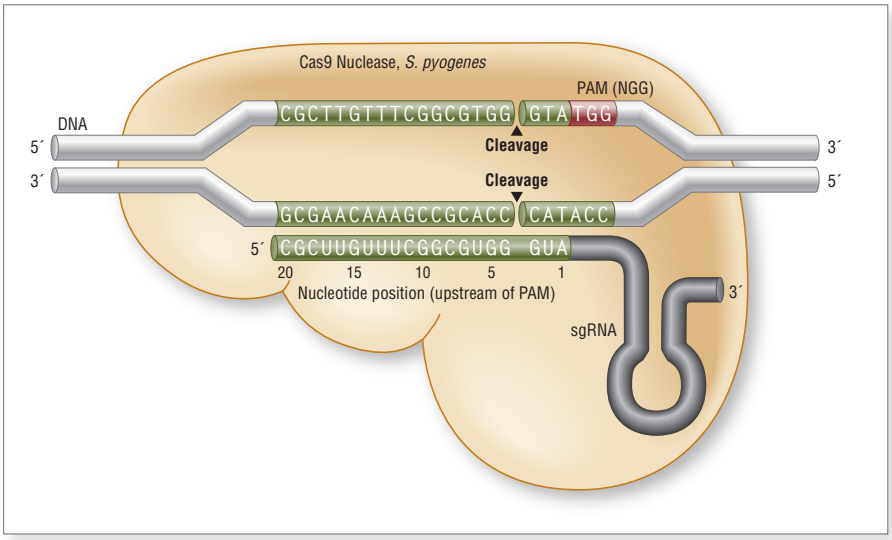
Protocol for using recombinant Cas9 Nuclease to assess locus modification in genome editing experiments

Introduction

In vitro digestion of PCR amplicons with Cas9 ribonucleoproteins (Cas9 Nuclease) is a sensitive assay for detecting indels. Unlike mismatch detection assays, Cas9 has the additional advantage of determining targeting efficiencies above 50%. This is of value as targeting efficiency in genome editing experiments increases and for detection of biallelic editing in isolated cell colonies or tissues, and was previously only achievable using specialized PCR or amplicon sequencing approaches.

Figure 1. Schematic representation of Cas9 Nuclease, *S. pyogenes* sequence recognition and DNA cleavage



Cas 9 Nuclease catalyzes site-specific cleavage of double-stranded DNA. Cleavage occurs 3 bases from the PAM (NGG) site.

Materials

- Q5® Hot Start High-Fidelity DNA 2X Master Mix (NEB #M0494)
- Purified genomic DNA from targeted cells
- PCR primers to amplify a 1 kb region containing the target site (see note)
- Thermocycler with programmable temperature ramp rate
- Spectrophotometer, fluorometer, or other DNA quantitation method
- Agilent Bioanalyzer®, Qiagen QIAxcel®, or agarose gel electrophoresis setup
- Cas9 Nuclease, *S. pyogenes* (NEB #M0386S/L)
- 10X Cas9 Nuclease Reaction Buffer
- sgRNA containing a target sequence in the region of interest (see note)

Notes:

Primers – The target site should be offset from the center of the amplicon so that digestion produces easily resolvable DNA fragments. PCR primer design is critical. Please use the NEB Tm Calculator (TmCalculator.neb.com).

sgRNA – sgRNAs can be generated by *in vitro* transcription using the HiScribe™ T7 Quick High-Yield RNA synthesis Kit (NEB #M2050S), or synthethized RNA oligonucelotides. sgRNAs must contain the target sequences (20 nucleotides) adjacent to the Protospacer Adjacent Motif (PAM, NGG) in the target DNA. (1,2)

Protocol

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: https://www.neb.com/tools-and-resources/usage-guidelines/avoiding-ribonuclease-contamination
- Reactions are typically 20 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 or near 10:10:1 to obtain the best cleavage efficiency. A calculator can be found here: nebiocalculator.neb.com
- Prepare 2 µM sgRNA by diluting the stock with nuclease-free water on ice.

PCR

1. Set up a 50 µl PCR reaction using ~100 ng of genomic DNA as a template. For each amplicon set up 3 PCR reactions using the following templates:
   a. gDNA from targeted cells (e.g., Cas9 or TALEN transfected cells)
   b. gDNA from negative control cells (e.g., non-specific DNA transfected cells)
   c. water (e.g., no template control)

COMPONENT	50 µL REACTION	FINAL CONCENTRATION
Q5® Hot Start High-Fidelity 2X Master Mix	25 µl	1X
10 µM forward primer	2.5 µl	0.5 µM
10 µM reverse primer	2.5 µl	0.5 µM
Template DNA	variable	~100 ng
Nuclease-free water	to 50 µl	–

2. Gently mix the reaction. Collect all liquid to the bottom of the tube by a quick spin if necessary. Transfer PCR tubes to a PCR machine and begin thermocycling. Q5 Hot Start High Fidelity 2X Master Mix does not require a separate activation step. Standard Q5 cycling conditions are recommended.

STEP	TEMP. (°C)	TIME
Initial denaturation	98	30 seconds
35 cycles	98	5 seconds
	*50-72	10 seconds
	72	20 seconds
Final extension	72	2 minutes
Hold	4	–

* Use of NEB Tm Calculator (TmCalculator.neb.com) is strongly recommended.

3. Analyze a small amount of the PCR product to verify size and appropriate amplification.
4. Measure the concentration of the purified PCR products by Qubit® dsDNA BR Assay or other relevant system. The yield at >25 ng DNA/µL is sufficient.

Note: Cas9 Nuclease cleavage efficiency may be altered in the presence of certain PCR buffers. In general, efficiency tends to decrease as the amount of buffer increases. If >2 µl of PCR product will be necessary for the cleavage reaction, consider purifying the PCR product prior to digestion.

Cas9 digestion with sgRNA

1. Pre-loading of sgRNA to Cas9 Nuclease:

COMPONENT	20 µL REACTION
10X Cas9 Nuclease Reaction Buffer	2 µl
sgRNA (2 µM)	2 µl (200 nM final)
Cas9 Nuclease, *S. pyogenes* (1 µM)	2 µl (100 nM final)
Nuclease-free water	14-x µl
Incubation time & temperature	5-10 minutes at room temperature

2. Digestion of PCR product:

COMPONENT	20 µL REACTION
Reaction from step 1	20-x µl
PCR product	x µl (50-200 ng DNA)*
Incubation time & temperature	30 minutes at 37°C

* <2 µl PCR product per 20 µl is recommended. The ratio of Cas9:sgRNA:target should be near 10:10:1.

Purification is optional. If desired, we recommend either digestion with with 1 µl Proteinase K, Molecular Biology Grade (NEB#P8107S) for 15-30 minutes at 37°C or column purification. These steps can be of use if nonspecific binding or high background interfere with fragment analysis.

Analysis

1. Analyze the fragmented PCR products by gel electrophoresis or other fragment analysis method.
2. Calculate the estimated gene modification using the following formula:
   % modification = 100 x ([uncut DNA] / [uncut DNA]+[fragment1]+[fragment2])

Ordering Information

PRODUCT	NEB #	SIZE
Q5 Hot Start High-Fidelity DNA 2X Master Mix	M0494S/L	100 reactions (50 µl vol)/ 500 reactions (50 µl vol)
Cas9 Nuclease, *S. pyogenes*	M0386S/L/M	50/250/500 pmol

References:
1. Jinek et al. (2012) *Science* 337 (6096) 816-821.
2. Larson et al. (2013) *Nature Protocol* (8) 2180-2196.

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