In vitro digestion of DNA with Cas9 Nuclease, S. pyogenes (M0386) Version 3

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Abstract

Cas9 Nuclease, *S. pyogenes* (Cas9), is a double-stranded DNA endonuclease that is guided to its target by sequence complementarity of a small RNA loaded into the protein. This protocol describes how to digest double-stranded DNA in vitro using Cas9 and a single guide RNA (sgRNA).

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Guidelines

OVERVIEW:
Cas9 Nuclease, S. pyogenes, (Cas9) is a double-stranded DNA endonuclease that is guided to its target by sequence complementarity of a small RNA loaded into the protein. This protocol describes how to digest double-stranded DNA in vitro using Cas9 and a single guide RNA (sgRNA).

REQUIRED MATERIALS:

- Cas9 Nuclease, S. pyogenes (NEB #M0386)
- 10X Cas9 Nuclease Reaction Buffer
- Nuclease-free water
- sgRNA containing the targeting sequence in the region of interest
- sgRNAs can be generated by in vitro transcription using the HiScribe T7 Quick High-Yield RNA synthesis Kit (NEB #E2050) using linearized plasmid, PCR products, or oligonucleotides as templates
- sgRNAs must contain sequence complementary to the target DNA (1,2)For information on design of sgRNA transcription templates please visit Addgene
- DNA substrate containing the target sequence
- The substrate DNA can be circular or linearized plasmid, PCR products, or synthesized oligonucleotides
- Proteinase K, Molecular Biology Grade (NEB #P8107S)

OPTIONAL MATERIALS:
Apparatus and reagents for DNA fragment analysis

- E. g. Agarose gel electrophoresis apparatus
- DNA Loading Dye (e.g. Gel Loading Dye, Purple (6X) NEB #B7024S)
- E.g. Agilent Bioanlyzer or similar

BEFORE YOU START:
We strongly recommend wearing gloves and using nuclease-free tubes and reagents to avoid RNase contamination. Further recommendations for avoiding ribonuclease contamination can be found here.
Reactions are typically 30 µl but can be scaled up as needed. Reactions should be assembled in nuclease-free microfuge tubes or PCR strip tubes.
It is essential to keep the molar ratio of Cas9 and sgRNA per target site at 10:10:1 or higher to obtain the best cleavage efficiency. A calculator can be found here.
Prepare 300nM sgRNA by diluting the stock with nuclease-free water on ice.
Prepare 30nM substrate DNA with a single target sequence by diluting the stock with nuclease-free water on ice.

PROCEDURE:
1. Assemble the reaction at room temperature in the following order:

Component	**Volume** (for 30 µl reaction)
Nuclease-free water	20 µl
10X Cas9 Nuclease Reaction Buffer	3 µl
300nM sgRNA	3 µl (30nM final)
1 µM Cas9 Nuclease, S.pyogenes (M0386S)	1 µl (~30nM final)
Reaction volume	27 µl
Pre-incubate for 10 minutes at 25ºC	
30nM substrate DNA	3 µl (3nM final)
Total reaction volume	30 µl

*The substrate DNA and sgRNA, and nuclease-free water are not included.

2. Mix thoroughly and pulse-spin in a microfuge.
3. Incubate at 37°C for 1 hour.
4. Proceed with fragment analysis.

REFERENCES:
1. Jinek et al. (2012) Science 337 (6096) 816-821.
2. Larson et al. (2013) Nature Protocol 8 (2180-2196).
3. Mali et al. (2013) Science 339 (6121): 823-826.

Before start

We strongly recommend wearing gloves and using nuclease-free tubes and reagents to avoid RNase contamination. Further recommendations for avoiding ribonuclease contamination can be found here.

Protocol

Step 1.
Prepare 300nM sgRNA by diluting the stock with nuclease-free water on ice.

Step 2.
Prepare 30nM substrate DNA with a single target sequence by diluting the stock with nuclease-free water on ice.

Step 3.
Assemble the reaction at room temperature in the following order (total volume **30 µl**)

PROTOCOL

. Cas9 M0386 Mixture

CONTACT: New England Biolabs

NOTES
New England Biolabs 10 May 2017
Reactions are typically 30 µl but can be scaled up as needed. Reactions should be assembled in nuclease-free microfuge tubes or PCR strip tubes.
New England Biolabs 10 May 2017
It is essential to keep the molar ratio of Cas9 and sgRNA per target site at 10:10:1 or higher to obtain the best cleavage efficiency. A calculator can be found here.
New England Biolabs 10 May 2017
The substrate DNA and sgRNA, and nuclease-free water are not included.

Step 3.1.
Nuclease-free water **20 µl**

Step 3.2.
10X Cas9 Nuclease Reaction Buffer **3 µl**

Step 3.3.
300nM sgRNA **3 µl** (30nM final)

Step 3.4.
1 µM Cas9 Nuclease, S.pyogenes (30nM final)

AMOUNT
1 µl Additional info:

REAGENTS
Cas9 Nuclease, S. pyogenes - 70 pmol M0386S by New England Biolabs

Step 4.

Pre-incubate for 10 minutes at 25 ºC

DURATION
00:10:00

Step 5.
Add 30nM substrate DNA

Step 6.
Mix thoroughly.

Step 7.

Incubate at 37°C for 15 minutes.

DURATION

01:00:00

Step 8.

Add 1 **µ**l of Proteinase K to each sample. Mix thoroughly and pulse-spin in a microfuge.

Step 9.

Incubate at room temperature for 10 minutes.

Step 10.
Proceed with fragment analysis.