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

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We use this protocol and it's working

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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:

5'-TTTCTTAGCTGCCTATACGGCAGTGAA**CCTATAGTGAGT**

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

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/ μ l) **New England Biolabs**
- ✕ Phenol-chloroform solution

Protocol materials

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

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 ₂ O	to 10mL
store at -20 °C	

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	
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	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 uM hybridized template

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

 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 μ l of RNase-free H₂O to the 70-80 mg of each NTP.

 500 μ L RNase-free H₂O

- 6 Bring pH of each NTP to 6.8-7.2 by adding 5 μ l of 5N NaOH followed by 10-90 μ l 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 μ l to 1.5 ml of H₂O.

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

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

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

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

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

Transcription reaction

9 Mix the following in a 100 μ l reaction:

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

Note

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

🧪 5 μ L 100 mM ATP

🧪 5 μ L 100 mM CTP

🧪 5 μ L 100 mM GTP

🧪 5 μ L 100 mM UTP

🧪 1 μ L 1M DTT

🧪 0.1 μ L 1 mg/mL pyrophosphatase (Roche)

🧪 10 μ L 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 μ L) RNase-free DNase directly to reaction.
2. Incubate at 37 °C, 30 minutes.

🧪 RNase-free DNase **Promega**

🧪 5 μ L RNase-free DNase

🌡️ 37 °C Incubation

🕒 00:30:00 Incubation

Note

This step is optional and may be omitted.

12

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

🧪 100 μ L 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 H₂O (or buffer) and store at -20 °C.

🧴 300 µL DEPC H₂O (or buffer)

🌡️ -20 °C Storage