Part I
RNA Synthesis

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I.1
Enzymatic RNA Synthesis, Ligation and Modification

1
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 1984 [1]. In contrast to multi-subunit DNA-dependent RNA polymerases from eukaryotes and prokaryotes, T7 RNAP consists of a single subunit of about 100 kDa [2]. The subdomains adopt a hand-like shape with the 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 to the active site [3].

T7 RNAP is highly specific for its own promoters and exhibits no affinity even to closely related phage T3 promoters, although the 23-bp consensus sequences are very similar (Fig. 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 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 will describe protocols that have been used routinely for T7 transcription. 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 transcription protocols given 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 transcription conditions. We also draw the reader's attention to the paper by Milligan and Uhlenbeck [7], which briefly discusses many fundamental aspects of T7 transcription and is still handed out to every new member of our groups.

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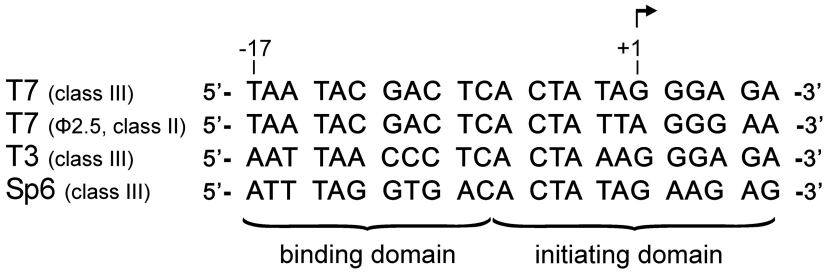
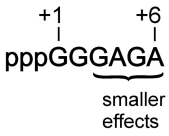
A



B



Nucleotide +1	Relative yield	Nucleotide +2	Relative yield
C	0.1	C	0.5
A	0.2	A	0.5
U	n.d.	U	n.d.
G	1.0	G	1.0

C

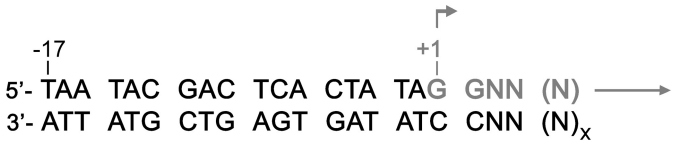


Fig. 1.1. (A) Consensus sequences of class III promoters of bacteriophages T7, T3 and SP6, and sequence of the T7 ϕ2.5 class II promoter [5, 34–36]. Phage polymerase initiating domains also include the first 5–6 nt of the transcribed template strand. The transcription start (position +1) is indicated 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 10 T7 class II promoters plus one more replication promoter (ϕOL); among these 11 promoters, which display some sequence variation within the −17 to +6 region, only the ϕ2.5 and ϕOL promoter initiate transcription with an A instead of G residue [35] (B) Effect of sequence variations in the +1 to +6 region of the T7 class III promoter on transcription efficiency (adapted from Milligan and Uhlenbeck [7]); n.d.: not determined. (C) T7 class III promoter region with the recommended G identities at positions +1 and +2 of the RNA transcript shown in grey.

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 [7, 8]. 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 (Fig. 1B and C [7]).

1.2.1 Templates

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

Strategy (a) 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 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 (Fig. 1.1B and C). The plasmid-encoded RNA expression cassette ought to be followed by a single restriction site (avoid restriction enzymes yielding 3′ overhangs [7]) for producing run-off transcripts. Templates with 5′ overhangs have successfully been used in several laboratories. Among those have been templates linearized with restriction enzymes that cleave several residues away from their binding/recognition site, such as *Fok*I. The advantage of using this type of restriction enzymes is independence from the sequence at the cleavage site. This permits the design of RNA transcript 3′ ends of complete identity to natural counterparts. Individual steps of template preparation are (1) ligation of (PCR) insert into plasmid, (2) cloning in *E. coli*, purification and sequencing of plasmid, (3) linearization of plasmid DNA for run-off transcription, (4) phenol/chloroform extraction and ethanol precipitation of template DNA before (5) use in T7 transcription assays.

Strategy (b) 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.

Strategy (c) 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 31mer for 5′-end-labeling purposes (see Protocol 6).

