In vitro transcription of S. pyogenes Cas9 sgRNAs from two complementary oligos

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Abstract

A protocol to in vitro transcribe sgRNAs from oligos for S. pyogenes Cas9.

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Guidelines

The protocol workflow is as follows:

1) Order oligos
2) Anneal oligo, extend & amplify using PCR
3) Gel purify PCR products (optional)
4) Transcribe
5) Bead cleanup or gel purify RNA

Note that there are two G's inserted at the 5' end of all sgRNAs generated using this protocol that are outside the protospacer. These do not affect cleavage biochemically and are trimmed inside cells if nucleofected as an RNP.

It's recommended to start with a 100 µL transcription because the volumes are convenient and this is typically enough sgRNA. Scale up as needed.

References:
Lin & Staahl, eLife 2014.
RNA: A Laboratory Manual (CSHL Press) Changelog: 1.01 - added references 1.1 - added bead cleanup

Before start

10X transcription buffer

- 300mM Tris-Cl pH 8.1
- 250mM MgCl2
- 0.1% Triton X-100
- 20mM spermidine
- 100mM DTT

2x Denaturing gel loading buffer

- 95% deionized formamide

- 20mM EDTA pH 8.0
- Dye (I typically use Bromophenol blue, Xylene Cyanol and Orange G in this. None overlap with sgRNA)

6x Agarose gel loading buffer

- 25% Ficoll
- Enough orange G so that the loading dye looks brighter than Orange Crush soda (~0.1%).

Gel extraction buffer

- 1mM EDTA
- 300mM NaOAc pH 5.2
- 0.5 w/v SDS

Materials

RQ1 RNase-Free DNase, 1,000u M6101 by Promega

✓ Oligos by Contributed by users

✓ Phusion polymerase (or other high fidelity PCR polymerase) by Contributed by users

T7 polymerase (We use homemade T7, but you can also use the Ambion MEGAshortscript kit) AM1354M by Thermo Fisher Scientific

✓ Large gel apparatus by Contributed by users

✓ 10X transcription buffer by Contributed by users

✓ 25mM ATP/CTP/GTP/UTP mix (pH 8.0) by Contributed by users

Pyrophosphatase by Roche

✓ 2x Denaturing gel loading buffer by Contributed by users

✓ 6x Agarose gel loading buffer by Contributed by users

✓ Gel extraction buffer by Contributed by users

Protocol

Designing oligo templates for sgRNAs

Step 1.

Order two oligos that have 20nt complementarity in the middle that span the guide RNA, with a T7 promoter at the 5' end. The sequence of the T7 promoter is TAATACGACTCACTATAGG.

Note that the first two G's here are added onto the template.

NOTES

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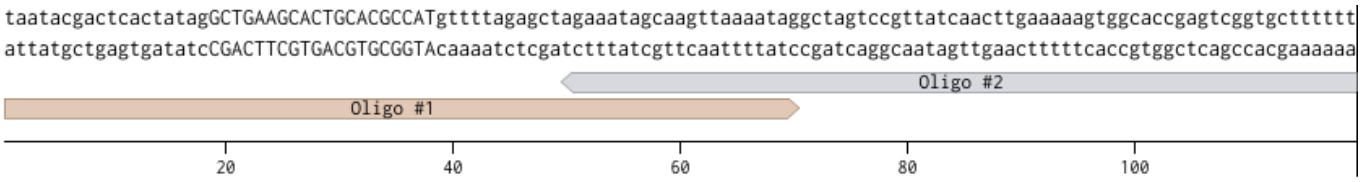
If your target sequence starts with G or GG, replace the GG at the end of the T7 promoter with the protospacer nucleotides.

Designing oligo templates for sgRNAs

Step 2.

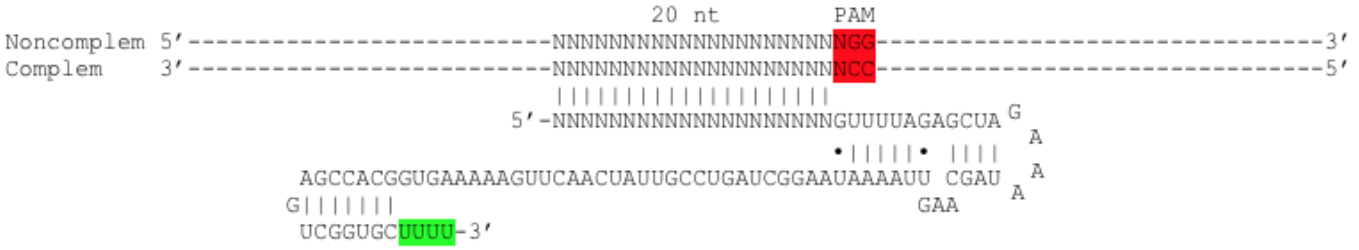
Oligo design - capital letters are the protospacer:



Designing oligo templates for sgRNAs

Step 3.

