

In vitro transcription of guide RNAs Version 6

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Abstract

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

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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

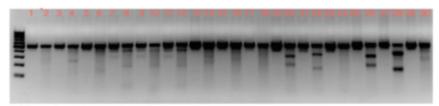
T7RevLong:

AAAAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTAACTTGCTATTTCTAGCTC
TAAAAC

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.

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.

 $1 \mu l 10 mM dNTPs$

Step 1.4.

1 μ l T7FwdVar (1 μ M) \leftarrow 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 μΙ	10x Buffer 1x
2 μΙ	ATP (100 mM), 10 mM
2 μΙ	GTP (100 mM), 10 mM
2 μΙ	CTP (100 mM), 10 mM
2 μΙ	UTP (100 mM), 10 mM
8 μl	DNA template (85 ng/µl) ←PCR PDTS 25 ng/µ
2 μΙ	T7 RNA polymerase mix

O 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 μI DNA template (85 ng/μI) ←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

O DURATION

18:00:00

In vitro T7 transcription

Step 5.

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

O DURATION

00:20:00

SPRI Beads clean-up of sqRNAS (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 sqRNA)= 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

O DURATION

00:05:00

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

Step 11.

Discard supernatant

SPRI Beads clean-up of sqRNAS (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.

O DURATION

00:02:00

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

Step 14.

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

O 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

O DURATION

00:05:00

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

Step 18.

Keep Supernatant. Transfer to a new plate / tubes.