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 [7, 9]. Also, 5′ end heterogeneity may become a problem when the template encodes a transcript with more than three consecutive guanosines at the 5′ end [10], as well as in the case of unusual 5′-terminal sequences, such as 5′-CACUGU, 5′-CAGAGA or 5′-GAAAAA [11]. Yet, 5′ end heterogeneity seems to be a problem associated with T7 class III promoters (Fig. 1.1A). Almost complete 5′ end homogeneity of T7 transcripts has been achieved with templates directing transcription from the more rarely used T7 ϕ2.5 class II promoter (Fig. 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 [12].

However, for the production of RNAs with 100% 5′ and 3′ end homogeneity, several methods are available (see Chapters 2 and 3). For example, hammerhead or Hepatitis delta virus (HDV) ribozymes can be tethered to the RNA of interest on one or both sides (see 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′-end-labeling (see 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 synthesis of large amounts of smaller RNAs. Chemical synthesis and purification of 10 mg of, for example, an RNA 15mer by a commercial supplier can be quite expensive. In such a case, a cheaper alternative would be to transcribe the 15mer sandwiched between two *cis*-cleaving ribozymes, resulting in post-transcriptional release of the 15mer with uniform 5′ and 3′ ends. Purification of the 15mer (and separation from the released ribozyme fragments) can then be achieved either by denaturing polyacrylamide gel electrophoresis (PAGE), UV shadowing or staining and gel elution (see Chapters 3 and 9), or by preparative HPLC if available (see Chapters 7 and 27). If T7 RNAP is self-prepared according to the protocol described in this article, synthesis of 10 mg of a 15mer 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 end-labeling with ^{32}P) or 5′-GMP (to generate 5′-monophosphates), when these components are added to reaction mixtures in excess over GTP [13]. RNA transcripts with 5′-GMP ends are preferred when the RNA is used for ligation with other RNA molecules.

Tab. 1.1. Modified substrates for T7 transcription.

NTP	*wt T7 RNAP*	*Reference*
NTPαS (*S*p)	+	14
NTPαS (*R*p)	−	14
5-Br-UTP	+	7
5-F-UTP	+	7
5-Hexamethyleneamino-UTP	+	7
6-Aza-UTP	+	7
4-Thio-UTP	+	7
Pseudo-UTP	+	7
8-Br-ATP	+	7
7-Me-GTP	−	7
ITP (with initiator)[1]	+	15
2′-dNTP	+/−	7
2′-dNTPαS	+/−	16, 17
2′-*O*-Me-NTP or -NTPαS	+/−	16
GTPγS	+	18

+/−: low incorporation efficiency.
[1] 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.

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 [7] and expanded by addition of more recent information.

Due to discrimination of rNTPs and dNTPs by wt T7 RNAP, the polymerase incorporates rNTPs 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 [19] was shown to have only about 4-fold higher preference for rNTPs than dNTPs [19, 20] and thus permits more efficient incorporation of substrates lacking the ribose 2′-hydroxyl, such as 2′-deoxy-2′-fluoro or 2′-deoxy-2′-amino nucleotides [20]. 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+} [16]. Likewise, dNTPαS analogs were partially incorporated into RNase P RNA in a sequence-specific manner under mixed metal ion conditions (Mg^{2+}/Mn^{2+} [21]). Despite these achievements, the Y639F mutant T7 RNAP is nowadays the enzyme of choice for the incorporation of all nucleotides with 2′-ribose modifications (available from Epicentre, WI, USA). For detailed protocols, the reader is referred to [20, 22].

Further modifications can be introduced into transcripts by tailored 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 [23]. 5′-Terminal

incorporation varied between 20% (hexamer) and 80–95% in the case of 5′-ApG or a 5′-biotinylated ApG (e.g. custom-synthesized by IBA, Göttingen, Germany). 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 [24].

