In vitro digestion of DNA with EnGen Spy Cas9 NLS (M0646)

Overview

EnGen Spy Cas9 NLS is a double-stranded DNA endonuclease that is guided to its target by sequence complementarity of a small RNA loaded into the protein. EnGen Cas9 NLS, *S. pyogenes* contains Simian virus 40 (SV40) T antigen nuclear localization sequence (NLS) on the N- and C- termini of the protein. This protocol describes how to digest double-stranded DNA *in vitro* using EnGen Spy Cas9 NLS and a single guide RNA (sgRNA).

Required Materials:

- EnGen Spy Cas9 NLS (NEB #M0646)
- 10X NEBuffer r3.1
- Nuclease-free water
- Proteinase K, Molecular Biology Grade (NEB #P8107)
- 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) or using the EnGen® sgRNA Synthesis Kit, *S. pyogenes* (NEB #E3322S)
    - sgRNAs must contain sequence complementary to the target DNA
    - 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 Bioanalyzer 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 EnGen Spy Cas9 NLS and sgRNA per target site at 10:10:1 or higher to obtain the best cleavage efficiency. A calculator can be found here.
- Prepare 300 nM sgRNA by diluting the stock with nuclease-free water on ice.
- Prepare 30 nM substrate DNA with a single target sequence by diluting the stock with nuclease-free water on ice.
- Prepare 1 µM EnGen Spy Cas9 NLS by diluting the enzyme stock (M0646Tor M0646M) with Diluent B (NEB #B8002S).

Procedure

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

Component	30 µl reaction
Nuclease-free water	20 µl
10XNEBuffer r3.1	3 µl
300nM sgRNA	3 µl (30 nM final)
1 µM EnGen Spy Cas9 NLS	1 µl (30 nM final)
Reaction volume	27 µl
Pre-incubate for 10 minutes at 25ºC	
30nM substrate DNA	3 µl (3 nM 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 15 minutes.
4. Add 1 µl of Proteinase K to each sample, Mix thoroughly and pulse-spin in a microfuge.
5. Incubate at room temperature for 10 minutes.
6. Proceed with analysis.