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In vitro digestion of DNA with Cas9 Nuclease, S. pyogenes (M0386) V.4

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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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https://www.neb.com/protocols/2014/05/01/in-vitro-digestion-of-dna-with-cas9-nuclease-s-pyogenes-m0386

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Cas9, S. Pyogenes, in vitro digestion, sgRNA

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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.

MATERIALS

Cas9 Nuclease, S. pyogenes - 70 pmol **New England Biolabs Catalog #M0386S**

Proteinase K, Molecular Biology Grade - 2 ml **New England Biolabs Catalog #P8107S**

HiScribe T7 Quick High Yield RNA Synthesis Kit - 50 rxns **New England Biolabs Catalog #E2050S**

Nuclease-free Water **New England Biolabs Catalog #E7764**

REQUIRED MATERIALS:

- Cas9 Nuclease, S. pyogenes (NEB #M0386)
- NEBuffer 3.1
- Nuclease-free water
- Proteinase K, Molecular Biology Grade (NEB#P8107S)
- 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

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

Please refer to the Safety Data Sheets (SDS) for health and environmental hazards.

- 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.
- If planning to use higher concentration Cas9 Nuclease, *S. pyogenes* (NEB #M0386T and NEB #M0386M) for *in vitro* digestion of DNA, the enzyme can be diluted to

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[M]**1 Micromolar (µM)** in [M]**1 X Buffer 3.1** and used immediately. If the 1 µM dilution will be stored at **-20 °C**, it should be diluted using Diluent B (NEB #B8002S):
[M]**300 Milimolar (mM) NaCl**, [M]**10 Milimolar (mM) Tris-HCl**,
[M]**0.1 Milimolar (mM) EDTA**, [M]**1 Milimolar (mM) DTT**, [M]**500 µg/ml BSA** and
[M]**50 % glycerol** (pH**7.4** @ **25 °C**) prior to the reaction assembly.

1 Prepare [M]**300 Nanomolar (nM) sgRNA** by diluting the stock with nuclease-free water **On ice**.

2 Prepare [M]**30 Nanomolar (nM) substrate DNA** with a single target sequence by diluting the stock with nuclease-free water **On ice**.

3 Assemble the reaction at **Room temperature** in the following order:

A	B
COMPONENT	**VOLUME (for 30 µl reaction)**
Nuclease-free water	20 µl
NEBuffer 3.1	3 µl
300 nM sgRNA	3 µl (30 nM final)
1 µM Cas9 Nuclease, S.pyogenes (M0386S)	1 µl (~30 nM final)
Reaction volume	*27 µl*

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

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Pre-incubate for **00:10:00** at **25 °C**.

5 

Add **3 µL 30 nM substrate DNA** (3 nM final).

6 

Mix thoroughly and pulse-spin in a microfuge.

7 

Incubate at **37 °C** for **00:15:00**.

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

9 

Incubate at **Room temperature** for **00:10:00** .

10 Proceed with fragment analysis.

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