Protocol for in vitro transcription of DNA oligos by T7 polymerase Version 2

CGER

Abstract

This is a protocol for *in vitro* transcription of DNA oligos by T7 polymerase from the Doudna Lab.

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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.

Before start

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_2O	to 10mL
store at -20 °C	

10x TBE	1 L
Tris base	108 g
Boric acid	55 g
0.5 M Na_2EDTA pH 8.0	40 ml
H_2O	to 1 L

0.5 M Na_2EDTA	0.5 L
Disodium ethylenediaminetetraacetate $2H_2O$	93 g
NaOH to get pH 8.0	10 g
H_2O	to 500 ml
autoclave	

10 % (w/v) APS	50 ml
Ammonium persulfate	5 g
H_2O	to 50 ml
store at -20 °C	

1 M spermidine

Spermidine	0.7 g
H_2O	to 5 ml

store at -20 °C

5N NaOH	25 ml
NaOH	5 g
H_2O	to 25 ml

1N NaOH	20 ml
5N NaOH	5 ml
H_2O	to 20 ml

10 % Triton X-100	10 ml
Triton X-100	1 ml
H_2O	9 ml

store at -20 °C

1% (w/v) bromophenol blue	10 ml
bromophenol blue	100 mg
H_2O	to 10 ml

1% (w/v) xylene cyanol	10 ml
xylene cyanol	100 mg
H_2O	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	

Protocol

Preparing 10 uM hybridized template

Step 1.

Mix the following in a final volume of 100 µl:

- 20 µl 5x transcription buffer
- 1.5 nmol T7oligo (15 uM final)
- 1.0 nmol template (10 uM final)
- DEPC H_2O to 100 µl

REAGENTS

- 5x transcription buffer by Contributed by users
- 1.5 nmol T7oligo by Contributed by users
- 1.0 nmol template by Contributed by users
- DEPC H2O by Contributed by users

Preparing 10 uM hybridized template

Step 2.

Heat at 70-80 °C for 2 minutes.

TEMPERATURE

70 °C : Heating

DURATION

00:02:00 : Heating

Preparing 10 uM hybridized template

Step 3.

Slow cool on bench-top.

Preparing 10 uM hybridized template

Step 4.

Use immediately or store at -20 °C.

TEMPERATURE
-20 °C : Storage

NOTES

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.

Preparation of NTPs

Step 5.

Add 500 µl of RNase-free H_2O to the 70-80 mg of each NTP.

AMOUNT
500 µl : RNase-free H2O

Preparation of NTPs

Step 6.

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.

REAGENTS
- 5N NaOH by Contributed by users
- 1N NaOH by Contributed by users

Preparation of NTPs

Step 7.

Measure absorbance of 500 fold dilution: 3 µl to 1.5 ml of H_2O.

ATP λ_{max} 259 nm, ε_{max} 1.59×10^4 cm^{-1} M^{-1}

CTP λ_{max} 271 nm, ε_{max} 0.9×10^4 cm^{-1} M^{-1}

GTP λ_{max} 253 nm, ε_{max} 1.37×10^4 cm^{-1} M^{-1}

UTP λ_{max} 259 nm, ε_{max} 1.59×10^4 cm^{-1} M^{-1}

Preparation of NTPs

Step 8.

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

Transcription reaction

Step 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_2O to 100 µl	

AMOUNT
5 µl : 100 mM ATP
AMOUNT
5 µl : 100 mM CTP
AMOUNT
5 µl : 100 mM GTP
AMOUNT
5 µl : 100 mM UTP
AMOUNT
1 µl : 1M DTT
AMOUNT
0.1 µl : 1 mg/mL pyrophosphatase (Roche)
AMOUNT
10 µl : 1 mg/mL T7 polymerase

NOTES

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.

Transcription reaction

Step 10.

Incubate at 37 °C, overnight.

TEMPERATURE
37 °C : Incubation
DURATION
16:00:00 : overnight incubation

Transcription reaction

Step 11.

