In vitro transcription of guide RNAs and 5'-triphosphate removal V.16

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ABSTRACT

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

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Protocol status: Working
We use this protocol and it's working

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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:

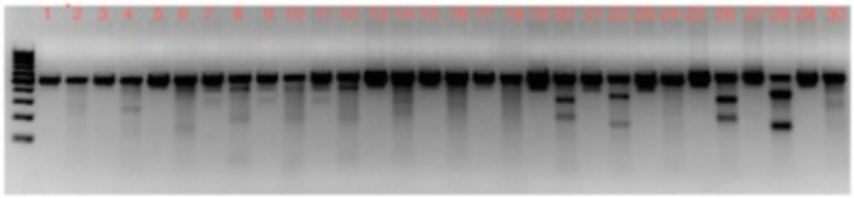
T7FwdVarV2 oligo (5'-TAATACGACTCACTATA**G**--protospacer sequence—GTTTCAGAGCTATGCTGGAAAC-3')

T7RevLongV2 oligo (5'-AAAAAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTCAACTTGCTATGCTGTTTCCAGCATAGCTCTGA-3')

Amplification Primers:

T7FwdAmpV2 primer (5'-TAATACGACTCACTATAG-3')

T7RevAmpV2 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.

MATERIALS

STEP MATERIALS

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

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

Phusion High-Fidelity PCR Kit - 50 rxns (50 ul vol) **New England Biolabs Catalo #E0553S**

miRNeasy mini Kit **Contributed by users Catalog #217004**

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

Design sgRNA and order PCR oligos

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')

Note

These V2 oligos differ from a previous version due to reported benefits using modified sgRNA structures that might enhance binding to Cas9 or increase sgRNA stability (see https://doi.org/10.1186/s13059-015-0846-3).

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).

Phusion High-Fidelity PCR Kit - 50 rxns (50 ul vol) **New England Biolabs Catalog #E0553S**

In vitro transcription DNA template PCR

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

	A	B
	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 #E2040**

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

18:00:00

37 °C

DNA template removal

5 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

sgRNA purification

6 sgRNAs need to be purfied before dephosphorylation. There are different methods one could purify their sgRNAs. We therefore tested different purification kits and found that the **Qiagen miRNeasy Tissue/Cells Advanced Mini Kit** gives the most consistent and highest yields. To purify our sgRNAs with this kit, we follow the manufacturer's instructions with the following modifications:

miRNeasy mini Kit **Contributed by users Catalog #217004**

Note

Yields of from column purification using the Qiagen RNeasy Mini kit can be low as the sgRNAs are small and most kits are designed to bind longer RNAs. Our yields are consistently higher when using the miRNeasy Tissue/Cells Advanced Mini Kit from Qiagen.

6.1 Adjust sgRNA sample to a volume of 100 µl with RNase-free water.
Add 350 µl RLT Buffer to the sample and mix well by pipetting

6.2 Add 450 µl Isopropanol and mix well by pipetting.

6.3 Transfer sample (~900 µl) to an RNeasy mini spin column; spin for 15 sec at 10.000 g.
Discard the flow-through.

6.4 Add 700 µl RWT Buffer; Spin for 15 sec at 10.000 g.
Discard the flow-through.

6.5 Add 500 µl RPE Buffer; Spin for 2 min at 10.000 g.

6.6 Move spin column to a new collection tube and spin for 1 min at 10.000 g to dry the membrane completely.

6.7 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 on the column and increase RNA concentration.

Removal of 5'-triphosphate groups

1d 6h

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

Note

Removal of the 5'-triphosphate group is not always necessary. We prefer the removal when working with primary cells.

7.1 For a 40µl reaction:
Add 4 ul of NEB CutSmart Buffer (comes with the Quick CIP enzyme)
Add 2 ul (2 units) of Quick CIP
Add X µl of H2O to a final reaction volume of 40 µl

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

7.2 Mix well and incubate at 37C for 3h

3h

03:00:00

37 °C

2nd sgRNA purification

1d 6h

8 sgRNAs need to be purified before editing. There are different methods one could purify their sgRNAs. We therefore tested different purification kits and found that the **Qiagen miRNeasy Tissue/Cells Advanced Mini Kit** gives the most consistent and highest yields. To purify our sgRNAs with this kit, we follow the manufacturer's instructions with the following modifications:

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8.4 Add 700 µl RWT Buffer; Spin for 15 sec at 10.000 g.
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8.5 Add 500 µl RPE Buffer; Spin for 2 min at 10.000 g.

8.6 Move spin column to a new collection tube and spin for 1 min at 10.000 g to dry the membrane completely.

8.7 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 on the column and increase RNA concentration.

sgRNA measurement/QC

9 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 -80 °C