Illustration of the sgRNA in context of a target:



Designing oligo templates for sgRNAs

Step 4.

Oligo sequences:

Top: 5' - TAATACGACTCACTATAG*NNNNNNNNNNNNNNNNNNNN*GTTTTAGAGCTAGAAATAGCAAGTTAAAATAG - 3'

Bottom: 5' - AAAAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTATTTTAACTTGCTATTTCT - 3'

sgRNA fwd primer: GGATCCTAATACGACTCACTATAG

sgRNA rev primer: AAAAAAGCACCGACTCGG

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Only change the sequence of the protospacer (N's above). The bottom oligo and fwd/rev primers are always the same and only needs to be ordered once.

Extend the oligos to a double-stranded DNA template by PCR (~1 hour)

Step 5.

Resuspend oligos to 100µM.

x nmol oligo * 10 = ul ddH_2O to add to yield 100µM

Extend the oligos to a double-stranded DNA template by PCR (~1 hour)

Step 6.

Set up two 100µL PCRs per sgRNA template. If just making one sgRNA or a small number, use the single-reaction setup. If making many, make the master mix and add 95µL of the master mix to each tube and 5µL of the sgRNA-specific top oligo.

Reagent	Volume (µl)	Master mix volume (µl)	Number of reactions (2 * # of sgRNAs)
5x HF buffer	20	44	2
1 uM top oligo (sgRNA-specific)	2	-	

1 uM bottom oligo (always the same)	2	4.4
100 uM sgRNA fwd	2	
100 uM sgRNA rev	2	
dNTPs	2	4.4
phusion polymerase	1	2.2
ddH_2O	69	151.8

Extend the oligos to a double-stranded DNA template by PCR (~1 hour)

Step 7.

Run a PCR with the following program:

98°C for 2 minutes

98°C for 30 seconds

60°C for 30 seconds

72°C for 30 seconds

repeat for 30 cycles

72°C for 1 minute

4°C forever

Analytical gel of the PCR(s) (~1 hour; ~2 hrs if gel purification required)

Step 8.

Pour a 2% agarose gel with SYBR Safe dye and using orange G dye in the loading buffer.

Analytical gel of the PCR(s) (~1 hour; ~2 hrs if gel purification required)

Step 9.

Load 5µL of each PCR and 5µL of the Lonza DNA Marker 50-1,000bp (50461).

AMOUNT

5 µl Additional info: of each PCR

AMOUNT

5 µl Additional info: Lonza DNA Marker 50-1,000bp

Analytical gel of the PCR(s) (~1 hour; ~2 hrs if gel purification required)

Step 10.

Run at 100V until the orange G band is 2/3rds through the gel.

Analytical gel of the PCR(s) (~1 hour; ~2 hrs if gel purification required)

Step 11.

Image the gel. If there is one major band around 100 nt, perform a PCR cleanup (Qiagen) and proceed directly to in vitro transcription. If there are multiple bands, pour a preparative 2% agarose gel(s) with comb large enough to hold the entire 200µL PCR volume, run the gel and gel purify (Qiagen)

In vitro transcription (~5 hours to overnight)

Step 12.

Prepare the transcription templates from above at 100ng/µL or adjust the volume of template versus DEPC H_2O in the table below. The amount of template is variable, and even 500 nanograms for a 100µL reaction should be plenty.

In vitro transcription (~5 hours to overnight)

Step 13.

