 RNAP

1) Inoculate 25 ml LB containing 50–100 μg ml^{-1} ampicillin with a colony from a fresh plate culture and grow overnight at 37 °C.
2) Two 2 l flasks with 500 ml of the same medium are inoculated 1 : 50 from this culture; grow at 37 °C under vigorous shaking.
3) When the cultures have reached an OD_{600} of about 0.6 (which should take not longer than 3 h), transfer 1 ml to an Eppendorf tube, centrifuge for 5 min at 5000 rpm in a desktop centrifuge, and keep the sediment as a control.
4) Then induce T7 RNAP expression in the remaining culture by addition of IPTG to a final concentration of 1 mM and shake the culture for 3 h at 37 °C.
5) Take a 1 ml sample as in step 3; harvest the bulk of the culture by centrifugation (10 min, 3000–5000 *g*), wash once with TEN buffer, shock-freeze in liquid N_2 or dry ice, and keep at −80 °C until needed.
6) Analyze the 1 ml samples from steps 3 and 5 for expression of T7 RNAP as follows: resuspend the cell sediment in an appropriate volume (example: if OD_{600} before induction was 0.6, suspend pellet in 60 μl and if OD_{600} after induction was 2.5, suspend pellet in 250 μl) of 1 x Laemmli gel loading buffer (note that this buffer is usually prepared as a more concentrated stock solution; see Section 1.5.1.2) and denature for 2 min at 95 °C. Then load 10 μl of each sample onto an SDS-10% polyacrylamide gel with appropriate size markers. If expression induction has been successful (a strong band of about 100 kDa should appear 2–3 h after induction), proceed with enzyme purification.

1.5.2.2 **Purification of T7 RNAP**

Generally, all steps are performed on ice or at 4 °C, and all buffers are supplemented with the protease inhibitor PMSF (20 μg ml^{-1}, if not stated otherwise). From each purification step, a small sample should be retained for SDS-PAGE monitoring of the purification process. All buffers used for FPLC must be filtered through a 0.2 μm filter.

1) Resuspend cells in 24 ml of TEN buffer supplemented with 50 μl of PMSF and 20 μl of leupeptin stock solutions (Section 1.5.1.2).
2) Add 2.5 ml of 0.8% Na-deoxycholate solution and incubate for 20 min.
3) Sonicate the sample to further promote cell lysis and to shear the DNA (15 min; duty cycle 50%; output 4; in an ice-water bath).
4) Centrifuge to clear the supernatant (15 min, 18 000 *g*, 4 °C).
5) Add 5 ml 2 M ammonium sulfate and adjust the total volume to 50 ml with TEN buffer.
6) Precipitate DNA by slow addition of 5 ml Polymin P and stirring for 20 min.
7) After centrifugation (15 min, 18 000 *g*, 4 °C), keep the supernatant and determine its volume.
8) Precipitate the enzyme from the supernatant by slow addition of 0.82 vol. of saturated ammonium sulfate and stirring for another 15 min.
9) After centrifugation for 15 min at 18 000 *g*, 4 °C, resuspend the sediment in 10 ml buffer C-100, and dialyze 3 × against 1 l of the same buffer (1 × 1 h, 1 × 1 h, 1 × overnight). Dialysis should be extensive in order to completely remove the ammonium sulfate contained in the sediment; otherwise, T7 RNAP will not bind to the cation exchange column.
10) Remove insoluble material by centrifugation as in step 9.
11) Filtrate the supernatant using a syringe filter (Sarstedt, Filtropur S 0.45).
12) Apply the filtrate to the EMD-SO_3^--column at a flow rate of 1–2 ml min^{-1}. Wash the column with 10 vol. of buffer C-100 or until protein is no longer detectable in the flow-through. Then apply a 500 ml gradient from 100 to 1000 mM NaCl in buffer C and collect 3 ml fractions. T7 RNAP elutes between 150 and 250 mM NaCl from the EMD-SO_3^--column, as visible by the high protein content in these fractions. Finally, wash the column with buffer C-1000; the resin can be reused after equilibration in buffer C-100.
13) Five microliters of each fraction and the flow-through, and 2–5 μl of the input material loaded in step 12, are then analyzed by SDS-PAGE.
14) The fractions containing the highest amounts of T7 RNAP (around 13 fractions each 3 ml) are pooled and dialyzed 3 × against 1 l of buffer C-10 (1 × 1 h, 1 × 1 h, 1 × overnight).
15) After dialysis, centrifuge (15 min at 18 000 *g*, 4 °C). It depends on the T7 RNAP concentration in the dialyzed sample if the enzyme will precipitate at this point or not. If there is massive precipitation, proceed to step 17; if no pellet is visible, concentrate the supernatant as described in step 16.
16) The supernatant is concentrated with centrifugal filter devices (Amicon Ultra-15, Millipore) to about 0.5–1 ml, followed by addition of an equal volume of

