EnGen® sgRNA Synthesis Kit, *S. pyogenes*

NEB #E3322V/S

10/20 reactions

Version 5.0_8/25

Table of Contents

The EnGen sgRNA Synthesis Kit, *S. pyogenes* Includes:

All kit components should be stored at –20°C. Each standard reaction yields between 4–25 µg of sgRNA.

EnGen sgRNA Enzyme Mix

EnGen 2X sgRNA Reaction Mix, *S. pyogenes*

DNase I (RNase-free)

EnGen sgRNA Control Oligo, *S. pyogenes*

Dithiothreitol (DTT) (0.1 M)

Required Materials Not Included:

Target-specific DNA oligo(s)

Thermocycler/37°C heat block/incubator

Nuclease-free water

Equipment and reagents for RNA quantitation

Spin columns for RNA cleanup (e.g., NEB #T2040)

RNase-free tubes, aerosol tips

Microcentrifuge

Optional Materials:

Alkaline Phosphatase, Calf Intestinal (CIP)

Cas9 Nuclease, *S. pyogenes*

EnGen Spy Cas9, NLS

Gels, running buffer, gel loading dye, RNA and DNA ladders, gel box

Introduction

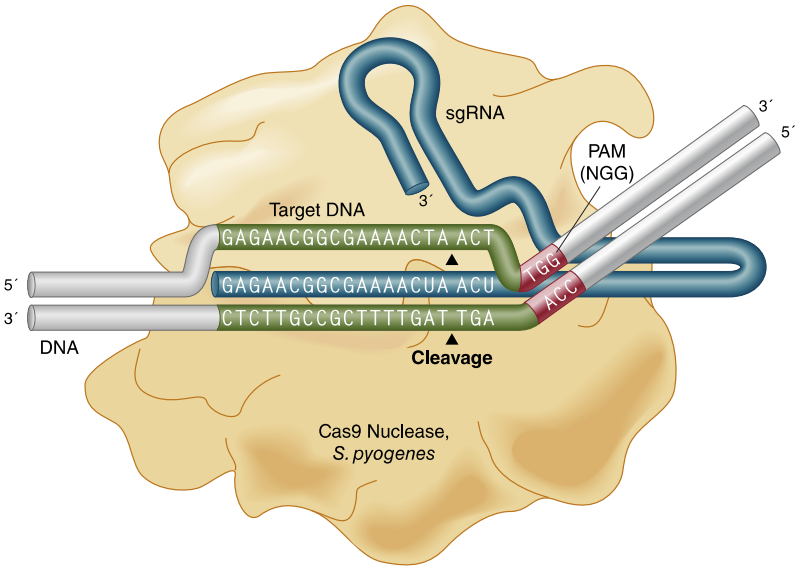
CRISPR is an acronym for Clustered Regularly Interspaced Short Palindromic Repeats which are genomic loci found in many bacteria and archaea. The CRISPR/Cas9 pathway, which naturally allows for the elimination of genomic material from invading sources in bacteria, has recently been adapted as a molecular biology tool to edit genomes in a target-specific manner. Cas9 (CRISPR-associated protein 9) is a double-stranded DNA endonuclease which forms an active ribonucleoprotein (RNP) when complexed with guide RNAs (gRNAs) encoded at the CRISPR loci. gRNAs provide sequence specificity to the RNP, directing the Cas9 nuclease to DNA targets resulting in double-strand breaks in the DNA.

In nature, *S. pyogenes* Cas9 is programmed with two separate RNAs, the crRNA and tracrRNA. The crRNA, or CRISPR RNA sequence contains approximately 20 nucleotides of homology complementary to the strand of DNA opposite and upstream of a PAM (Protospacer Adjacent Motif) (NGG) sequence. The tracrRNA, or transactivating crRNA, contains partial complementary sequence to the crRNA as well as the sequence and secondary structure that is recognized by Cas9. These sequences have been adapted for use in the lab by combining the tracrRNA and crRNA into one long single guide RNA (sgRNA) (1) species capable of complexing with Cas9 to recognize and cleave the target DNA (Figure 1).

