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 We use this protocol and it's working

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Protocol for *in vitro* transcription of DNA oligos by T7 polymerase V.2

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ABSTRACT

This is a protocol for *in vitro* transcription of DNA oligos by T7 polymerase from the [Doudna Lab](https://doudnalab.org/).

ATTACHMENTS

[RNA_in_vitro_transcription.pdf](#)

GUIDELINES

Template design:

T7 polymerase requires a double-stranded promoter region for efficient template binding, but can then transcribe a single-stranded DNA template. Therefore, the following protocol uses an approach, whereby two oligos are annealed in order to generate a dsDNA promoter, followed by a single-stranded overhang that contains the reverse complement of the desired RNA sequence.

The reverse complement of the desired RNA sequence is ordered as a DNA oligo (assuming the length does not exceed ~80 nt), to which the reverse complement of the T7 promoter is appended at the 3' end. Example template:

5'-TTTCTTAGCTGCCTATACGGCAGTGAA**CCTATAGTGAGTCGTATTA**-3'

- **XXX** = reverse complement of T7 promoter
- **XXX** = reverse complement of the desired RNA sequence
- **Note:** the consensus T7 promoter sequence contains GGGAGA as the first 6 nucleotides that are transcribed. However, only the GG dinucleotide is required for high efficiency transcription (Ambion).

A separate, synthetic DNA oligo is ordered that contains the T7 promoter in the forward direction, which can be annealed with the above construct:
 T7oligo: 5'-TAATACGACTCACTATA-3'

Note: according to Ambion, for transcription of synthetic oligonucleotides, only the -17 to -1 positions of the promoter need to be double-stranded. This oligo takes this into account.

MATERIALS

STEP MATERIALS

- ☒ 5x transcription buffer **Contributed by users**
- ☒ 1.5 nmol T7oligo **Contributed by users**
- ☒ 1.0 nmol template **Contributed by users**
- ☒ DEPC H₂O **Contributed by users**
- ☒ 5N NaOH **Contributed by users**
- ☒ 1N NaOH **Contributed by users**
- ☒ RNase-free DNase **Promega**
- ☒ EtOH **Contributed by users**
- ☒ Silanization Solution I **Contributed by users**
- ☒ 15% urea polyacrylamide gel **Contributed by users**
- ☒ 40% Acrylamide-bisacrylamide (19:1) **Contributed by users**
- ☒ Urea **Contributed by users**
- ☒ Water **Contributed by users**
- ☒ 10% APS **Contributed by users**
- ☒ 1x TBE **Contributed by users**
- ☒ 1x TBE buffer **Contributed by users**
- ☒ RNase-free water **Contributed by users**
- ☒ Corning Spin-X filter tube **Contributed by users**
- ☒ 10x NEBuffer 3 **Contributed by users**
- ☒ CIP (10 U/μl) **New England Biolabs**
- ☒ Phenol-chloroform solution **Contributed by users**
- ☒ 5x transcription buffer **Contributed by users**
- ☒ 1.5 nmol T7oligo **Contributed by users**
- ☒ 1.0 nmol template **Contributed by users**
- ☒ DEPC H₂O **Contributed by users**
- ☒ 5N NaOH **Contributed by users**
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- ☒ Silanization Solution I **Contributed by users**
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- ☒ 1x TBE buffer **Contributed by users**
- ☒ RNase-free water **Contributed by users**
- ☒ Corning Spin-X filter tube **Contributed by users**
- ☒ 10x NEBuffer 3 **Contributed by users**
- ☒ CIP (10 U/μl) **New England Biolabs**
- ☒ Phenol-chloroform solution **Contributed by users**

SAFETY WARNINGS

- ⓘ Please refer to the SDS (Safety Data Sheet) for safety warnings and hazard information.

BEFORE START INSTRUCTIONS

Stock solutions:

5x transcription buffer:	10 ml
150 mM Tris-Cl, pH 8.1	1.5 ml
125 mM MgCl ₂	1.25 ml
0.05% Triton X-100	50 μl
10 mM spermidine	100 μl
H ₂ O	to 10mL

store at -20 °C	
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10x TBE	1 L
Tris base	108 g
Boric acid	55 g
0.5 M Na ₂ EDTA pH 8.0	40 ml
H ₂ O	to 1 L

0.5 M Na₂EDTA	0.5 L
Disodium ethylenediaminetetraacetate 2H ₂ O	93 g
NaOH to get pH 8.0	10 g
H ₂ O	to 500 ml
autoclave	

10 % (w/v) APS	50 ml
Ammonium persulfate	5 g
H ₂ O	to 50 ml
store at -20 °C	

1 M spermidine	
Spermidine	0.7 g
H ₂ O	to 5 ml
store at -20 °C	

5N NaOH	25 ml
NaOH	5 g
H ₂ O	to 25 ml

1N NaOH	20 ml
5N NaOH	5 ml
H ₂ O	to 20 ml

10 % Triton X-100	10 ml
Triton X-100	1 ml
H ₂ O	9 ml
store at -20 °C	

1% (w/v) bromophenol blue	10 ml
bromophenol blue	100 mg
H ₂ O	to 10 ml

1% (w/v) xylene cyanol	10 ml
xylene cyanol	100 mg
H ₂ O	to 10 ml

2x RNA Loading Dye	30 ml
95% formamide	28.5 ml
1 mM EDTA	60 µl of 0.5 M

0.025% SDS	75 µl of 10%
0.025% bromophenol blue	750 µl of 1%
0.01% xylene cyanol	300 µl of 1%
store at -20 °C	