1.3 Transcription Protocols

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 1–6 (Hartmann lab), T7 RNAP from MBI Fermentas has been used. These protocols may be suboptimal with T7 RNAP from other sources. This has been accounted for by including protocols from the Kirsebom (Protocols 7 and 8) and Schön (Protocol 9) labs. Nevertheless, 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, which may require repeated synthesis. In addition to commercially available T7 RNAP, protocols for partial purification of T7 RNAP from bacterial overexpression strains are available ([1, 25, 26] and 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 different basic protocols (e.g. Protocols 1–3). 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 µg/ml (final assay concentration) are used as a rule of thumb, whereas a PCR template of 400 bp is adjusted to about 5 µg/ml final assay concentration. In the Hartmann lab, 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) of T7 RNAP after 2 h at 37 °C, followed by a further 2-h incubation period at 37 °C. In transcriptions according to Protocol 3, a white precipitate will appear because of pyrophosphate accumulation. This is avoided in Protocols 1 and 2 where pyrophosphate is hydrolyzed due to the presence of pyrophosphatase. Extension of incubation periods beyond 4–6 h did not prove 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 concen-

tration may drop under a critical limit, thereby favoring RNA degradation [27]. Protocol 4 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.

We like to note here that transcription from the T7 ϕ2.5 class II promoter is initiated with A instead of G (Fig. 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 [12, 24].

As an alternative to starting class III-promoter-directed transcripts with guanosine, the dinucleotide 5′-ApG (see above) may be employed. The 5′-terminal incorporation of the dinucleotide leads to a −1 adenosine extension of the transcript (Protocol 5). ApG, which is more convenient to use than guanosine, is also available from Sigma, but about 10 000-fold more expensive than guanosine. Transcripts can further be initiated with 5′-GMP if present in excess over 5′-GTP (15 versus 3.75 mM). Although the majority of RNA products should possess a 5′-terminal monophosphate, a dephosphorylation/phosphorylation strategy (see Chapter 3) may be preferred to obtain RNA products with 100% 5′-monophosphates.

Protocol 6 is a quick protocol for the synthesis of small amounts of shorter RNAs for 5′-end-labeling 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 addition of T7 RNAP.

Protocol 7 (from the Kirsebom lab) has the characteristics of high T7 RNAP concentrations and the presence of RNase inhibitor, suitable for large-scale transcriptions. Protocol 8 is used in the Kirsebom lab for the production of internally labeled RNA.

Protocol 9 (from the Schön lab) 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: Hartmann lab

	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	22 mM	7.3 µl
Spermidine 100 mM	1 mM	10 µl
BSA[1] 20 mg/ml	0.12 mg/ml	6 µl
rNTP mix (25 mM each)	3.75 mM (each)	150 µl
Template (linearized plasmid 3.2 kb) 1 µg/µl	40 µg/ml	40 µl
Pyrophosphatase[2] 200 U/ml	1 U/ml	5 µl
T7 RNAP 200 U/µl	1000–2000 U/ml	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 according to this protocol and Protocols 2–5, 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/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.

[1] BSA (Sigma, minimum purity 98% based on electrophoretic analysis, pH 7).

[2] Pyrophosphatase from yeast (Roche, EC 3.6.1.1, 200 U/mg, <0.01% ATPase and phosphatases each).

Protocol 2: Hartmann lab

	Final concentration	1000 µl
HEPES pH 7.5, 1 M	80 mM	80 µl
DTT 100 mM	15 mM	150 µl
$MgCl_2$ 3 M	33 mM	11 µl
Spermidine 100 mM	1 mM	10 µl
rNTP mix (25 mM each)	3.75 mM (each)	150 µl
Template (linearized plasmid 3.2 kb) 1 µg/µl	80 µg/ml	80 µl
Pyrophosphatase 200 U/ml	2 U/ml	10 µl
T7 RNAP 200 U/µl	2000–3000 U/ml	10–15 µl
RNase-free water		to 1000 µl

For incubation, see Protocol 1.

Protocol 3: Hartmann lab

	Final concentration	1000 µl
5 × transcription buffer (MBI)[1]	1 × buffer	200 µl
$MgCl_2$ 3 M	40 mM	13.3 µl
rNTP mix (25 mM each)	3 mM (each)	120 µl
Template (linearized plasmid 3.2 kb) 1 µg/µl	80 µg/ml	80 µl
T7 RNAP 200 U/µl	2000–3000 U/ml	10–15 µl
RNase-free water		to 1000 µl

[1] 5 × transcription buffer (MBI Fermentas): 200 mM Tris–HCl (pH 7.9 at 25 °C), 30 mM $MgCl_2$, 50 mM DTT, 50 mM NaCl and 10 mM spermidine. For incubation, see Protocol 1.