When gRNA-programmed Cas9 RNPs are introduced into eukaryotic cells, dsDNA breaks are introduced at target sites. Activation of the cellular double-strand break machinery can lead to insertions and/or deletions (indels) through Non-Homologous End Joining (NHEJ), resulting in disruption of the gene at that specific locus. In the presence of a homologous repair template, the homology-directed repair (HDR) pathway can be activated leading to the introduction of specific changes to the DNA at the targeted site.

1. Jinek, M. et al. (2012) *Science* 816–821. PubMed ID: 22745249.

Figure 1. Cas9 Nuclease, *S. pyogenes*



Cas9 nuclease, S. pyogenes, complexed with an sgRNA. Cleavage occurs three nucleotides upstream of the PAM sequence (shown in red). sgRNAs are complimentary to the strand of DNA opposite of the PAM.

The EnGen sgRNA Synthesis Kit, *S. pyogenes* combines an *S. pyogenes* Cas9-specific Scaffold Oligo (included in the EnGen 2X sgRNA Reaction Mix) that is partially complementary to the target-specific oligos designed by the user. The two oligos anneal at the overlapping region and are filled in by the DNA polymerase, creating a double-stranded DNA (dsDNA) template for transcription. Synthesis of the dsDNA template and transcription of RNA occur in a single reaction, resulting in the generation of a functional sgRNA.

The EnGen sgRNA Synthesis Kit, *S. pyogenes* provides a simple and quick method for transcribing high yields of sgRNA in a single 30 minute reaction, using the supplied reagents and target-specific DNA oligos designed by the user.

Figure 2. General workflow for the EnGen sgRNA Synthesis Kit, *S. pyogenes*

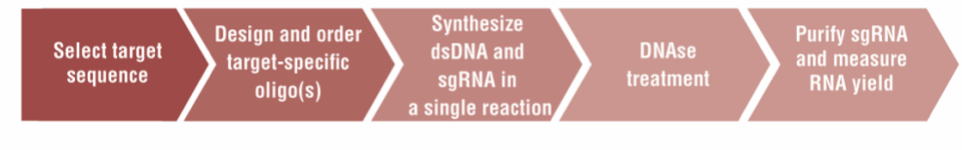
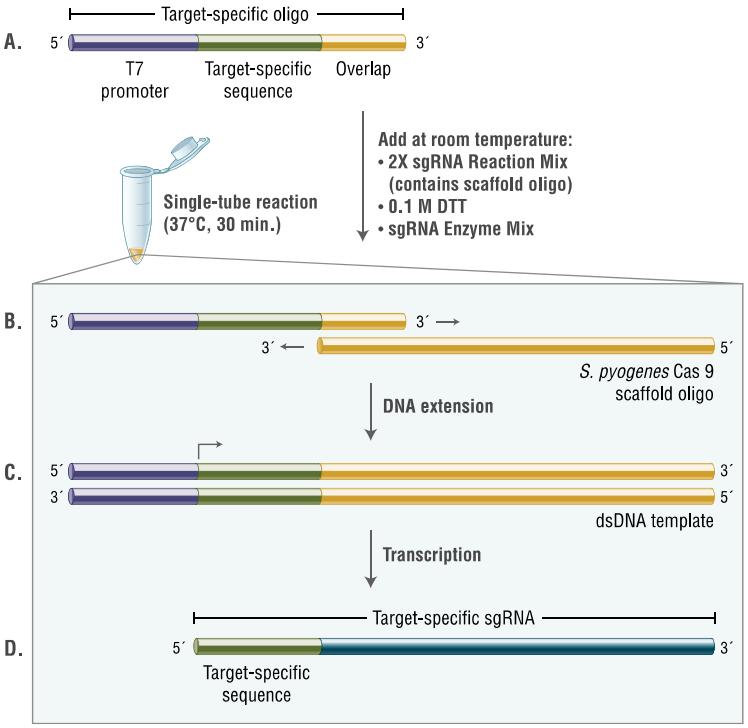


