sgRNA Synthesis Using the HiScribe® Quick T7 High Yield RNA Synthesis Kit (NEB #E2050)

Materials Required but not Supplied

HiScribe® T7 Quick High Yield RNA Synthesis Kit

- Nuclease-free Water (NEB #B1500)

Overview

This protocol serves as a starting point to synthesize single guide RNAs (sgRNAs) using the HiScribe® Quick T7 High Yield RNA Synthesis Kit (NEB #E2050) and a user-supplied, linear, dsDNA template containing the T7 RNA Polymerase promoter sequence immediately followed by one or more G residues, a ~20 nt target-specific sequence and an ~80 bp constant sequence that is specific to the Cas homolog being loaded (e.g. Spy, Seq, or Sau). The generated sgRNAs are incorporated into Cas9 ribonucleoprotein complexes (RNPs) and function to direct sequence-specific DNA binding. The resulting programmed Cas9 RNPs can be used to cleave (or bind, or nick) dsDNA.

This protocol is recommended when you need high yield (>25 µg) of a single sgRNA. When screening sgRNAs for Spy Cas9, we recommend our EnGen® sgRNA Synthesis Kit, *S. Pyogenes* (NEB #E3322) for convenient synthesis of 4-25 µg from an ssDNA oligonucleotides template.

Before starting

Template Sequence:

This protocol utilizes a double-stranded DNA template. Target-specific oligos are designed by the user; we recommend using a target DNA selection program, such as ChopChop.

Below are sgRNA sequences for the different Cas9 homologs sold by NEB. We recommend ordering synthetic sgRNA for Cas12a instead of making it by IVT.

- The 5' "GAAT" serves as a footprint that stabilizes T7 RNA Polymerase to the promoter.
- The **purple sequence** on the 5' end is the T7 promoter sequence. The first two nucleotides immediately following the T7 promoter should be guanosines to ensure that T7 RNA polymerase will initiate effectively.
- The underlined 20-nucleotide spacer sequence is specific to the desired DNA target region immediately upstream of the protospacer adjacent motif (PAM). The spacer sequence does not contain the PAM sequence itself.
- The 3' portion of the sgRNA that is not underlined is the scaffold specific for ribonucleoprotein complex formation with that particular Cas9 homolog.

The single guide RNA for use with Cas9 Nuclease, S. *pyogenes* (NEB #M0386), EnGen® Spy Cas9 nucleases (NEB #M0646, #M0650, #M0652, #M0667), or EnGen® SpRY Cas9 (NEB #M0669) has the following sequence:

5’-
GAAT**TTCTAATACGACTCACTATAGG**NNNNNNNNNNNNNNNNNNNGUUUUAGAGCUAGAAAUAGCAAGUUAAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUUU -3’.

We also offer a separate product and protocol, EnGen® sgRNA Synthesis Kit, S. *pyogenes* (NEB #E3322). This product contains a premixed S. *pyogenes* scaffold sequence and only requires an ssDNA oligo containing the target sequence as template.

The single guide RNA for use with EnGen Seq1 Cas9 (NEB #M0668) has the following sequence:

5’- GAAT**TTCTAATACGACTCACTATAGG**NNNNNNNNNNNNNNNNNNNGUUUUAGAGCUGUGUUGGAAACAACACAGCGAGUUAAAAUAAGGCUUUGUCCGUACACAACUUGUAAAAGUGGCACCCGAUUCGGGUGCAUUUUUU -3’.

The single guide RNA for use with EnGen Sau Cas9 (NEB #M0654) has the following sequence:

5’- GAAT**TTCTAATACGACTCACTATAGG**NNNNNNNNNNNNNNNNNNNGTTTTAGTACTCTGGAAACAGAATCTACTAAAACAAGGCAAAATGCCGTGTTTATCTCGTCAACTTGTTGGCGAGATTT -3’.

Linearized plasmid DNA, PCR products, or double-stranded synthetic DNA oligonucleotides can be used as templates for *in vitro* transcription with the HiScribe T7 Quick High Yield RNA Synthesis Kit to make sgRNA.

Synthetic DNA Oligonucleotide Templates:

- Synthetic DNA Oligonucleotides are a rapid and convenient way to generate sgRNA transcription templates. You may consider the Elegen IVT Ready DNA service. Other good sources for synthetic transcription templates are gBlocks® Gene Fragments double-stranded, synthetic DNA fragments or duplexed Ultramer® Oligonucleotides. (Note that it is important to order both strands when ordering Ultramers). We have tested all levels of oligonucleotide purity (standard desalting, column purification, PAGE purification) and observed no difference in yield or quality of sgRNA.

- We recommend that the DNA oligonucleotide templates be completely double-stranded and have a few extra nucleotides on the 5’ end. Partially double-stranded oligonucleotide templates with a double-stranded T7 promoter sequence may be used in the T7 Quick High Yield RNA Synthesis Kit, but yields may be variable and, in general, are lower than fully double-stranded templates.