Protocol 4: Hartmann lab

	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	22 mM	7.3 µl

Spermidine 100 mM	1 mM	10 µl
BSA 20 mg/ml	0.12 mg/ml	6 µl
rNTP mix (25 mM each)	3.75 mM (each)	150 µl
Template (linearized plasmid 3.2 kb) 1 µg/µl	40 µg/ml	40 µl
Pyrophosphatase 200 U/ml	5 U/ml	25 µl
RNase-free water		321.7 µl
• Prewarm mixture to 37 °C, then add:		
guanosine (30 mM, kept at 75 °C)	9 mM	300 µl
T7 RNAP 200 U/µl	2000 U/ml	10 µl

For incubation, see Protocol 1.

Protocol 5: Initiation with 5′-GMP or 5′-ApG (Hartmann lab)

	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	22 mM	7.3 µl
Spermidine 100 mM	1 mM	10 µl
BSA 20 mg/ml	0.12 mg/ml	6 µl
rNTP mix (25 mM each)	3.75 mM (each)[1]	150 µl
5′-GMP 100 mM (initiator)[1]	15 mM	150 µl
Template (linearized plasmid 3.2 kb) 1 µg/µl	40 µg/ml	40 µl
Pyrophosphatase 200 U/ml	5 U/ml	25 µl
T7 RNAP 200 U/µl	2000 U/ml	10 µl
RNase-free water		to 1000 µl

[1] When 5′-GMP is replaced with the dinucleotide 5′-ApG for transcription initiation, adjust 5′-ApG to 7.5 mM and rNTPs to 2.5 mM each (final concentrations). For incubation, see Protocol 1.

Protocol 6: 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
rNTP mix (25 mM each)	3.75 mM (each)[3]	75 µl
Promoter DNA oligonucleotide 3.3 µg/µl[1]	132 µg/ml	20 µl
Template DNA oligonucleotide 2.1 µg/µl[2]	84 µg/ml	20 µl
BSA 20 mg/ml	0.12 mg/ml	3 µl
Pyrophosphatase 200 U/ml	2 U/ml	5 µl

RNase-free water		51.5 µl
• Prewarm mixture to 37 °C		
Then add guanosine (30 mM, kept at 75 °C)	9 mM	150 µl
• Mix and preincubate for 1 h at 37 °C		
Then add T7 RNAP 200 U/µl	4000 U/ml	10 µl

Incubate at 37 °C for 4 h.

[1]Promoter DNA oligonucleotide: 5′-TAA TAC GAC TCA CTA TAG.

[2]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.

[3]To increase the percentage of transcripts initiated with guanosine, the ratio of guanosine to rNTPs may be increased, e.g. by reducing the rNTP concentration to 1.5 mM each.

10 × transcription buffer (TRX), Kirsebom lab, for transcription Protocols 7 and 8

	Final concentration	1000 µl
Tris–HCl pH 7.5, 1 M	200 mM	200 µl
Tris–HCl pH 7.9, 1 M	200 mM	200 µl
$MgCl_2$ 3 M	240 mM	80 µl
Spermidine 100 mM	20 mM	200 µl
RNase-free water		320 µl

Protocol 7: Kirsebom lab, non-radioactive transcription, volume sufficient for 4 reactions; however, note that the mix is prepared for 4.5 reactions

	Final concentration[1]	432 µl
10 × TRX	1×	45 µl
DTT 0.5 M	10 mM	9 µl
0.2% Triton X-100	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 24 U/µl	32 U/ml	0.6 µl
T7 RNAP 200 U/µl	10 000 U/ml	22.5 µl
RNase-free water		296.4 µl
• To 96 µl of this mix add:		
template (linearized plasmid ≈ 3.2 kb) 1 µg/µl	40 µg/ml	4 µl

Incubate at 37 °C for ≤10 h.

[1,2,3] Final concentrations after addition of template.

Protocol 8: Kirsebom lab; internal radioactive labeling mix, volume sufficient for 9 reactions; however, note that the mix is prepared for 10 reactions

	Final concentration[1]	230 µl
10 × TRX	1×	25 µl
DTT 0.5 M	10 mM	5 µl
0.2% Triton X-100	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 (20 mCi/ml)	10 Ci/mmol	25 µl
RNase inhibitor 24 U/µl	31.7 U/ml	0.33 µl
T7 RNAP 200 U/µl	10 000 U/ml	12.5 µl
RNase-free water		84.7 µl
• To 23 µl of this mix add:		
template (linearized plasmid ≈ 3.2 kb) 1 µg/µl	80 µg/ml	2 µl

Incubate at 37 °C for ≤ 10 h.
[1] Final concentrations after addition of template.