Figure 3. EnGen sgRNA Synthesis Kit Overview



A. The target-specific oligo contains the T7 promoter sequence, ~20 nucleotides of target-specific sequence and a 14 nucleotide overlap sequence complementary to the S. pyogenes Cas9 Scaffold Oligo supplied in the reaction mix. Target-specific oligos (or EnGen sgRNA Control Oligo, S. pyogenes) are mixed with the EnGen 2X sgRNA Reaction Mix (NTPs, dNTPs, S. pyogenes Cas9 Scaffold Oligo), 0.1 M DTT and the EnGen sgRNA Enzyme Mix (DNA and RNA polymerases) at room temperature. B. At 37°C the two oligos anneal at the 14 nucleotide overlap region of complementarity. C. The DNA polymerase extends both oligos from their 3′ ends creating a double-stranded DNA template. D. The RNA polymerase recognizes the double-stranded DNA of the T7 promoter and initiates transcription. The resulting sgRNA contains the target-specific/crRNA sequence as well as the tracrRNA. All steps occur in a single reaction during a 30 minute incubation at 37°C.

Protocols

Target-specific Oligo Design

This kit contains the *S. pyogenes* Cas9 Scaffold Oligo within the EnGen 2X sgRNA Reaction Mix, *S. pyogenes*. Target-specific oligos are designed by the user as follows:

1. Select 20 nucleotide target sequence (not including the PAM (NGG) sequence).

Use of a target DNA selection program is recommended.
We recommend Desktop Genetics at www.deskgen.com/landing or ChopChop at link https://chopchop.cbu.uib.no/

2. Check input sequence for presence of "G" at the 5′ end.

   If there are no "G's" at the 5′ end, add one "G" (making it a total ofat least one G at the 5′ end).

3. To the 5′ end; append T7 promoter sequence:
   TTCTAATACGACTCACTATA

4. To the 3′ end; append 14 nucleotide overlap sequence:
   GTTTTAGAGCTAGA

5. Check complete oligo sequence:
   5′ TTCTAATACGACTCACTATAG(N)$_{20}$GTTTTAGAGCTAGA 3′

Example

1. Select target-specific DNA sequence (we recommend using a target DNA selection program):
   Example:
   TGCAACCTTCATTTCCCTGCTGG

The PAM sequence (NGG, underlined) is required for Cas9 recognition of the target sequence and is **NOT** part of the sgRNA sequence (Figure 1B). Remove these three nucleotides:
5′ TGCAACCTTCATTTCCCTGC

2. Check for "G" at the 5′ end of the target sequence (at least one G is necessary for transcription). If no G is present, add one **G**:
5′ GTGCAACCTTCATTTCCCTGC 3′

3. To the 5′ end, append T7 promoter sequence (shown in red):
5′ TTCTAATACGACTCACTATAGTGCAACCTTCATTTCCCTGC 3′

4. To the 3′ end, append the 14 nucleotide overlap sequence (shown in blue):
5′ TTCTAATACGACTCACTATAGTGCAACCTTCATTTCCCTGCGTTTTAGAGCTAGA 3′

This represents the oligo sequence to be ordered.

The sequence of the *S. pyogenes* Cas9 Scaffold Oligo [provided as a component of the EnGen 2X sgRNA Reaction Mix] is as follows (5′ to 3′, overlap in blue):

5′ AAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTAACTTGC
TATTTCTAGCTCTAAAAC 3′

The overlapped oligos:

5′ TTCTAATACG ACTCACTATA GTGCAACCTT CATTTCCCTG CGTTTTAGAG CTAGA-----
3′ ---------- ---------- ---------- ---------- -CAAAATCTC GATCTTTATC

---------- ---------- ---------- ---------- ---------- ---------- - 3′
GTTCAATTTT ATTCCGATCA GGCAATAGTT GAACTTTTTC ACCGTGGCTC AGCCACGAAA A 5′

The dsDNA product following fill in by the DNA polymerase:

5′ TTCTAATACG ACTCACTATA GTGCAACCTT CATTTCCCTG CGTTTTAGAG CTAGAAATAG
3′ AAGATTATGC TGAGTGATAT CACGTTGGAA GTAAAGGGAG CCAAAATCTC GATCTTTATC

CAAGTTAAAA TAAGGCTAGT CCGTTATCAA CTTGAAAAAG TGGCACCGAG TCGGTGCTTT T 3′
GTTCAATTTT ATTCCGATCA GGCAATAGTT GAACTTTTTC ACCGTGGCTC AGCCACGAAA A 5′

In this example, the final sgRNA sequence would be:

5′ GUGCAACCUUCAUUUCCCUGCGUUUUAGAGCUAGAAAUAGCAAGUUAAAAUAAGGCUAGUCC
GUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUUU 3′

sgRNA Synthesis Protocol
We strongly recommend wearing gloves and using nuclease-free tubes and reagents. Reactions should be assembled in microfuge tubes or PCR strip tubes.