Preparing 10 µM hybridized template

- Mix the following in a final volume of 100 µl:
20 µl 5x transcription buffer
1.5 nmol T7oligo (15 µM final)
1.0 nmol template (10 µM final)
DEPC H₂O to 100 µl

☒ 5x transcription buffer **Contributed by users**

☒ 1.5 nmol T7oligo **Contributed by users**

☒ 1.0 nmol template **Contributed by users**

☒ DEPC H₂O **Contributed by users**

- Heat at 70-80 °C for 2 minutes.

🔥 70 °C Heating

🕒 00:02:00 Heating

- Slow cool on bench-top.

- Use immediately or store at -20 °C.

Note

For some applications, prior gel purification of the DNA template may be desirable. However, for my transcriptions, the oligos are not very long and I immediately gel purify the RNAs after transcription, so purity of the DNA oligo is not a major concern.

🔥 -20 °C Storage

Preparation of NTPs

- Add 500 µl of RNase-free H₂O to the 70-80 mg of each NTP.

🧴 500 µL RNase-free H₂O

- Bring pH of each NTP to 6.8-7.2 by adding 5 µl of 5N NaOH followed by 10-90 µl of 1N NaOH. Monitor pH in the stripes with 6.5-10 range. Most adjustment will be needed for ATP, least - for UTP.

☒ 5N NaOH **Contributed by users**

☒ 1N NaOH **Contributed by users**

-

Measure absorbance of 500 fold dilution: 3 µl to 1.5 ml of H₂O.

ATP $\lambda_{\max}$ 259 nm, $\epsilon_{\max}$ $1.59 \times 10^4 \text{ cm}^{-1} \text{ M}^{-1}$

CTP $\lambda_{\max}$ 271 nm, $\epsilon_{\max}$ $0.9 \times 10^4 \text{ cm}^{-1} \text{ M}^{-1}$

GTP $\lambda_{\max}$ 253 nm, $\epsilon_{\max}$ $1.37 \times 10^4 \text{ cm}^{-1} \text{ M}^{-1}$

UTP $\lambda_{\max}$ 259 nm, $\epsilon_{\max}$ $1.59 \times 10^4 \text{ cm}^{-1} \text{ M}^{-1}$

- Calculate concentrations and required dilutions to reach 100 mM concentration each.

Transcription reaction

9 Mix the following in a 100 µl reaction:

	Final concentrations:
20 µl 5X transcription buffer	(1X)
5 µl 100 mM ATP	(5 mM)
5 µl 100 mM CTP	(5 mM)
5 µl 100 mM GTP	(5 mM)
5 µl 100 mM UTP	(5 mM)
1 µl 1M DTT	(10 mM)
0.1 µl 1 mg/mL pyrophosphatase (Roche)	(1 ug/ml)
10 µl 1 mg/mL T7 polymerase	(100 ug/ml)
10 µl 10 uM hybridized template	(1 uM)
DEPC H ₂ O to 100 µl	

Note

My typical final yields on transcription reactions of this size are roughly 30 µl of 10-20 uM (300-600 pmol). For my biochemical purposes, this is more than enough material. However, the above reaction can easily be scaled up.

🧪 5 µL 100 mM ATP

🧪 5 µL 100 mM CTP

🧪 5 µL 100 mM GTP

🧪 5 µL 100 mM UTP

🧪 1 µL 1M DTT

🧪 0.1 µL 1 mg/mL pyrophosphatase (Roche)

🧪 10 µL 1 mg/mL T7 polymerase

10 Incubate at 37 °C, overnight.

🔥 37 °C Incubation

🕒 16:00:00 overnight incubation

11 DNase treat:

1. Add 5 units (5 µl) RNase-free DNase directly to reaction.

2. Incubate at 37 °C, 30 minutes.

🧪 RNase-free DNase **Promega**

🧪 5 µL RNase-free DNase

🔥 37 °C Incubation

🕒 00:30:00 Incubation

Note

This step is optional and may be omitted.

12

Add equal volume (100 µl) gel loading buffer directly to transcription reaction.

🧪 100 µL Gel loading buffer

Note

My RNAs run very close to the xylene cyanol dye. Therefore, I use a gel loading buffer that contains only bromophenol blue.

- 13 Gel-purify transcribed RNAs on a medium thickness, denaturing urea-polyacrylamide gel.

Note

For my RNAs (~30 nt) I use a 15% gel. At this polyacrylamide concentration, with RNA from a 100 µl transcription reaction, I get excellent separation of the desired RNA length from n+1 and n-1 contaminants (these arise from template-independent addition of nts by T7 polymerase; and synthetic template oligos that are not the full length). Optimization of the gel running time and acrylamide concentration will be necessary for gel-purifying RNAs of different length.

