Jun 25, 2019

In vitro transcription, capping, and 2'-O methylation of long RNAs

Stephen Floor[1]

[1]University of California, San Francisco

Working dx.doi.org/10.17504/protocols.io.4pwgvpe

Stephen Floor Lab

Stephen Floor
University of California, San Francisco

ABSTRACT

This protocol is for in vitro transcription of long RNAs off plasmid or PCR product templates. It is assumed that the template has been gel purified, is the right size, has a T7 promoter and a 3' polyA tail (typically A60). Following this protocol the RNA will be ready for transfection into mammalian cells or in vitro translation. Significant amounts of template are necessary: at least 1ug of template per 100ul reaction; ideally closer to 5ug of template, especially for very long templates. Use RNase sensitive protocols and reagents for all steps of this procedure.

The basic protocol is:
- PCR amplification of the template
- in vitro transcription
- capping and 2'-O-Methylation
- quality control and optional purification

Based on protocols from Kaihong Zhou (Doudna lab) and RNA: A Laboratory Manual (Rio, Ares, Hannon, Nilsen).

THIS PROTOCOL ACCOMPANIES THE FOLLOWING PUBLICATION

Stephen N. Floor and Jennifer A. Doudna (2016). “Tunable protein synthesis by transcript isoforms in human cells.” eLife 5, e10921.

GUIDELINES

Use RNase sensitive protocols and reagents for all steps of this procedure.

MATERIALS

NAME	CATALOG #	VENDOR
E.coli Poly (A) Polymerase - 100 units	M0276S	New England Biolabs
mRNA Cap2'-O-Methyltransferase - 2,000 units	M0366S	New England Biolabs
Vaccinia Capping System - 400 units	M2080S	New England Biolabs
GlycoBlue™ Coprecipitant	AM9516	Thermo Scientific
MEGAscript® T7 Transcription Kit	AM1334	Thermo Scientific
RQ1 RNase-Free DNase, 1,000u	M6101	Promega
RNasin(R) Plus RNase Inhibitor, 10,000u	N2615	Promega
Ambion NorthernMAX glyoxal loading dye	AM8551	Thermo Fisher Scientific
RNA clean & concentrator-25	R1017	Zymo Research
RNA Gel Recovery Kit	R1011	Zymo Research

MATERIALS TEXT

10X transcription buffer
- 300 mM Tris-Cl pH (RT) = 8.1
- 250 mM MgCl2
- 0.1% Triton X-100
- 20 mM spermidine
- 100 mM DTT

0.3 M NaOAc pH 5.2

50 mM EDTA

One of these two RNA ladders:
- https://www.neb.com/products/n0362-ssrna-ladder (for transcripts > 2kb)
- https://www.thermofisher.com/order/catalog/product/15623100 (for transcripts < 2kb)

note: we typically use T7 polymerase we have purified but the Megascript kit above or similar should also work. If using a kit, perform the in vitro transcription according to the kit instructions.

note: The RNA Gel Recovery Kit is optional and only necessary if the transcription has more than one product. the polyA polymerase is only required if your template does not contain a polyA stretch.

PCR amplification of template

1 Primer design:

pA60 txn rev TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT CTG CAG
pA60 txn fwd CGG CCA GTG AAT TCG AGC TCT AAT ACG ACT CAC TAT AGG

note: if using a different template, substitute the reverse and forward primers for primers that will amplify the forward and reverse of your template. The forward primer should have the T7 promoter in it. If the template does not have a genetically encoded polyA tail, as this one does, then your reverse primer will not have all these T's and instead you will have to polyA tail the RNAs with this: https://www.neb.com/products/m0276-ecoli-poly-a-polymerase

2 PCR setup (4x 100ul for each template, four replicate reactions1):

Reagent	Amount per 100 ul reaction
HF buffer	10 ul
dNTP (10 mM)	2 ul
forward primer (100 uM)	0.5 ul
reverse primer (100 uM)	2 ul
phusion polymerase	1 ul
template (50 ng total)	variable
water	to 100 ul

3 Run PCR with a two step protocol:
72 degrees annealing/extension for 3 minutes
98 degrees melting for 30 seconds
repeat 30 cycles
10 minute final extension time at 72

4

Load all of the PCRs into an agarose gel and verify then size of the template band and then gel purify it

In vitro transcription of RNA

5 Warm all reagents except T7 polymerase and Superase:IN to room temperature and assemble the reaction at RT.

warning: Adding cold spermidine to template can cause precipitation of the template.

