In vitro transcription of guide RNAs Version 5

Mark Dewitt, Julia Wong

Abstract

sgRNA template assembly, in vitro T7 transcription, and SPRI bead cleanup

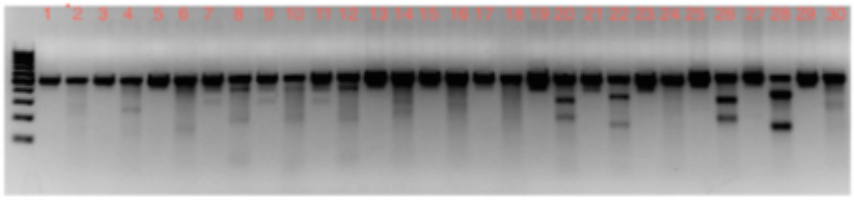
Citation: Mark Dewitt, Julia Wong In vitro transcription of guide RNAs. **protocols.io**
dx.doi.org/10.17504/protocols.io.exabfie
Published: 26 May 2016

Guidelines

The primers used are: one long, variable oligo that carries the T7 promoter and desired guide sequence; an 82-nt constant oligo that carries the 3' end of the sgRNA; two short external primers for amplification.

Assembly Oligos:
T7FwdVar:
GGATCCTAATACGACTCACTATAG---guide-sequence---GTTTTAGAGCTAGAA
T7RevLong:
AAAAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAAC

Amplification Primers:
T7FwdAmp: GGATCCTAATACGACTCACTATAG
T7RevAmp: AAAAAAGCACCGACTCGG



Odd numbers: just PCR product
Even numbers: with T7E1 treatment
Here's a gel that shows some good guides and some that didn't work; examples of more promising looking guides would be #'s 20, 22, 26, and 28.

Designing the guide sequence: The T7 transcript starts with the G before the dashes in the Fwd assembly oligo. If your target calls for a G at the 5' end of the guide, use the one already included in T7FwdVar design. If your target calls for a different base at the 5' end, put the whole guide sequence in T7FwdVar; there will be an extra G on the end that should have minimal effect, unless your guide is quite short.
Making the template: we have used the Phusion-HF DNA polymerase. Although the amplification primers have quite different Tm's, they work together fine.

Protocol

Making in vitro transcription DNA template

Step 1.
see guidelines for the primers

Set up the following 50 µl reaction

AMOUNT
50 µl Additional info:

PROTOCOL

. T7 PCR Template for 1sgRNA Mixture

CONTACT: Jacob Corn
Step 1.1.
35.5 µl H2O

Step 1.2.
10 µl 5x Phusion HF Buffer

Step 1.3.
1 µl 10 mM dNTPs

Step 1.4.
1 µl T7FwdVar (1 µM) ← THIS IS THE PART WE DESIGN

Step 1.5.
1 µl T7RevLong (1 µM)

Step 1.6.
0.5 µl T7FwdAmp (100 µM)

Step 1.7.
0.5 µl T7RevAmp (100 µM)

Step 1.8.
0.5 µl Phusion HF DNA polymerase (2u/µl)

Making in vitro transcription DNA template

Step 2.
Run PCR:
95˚ 30 sec
95˚ 10 sec
57˚ 10 sec
72˚ 10 sec
30x steps 2-4
72˚ 2 min
4˚ hold

No PCR cleanup necessary at this point

In vitro T7 transcription

Step 3.

Mix the following to make **20 µl** total T7 transcription mix

volume	reagent
2 µl	10x Buffer 1x
2 µl	ATP (100 mM), 10 mM
2 µl	GTP (100 mM), 10 mM
2 µl	CTP (100 mM), 10 mM
2 µl	UTP (100 mM), 10 mM
8 µl	DNA template (85 ng/µl) ←PCR PDTS 25 ng/µ
2 µl	T7 RNA polymerase mix

DURATION
00:18:00

PROTOCOL

. T7 transcription mix for guide RNAs protocol

CONTACT: Jacob Corn
Step 3.1.
2 µl 10x Buffer 1x

Step 3.2.
2 µl ATP (100 mM) 10 mM

Step 3.3.
2 µl GTP (100 mM) 10 mM

Step 3.4.
2 µl CTP (100 mM) 10 mM

Step 3.5.
2 µl UTP (100 mM) 10 mM

Step 3.6.
8 µl DNA template (85 ng/µl) ←PCR PDTS 25 ng/µ

Step 3.7.
2 µl T7 RNA polymerase mix

In vitro T7 transcription

Step 4.
Incubate transcription mix for 18 hours at 37˚ in a thermalcycler

DURATION
18:00:00

In vitro T7 transcription

Step 5.

Add 1 µl of RNase-free DNase; incubated 20 min, room Temp

DURATION
00:20:00

SPRI Beads clean-up of sgRNAS (96-well plate):

Step 6.
Bring volume to 150 uL with 100% EtOH (this helps binding of small fragments)

SPRI Beads clean-up of sgRNAS (96-well plate):

Step 7.

Add 5X SPRI (we use homemade SeraPure beads for RNA binding)
5*10 (IVT sgRNA)= 50 uL of SPRI Beads
5*20 (IVT sgRNA)= 100 uL SPRI Beads

REAGENTS

Agencourt AMPure XP A63880 by Beckman Coulter

SPRI Beads clean-up of sgRNAS (96-well plate):

Step 8.

Pipette to mix 10 times

SPRI Beads clean-up of sgRNAS (96-well plate):

Step 9.

Incubate 5 minutes at room temperature

DURATION

00:05:00

SPRI Beads clean-up of sgRNAS (96-well plate):

Step 10.

Place on magnetic stand, 5 min

DURATION

00:05:00

SPRI Beads clean-up of sgRNAS (96-well plate):

Step 11.

Discard supernatant

SPRI Beads clean-up of sgRNAS (96-well plate):

Step 12.

Wash#1 Add 200 uL, 80% EtOH. Wait 2 min. Remove EtOH.

DURATION

00:02:00

SPRI Beads clean-up of sgRNAS (96-well plate):

Step 13.

Wash #2: Add 200 uL, 80% EtOH. Wait 2 min. Remove EtOH.

DURATION

00:02:00

SPRI Beads clean-up of sgRNAS (96-well plate):

Step 14.

Air dry 5-10 min (pellet will change from a glossy/wet to matte/dry.)

DURATION

00:10:00

SPRI Beads clean-up of sgRNAS (96-well plate):

Step 15.

Elute 20 uL of water or TE. Pipette mix 10 times.

SPRI Beads clean-up of sgRNAS (96-well plate):

Step 16.

Incubate 2 minutes at room temperature

DURATION

00:02:00

SPRI Beads clean-up of sgRNAS (96-well plate):

Step 17.

Place on magnetic stand, 5 min

DURATION

00:05:00

SPRI Beads clean-up of sgRNAS (96-well plate):

Step 18.

Keep Supernatant. Transfer to a new plate / tubes.