Set up one of the reactions below depending on desired yield:

Reagent	100 ul reaction	1 ml reaction	5 ml reaction	Final concentration
10X transcription buffer	10	100	500	1X
DTT (1 M)	2	20	50	20 mM
25 mM A/C/G/U mix	20	200	1000	5 mM
Pyrophosphatase (Roche)	0.5	1	5	1 ug/ml
T7 polymerase	10	100	500	100 ug/ml
Template (100 ng/ul)	10	100	500	1 uM
DEPC H2O	49.5	499	3 * 832 (2495)	
Expected yield	50 micrograms	500 micrograms	2 mg	
Expected yield (sgRNA molarity)	15 ul of 100 uM	150 ul of 100 uM	> 500 ul of 100 uM	

NOTES

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I typically ***do not*** add pyrophosphatase until the reaction starts turning cloudy (after 30 minutes to 1 hour). This is a positive control that the transcription is working, and the PPIase then stops accumulation of PPI

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CRITICAL It is important to keep all reagents on ice but **set up the reaction at room temperature in the order indicated**. Cold spermidine (in the transcription buffer) can precipitate the template DNA.

In vitro transcription (~5 hours to overnight)

Step 14.

Incubate in a water bath or air incubator at 37°C for 5 hours to overnight.

TEMPERATURE

37 °C Additional info:

Incubation

NOTES

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It is not recommended to use a heat block since the temperature fluctuates too much (and may exceed 37°C).

In vitro transcription (~5 hours to overnight)

Step 15.

After transcription, add 1µL RQ1 RNase-free DNase per 100µL of transcription and continue to incubate at 37°C for 30 minutes.

TEMPERATURE

37 °C Additional info:

Incubation

In vitro transcription (~5 hours to overnight)

Step 16.

End the transcription by adding half transcription volume of 2x denaturing loading dye (e.g. 50µL for a 100µL transcription).

NOTES

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Using this reduced amount of loading dye facilitates loading more RNA into a gel and is sufficient to denature short RNAs.

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PAUSE RNA is stable in denaturing loading dye overnight if necessary - immediate purification is advised.

Cleanup

Step 17.

Please select one of the following two Cleanup options:

Cleanup option #1: bead cleanup (much faster, may purify truncated products or nucleotides)

Cleanup option #2: Gel purify transcribed RNA (more difficult, higher purity)

Cleanup option #1: bead cleanup

Step 18 - Cleanup option #1: bead cleanup (much faster, may purify truncated products or nucleotides).

CRITICAL Incubate RNAclean or homemade SPRI beads at room temperature for 1 hour to bring to RT.

Cleanup option #1: bead cleanup

Step 19 - Cleanup option #1: bead cleanup (much faster, may purify truncated products or nucleotides).

Add 900µL 100% EtOH to the transcription.

AMOUNT

900 µl Additional info:
100% EtOH

NOTES

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This and all following steps assume a 100µL transcription. Scale up accordingly if using a larger transcription.

Cleanup option #1: bead cleanup

Step 20 - Cleanup option #1: bead cleanup (much faster, may purify truncated products or nucleotides).

Add 500µL SPRI beads.

AMOUNT

500 µl Additional info:
500µL SPRI beads

Cleanup option #1: bead cleanup

Step 21 - Cleanup option #1: bead cleanup (much faster, may purify truncated products or nucleotides).

Immediately pipet ten times to mix.

Cleanup option #1: bead cleanup

Step 22 - Cleanup option #1: bead cleanup (much faster, may purify truncated products or nucleotides).

Incubate 5 minutes at room temperature.

Cleanup option #1: bead cleanup

Step 23 - Cleanup option #1: bead cleanup (much faster, may purify truncated products or nucleotides).

Move tube to magnetic stand for five minutes or until clear.

Cleanup option #1: bead cleanup

Step 24 - Cleanup option #1: bead cleanup (much faster, may purify truncated products or nucleotides).

Aspirate supernatant without disturbing beads.

Cleanup option #1: bead cleanup

Step 25 - Cleanup option #1: bead cleanup (much faster, may purify truncated products or nucleotides).

Keep tube on magnet, add 1mL 80% EtOH. (1/3)

AMOUNT

1 ml Additional info: 80% EtOH

NOTES

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Use freshly prepared 80% EtOH as it is hygroscopic.

Cleanup option #1: bead cleanup

Step 26 - Cleanup option #1: bead cleanup (much faster, may purify truncated products or nucleotides).

Wait 1 minute. (1/3)

Cleanup option #1: bead cleanup

Step 27 - Cleanup option #1: bead cleanup (much faster, may purify truncated products or nucleotides).

Aspirate wash. (1/3)

Cleanup option #1: bead cleanup

Step 28 - Cleanup option #1: bead cleanup (much faster, may purify truncated products or nucleotides).

