In vitro transcription of guide RNAs Version 4

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Abstract

In vitro T7 template synthesis and transcription
sgRNA protocol with SPRI beads

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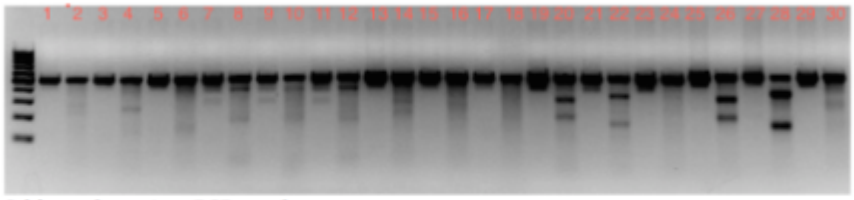
Guidelines

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:
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 the Template (for 1 sgRNA)

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)

PCR

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

Making template

Step 3.

Mix: Final conc.
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
l2 µl T7 RNA polymerase mix
20 µl total

Incubated this mix for 18 hours at 37˚ in a thermalcycler.Added 1 µl of RNase-free DNase; incubated 20 min, room T.

DURATION
00:18:00

Run PCR

Step 4.
Run PCR
95C 30s
95C 10s
57C 10s
72C 10s
steps 2-4, 30 cycles
72 2min
4C hold

No PCR cleanup necessary at this point

T7 transcription

Step 5.
Make 20 µl total T7 transcription mix

AMOUNT
20 µl Additional info:

PROTOCOL

. T7 transcription mix

CONTACT: Jacob Corn

T7 transcription

Step 6.
Incubate transcription mix for 18 hours at 37˚ in a thermalcycler

DURATION
18:00:00

T7 transcription

Step 7.
Add 1 µl of RNase-free DNase; incubated 20 min, room T

DURATION
00:20:00

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

Step 8.

IVT sgRNAs to 20uL (or 10uL)

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

Step 9.

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

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

Step 10.

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 11.

Pipette to mix 10 times

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

Step 12.

Incubate 5 minutes at room temperature

DURATION
00:05:00

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

Step 13.

Place on magnetic stand, 5 min

DURATION
00:05:00

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

Step 14.

Discard supernatant

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

Step 15.

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 16.

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 17.

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 18.

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

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

Step 19.

Incubate 2 minutes at room temperature

DURATION

00:02:00

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

Step 20.

Place on magnetic stand, 5 min

DURATION

00:05:00

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

Step 21.

Keep Supernatant. Transfer to a new plate / tubes.