

## ***In Vitro* Transcription of RNA Oligonucleotides with T7 Polymerase**

### **References:**

J. F. Milligan, D.R. Groebe, G.W. Witherell, and O.C. Uhlenbeck (1987) *Nucl. Acids Res.* 15, 8783-98.

J. F. Milligan and O.C. Uhlenbeck (1989) *Meth. Enzym.* 180, 51-62

### **Basic Transcription Reaction Conditions:**

1x Buffer:	40 mM	Tris [pH 8.3]
	5 mM	DTT
	1 mM	spermidine
	0.01 %	Triton X-100
	50 mg/ml	PEG-8000
	200 nM	Template (1:1 - T7 primer: template sequence)
	0.1 mg/ml	T7 RNA polymerase
	2.5 U/ml	PiPase
	25 mM	MgCl <sub>2</sub> (Optimize - template dependent)
	4 mM	Each NTP (ATP, GTP, CTP, UTP)

Incubate 4-6 hours at 37° C.

### **Stock Solutions:**

1 M Tris [pH = 8.3]

40% w/v PEG (Avg. Mol. Wt 8000)

1% v/v NP-40 or Triton-X100

1 M MgCl<sub>2</sub>

1 M DTT ( stored in 0.5 ml aliquots at -20° C)

100 mM Spermidine (stored in 0.5 ml aliquots at -20° C)

100 mM NTP (ATP, GTP, UTP, CTP made as separate stocks and stored at -20°C)

DEPC treated ddH<sub>2</sub>O is made by adding 0.01% (v/v) DEPC to ddH<sub>2</sub>O, mixing and allowing the ddH<sub>2</sub>O to stand overnight. The ddH<sub>2</sub>O is then autoclaved.

The Tris, MgCl<sub>2</sub> and PEG solutions are made with DEPC treated ddH<sub>2</sub>O and then autoclaved.

The DTT and Spermidine solutions are made with DEPC treated ddH<sub>2</sub>O but are **NOT** autoclaved. They are stored as 1 ml aliquots.

The 1% NP-40 or Triton-X100 stock is made with DEPC treated ddH<sub>2</sub>O but is **NOT** autoclaved.

The NTP stocks (Pharmacia) are made with DEPC treated ddH<sub>2</sub>O and are **NOT** autoclaved. The pH of the NTP stocks is adjusted to pH=8.0 with 1 M NaOH and they are stored as 1 ml aliquots.

### Template:

DNA templates are chemically synthesized, purified and stored in TE [pH=7.0]. A 1 μmole DNA synthesis should provide enough DNA template (~200-400 nmoles) for many (~300) large scale transcriptions (~50 ml).

The template top strand contains the -17 to +1 region of the T7 primer, while the bottom strand contains the complement of these nucleotides attached to the coding sequence for the RNA Oligonucleotide.

The most important factor in the yield and fidelity of your transcription is your choice of sequence! However, making changes in the sequence (apart from altering the first few nucleotides) is usually not an option. In general, transcripts which transcribe best begin with the following three nucleotides:



### Enzymes:

T7 polymerase is stored in 1 ml aliquots at -70°C.

### Optimization

Before proceeding to large-scale reactions, it is a good idea to optimize the reaction conditions to obtain the best transcription yields for your particular template. Optimization of T7 RNA transcriptions is performed using several small scale (40 μL) reactions. Typically, 20 to 30 individual small scale reactions are tried where only one ingredient is varied. The yield of RNA from these reactions is then assayed by running 10 μL of each test mixture on analytical 20% 8M Urea polyacrylamide gels and visualized either by UV shadowing or ethidium bromide staining. The optimal concentration (or pH with respect to Tris) for a given ingredient is determined simply by comparing RNA band intensities from the gel.

### Standard 40 λ Reaction:

<u>Final Concentration</u>	<u>Ingredient</u>
40 mM	Tris [pH 8.3]
25 mM	MgCl <sub>2</sub>
5 mM	DTT
1 mM	Spermidine
0.01%	Triton X-100
4 mM	NTPs ( <b>***4mM of ATP, UTP, GTP and CTP***</b> )
0.2 μM	DNA Template
4%	PolyEthyleneGlycol 8000 ave.mol. weight
0.1 mg/mL	T7 RNA Polymerase
add ddi H <sub>2</sub> O to a final volume of 40 μL	
Incubate 4-8 hours at 37°C.	

<b>Ingredients to Optimize</b>	<b>Varying Conditions to Try</b>							
MgCl <sub>2</sub>	20	25	30	35	(mM)			
pH of Tris	7.0	7.5	8.0	8.5				
DNA Template	0.2	0.4	0.8	1.0	1.2	1.5	2.0	(μM)
unlabeled NTPs*	2	3	4	(mM each)				
labeled NTPs*	2	3	4	(mM each)				
8000-PEG	0	2	4	(% w/v)				
T7 polymerase**	0.025	0.05	0.075	0.1	(mg/mL)			

\* When optimizing for any labeled NTPs, it is important to run at least one 40 μL test reaction with unlabeled, store-bought NTPs as a standard for comparison. Generally, store-bought NTPs are of higher purity and quality and will should give a higher yield than labeled NTPs that are synthesized in house.