Keep tube on magnet, add 1mL 80% EtOH. (2/3)

AMOUNT

1 ml Additional info: 80% EtOH

Cleanup option #1: bead cleanup

Step 29 - Cleanup option #1: bead cleanup (much faster, may purify truncated products or nucleotides).

Wait 1 minute. (2/3)

Cleanup option #1: bead cleanup

Step 30 - Cleanup option #1: bead cleanup (much faster, may purify truncated products or nucleotides).

Aspirate wash. (2/3)

Cleanup option #1: bead cleanup

Step 31 - Cleanup option #1: bead cleanup (much faster, may purify truncated products or nucleotides).

Keep tube on magnet, add 1mL 80% EtOH. (3/3)

AMOUNT

1 ml Additional info: 80% EtOH

Cleanup option #1: bead cleanup

Step 32 - Cleanup option #1: bead cleanup (much faster, may purify truncated products or nucleotides).

Wait 1 minute. (3/3)

Cleanup option #1: bead cleanup

Step 33 - Cleanup option #1: bead cleanup (much faster, may purify truncated products or nucleotides).

Aspirate wash. (3/3)

Cleanup option #1: bead cleanup

Step 34 - Cleanup option #1: bead cleanup (much faster, may purify truncated products or nucleotides).

Remove any residual ethanol.

Cleanup option #1: bead cleanup

Step 35 - Cleanup option #1: bead cleanup (much faster, may purify truncated products or nucleotides).

Air dry pellet on magnet for 5-10 minutes.

Cleanup option #1: bead cleanup

Step 36 - Cleanup option #1: bead cleanup (much faster, may purify truncated products or nucleotides).

Remove from magnetic rack.

Cleanup option #1: bead cleanup

Step 37 - Cleanup option #1: bead cleanup (much faster, may purify truncated products or nucleotides).

Add 50µL water.

AMOUNT

50 µl Additional info: Water

Cleanup option #1: bead cleanup

Step 38 - Cleanup option #1: bead cleanup (much faster, may purify truncated products or nucleotides).

Pipet 5 times to mix.

Cleanup option #1: bead cleanup

Step 39 - Cleanup option #1: bead cleanup (much faster, may purify truncated products or nucleotides).

Return to magnetic rack, wait until clear.

Cleanup option #1: bead cleanup

Step 40 - Cleanup option #1: bead cleanup (much faster, may purify truncated products or nucleotides).

Transfer supernatant to new tube - this contains your RNA.

Cleanup option #1: bead cleanup

Step 41 - Cleanup option #1: bead cleanup (much faster, may purify truncated products or nucleotides).

It is recommended to measure the concentration of this using a qubit

Cleanup option #2: Gel purify transcribed RNA

Step 18 - Cleanup option #2: Gel purify transcribed RNA (more difficult, higher purity).

Pour a 7M urea, 10% 29:1 polyacrylamide denaturing gel of sufficient size to hold all of the transcription(s).

Cleanup option #2: Gel purify transcribed RNA

Step 19 - Cleanup option #2: Gel purify transcribed RNA (more difficult, higher purity).

Run gel until the xylene band is midway through the gel (a few hours).

Cleanup option #2: Gel purify transcribed RNA

Step 20 - Cleanup option #2: Gel purify transcribed RNA (more difficult, higher purity).

Image gel using UV shadowing to visualize band. Band should be just above the xylene.

Cleanup option #2: Gel purify transcribed RNA

Step 21 - Cleanup option #2: Gel purify transcribed RNA (more difficult, higher purity).

Excise gel slice with razor blade and place in Eppendorf tube (small txn) or conical tube (large txn).

Cleanup option #2: Gel purify transcribed RNA

Step 22 - Cleanup option #2: Gel purify transcribed RNA (more difficult, higher purity).

Crush gel slice with a P_{1000} tip or glass rod.

Cleanup option #2: Gel purify transcribed RNA

Step 23 - Cleanup option #2: Gel purify transcribed RNA (more difficult, higher purity).

Add enough gel elution buffer to cover gel bits so they can move around when rocked (typically

500µL per 100µL transcription).

Cleanup option #2: Gel purify transcribed RNA

Step 24 - Cleanup option #2: Gel purify transcribed RNA (more difficult, higher purity).
Freeze the resuspended gel bits for 30 minutes on dry ice or at -80°C.

