In vitro transcription of guide RNAs Version 7

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Abstract

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

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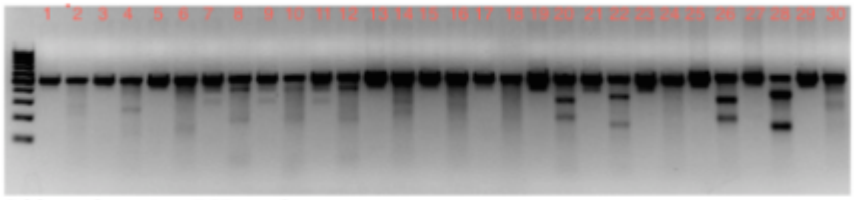
Guidelines

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:
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

Design sgRNA and order PCR oligos.

Step 1.

Add the desired protospacer sequence to the T7FwdVar oligo and order the oligo from your favorite oligonucleotide supplier. There are many programs available for protospacer design that attempt to optimize on- and/or off-target activity. Which program is most useful depends upon many factors including type of editing, organism being edited, etc. Choice of protospacer design program is beyond the scope of this protocol.

The transcript will start with the bolded G just 5' of the dashes in the T7FwdVar oligo. T7 RNA polymerase requires a 5' G for proper transcript initiation. If your protospacer has a G at the 5' end, you can omit it from the T7FwdVar design to avoid duplication of the G. If your protospacer has a C, T, or A at the 5' end, add the whole protospacer sequence to T7FwdVar. In this case, there will be an extra G added to the 5' end of the protospacer, but literature indicates this will have minimal effect unless your guide is very short.

Primers:

T7FwdVar oligo (5'-GGATCCTAATACGACTCACTATA**G**--protospacer sequence—GTTTTAGAGCTAGAA-3')
T7RevLong oligo (5'-AAAAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAAC-3')
T7FwdAmp primer (5'-GGATCCTAATACGACTCACTATAG-3')
T7RevAmp primer (5'-AAAAAAGCACCGACTCGG-3')

Making in vitro transcription DNA template

Step 2.

For each T7FwdVar oligo you designed, set up the following PCR (total volume should be 20.0 µL). Make sure everything is RNase free and filter tips are used. Furthermore, whipe down everything (in every step of protocol) with RNase Away to ensure no contamination.

13.4 µl DEPC-treated H_2O
4.0 uL 5x Phusion HF Buffer
0.8 µl 10 mM dNTPs
0.4 µl T7FwdVar (1 µM)
0.4 µl T7RevLong (1 µM)
0.4 µl T7FwdAmp (100 µM)
0.4 µl T7RevAmp (100 µM)
0.2 µl Phusion HF DNA polymerase (2u/µl)

If making multiple sgRNA templates, prepare a master mix with all components except T7FwdVar. Include a no template control (omit T7FwdVar).

Primers:

T7FwdVar oligo (5'-GGATCCTAATACGACTCACTATA**G**--protospacer sequence—GTTTTAGAGCTAGAA-3')
T7RevLong oligo (5'-AAAAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAAC-3')
T7FwdAmp primer (5'-GGATCCTAATACGACTCACTATAG-3')
T7RevAmp primer (5'-AAAAAAGCACCGACTCGG-3')

Step 3.

Run PCR:
95˚ 30 sec
95˚ 10 sec
57˚ 10 sec
72˚ 10 sec
34x steps 2-4
72˚ 2 min
4˚ hold

No PCR cleanup necessary at this point

In vitro T7 transcription

Step 4.

In vitro T7 transcription

Step 5.

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

DURATION
18:00:00

In vitro T7 transcription

Step 6.

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 7.

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

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

Step 8.

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 9.

Pipette to mix 10 times

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

Step 10.

Incubate 5 minutes at room temperature

DURATION

00:05:00

Step 11.

Place on magnetic stand, 5 min

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

Step 12.

Discard supernatant

Step 13.

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

DURATION

00:02:00

Step 14.

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

Step 15.

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 16.

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

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

Step 17.

Incubate 2 minutes at room temperature

DURATION

00:02:00

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

Step 18.

Place on magnetic stand, 5 min

DURATION

00:05:00

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

Step 19.

Keep Supernatant. Transfer to a new plate / tubes.