1. Thaw EnGen 2X sgRNA Reaction Mix, *S. pyogenes*, 0.1 M DTT and customer-supplied target-specific oligo (1 µM). Mix and pulse-spin each component in a microfuge prior to use. **Store enzyme mix on ice but assemble reaction at room temperature.**

2. Assemble the reaction at **room temperature in the order listed**. Avoid master mixes, and add the enzyme last to each reaction:

REAGENT	AMOUNT
Nuclease-free water	2 µl
EnGen 2X sgRNA Reaction Mix, *S. pyogenes*	10 µl
Target-specific DNA Oligo (1 µM)	5 µl
DTT (0.1 M)	1 µl
EnGen sgRNA Enzyme Mix	2 µl
Total Volume	20 µl

3. Mix thoroughly and pulse-spin in a microfuge. Incubate at 37°C for 30 minutes.

4. Transfer reaction to ice.

5. For DNase treatment, bring volume to 50 µl by adding 30 µl of nuclease-free water. Add 2 µl of DNase I (RNase-free, provided), mix and incubate at 37°C for 15 minutes.

6. Proceed with purification of RNA or analysis by gel electrophoresis.

NOTE: sgRNAs synthesized using *in vitro* transcription methods from a DNA template and a bacteriophage polymerase will possess a 5′ triphosphate.

Optional: To remove the 5′ triphosphate (leaving a 5′ OH), treatment of the sgRNA with Quick CIP (NEB #M0525) can be performed. Phenol:chloroform extraction and/or cleanup by spin column is recommended to inactivate and remove the phosphatase.

Purification of sgRNAs

Spin columns will remove proteins, salts and most unincorporated nucleotides. Please ensure that spin columns are compatible with the size of sgRNAs (~100 nts) and expected RNA yields (4–25 µg). Follow manufacturer's instructions. We recommend Monarch RNA Cleanup Kit (50 µg) (NEB #T2040) for purification.

Evaluation of sgRNA Transcription Products

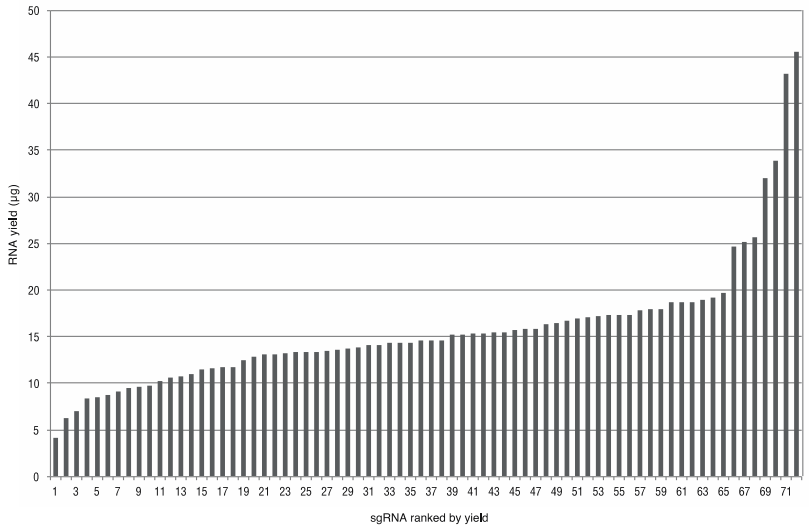
Quantitation by UV Light Absorbance

sgRNA concentration following purification can be determined by measuring absorbance at 260 nm on a standard UV spectrophotometer following dilution of the sample, or read directly on a NanoDrop® (recommended). 1 OD_{260} unit for single-stranded RNA (ssRNA) is 40 µg/ml. RNA concentration can be determined as follows:

A_{260} x dilution factor x 40 = "x" µg/ml ssRNA.