TEMPERATURE

-80 °C Additional info:
Freezing gel bits

NOTES

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This fractures the gel bits and increases yield.

Cleanup option #2: Gel purify transcribed RNA

Step 25 - Cleanup option #2: Gel purify transcribed RNA (more difficult, higher purity).
PAUSE Rock overnight at 4°C.

TEMPERATURE

4 °C Additional info:
Rocking overnight

Cleanup option #2: Gel purify transcribed RNA

Step 26 - Cleanup option #2: Gel purify transcribed RNA (more difficult, higher purity).
For small scale transcription, filter the gel bits through a 0.45 micron filter. Use a P-1000 tip with the end cut off using a razor blade to collect all the gel bits into the filter.

NOTES

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The SDS may precipitate during this process, especially if left on ice. If so, repeat filtration using a new filter.

Cleanup option #2: Gel purify transcribed RNA

Step 27 - Cleanup option #2: Gel purify transcribed RNA (more difficult, higher purity).
For large scale transcription, vortex the gel bits and pour all of it onto a flip-top conical tube filter. Allow to vacuum filter until no more drops are retained.

Cleanup option #2: Gel purify transcribed RNA

Step 28 - Cleanup option #2: Gel purify transcribed RNA (more difficult, higher purity).
Ethanol precipitate the RNA by adding 1/1000 volumes glycoblue and 2.5 volumes 100% ethanol.

Cleanup option #2: Gel purify transcribed RNA

Step 29 - Cleanup option #2: Gel purify transcribed RNA (more difficult, higher purity).
Incubate at -80°C for at least 1 hour but note that RNA under EtOH is stable for months at -80°C.

TEMPERATURE

-80 °C Additional info:
Incubation

Cleanup option #2: Gel purify transcribed RNA

Step 30 - Cleanup option #2: Gel purify transcribed RNA (more difficult, higher purity).
Spin down at 16k g (small-scale) or 5k g (large-scale) for 25 minutes at 4°C.

TEMPERATURE

4 °C Additional info: Spin
down

NOTES

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For large-scale transcription, resuspend the pellet from above in 500µL of -20 degree 100% EtOH and transfer to a microfuge tube. The pellet will remain chunky - collect all the chunks with more ethanol if needed. Spin again at 16k g for 5 minutes to re-pellet.

Cleanup option #2: Gel purify transcribed RNA

Step 31 - Cleanup option #2: Gel purify transcribed RNA (more difficult, higher purity).
Aspirate supernatant and discard, being careful not to dislodge pellet.

Cleanup option #2: Gel purify transcribed RNA

Step 32 - Cleanup option #2: Gel purify transcribed RNA (more difficult, higher purity).
Wash pellet with 500µL -20°C 70% ethanol.

AMOUNT

500 µl Additional info:
-20°C 70% ethanol

NOTES

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CRITICAL Pipet the 70% ethanol gently not onto the pellet. Keep the tube cold to avoid resuspending the RNA in the 70% ethanol.

Cleanup option #2: Gel purify transcribed RNA

Step 33 - Cleanup option #2: Gel purify transcribed RNA (more difficult, higher purity).
Spin at 16k g for 5 minutes at 4°C.

TEMPERATURE

4 °C Additional info: Spin

Cleanup option #2: Gel purify transcribed RNA

Step 34 - Cleanup option #2: Gel purify transcribed RNA (more difficult, higher purity).
Aspirate supernatant carefully and discard.

Cleanup option #2: Gel purify transcribed RNA

Step 35 - Cleanup option #2: Gel purify transcribed RNA (more difficult, higher purity).
Quick spin in tabletop centrifuge.

Cleanup option #2: Gel purify transcribed RNA

Step 36 - Cleanup option #2: Gel purify transcribed RNA (more difficult, higher purity).
Aspirate remaining ethanol and discard.

Cleanup option #2: Gel purify transcribed RNA

Step 37 - Cleanup option #2: Gel purify transcribed RNA (more difficult, higher purity).
Air dry at room temperature for 5 minutes with the tube cap open.

Cleanup option #2: Gel purify transcribed RNA

Step 38 - Cleanup option #2: Gel purify transcribed RNA (more difficult, higher purity).
Resuspend pellet in 30µL DEPC H_2O per 100µL transcription and quantify using nanodrop or qubit.

Warnings

Please refer to the SDS (Safety Data Sheet) for safety warnings and hazard information.