Aug 22, 2018 Version 3

Protocol for *in vitro* transcription of DNA oligos by T7 polymerase V.3

DOI
dx.doi.org/10.17504/protocols.io.ssmeec6

Audrey Lapinaite[1]

[1]University of California, Berkeley

The Center for Genome ...

Meredith Triplet

DOI: dx.doi.org/10.17504/protocols.io.ssmeec6

Protocol Citation: Audrey Lapinaite 2018. Protocol for in vitro transcription of DNA oligos by T7 polymerase. **protocols.io https://dx.doi.org/10.17504/protocols.io.ssmeec6**

Protocol status: Working

Created: August 22, 2018

Last Modified: August 22, 2018

Protocol Integer ID: 14893

Abstract

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

Attachments

RNA_in_vitro_transcr...

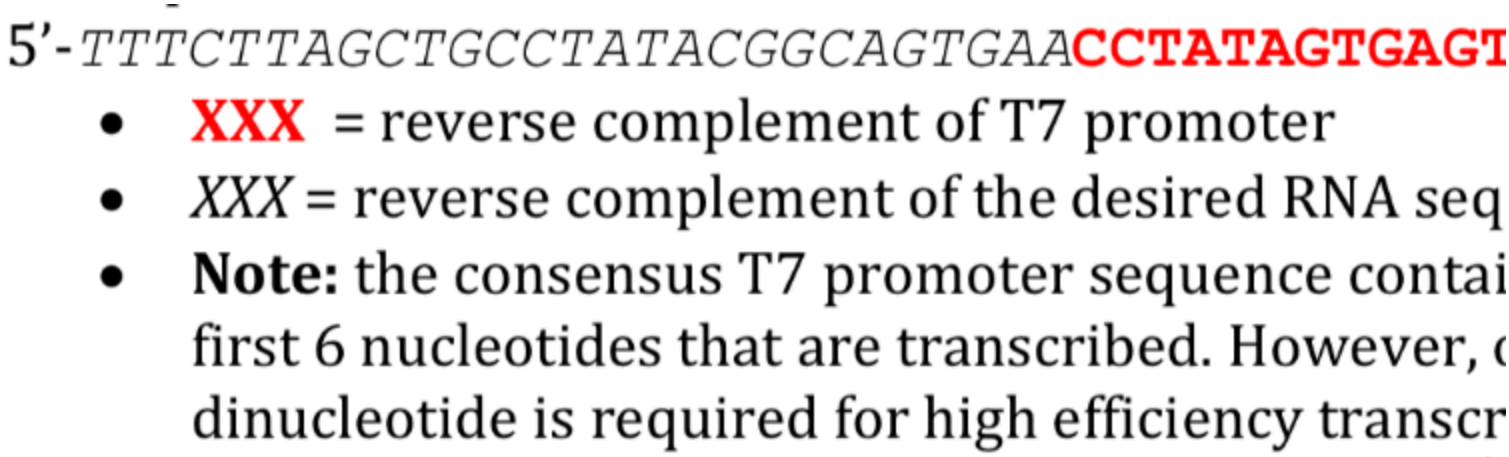
226KB

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:



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

- Silanization Solution I
- 15% urea polyacrylamide gel
- 40% Acrylamide-bisacrylamide (19:1)
- Urea
- Water
- 10% APS
- 1x TBE
- 1x TBE buffer
- RNase-free water
- Corning Spin-X filter tube
- 10x NEBuffer 3
- CIP (10 U/µl) **New England Biolabs**
- Phenol-chloroform solution

Protocol materials

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

Corning Spin-X filter tube
10x NEBuffer 3
40% Acrylamide-bisacrylamide (19:1)
15% urea polyacrylamide gel
1.5 nmol T7oligo
1x TBE
10x NEBuffer 3
EtOH
Silanization Solution I
RNase-free DNase **Promega**
1N NaOH
1N NaOH
1.5 nmol T7oligo
1.0 nmol template
DEPC H2O
5x transcription buffer
5N NaOH
1N NaOH
RNase-free DNase **Promega**
EtOH
Silanization Solution I
15% urea polyacrylamide gel
Water
10% APS
40% Acrylamide-bisacrylamide (19:1)
Urea
1x TBE
1x TBE buffer
RNase-free water
Corning Spin-X filter tube
10x NEBuffer 3

CIP (10 U/µl) **New England Biolabs**

Phenol-chloroform solution

Safety warnings

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

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	

Preparing 10 uM hybridized template

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

5x transcription buffer
1.5 nmol T7oligo
1.0 nmol template
DEPC H2O

2 Heat at 70-80 °C for 2 minutes.

70 °C Heating
00:02:00 Heating

3 Slow cool on bench-top.

4 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

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

500 µL RNase-free H2O

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.

5N NaOH

1N NaOH

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}

8 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_2O 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	Bro
30	2-8	
20	8-25	
10	25-35	
8	35-45	
6	45-70	
5	70-300	
4	100-500	

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

EtOH

Silanization Solution I

200 µL Silanization Solution I

15 Assemble glass plates.

16 Prepare 15% urea polyacrylamide gel.

15% urea polyacrylamide gel

17 Denaturing Urea Polyacrylamide Gel (300 ml for a gig gel: 35 × 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)

Urea

Water

10% APS

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

22 Rinse wells with 1x TBE buffer.

1x TBE buffer

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 ¼ 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

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

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

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

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 H_2O (or buffer) and store at –20 °C.

300 µL DEPC H2O (or buffer)

-20 °C Storage