Expected yields following purification can be 4–25 µg.

Figure 4. Range of sgRNA yield from the EnGen sgRNA Synthesis Kit, *S. pyogenes*.



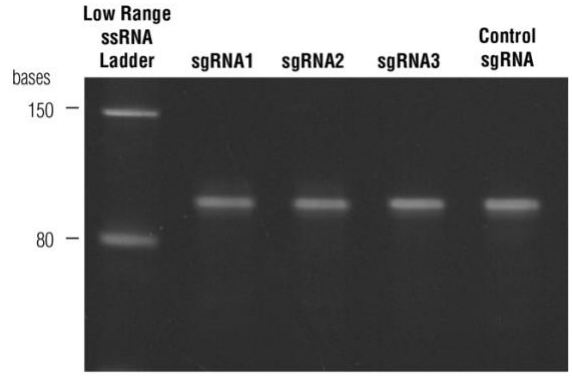
72 different target-specific oligos were designed and tested for RNA yield following DNase I treatment and column purification as determined by NanoDrop. RNA yields may be dependent on specific oligo sequence, with most falling between 4–25 µg.

Analysis of sgRNAs by Gel Electrophoresis

Assessment of sgRNA quality and length can be evaluated by gel electrophoresis. Gels should be run under denaturing conditions to avoid formation of secondary structure.

1. Mix an aliquot of RNA with RNA Loading Dye, (2X) (NEB #B0363) or Urea Dye.
2. Denature RNA according to manufacturer's recommendation and load onto a TBE-urea gel, such as Novex® TBE-urea gels.
3. Visualize RNA by staining the gel with SYBR® Gold or ethidium bromide.

Figure 5. Examples of sgRNAs synthesized using the EnGen sgRNA Synthesis Kit, *S. pyogenes* and multiple different target-specific oligos including the EnGen sgRNA Control Oligo, *S. pyogenes*.



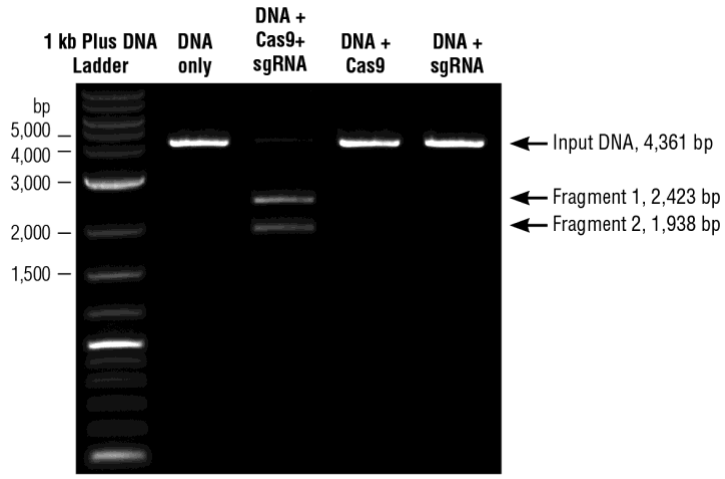
RNA was run under denaturing conditions on a 10% Novex TBE-Urea gel and post-stained with SYBR Gold.

***In-vitro* Cas9 Nuclease Assay**

S. pyogenes Cas9 double-stranded DNA nuclease is guided to the target DNA based on sequence complementarity to the sgRNA that is loaded into the protein. Site-specific DNA cleavage occurs three nucleotides upstream of the PAM sequence (NGG), within the complementary region. sgRNAs synthesized with this kit can be used with Cas9 Nuclease, *S. pyogenes* (NEB #M0386) or EnGen Spy Cas9, NLS (NEB #M0646) for *in vitro* cleavage reactions.

For a detailed protocol, please visit **www.neb.com/M0386** and click on the Protocols tab.

Figure 6. Example of an *in vitro* Cas9 nuclease assay.