Gel-purification

- 14

PAGE Fragment Resolution: Denaturing Conditions (6M Urea)

% Acrylamide	Fragment Size	Bromophenol Blue	Xylene Cyanol
30	2-8	6	20
20	8-25	8	28
10	25-35	12	55
8	35-45	19	75
6	45-70	26	105
5	70-300	35	130
4	100-500	~50	~230

Clean glass plates with EtOH and coat with 200 µl Silanization Solution I.

☒ EtOH Contributed by users

☒ Silanization Solution I Contributed by users

🧴 200 µL Silanization Solution I

- 15 Assemble glass plates.

- 16 Prepare 15% urea polyacrylamide gel.

☒ 15% urea polyacrylamide gel **Contributed by users**

- 17 Denaturing Urea Polyacrylamide Gel (300 ml for a gig gel: 35 x 25 cm)

	10%	12.5%	15%
10x TBE	30 ml	30 ml	30 ml
40% Acrylamide-bisacrylamide (19:1)	75 ml	93.75 ml	112.5 ml
Urea	108 g	108 g	108 g
Water	192 ml	173 ml	155 ml
Before casting add:			
10% APS	1.5 ml	1.5 ml	1.5 ml
TEMED	600 µl	600 µl	600 µl

After addition of APS and TEMED to Urea-Polyacrylamide mixture pour the gel immediately.

☒ 40% Acrylamide-bisacrylamide (19:1) **Contributed by users**

☒ Urea **Contributed by users**

☒ Water **Contributed by users**

☒ 10% APS **Contributed by users**

- 18 Place comb and polymerize for 60 min.

⌚ 01:00:00 Polymerize

- 19 Assemble electrophoresis apparatus.

- 20 Remove comb.

- 21 Fill inner and outer buffer chambers with 1x TBE.

☒ 1x TBE **Contributed by users**

- 22 Rinse wells with 1x TBE buffer.

☒ 1x TBE buffer **Contributed by users**

- 23 Pre-run the gel for 30 min to heat-up and remove remaining urea. Set constant P=40 W, 3000 V, 400 mA.

⌚ 00:30:00 Pre-running gel

- 24 Load sample.

- 25 Run P=40 W, 3000 V, 400 mA for around 3 h, until the bromophenol blue band is migrating in the last 1/4 of the gel.

⌚ 03:00:00 Running gel

- 26 Remove one of the glass plates and cover the gel with saran wrap. Flip. Remove another glass plate and cover all gel with saran wrap.
- 27 UV shadow band and excise with sterile razor and place in Falcon tube.
- 28 Break gel slices with p-1000 tip.
- 29 Crush and soak gel slices in equal volume of RNase-free water.
☒ RNase-free water **Contributed by users**
- 30 Elute RNA overnight by rocking at 4 °C.
🔥 4 °C Eluting RNA

Next day

- 31 Filter out gel pieces by spinning sample through Corning Spin-X filter tube. Centrifuge at top speed for 5 minutes, 4 °C.

🔥 4 °C Centrifugation

🕒 00:05:00 Centrifugation

Note

I cut the top off a P-1000 tip so that I can pipette as much as possible of the water-gel slurry into the Corning filter tube.

☒ Corning Spin-X filter tube **Contributed by users**

- 32 Add 30 µl 10x NEBuffer 3 to each sample, then 1 µl CIP (10 U/µl). Incubate at 37 °C for one hour.

☒ 10x NEBuffer 3 **Contributed by users**

🧴 30 µL 10x NEBuffer 3

☒ CIP (10 U/µl) **New England Biolabs**

🧴 1 µL CIP

🔥 37 °C Incubation

🕒 01:00:00 Incubation

Note

This step is only required if removal of the 5' triphosphate is required (e.g. if the RNA will subsequently be 5'-radiolabeled with T4 PNK.)

- 33 Phenol/chloroform extract with 300-500 µl phenol-chloroform solution, pH 8.0.

☒ Phenol-chloroform solution **Contributed by users**

🧴 300 µL Phenol-chloroform solution

34

Ethanol precipitate the RNA, by adding 1/10 volume 3 M NaOAc (pH 5.2), 1/100 volume 100x linearized acrylamide and 3 volumes 100% ethanol. [described in step 35-40 below]

35

Incubate at -80 °C, at least 1 hour

🔥 -80 °C Incubation

🕒 01:00:00 Incubation

36

Spin down at top speed, 20 minutes, 4 °C.

🔥 4 °C Spinning down

🕒 00:20:00 Spinning down

37

Remove supernatant.

38

Wash pellet with 70% ethanol.

39

Spin down again and remove supernatant.

40

Dry pellets in speed vacuum, ~10 minutes.

🕒 00:10:00 Drying pellets

41

Resuspend pellets in 30 µl DEPC H₂O (or buffer) and store at -20 °C.

🧴 300 µL DEPC H₂O (or buffer)

🔥 -20 °C Storage