sterile glycerol; store in aliquots at −20 °C. Yet, if the enzyme precipitates during this concentration process (and clogs the filter), then disperse the supernatant in the filter unit by pipetting up and down and transfer it to a centrifugation tube, followed by centrifugation (15 min at 18 000 *g*, 4 °C). The pellet is handled as described in step 17. The supernatant is concentrated in a new centrifugal filter to about 0.3–0.5 ml, followed by addition of an equal volume of sterile glycerol and storage in aliquots at −20 °C.

17) The pellet containing T7 RNAP is dissolved in 0.3–0.5 ml C-300 buffer; then add an equal volume of sterile glycerol and store the enzyme preparation in aliquots at −20 °C.

Specific activity of T7 RNAP may be determined by incorporation of ^{32}P-labeled nucleotides into acid-precipitable material and can reach 400 000 U mg^{-1} (one U is defined as the incorporation of 1 nmol AMP into acid-precipitable material in 1 h at 37 °C [12]). For most purposes, it is sufficient to titrate the amount of enzyme preparation needed to give good transcription yields without too many side products. To analyze a typical preparation of T7 RNAP (5–10 µg µl^{-1} total protein content), we usually test 1 µl and 1 µl of a 1 : 10 dilution according to Protocol 1 scaled down to 50 µl and containing a standard template DNA (about 50% G + C). Transcription efficiency and quality are then evaluated by analyzing 5 µl (1/10th) of the test transcription on a denaturing PAA gel followed by gel staining with ethidium bromide. Alternatively, for easier detection of abortive transcripts and degradation products, a radioactive tracer (e.g., 10 µCi of [α-^{32}P]UTP or [α-^{32}P]GTP) can be added to the transcription reaction and the products visualized on a Phosphorimager or by autoradiography (Protocol 7).

1.5.3 Notes and Troubleshooting

1) We advise not to use lysozyme for cell disruption (Section 1.5.2.2, steps 2 and 3). In our hands, lysozyme treatment caused T7 RNAP to appear entirely in the insoluble fraction, for reasons not understood at present.
2) If protein gel electrophoresis of crude cell samples (Section 1.5.2.1, step 6) yields smeared bands, try to shear the DNA by sonication as described for the enzyme purification (Section 1.5.2.2, step 3). Alternatively, samples can be squeezed with a syringe through a thin (0.7 mm, or 22-gauge) needle.
3) If expression of T7 RNAP is insufficient, start again with a fresh cell clone picked from an LB-ampicillin agar plate; if this is unsuccessful, prepare a new IPTG stock solution, vary the IPTG concentration used for induction, change growth times before and after induction, or switch to other growth media, such as TB or 2 x YT [48], or M9 Minimal Medium supplemented with trace elements [49].
4) The Fractogel EMD-SO_3^--column can be substituted by any strong cation exchanger of the SO_3^--type. However, with most other column matrices, T7 RNAP elutes at different NaCl concentrations.