DNase treat:

1. Add 5 units (5 µl) RNase-free DNase directly to reaction.
2. Incubate at 37 °C, 30 minutes.

AMOUNT
5 µl : RNase-free DNase
TEMPERATURE
37 °C : Incubation
REAGENTS
RNase-free DNase by Promega
DURATION
00:30:00 : Incubation
NOTES

This step is optional and may be omitted.

Transcription reaction

Step 12.

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

AMOUNT
100 µl : Gel loading buffer
NOTES

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

only bromophenol blue.

Transcription reaction

Step 13.

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

NOTES

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

Step 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.

AMOUNT

200 µl : Silanization Solution I

REAGENTS

- EtOH by Contributed by users
- Silanization Solution I by Contributed by users

Gel-purification

Step 15.

Assemble glass plates.

Gel-purification

Step 16.

Prepare 15% urea polyacrylamide gel.

REAGENTS

- 15% urea polyacrylamide gel by Contributed by users

Gel-purification

Step 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.

REAGENTS

- 40% Acrylamide-bisacrylamide (19:1) by Contributed by users
- Urea by Contributed by users
- Water by Contributed by users
- 10% APS by Contributed by users

Gel-purification

Step 18.

Place comb and polymerize for 60 min.

DURATION

01:00:00 : Polymerize

Gel-purification

Step 19.

Assemble electrophoresis apparatus.

Gel-purification

Step 20.

Remove comb.

Gel-purification

Step 21.

Fill inner and outer buffer chambers with 1x TBE.

REAGENTS

- 1x TBE by Contributed by users

Gel-purification

Step 22.

Rinse wells with 1x TBE buffer.

REAGENTS

- 1x TBE buffer by Contributed by users

Gel-purification

Step 23.

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

3000 V, 400 mA.

DURATION

00:30:00 : Pre-running gel

Gel-purification

Step 24.

Load sample.

Gel-purification

Step 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.

DURATION

03:00:00 : Running gel

Gel-purification

Step 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.

Gel-purification

Step 27.

UV shadow band and excise with sterile razor and place in Falcon tube.

Gel-purification

Step 28.

Break gel slices with p-1000 tip.

Gel-purification

Step 29.

Crush and soak gel slices in equal volume of RNase-free water.

REAGENTS

- RNase-free water by Contributed by users

Gel-purification

Step 30.

Elute RNA overnight by rocking at 4 °C.

TEMPERATURE

4 °C : Eluting RNA

Next day

Step 31.

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

TEMPERATURE

4 °C : Centrifugation

REAGENTS

- Corning Spin-X filter tube by Contributed by users

DURATION

00:05:00 : Centrifugation

NOTES

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.

Next day

Step 32.

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

AMOUNT
30 µl : 10x NEBuffer 3
AMOUNT
1 µl : CIP
TEMPERATURE
37 °C : Incubation
REAGENTS
10x NEBuffer 3 by Contributed by users
CIP (10 U/µl) by New England Biolabs
DURATION
01:00:00 : Incubation
NOTES

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.)

Next day
Step 33.

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

AMOUNT
300 µl : Phenol-chloroform solution
REAGENTS
Phenol-chloroform solution by Contributed by users

Next day
Step 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]

Next day
Step 35.

Incubate at -80 °C, at least 1 hour

TEMPERATURE

-80 °C : Incubation

DURATION

01:00:00 : Incubation

Next day

Step 36.

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

TEMPERATURE

4 °C : Spinning down

DURATION

00:20:00 : Spinning down

Next day

Step 37.

Remove supernatant.

Next day

Step 38.

Wash pellet with 70% ethanol.

Next day

Step 39.

Spin down again and remove supernatant.

Next day

Step 40.

Dry pellets in speed vacuum, 10 minutes.

DURATION

00:10:00 : Drying pellets

Next day

Step 41.

Resuspend pellets in 30 µl DEPC H_2O (or buffer) and store at –20 °C.

AMOUNT

300 µl : DEPC H2O (or buffer)

TEMPERATURE

-20 °C : Storage

Warnings

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