Plasmid Templates:

- We do not offer plasmid templates for sgRNA synthesis, which can be obtained from a number of sources, such as Addgene. Empty gRNA vectors can be modified to encode target-specific sgRNAs using the Q5® Site-Directed Mutagenesis Kit (NEB #E0554), traditional cloning, or by bridging dsDNA with a ssDNA Oligo and using NEBuilder® HiFi DNA Assembly.

- Quality of the template DNA affects transcription efficiency, as well as the integrity of the RNA synthesized. It is therefore critical to begin the protocol with highly purified DNA. Any plasmid purification method may be used as long as the product is predominantly supercoiled and free of contaminating RNase, protein, RNA, and salts.

- To produce an RNA transcript of defined length, plasmid DNA must be completely linearized with a restriction enzyme downstream of the insert to be transcribed. NEB offers a large selection of restriction enzymes for this purpose; we recommend selecting a high-fidelity (HF) restriction enzyme that generates blunt ends or 5 ′ overhangs.

- We recommend purifying the linearized template DNA by phenol: chloroform extraction:

- Extract DNA with an equal volume of 1:1 phenol: chloroform mixture.
- Extract twice with an equal volume of chloroform to remove residual phenol.
- Precipitate the DNA by adding 1/10th volume of 3 M sodium acetate, pH 5.2, and two volumes of ethanol. Incubate at –20°C for at least 30 minutes.
- Pellet the DNA in a microcentrifuge for 15 minutes at top speed. Carefully remove the supernatant.
- Rinse the pellet by adding 500 µl of 70% ethanol and centrifuge for 15 minutes at top speed. Carefully remove the supernatant.
- Air dry the pellet and resuspend it in nuclease-free water at a concentration of 0.5–1 µg/µl.

PCR Templates:

- Transcription templates for sgRNA synthesis can be amplified by PCR from plasmid or synthetic oligonucleotide templates and appropriate PCR primers. We recommend the use of Q5 High Fidelity Hot Start 2X Master Mix (NEB #M0494) for generating PCR templates.

- PCR products should be run on an agarose gel to estimate concentration and to confirm amplicon size prior to its use as a template in this protocol.

- PCR products may be used directly if diluted at least 10X in the transcription reaction. However, better yields will be obtained with purified PCR products. PCR products can be purified according to the phenol: chloroform protocol for linearized plasmid (above), or with commercially available spin columns such as our Monarch® Spin PCR & DNA Cleanup Kit (5 µg) (NEB #T1130).

Reaction Considerations:

- We strongly recommend wearing gloves and using nuclease-free tubes (microfuge tubes or PCR strip tubes) and reagents to avoid RNase contamination.

- Unlike the standard IVT protocol with this kit, which calls for a final reaction volume of 20 µl, reactions for short RNAs (<300 nts) are typically 30 µl. This reaction may be scaled linearly as needed.

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sgRNA Synthesis Protocol:

1. Thaw the necessary components at room temperature. Keep the T7 RNA Polymerase Mix on ice.

2. Mix and pulse-spin in a microfuge to collect the solutions to the bottom of the tubes.

3. Set up the reaction at **room temperature** in the order listed in the table below:

COMPONENTS	30 µl REACTION	FINAL AMOUNT
Nuclease-free Water	X µl	-
NTP Buffer Mix	10 µl	6.7 mM each NTP

COMPONENTS	30 µl REACTION	FINAL AMOUNT
Linear Template DNA	X µl	1 µg Plasmid, 100 ng PCR product, or 100 ng Synthetic dsDNA
DTT (0.1M)	X µl	5 mM
T7 RNA Polymerase Mix	2 µl	-

4. Mix thoroughly by pipetting and pulse-spin in a microfuge. Incubate at 37°C for 4 hours or longer in a dry air incubator or thermocycler to prevent evaporation. It is safe to incubate the reaction for 16 hours (overnight).

   Optional: To remove template DNA, add 20 µl nuclease-free water and 2 µl of DNase I (RNase-free) (NEB #M0303), mix, and incubate for 15 minutes at 37°C. Alternatively, 2 µl of DNase I-XT (NEB# M0570) can be added directly to the IVT product and incubated for 15 minutes at 37°C.

5. Proceed with purification of synthesized RNA and/or evaluation of transcription product yield and/or length. For purification, we recommend the 500 µg capacity Monarch RNA Spin Cleanup Kit (NEB# T2050). If using other purification columns, please ensure that they are compatible with the size of your synthesized sgRNA (~80-100 nt) and follow the manufacturer's instructions.

Related Resources

- CRISPR/Cas9 & Targeted Genome Editing: New Era in Molecular Biology
- Avoiding Ribonuclease Contamination
- A Practical Guide to Analyzing Nucleic Acid Concentration and Purity with Microvolume Spectrophotometers