To account for a severely biased nucleotide composition of the template, such as in RNase P RNAs from the *Cyanophora paradoxa* cyanelle [28] or from a plant-pathogenic phytoplasma [29], the relative concentrations of rNTPs are adjusted accordingly. For phytoplasma RNase P RNA (around 73% A + U), the composition of the nucleotide mix was calculated as follows:

	Calculated mol% of each nucleotide in transcript	Concentration of each rNTP in nucleotide mix (mM)	Final concentration of each rNTP in reaction mix (mM)
rATP	41.08	33	3.3
rCTP	11.06	9	0.9
rGTP	16.03	12.5	1.25
rUTP	31.83	25.5	2.55
Total	100	80	8.0

The following sample protocol is routinely used for 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" rNTP mix (20 mM each rNTP) is replaced by the template-specific rNTP mix with adjusted nucleotide concentrations.

Protocol 9: Preparative transcription, nucleotide composition from the example above (Schön lab)

	Final concentration	250 µl
10 × transcription buffer[1]	1 × buffer	25 µl
rNTP mix (here: 33 mM ATP/9 mM CTP/12.5 mM GTP/25.5 mM UTP)	0.1 × rNTP mix	25 µl
Template (linearized plasmid 3.2 kb) 0.1 µg/µl	50 µg/ml	125 µl
T7 RNAP (own preparation, 5–10 µg/µl total protein; see Section 1.5)	40–200 µg/ml[2]	2–5 µl[2]
RNase-free water		to 250 µl

[1] 10 × 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.
[2] Depending on the specific activity of the individual T7 RNAP preparation; see Section 1.5.2.2.
For phytoplasma RNase P RNA, a 250-µl reaction performed under these conditions results in up to 150 µg RNA after gel purification. Note that due to the high concentrations of rNTPs 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 the addition of template and polymerase. We let the reactions proceed for at least 2 h (preferably overnight) at 37 °C and quench the excess Mg^{2+} by addition of Na_2EDTA to a final concentration of 25 mM before phenol extraction and EtOH precipitation (see Section 1.3.3, Step 1).

After transcription, check product yield and quality (5–10-µl aliquot plus equal volume gel loading buffer) by PAGE in the presence of 8 M urea and stain the gel with 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 low 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 by a factor of only 5. 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 Pyrimidine Nucleotides

To produce nuclease-resistant 2′-fluoro-modified RNAs with the Y639F mutant T7 RNAP, we replaced rUTP and rCTP with the corresponding 2′-fluoro-analogs (IBA,

Göttingen, Germany; or Epicentre, WI, USA). Protocol 10 has been employed in an *in vitro* selection study 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: Transcription of 2′-fluoro-modified RNA (Hartmann lab)

		150 µl
10 × transcription buffer[1]	1 × 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
rATP, rGTP (10 mM each)	1.25 mM (each)	18.75 µl
[α-^{32}P]ATP 800 Ci/mmol (10 mCi/ml)	0.16 Ci/mmol	3 µl
PCR template	1–3.33 nmol/ml	0.15–0.5 nmol
Y639F mutant T7 RNAP (3.7 µg/µl)	93.73 µg/ml	3.8 µl
RNase-free water		to 150 µl

[1] 10 × transcription buffer: 400 mM Tris–HCl (pH 8.0), 200 mM $MgCl_2$, 10 mM spermidine, 0.1% Triton X-100.

1.3.3 Purification

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 gel purification of RNA is described in Chapter 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 Step 2 by centrifugation at 14 000 *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 (Tris-saturated, stabilized with hydroxy-quinoline, pH 7.7; Biomol), mix the sample with 0.5–1.0 volumes 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 0.5–1.0 volumes 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 NAP 10 columns (Amersham Biosciences, now part of 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 volumes ethanol, 0.1 volumes 3 M NaOAc (pH 4.7) and 1 µl glycogen (20 µg/µl); 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 [0.33 × TBE (see Step 6), 2.7 M urea, 67% formamide, 0.01% (w/v) each bromophenol blue (BPB) and xylene cyanol blue (XCB)].