DNA target is PvuII-linearized pBR322, sgRNA was synthesized using the EnGen sgRNA Control Oligo, S. pyogenes. Reactions were set up following NEB® protocols with a ratio of 20:20:1 (Cas9:sgRNA:target site) in 30 µl reactions. Aliquots of reactions were mixed with Gel Loading Dye, Purple (6X) (NEB #B7024). Cleavage products and 1 kb Plus DNA Ladder (NEB #N3200) were resolved on a 1% TBE agarose gel stained with ethidium bromide.

Troubleshooting

Positive Control Reaction

- The control oligo (EnGen sgRNA Control Oligo, *S. pyogenes*) provided with this kit can be used in place of the target-specific oligo to test the efficiency of the reaction. Following incubation for 30 minutes at 37°C, the control reaction should contain ≥ 15 µg sgRNA, post-purification, as determined by NanoDrop. Resulting sgRNA should run as a single band of ~100 nucleotides on a denaturing TBE-Urea gel under denaturing conditions.

Low Yield of RNA

- If the sgRNA yield using the control oligo is lower than expected, there may be issues during the reaction setup. It is very important to follow the directions exactly; components should be added in the exact order listed, making sure that the EnGen sgRNA Enzyme Mix is added last. Reactions should be set up at room temperature. Make sure that the heat block/incubator or thermocycler is at 37°C.

  If the sgRNA yield using the control oligo is at the level expected, then there may be an issue with the design of the target-specific oligo(s). Make sure to follow the design protocol, and double-check the sequence before ordering. It is important to have at least one G following the T7 promoter sequence and to have a 14 nucleotide overlap with the *S. pyogenes* Cas9 Scaffold Oligo. If a higher yield is required, a longer incubation at 37°C is reasonable (1–2 hours). Use of a dry air incubator or thermocycler is recommended for incubations > 30 minutes.

  As we have observed, transcription can be somewhat sequence dependent, though the yield of the majority of sgRNA synthesis reactions falls between 4–25 µg. Designing multiple sgRNAs per target is recommended.

sgRNA Smearing on Denaturing Gel

- We strongly recommend wearing gloves and using nuclease-free tubes and reagents. If the RNA appears degraded or smeared on a denaturing gel, it is likely that RNAse contamination has been introduced at some step during the reaction. The sgRNAs synthesized from this kit should yield a single band of ~100 nucleotides on a TBE-Urea gel.

Multiple Bands are Detected on Denaturing Gel

- If more than one band appears on the gel it is likely that the RNA is not fully denatured due to the presence of strong secondary structure. It is important to run these reactions under denaturing conditions (using urea dye or formamide dye and heating the sample according to the manufacturer's directions prior to loading).

  It is important to DNase treat the sample prior to gel analysis. DNA template present in the sgRNA synthesis reaction prior to DNase treatment may be detected on the gel as a slightly larger band compared to the RNA.

Ordering Information

NEB #	PRODUCT	SIZE
E3322V/S	EnGen sgRNA Synthesis Kit, *S. pyogenes*	10/20 reactions

COMPANION PRODUCTS

NEB #	PRODUCT	SIZE
M0386S/L	Cas9 Nuclease, *S. pyogenes*	70/350 pmol
M0386T/M	Cas9 Nuclease, *S. pyogenes*	300/600 pmol
M0646T/M	EnGen Spy Cas9, NLS	400/2,000 pmol
M0650S/T	EnGen Spy Cas9 Nickase	70/400 pmol
M0652S/T	EnGen Spy dCas9 (SNAP-tag®)	70/400 pmol
E3321S	EnGen Mutation Detection Kit	25 reactions
N0364S	Low Range ssRNA Ladder	25 gel lanes
B0363S	RNA Loading Dye (2X)	4 ml
B7024S	Gel Loading Dye, Purple (6X)	4 ml
N3200S/L	1 kb Plus DNA Ladder	200/1,000 gel lanes
M0290S/L	Alkaline Phosphatase, Calf Intestinal (CIP)	1,000/5,000 units
T2040S/L	Monarch RNA Cleanup Kit (50 µg)	10/100 preps

Revision History

REVISION #	DESCRIPTION	DATE
1.0	N/A	5/16
2.0		7/18
3.0		1/19
4.0	Apply new manual format and add a “V” size.	2/20
5.0	Apply new format and legal text and adjust an optional step to include Quick CIP	8/25