5) High enzyme concentrations and low salt favor the precipitation of T7 RNAP during dialysis. For this reason, the dialysis step is performed in buffer C-10 (Section 1.5.2.2, step 14). When performing the dialysis step in C-100, we did not observe any precipitation of T7 RNAP, even not during the volume reduction procedure with centrifugal filter devices (step 16), indicating that the enzyme concentration was not high enough for precipitation at 100 mM NaCl.

References

1. Davanloo, P., Rosenberg, A.H., Dunn, J.J., and Studier, F.W. (1984) Cloning and expression of the gene for bacteriophage T7 RNA polymerase. *Proc. Natl. Acad. Sci. U.S.A.*, **81**, 2035–2039.
2. Ebright, R.H. (2000) RNA polymerase: structural similarities between bacteria RNA polymerase and eukaryotic RNA polymerase II. *J. Mol. Biol.*, **304**, 687–698.
3. Cheetham, G.M.T. and Steitz, T.A. (2000) Insights into transcription: structure and function of single-subunit DNA-dependent RNA polymerases. *Curr. Opin. Struct. Biol.*, **10**, 117–123.
4. Diaz, G.A., Rong, M., McAllister, W.T., and Durbin, R.K. (1996) The stability of abortively cycling T7 RNA polymerase complexes depends upon template conformation. *Biochemistry*, **35**, 10837–10843.
5. Kochetkov, S.N., Rusakova, E.E., and Tunitskaya, V.L. (1998) Recent studies of T7 RNA polymerase mechanism. *FEBS Lett.*, **440**, 264–267.
6. Brakmann, S. and Grzeszik, S. (2001) An error-prone T7 RNA polymerase mutant generated by directed evolution. *ChemBioChem*, **2**, 212–219.
7. Huang, F., Bugg, C.W., and Yarus, M. (2000) RNA-catalyzed CoA, NAD, and FAD synthesis from phosphopantetheine, NMN, and FMN. *Biochemistry*, **39**, 15548–15555.
8. Dunn, J.J. and Studier, F.W. (1983) Complete nucleotide sequence of bacteriophage T7 DNA and the locations of T7 genetic elements. *J. Mol. Biol.*, **166**, 477–535.
9. Lee, S.S. and Kang, C. (1993) Two base pairs at -9 and -8 distinguish between the bacteriophage T7 and SP6 promoters. *J. Biol. Chem.*, **268**, 19299–19304.
10. Milligan, J.F. and Uhlenbeck, O.C. (1989) Synthesis of small RNAs using T7 RNA polymerase. *Meth. Enzymol.*, **180**, 51–63.
11. Pokrovskaya, I.D. and Gurevich, V.V. (1994) *In vitro* transcription: preparative RNA yields in analytical scale reactions. *Anal. Biochem.*, **220**, 420–423.
12. Milligan, J.F., Groebe, D.R., Witherell, G.W., and Uhlenbeck, O.C. (1987) Oligoribonucleotide synthesis using T7 RNA polymerase and synthetic DNA templates. *Nucleic Acids Res.*, **15**, 8783–8798.
13. Helm, M., Brulé, H., Giegé, R., and Florentz, C. (1999) More mistakes by T7 RNA polymerase at the 5′ ends of *in vitro*-transcribed RNAs. *RNA*, **5**, 618–621.
14. Pleiss, J.A., Derrick, M.L., and Uhlenbeck, O.C. (1998) T7 RNA polymerase produces 5′ end heterogeneity during *in vitro* transcription from certain templates. *RNA*, **4**, 1313–1317.
15. Coleman, T.M., Wang, G., and Huang, F. (2004) Superior 5′ homogeneity of RNA from ATP-initiated transcription under the T7 phi 2.5 promoter. *Nucleic Acids Res.*, **32**, e14.
16. Sampson, J.R. and Uhlenbeck, O.C. (1988) Biochemical and physical characterization of an unmodified yeast phenylalanine transfer RNA transcribed *in vitro*. *Proc. Natl. Acad. Sci. U.S.A.*, **85**, 1033–1037.
17. Griffiths, A.D., Potter, B.V., and Eperon, I.C. (1987) Stereospecificity of nucleases towards phosphorothioate-substituted RNA: stereochemistry of transcription by

T7 RNA polymerase. *Nucleic Acids Res*, **15**, 4145–4162.

