In vitro transcription of guide RNAs and 5'-triphosphate removal Version 10

Mark Dewitt, Julia Wong,Beeke Wienert

Abstract

sgRNA template assembly, in vitro T7 transcription, and sgRNA column cleanup to remove 5'-triphosphate groups

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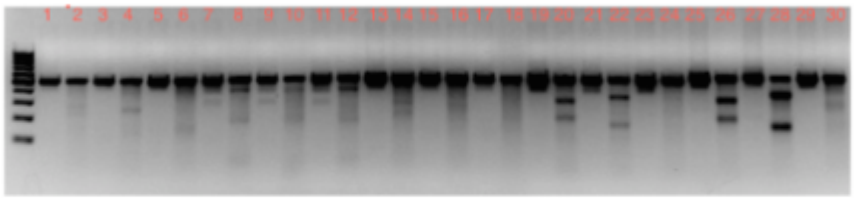
Guidelines

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')

Generate 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, wipe down everything (in every step of protocol) with RNase Away to ensure no contamination with RNAse.

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')

In vitro transcription DNA template PCR

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.

We like to use HiScribe T7 High Yield RNA Synthesis Kit but any T7 RNA synthesis kit should be fine.

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 from Step 3
2 µl	T7 RNA polymerase mix

REAGENTS

HiScribe™ T7 High Yield RNA Synthesis Kit E2040S by New England Biolabs

In vitro T7 transcription

Step 5.

Incubate transcription mix for 18 hours (over night) at 37˚ in a thermocycler

TEMPERATURE

37 °C Additional info:

DURATION

18:00:00

DNA template removal

Step 6.

Remove DNA template by adding 1 µl of RNase-free DNase; incubate 15 min at 37C in thermocycler

TEMPERATURE

37 °C Additional info:

REAGENTS

DNase I (RNase-free) - 1,000 units M0303S by New England Biolabs

Removal of 5'-triphosphate groups

Step 7.

T7 in vitro transcription results in RNA carrying a 5'-triphosphate group. This triggers a RIG-I-mediated innate immnue response in mammalian cells and can cause cell death, particularly in primary cells. We highly recommend treating your IVT sgRNA with Alkaline Calf Intestinal Phosphatase (CIP) before proceeding to the purification step. We found that CIP treatment must be rigorous to completely remove all 5-PPP groups from your RNA. However, CIP binds tightly to RNA and NEB recommends to only use the minimal amount needed.

Bring your IVT mix to a total volume of 88 ul with RNAse-free water

Removal of 5'-triphosphate groups

Step 8.

Add 10ul of NEB buffer 2.1 (comes with the CIP enzyme)

Add 2ul (20 units) of CIP

REAGENTS

Alkaline Phosphatase, Calf Intestinal (CIP) M0290S by New England Biolabs

Removal of 5'-triphosphate groups

Step 9.

Mix well and incubate at 37C for 3h

TEMPERATURE

37 °C Additional info:

sgRNA purification

Step 10.

sgRNAs need to be purfied before transfection. There are different methods one could purify their sgRNAs. We found that while SPRI bead clean-up of RNAs is quick and gives reliable yields (see older versions of protocol), SPRI bead purified sgRNAs still cause an elevated immune response even after CIP treatment. We therefore tested different column purification kits and found that column purification of sgRNAs completely eliminates the immune response after CIP treatment.

We use the Qiagen RNeasy Mini Prep Kit and follow the manufacturer's instructions with the following modifications:

NOTES

Jacob Corn 27 Feb 2018

Yields of from column purification can be low as the sgRNAs are small and most kits are designed to bind longer RNAs. We found the Qiagen RNeasy kit works ok, but others recommend the miRNeasy kit from Qiagen. It might be worth trying a few different kits to optimize your yield.

sgRNA purification

Step 11.

Add 350 µl RLT Buffer to the sample

sgRNA purification

Step 12.

Add 550 µl 100% ethanol

sgRNA purification

Step 13.

Tranfer 500 µl to an RNeasy mini spin column; spin for 15 sec

Then transferr the remainder onto the same column; spin for 15 sec

sgRNA purification

Step 14.

Add 500 µl RPE Buffer; spin 15 sec

Repeated this wash step

sgRNA purification

Step 15.

Moved spin column to a new collection tube and spin for 1 min to dry the membrane completely

sgRNA purification

Step 16.

Move spin column to an RNAse-free 1.5 ml microfuge tube

Add 20-30 µl DEPC-treated H2O; spin 1 min

Optional: Repeat the elution to collect any remaining RNA

sgRNA purification

Step 17.

Measure your RNA concentration by Nanodrop or Qubit. Store sgRNAs at -80C

ANNOTATIONS

Mark DeWitt 27 Feb 2018

Qubit works better