

***In vitro* T7 template synthesis and transcription**

This procedure is based on the Doudna Lab protocol (Lin *et al.* eLife, 2014) for making sgRNA templates and transcribing them with T7 RNA polymerase. The elements are: one long, variable oligo that carries the T7 promoter and your guide sequence; an 82-nt constant oligo that carries the 3' end of the sgRNA; two short external primers for amplification.

Assembly Oligos:

T7FwdVar: 5'-GGATCCTAATACGACTCACTATAG-----guideseq-----GTTTTAGAGCTAGAA

T7RevLong: 5'-

AAAAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTAACTTGCTATTTCTAGCTC
TAAAAC

Amplification Primers:

T7FwdAmp: 5'-GGATCCTAATACGACTCACTATAG

T7RevAmp: 5'-AAAAAAGCACCGACTCGG

Designing the guide sequence:

The T7 transcript starts with the G before the dashes in the T7FwdVar oligo. If your target calls for a G at the 5' end of the guide, use that one in your 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:

Although the amplification primers have quite different T_m 's, they work together fine.

Mix:		Final conc.
13.4 μ l	H ₂ O	
4.0 μ l	5x Phusion HF Buffer	1x
0.8 μ l	10 mM dNTPs	400 μ M
0.4 μ l	T7FwdVar (1 μ M)	20 nM
0.4 μ l	T7RevLong (1 μ M)	20 nM
0.4 μ l	T7FwdAmp (100 μ M)	2 μ M
0.4 μ l	T7RevAmp (100 μ M)	2 μ M
0.2 μ l	Phusion HF DNA polymerase (2u/ μ l)	0.4 unit
20.0 μ l		

PCR:	95°	30 sec	} 35x
	95°	10 sec	
	57°	10 sec	
	72°	10 sec	
	72°	2 min	
	4°	hold	

You can run 2 μ l of this product on a 2% agarose gel to make sure the synthesis went well.

T7 transcription:

Use the NEB HiScribe T7 High Yield RNA Synthesis Kit. The kit contains 10x Buffer, each of the individual NTPs at 100 mM, and the T7 RNA polymerase mix. You don't know the concentration of template DNA at this stage, so you just add as much of the foregoing PCR reaction as there is room for in a total of 20 μ l.

		<u>Final conc.</u>
Mix:	2 μ l 10x Buffer	1x
	2 μ l ATP (100 mM)	10 mM
	2 μ l GTP (100 mM)	10 mM
	2 μ l CTP (100 mM)	10 mM
	2 μ l UTP (100 mM)	10 mM
	8 μ l DNA template	?
	2 μ l T7 RNA polymerase mix	
	<u>20 μl</u>	

Incubate this mix overnight at 37° in a thermalcycler.

At the conclusion of the incubation, add 1 μ l of RNase-free DNase; incubate 20 min, room T. Isolate the RNA with a QIAgen RNeasy mini kit, using the modifications for small RNAs. All operations at room T, with standard RNA precautions:

- Add 350 μ l RLT Buffer to the sample
- Add 550 μ l 100% ethanol
- Transfer ~500 μ l to an RNeasy mini spin column; spin for 15 sec
- Transfer the remainder; spin for 15 sec
- Move spin column to a new collection tube
- Add 500 μ l RPE Buffer; spin 15 sec
- Repeat this wash step
- Move spin column to a new collection tube and spin for 1 min
- Move spin column to a 1.5 ml microfuge tube
- Add 30 μ l DEPC-treated H₂O; spin 1 min
- Repeat the 30 μ l elution to collect any remaining RNA

Assess sgRNA yield on the Nanodrop spectrophotometer.

Confirm the concentration and assess the quality of the sgRNA by running an aliquot (0.5–1.0 μ g) on a 10% polyacrylamide TBE-urea gel.

Dana Carroll, June 24, 2016