18. Axelrod, V.D. and Kramer, F.D. (1985) Transcription from bacteriophage T7 and SP6 RNA polymerase promoters in the presence of 3′-deoxyribonucleoside 5′-triphosphate chain terminators. *Biochemistry*, **24**, 5716–5723.
19. Aurup, H., Williams, D.M., and Eckstein, F. (1992) 2′-Fluoro- and 2′-amino-2′-deoxynucleoside 5′-triphosphates as substrates for T7 RNA polymerase. *Biochemistry*, **31**, 9636–9641.
20. Padilla, R. and Sousa, R. (1999) Efficient synthesis of nucleic acids heavily modified with non-canonical ribose 2′-groups using a mutant T7 RNA polymerase. *Nucleic Acids Res.*, **27**, 1561–1563.
21. Conrad, F., Hanne, A., Gaur, R.K., and Krupp, G. (1995) Enzymatic synthesis of 2′-modified nucleic acids: identification of important phosphate and ribose moieties in RNase P substrates. *Nucleic Acids Res.*, **23**, 1845–1853.
22. Gaur, R.K. and Krupp, G. (1993) Enzymatic RNA synthesis with deoxynucleoside 5′-O-(1-thiotriphosphates). *FEBS Lett.*, **315**, 56–60.
23. Hardt, W.D., Erdmann, V.A., and Hartmann, R.K. (1996) Rp-deoxy-phosphorothioate modification interference experiments identify 2′-OH groups in RNase P RNA that are crucial to tRNA binding. *RNA*, **2**, 1189–1198.
24. Veedu, R.N., Vester, B., and Wengel, J. (2008) Polymerase chain reaction and transcription using locked nucleic acid nucleotide triphosphates. *J. Am. Chem. Soc.*, **130**, 8124–8125.
25. Veedu, R.N., Burri, H.V., Kumar, P., Sharma, P.K., Hrdlicka, P.J., Vester, B., and Wengel, J. (2010) Polymerase-directed synthesis of C_5-ethynyl locked nucleic acids. *Bioorg. Med. Chem. Lett.*, **20**, 6565–6568.
26. Stengel, G., Urban, M., Purse, B.W., and Kuchta, R.D. (2010) Incorporation of the fluorescent ribonucleotide analogue tCTP by T7 RNA polymerase. *Anal Chem.*, **82**, 1082–1089.
27. Logsdon, N., Lee, C.G.L., and Harper, J.W. (1992) Selective 5′ modification of T7 RNA polymerase transcripts. *Anal. Biochem.*, **205**, 36–41.
28. Kim, Y.-H., Sung, H.J., Kim, S., Lee, J.W., Moon, J.Y., Choi, K., Jung, J.E., Lee, Y., Koh, S.S., Rhee, S.G., Heo, K., and Kim, I.H. (2011) An RNA aptamer that specifically binds pancreatic adenocarcinoma up-regulated factor inhibits migration and growth of pancreatic cancer cells. *Cancer Lett.*, **313**, 76–83, doi: 10.1016/j.canlet.2011.08.027.
29. Christian, E.L., McPheeters, D.S., and Harris, M.E. (1998) Identification of individual nucleotides in the bacterial ribonuclease P ribozyme adjacent to the pre-tRNA cleavage site by short-range photo-cross-linking. *Biochemistry*, **37**, 17618–17628.
30. Harris, M.E. and Christian, E.L. (1999) Use of circular permutation and end modification to position photoaffinity probes for analysis of RNA structure. *Methods*, **18**, 51–59.
31. Sousa, R. and Padilla, R. (1995) A mutant T7 RNA polymerase as a DNA polymerase. *EMBO J.*, **14**, 4609–4621.
32. Huang, Y., Eckstein, F., Padilla, R., and Sousa, R. (1997) Mechanism of ribose 2′-group discrimination by an RNA polymerase. *Biochemistry*, **36**, 8231–8242.
33. Ryder, S.P. and Strobel, S.A. (1999) Nucleotide analog interference mapping. *Methods*, **18**, 38–50.
34. Padilla, R. and Sousa, R. (2002) Y639F/H784A T7 RNA polymerase double mutant displays superior properties for synthesizing RNAs with non-canonical NTPs. *Nucleic Acids Res.*, **30**, e138.
35. Pitulle, C., Kleineidam, R.G., Sproat, B., and Krupp, G. (1992) Initiator oligonucleotides for the combination of chemical and enzymatic RNA synthesis. *Gene*, **112**, 101–105.
36. Huang, F. (2003) Efficient incorporation of CoA, NAD and FAD into RNA by *in vitro* transcription. *Nucleic Acids Res.*, **31**, e8.
37. Grodberg, J. and Dunn, J.J. (1988) *ompT* encodes the *Escherichia coli* outer membrane protease that cleaves T7 RNA polymerase during purification. *J. Bacteriol.*, **170**, 1245–1253.