(6) Preparative denaturing PAGE. A gel well, 6- to 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 × 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)/buffer B: 1 M NaOAc (pH 4.7)/buffer C (successfully used for the elution of phosphorothioate-modified RNAs): 1 M NH_4OAc (pH 7). 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.

(b) *Alternatively: electro-elution*. Excised gel pieces containing the RNA are placed in a BIOTRAP chamber (Schleicher & Schuell, Dassel, Germany; in USA and Canada: ELUTRAP) following the manufacturer's protocol. The RNA is eluted in 0.5 × 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 BIOTRAP chamber. This lid has to be closed when the BIOTRAP is running. To minimize evaporation/condensation, the BIOTRAP should not be run at higher voltage than 150 V overnight. After elution, the RNA is extracted once with phenol and twice with chloroform/isoamylalcohol, followed by ethanol precipitation.

(8) Quantification (UV spectroscopy, see Chapter 4 and Appendix) and quality check (denaturing PAGE).

1.4 Troubleshooting

1.4.1 Low or No Product Yield

- 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 50-µl scale; if unsuccessful as well, test different enzyme batches or enzymes from alternative suppliers. 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 at ambient temperature.
- 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 °C, and adjust stock solutions (in H_2O) to pH 7.0 or consider to buffer with 10–40 mM Tris–HCl adjusted to the pH used in transcription reactions; use lithium salts if available and be aware that diluted working solutions may degrade rapidly.
- Prepare new template DNA; take particular care to effectively remove salts 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 [28, 29]), adjust the composition of the rNTP solution according to the nucleotide ratio of the RNA. However, do not alter the total nucleotide concentration of the reaction mix.

1.4.2 Side-products and RNA Quality

- Usually we get the least artifact products, in addition to the correct RNA product, with Protocol 3, but redissolving the RNA after ethanol precipitation may become a severe problem due to pyrophosphate precipitates. To alleviate such solubility problems, see Section 1.3.3, Step 1.
- Gel entry problems or smear on gel: perform Steps 1–5 of Section 1.3.3 before proceeding to gel purification (Section 1.3.3, Step 6).

1.5 Rapid Preparation of T7 RNA Polymerase

This protocol is based on the publications of Grodberg and Dunn [30] and Zawadzki and Gross [25], 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 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 (obtained from F. W. Studier, Biology Department, Brookhaven National Laboratory, Upton, NY 11973, USA).

Medium
LB (Luria-Bertani) medium [31] supplemented with 50 μg/ml Ampicillin.

Buffers and solutions
100 mM IPTG
TEN buffer (50 mM Tris–HCl, pH 8.1; 2 mM Na_2EDTA; 20 mM NaCl)
Phenylmethylsulfonyl fluoride (PMSF), 20 mg/ml in isopropanol
Leupeptin, 5 mg/ml
Egg white lysozyme, 1.5 mg/ml in TEN (freshly prepared)
0.8% sodium 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 [20 mM sodium phosphate pH 7.7; 1 mM Na_2EDTA; 1 mM DTT; 5% glycerol (w/v)]
4 × 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) bromophenol blue; adjust pH before addition of bromophenol blue
Buffer C-10, C-100: buffer C supplemented with 10 or 100 mM NaCl, respectively.

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 supplemented with 50 μg/ml Ampicillin with a colony from a fresh plate culture and grow overnight at 37 °C.
(2) Four 2-l flasks with 500 ml of the same medium are inoculated 1:100 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 r.p.m. in a desktop centrifuge and keep the sediment as a control.
(4) Then induce the remaining culture for expression by addition of IPTG to a

final concentration of 0.5 mM. At 1.5, 2 and 3 h after induction, take 1-ml samples as in Step 3.

(5) Harvest the flask cultures by centrifugation (10 min, 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 4 for expression of T7 RNAP as follows: resuspend the cell sediment in 100 µl of 1 × concentrated Laemmli gel loading buffer (note that this buffer is usually prepared as a more concentrated stock solution; see Section 1.5.1) and denature for 2 min at 95 °C. Then load 10–25 µl of each sample onto an SDS–10% polyacrylamide gel with appropriate size markers. If expression is sufficient (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, if not stated otherwise). From each purification step, a small sample should be retained for the determination of protein concentration and purity. Protein concentrations are most conveniently determined by dye binding [32] or by direct measurement of extinction at 280 nm.