6 Mix the following together in a 1.5ml tube

Template DNA	1 to 5 ug
ACGU rNTP mix (25 mM each, pH 8)	30 ul
10X transcription buffer (RT)	10 ul
1M DTT	2 ul
1M MgCl2	1 ul
T7 polymerase	10 ul
Superase:IN	1 ul
DEPC H2O	to 100 ul

note: we typically use T7 polymerase we have purified but the Megascript kit in the materials or similar should also work. If using a kit, perform the in vitro transcription according to the kit instructions.

7 Incubate at 37 degrees C in a stable incubator - a water bath or stable dry incubator like a thermocycler. Warm rooms and heat blocks can have higher variability in temperature and T7 is a sensitive enzyme.

8 Wait 1 hour and check the reactions - they should be somewhat cloudy. If they are, add 1 ul of 1 mg/ml pyrophosphatase to each (Roche 10108987001; optional)

9 Allow the reactions to proceed for 3-4 hours.

10 Add 1 ul of RQ1 RNase-free DNase to each reaction and incubate at 37 for 30 minutes.

11 Precipitate by adding 200 ul 0.3M NaOAc pH 5.2, 1 ul GlycoBlue and 750 ul 100% EtOH.

12

[PAUSE] Incubate at -80 for >1 hour to overnight.

Resuspending EtOH precipitated RNA

13 Pellet precipitations by centrifugation at 16,000 g at 4 degrees for 25 minutes.

14 Aspirate supernatant and discard

15 Wash with 500 ul -20 degree 70% EtOH

16 Centrifuge at 16,000 g and 4 degrees for 5 minutes

17 Aspirate supernatant and discard

18 Quick-spin and remove residual ethanol with a P20 pipet

19 Allow pellet to air dry for 5 minutes; do not overdry

20 Resuspend pellet in 100 ul 50 mM EDTA

21 Purify RNA over Zymo RNA Clean & Concentrator 25 column following small RNA removal protocol and elute in 30 ul DEPC H2O

22 Recommended but optional: assess RNA integrity by running a Glyoxal gel before proceeding

23 Measure concentration of RNA on nanodrop

Capping and 2'-O-Methylation

24 Dilute 20 ug of RNA in 26 uL DEPC H2O

25 Prepare a 4mM stock of SAM from the supplied 32 mM stock (4 uL 32mM and 28 uL DEPC H2O for up to 15 reactions)

note: SAM is unstable and should not be freeze-thawed. If the SAM is degraded, there will not be mRNA capping or methylation.

26 Either add these directly to the tube above, or assemble a master mix according to the following recipe if performing many reactions (note all these reagents are supplied by the corresponding kits):

Reagent	Volume per 40 ul reaction
10X capping buffer	4 ul
10 mM GTP	2 ul
4 mM SAM	2 ul
Vaccinia capping enzyme	2 ul
2'-O-Me transferase	2 ul
RNasin plus (Promega)	2 ul

27 Add 14 uL of the master mix to each tube with RNA

28 Incubate at 37 deg for 60 minutes

29 Purify over zymo Clean and Concentrator 25 column following small RNA removal protocol and elute in 30 ul DEPC H2O

if your RNA is not A-tailed from the PCR template and you want it to have a polyA tail, you should A-tail it with E. coli poly-A polymerase at this stage. Follow the kit instructions in materials

Quality control: glyoxal gel

30 Run a glyoxal gel of all RNAs and verify their size.

31 Also glyoxylate the ladder and include (typically I use 5ul of either of the ladders listed above)

32 Use Image Lab to quantify the full-length band and use this to determine concentration in combination with nanodrop or qubit readings.

33 If significant truncated products are present I recommend agarose gel purification of full-length RNA using the Zymo Agarose Gel Zymoclean kit (R1011)