\*\* At a high enough concentration, T7 RNA polymerase will actually inhibit RNA transcription. It is important to check that this inhibitory concentration is not encroached, while at the same time ensuring enough enzyme is added to provide for efficient transcription.

### **1 mL Transcription Reaction**

Once the transcription of a particular template has been optimized, but before a large scale reaction is attempted, try a 1.0 ml test reaction. This size transcription provides enough RNA for melting and band-shift type experiments.

### **Large Scale Reactions**

With properly optimized conditions and a good transcribing template, a 20 mL (f.v.) reaction should be sufficient to generate NMR quantities of an RNA oligonucleotide (~2 mM in 300 vL). If the template is a poor transcriber, simply scale up the volume of the transcription reaction, after making sure that the optimization procedure was performed properly.

### **For 1 mL or greater Transcription Reaction:**

<u>Final Concentrations</u>	<u>Ingredient</u>
40 mM	TRIS at optimized pH
optimized value	MgCl <sub>2</sub>
5 mM	DTT
1 mM	Spermidine
0.01%	Triton X-100
optimized value	NTPs (***) <b>4mM of ATP, UTP, GTP and CTP</b> (***)
optimized value	DNA Template
optimized value	PolyEthyleneGlycol 8000 ave.mol. weight
optimized value	T7 RNA Polymerase

add ddi H<sub>2</sub>O to the final volume

Incubate 6-12 hours at 37° C.

## Purification of Large Scale Reactions

1. Stop the reaction by adding an amount of EDTA that is twice the concentration of  $\text{MgCl}_2$  in the transcription mixture and vortex thoroughly. The white precipitate (which is Mg-pyrophosphate) should go back into solution.
2. Precipitate the reaction by adding 3 to 4 volumes of 200 proof ethanol and 3M sodium acetate [pH 5.5] at a ratio of 50  $\mu\text{L}$  per mL of reaction (this would be 1 mL of 3M sodium acetate to a 20 mL transcription reaction). Incubate over night in the  $-20^\circ\text{C}$  freezer.
3. Pellet the precipitate by low speed centrifugation (2000 x g) for ~30 minutes. Decant the ethanol and allow the pellet air dry.
4. Try to dissolve all the RNA with a minimal volume of 0.5 M EDTA (a pellet from a 10 ml reaction should be soluble in ~ 500 - 1000  $\lambda$ ).
5. Bring the resuspended mixture up to 10 mL using DEPC treated  $\text{H}_2\text{O}$ . Desalt the solution using a Centriprep (MWC 2000) in the clinical centrifuge. The Centriprep holds up to 10 mL and can be used with a JA-14 rotor and spun at a maximum of 3,800 x g. At this speed 10 mL is reduced to approximately 2 ml in 15 hours/overnight. The 2 ml that remains should be lyophilized to dryness.
6. Dissolve the lyophilized reaction mixture in a minimal volume of DEPC treated dd $\text{H}_2\text{O}$  and then add an equal volume of formamide. Add dye (0.1% bromophenol blue which runs as an 8mer).
7. Run on 15 or 20% (19:1) acrylamide, 8 M urea, 1x TBE gel. The gel should be run until bromophenol dye reaching the bottom. One semi-preparative gel can be used to purify ~ 0.2 - 0.3  $\mu\text{moles}$  of RNA to nucleotide resolution which is about the yield from 10 ml of reaction. The larger sized preparative gels should be used for transcription reaction of 20 mL or larger volumes.
8. Visualize the RNA band by UV shadowing. Cut out the desired band and store the gel slices at  $-20^\circ\text{C}$ .
9. Elute purified RNA using the Elutrap. Run in 1/2X TBE at 250V. Collect fractions every hour for at least 4 hours. Measure the  $\text{OD}_{260}$  of each fraction to monitor the elution.
10. Combine fractions and lyophilize to dryness. Resuspend the RNA in ~1/2 ml of DEPC treated dd $\text{H}_2\text{O}$ .

## Dialysis for NMR

1. Once you have the sample resuspended in DEPC treated dd $\text{H}_2\text{O}$  dialyze against dd $\text{H}_2\text{O}$  for 8 hrs using the Pierce micro-dialyzer. Remember that the sample will swell upon dialysis.

2. Make a ~25x NMR buffer for dialysis. Dialysis against this buffer should compete off the remaining unwanted salts.
3. Dialyze the sample against 10x NMR buffer.
4. Dialyze the sample multiple times (2-3 times) against 1x NMR buffer.

In general, dialysis equilibrium for 2000 MWC dialysis tubing is reached after 8-10 hrs. The equilibrium time will scale inversely with the cut-off size.

**Typical NMR Buffers:**

1 - 5 mM buffer (cacodylate, Na- or K- phosphate,... pH [6.5 - 7.5])

Salts necessary for sample stability, for example:

25-100 mM NaCl  
1-5 mM Mg<sub>2</sub>Cl

A general rule of thumb is not to add salt unless it is necessary for sample stability. In general, high salt concentrations, especially Mg, tend to broaden NMR line-widths by causing non-specific aggregation of the RNA.