(1) Resuspend cells in 24 ml of TEN buffer supplemented with 50 µl of PMSF and 20 µl of leupeptin stock solutions (see Section 1.5.1).

(2) Add 6 ml lysozyme solution; after 20 min of incubation, add 2.5 ml of 0.8% sodium deoxycholate solution and incubate for another 20 min.

(3) Shear the DNA in this viscous lysate by sonication (4 × 15 s with an immersible probe; 2–5 min for sonication in a water bath).

(4) Add 5 ml 2 M ammonium sulfate and adjust the total volume to 50 ml with TEN buffer.

(5) Remove DNA by slow addition of 5 ml Polymin P and stirring for 20 min.

(6) After centrifugation (15 min, 39 000 *g*), keep the supernatant and determine its volume.

(7) Precipitate the enzyme from the supernatant by slow addition of 0.82 volumes of saturated ammonium sulfate and stirring for another 15 min.

(8) After centrifugation for 15 min at 12 000 *g*, resuspend the sediment in 15 ml buffer C-100, and dialyze for at least 8 h against 2 × 1 l of the same buffer. 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.

(9) Remove insoluble material by centrifugation as in Step 8, and apply the supernatant to the EMD-SO_3^- column at a flow rate of about 20 ml/h. Wash the column with 10 volumes of buffer C-100 or until protein is no longer detectable in the flow-through. Then apply a 250-ml gradient from 100 to 500 mM NaCl in buffer C and collect 5-ml fractions. T7 RNAP elutes between 300 and 400 mM NaCl from the EMD-SO_3^- column, as visible by the high

protein content in these fractions. Finally, wash the column with 1 M NaCl in buffer C; the resin can be reused after equilibration in buffer C-100.

(10) 10–20 µl of each fraction and the flow-through, and 2–5 µl of the applied sample, are then analyzed by SDS–PAGE as described in Section 1.5.2.1, Step 6.

(11) The fractions containing T7 RNAP are pooled and dialyzed for at least 8 h against 2 × 2 l buffer C-10.

(12) The resulting precipitate, enriched in T7 RNAP, is collected by centrifugation as in Step 8, resuspended in 1–2 ml of buffer C-100 and adjusted to 50% glycerol (w/v) for storage at −20 °C.

Specific activity of T7 RNAP may be determined by incorporation of ^{3}H- or ^{32}P-labeled nucleotides into acid-precipitable material and can reach 400 000 U/mg (1 U is defined as the incorporation of 1 nmol AMP into acid precipitable material in 1 h at 37 °C [9]). 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 test a typical preparation of T7 RNAP (5–10 µg/µl total protein content), we vary the amount of polymerase (usually between 0.1 and 1 µl) in a series of analytical transcriptions, for example, in a variation of Protocol 9 scaled down to 25 µl, containing 2 mM of each rNTP and a standard template DNA (around 50% G + C). Transcription products are then separated by gel electrophoresis and the efficiency of transcription is evaluated after toluidine blue staining of the gel (see Chapter 9). Alternatively, for easier detection of abortive transcripts and degradation products, a radioactive tracer (e.g. 10 µCi of [α-^{32}P]GTP) can be added to the transcription reaction and the products visualized on a PhosphoImager or by autoradiography (see Protocol 8).

1.5.3 Notes and Troubleshooting

(1) If protein gel electrophoresis of crude cell samples (Section 1.5.2.1, Step 6) yields badly smeared bands, try to shear the DNA by sonication as described for the enzyme purification. Alternatively, samples can be squeezed with a syringe through a thin (0.7 mm, or 22 gauge) needle.

(2) If expression of T7 RNAP is not sufficient, vary ampicillin or IPTG concentration, change growth times before and after induction, or switch to other growth media, such as TB or 2 × YT [31], or M9 Minimal Medium supplemented with trace elements [33].

(3) 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 much earlier (around 200 mM NaCl).

(4) If T7 RNAP does not precipitate after the dialysis step with buffer C-10 (Section 1.5.2.2, Step 11), repeat dialysis with fresh buffer C (without NaCl).

(5) If a substantial portion of the precipitate from Step 12 above (Section 1.5.2.2) cannot be re-dissolved in buffer C-100, one may gradually increase the NaCl concentration up to 500 mM.

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