38. Zawadzki, V. and Gross, H.J. (1991) Rapid and simple purification of T7 RNA polymerase. *Nucleic Acids Res.*, **19**, 1948.
39. Ellinger, T. and Ehricht, R. (1998) Single-step purification of T7 RNA polymerase with a 6-histidine tag. *BioTechniques*, **24**, 718–720.
40. Sastry, S.S. and Ross, B.M. (1997) Nuclease activity of T7 RNA polymerase and the heterogeneity of transcription elongation complexes. *J. Biol. Chem.*, **272**, 8644–8652.
41. Liu, F. and Altman, S. (1996) Requirements for cleavage by a modified RNase P of a small model substrate. *Nucleic Acids Res.*, **24**, 2690–2696.
42. Baum, M., Cordier, A., and Schön, A. (1996) RNase P from a photosynthetic organelle contains an RNA homologous to the cyanobacterial counterpart. *J. Mol. Biol.*, **257**, 43–52.
43. Wagner, M., Fingerhut, C., Gross, H.J., and Schön, A. (2001) The first phytoplasma RNase P RNA provides new insights into the sequence requirements of this ribozyme. *Nucleic Acids Res.*, **29**, 2661–2665.
44. Peng, Z.H., Sharma, V., Singleton, S.F., and Gershon, P.D. (2002) Synthesis and application of a chain-terminating dinucleotide mRNA cap analog. *Org. Lett.*, **4**, 161–164.
45. Aziz, R.B. and Soreq, H. (1990) Improving poor *in vitro* transcription from G,C-rich genes. *Nucleic Acids Res.*, **18**, 3418.
46. Studier, F.W., Rosenberg, A.H., Dunn, J.J., and Dudendorff, J.W. (1990) Use of T7 RNA polymerase to direct expression of cloned genes. *Meth. Enzymol.*, **185**, 60–89.
47. Bonner, G., Patra, D., Lafer, E.M., and Sousa, R. (1992) Mutations in T7 RNA polymerase that support the proposal for a common polymerase active site structure. *EMBO J.*, **11**, 3767–3775.
48. Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989) *Molecular Cloning*, Cold Spring Harbor Laboratory Press.
49. Mossakawska, D.E. and Smith, R.A.G. (1997) in *Protein NMR Techniques* (ed. D.G. Reid), Humana Press Inc., Totowa, NJ, 325–335.