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May 05, 2022

In vitro transcription of guide RNAs and 5'-triphosphate removal V.13

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dx.doi.org/10.17504/protocols.io.n2bvjyp5vk5w/v13

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

DOI

dx.doi.org/10.17504/protocols.io.n2bvjyp5vk5w/v13

https://doi.org/10.1371/journal.pbio.2005840

Mark Dewitt, Julia Wong, Beeke Wienert, Moritz F Schlapansky 2022. In vitro transcription of guide RNAs and 5'-triphosphate removal. **protocols.io** https://protocols.io/view/in-vitro-transcription-of-guide-rnas-and-5-triphos-bzjpp4mn Moritz Schlapansky

protocol

Wienert B, Shin J, Zelin E, Pestal K, Corn JE (2018) In vitro–transcribed guide RNAs trigger an innate immune response via the RIG-I pathway. PLoS Biol 16(7): e2005840. doi: 10.1371/journal.pbio.2005840

protocol ,

Oct 28, 2021

May 05, 2022

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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:

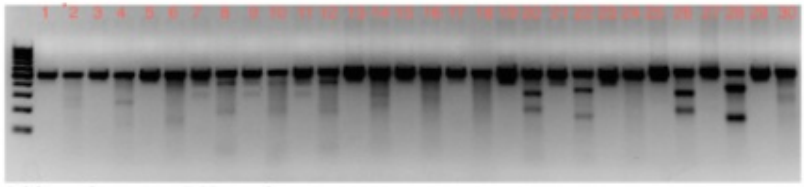
T7FwdVar oligo (5'-TAATACGACTCACTATA**G**--protospacer sequence—GTTTCAGAGCTATGCTGGAAAC-3')

T7RevLong oligo (5'-AAAAAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTCAACTTGCTATGCTGTTTCCAGCATAGCTCTGA-3')

Amplification Primers:

T7FwdAmp primer (5'-TAATACGACTCACTATAG-3')

T7RevAmp primer (5'-AAAAAAAGCACCGACTCGGTGC-3')



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.

STEP MATERIALS

DNase I (RNase-free) - 1,000 units **New England Biolabs Catalog #M0303S** Step 6

HiScribe T7 High Yield RNA Synthesis Kit - 50 rxns **New England Biolabs Catalog #E2040S** Step 4

Shrimp Alkaline Phosphatase (rSAP) - 500 units **New England Biolabs Catalog #M0371S**

Design sgRNA and order PCR oligos.

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1 Add the desired protospacer sequence to the T7FwdVarV2 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 T7FwdVarV2 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 T7FwdVarV2 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 T7FwdVarV2. 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:
T7FwdVarV2 oligo (5'-TAATACGACTCACTATA**G**--protospacer sequence—GTTTCAGAGCTATGCTGGAAAC-3')
T7RevLongV2 oligo (5'-AAAAAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTCAACTTGCTATGCTGTTTCCAGCATAGCTCTGA-3')
T7FwdAmpV2 primer (5'-TAATACGACTCACTATAG-3')
T7RevAmpV2 primer (5'-AAAAAAAGCACCGACTCGGTGC-3')

Generate in vitro transcription DNA template

2 For each T7FwdVarV2 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.

10.6 µl DEPC-treated H_2O
4.0 uL 5x Phusion HF Buffer
0.4 µl 10 mM dNTPs
0.4 µl T7FwdVarV2 (1 µM)
0.4 µl T7RevLongV2 (1 µM)
2 µl T7FwdAmpV2 (10 µM)
2 µl T7RevAmpV2 (10 µM)
0.2 µl Phusion HF DNA polymerase (2u/µl)

If making multiple sgRNA templates, prepare a master mix with all components except T7FwdVarV2. Include a no template control (omit T7FwdVarV2).

Primers:
T7FwdVarV2 oligo (5'-TAATACGACTCACTATA**G**--protospacer sequence—GTTTCAGAGCTATGCTGGAAAC-3')
T7RevLongV2 oligo (5'-AAAAAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTCAACTTGCTATGCTGTTTCCAGCATAGCTCTGA-3')
T7FwdAmpV2 primer (5'-TAATACGACTCACTATAG-3')
T7RevAmpV2 primer (5'-AAAAAAAGCACCGACTCGGTGC-3')

In vitro transcription DNA template PCR

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3 Run PCR:
98˚ 30 sec
98˚ 10 sec
51˚ 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

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

A	B
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, (usually ~4µg, 50 pmol)
2 µl	T7 RNA polymerase mix

HiScribe T7 High Yield RNA Synthesis Kit - 50 rxns **New England Biolabs Catalog #E2040S**

5 Incubate transcription mix for ~18 hours (over night) at 37˚ in a thermocycler
18:00:00
37 °C

DNA template removal

6 Remove DNA template by adding 1 µl of RNase-free DNase; incubate 15 min at 37C in thermocycler
00:15:00 37C incubation
DNase I (RNase-free) - 1,000 units **New England Biolabs Catalog #M0303S**
37 °C

Removal of 5'-triphosphate groups

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.

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We highly recommend treating your IVT sgRNA with a heat-labile version of calf intestinal alkaline phosphatase (Quick CIP) before proceeding to the purification step. We found that Quick CIP treatment must be rigorous to completely remove all 5-PPP groups from your RNA. However, Quick CIP binds tightly to RNA and NEB recommends to only use the minimal amount needed.

8 Add 3ul of NEB CutSmart Buffer (comes with the Quick CIP enzyme)
Add 4µl of H2O
Add 2ul (2 units) of Quick CIP

Quick CIP **New England Biolabs Catalog #M0525**

9 Mix well and incubate at 37C for 3h

03:00:00

37 °C

sgRNA purification

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 Phosphatase treatment. We therefore tested different column purification kits and found that column purification of sgRNAs completely eliminates the immune response after Phosphatase treatment.

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

> 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.

11 Adjust sgRNA sample to a volume of 100 µl with RNase-free water.
Add 350 µl RLT Buffer to the sample

12 Add 250 µl 100% ethanol and mix well by pipetting.

13 Tranfer the sample (~700 µl) to an RNeasy mini spin column; spin for 15 sec at >8000 g
Then transfer the remainder onto the same column; spin for 15 sec

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14 Add 500 µl RPE Buffer; spin 15 sec
Repeated this wash step

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

16 Move spin column to an RNAse-free 1.5 ml microfuge tube
Add 33 µl DEPC-treated H2O; spin 1 min
Optional: Repeat the elution to collect any remaining RNA

17 Measure your RNA concentration by Nanodrop or Qubit. You can also check integrity/corrrect size of the sgRNA(s) on the RNA tapestation. Store sgRNAs at -80C

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