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PROTOCOL

CRISPR/Cas9 editing: mutation detection with mismatch cleavage assay

INTRODUCTION

Genome editing using CRISPR/Cas9 is used for targeted mutagenesis. But because genome editing does not target all loci with similar efficiencies, the mutation hit-rate at a given locus needs to be evaluated. The analysis of mutants obtained using CRISPR/Cas9 requires specific methods for mutation detection, and the enzyme mismatch cleavage method is used commonly for this purpose.

This CRISPR/Cas9 protocol describes how to use T7 Endonuclease I (T7E1) to detect on-target CRISPR/Cas9 editing events in cells. In this method, a sample of the edited cell population is used as a direct PCR template for amplification with primers specific to the targeted region. The PCR product is then denatured and reannealed to produce heteroduplex mismatches where double-strand breaks have occurred, resulting in insertion/deletion (indel) introduction. These mismatches are recognized and cleaved by T7EI, and the cleavage is easily detectable and quantifiable by gel analysis.

We currently recommend using T7EI instead of the Surveyor nuclease for CRISPR mutation detection because the T7EI method is more sensitive. T7EI is compatible with a broader range of PCR buffers and does not require purification of the PCR product prior to digestion.

REQUIRED MATERIALS (NOT PROVIDED)

- MethylTaq polymerase (Diagenode, Cat. No. C09010010)
- Gloves to wear during all steps
- T7 Endonuclease I (e.g., NEB, Cat. No. M0302)
- 0.5 M EDTA pH 8.0
- Purified genomic DNA from targeted and control cells
- PCR primers to amplify ~700bp region containing the target site
- dNTPs
- Thermocycler with programmable temperature ramp rate
- Apparatus to quantitate DNA (spectrophotometer of fluorometer)
- Apparatus to analyze DNA fragments (e.g. Agilent Bioanalyzer or standard agarose gel electrophoresis)
- Optional: DNA purification system (e.g. High Pure PCR Product Purification Kit, Roche)

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Europe Diagenode sa / LIEGE SCIENCE PARK // Rue du Bois Saint-Jean, 3 // 4102 Seraing (Ougrée) // Belgium // Phone: (+32) 4 364 20 50 // E-mail: info@diagenode.com
USA Diagenode Inc. / 400 Morris Avenue, Suite 101 // Denville, NJ 07834 // USA // Phone: (+1) 862 209-4680 // Mail: info.na@diagenode.com

REMARKS BEFORE STARTING

- For this protocol you will need to design PCR primers that amplify your experimental target site and adjacent sequence. We recommend that you generate a 600-1000 bp amplicon. The CRISPR/Cas9 target site should be offset from the center of the amplicon so that digestion produces easily resolvable DNA fragments. PCR conditions should be carefully optimized, and a single PCR product should be confirmed by electrophoresis. The purification of PCR product prior to T7 Endonuclease I is optional.
- MethylTaq DNA polymerase is a high performance Hot Start thermostable recombinant DNA polymerase. MethylTaq is an extremely robust modified Taq DNA polymerase that requires a 10 minute activation step at 95°C to reach maximal initial activity. This highly robust enzyme produces excellent results in demanding applications and is recommended when genomic DNA is used as template.
- Users are encouraged to perform PCR on genomic DNA from CRISPR/Cas9 targeted cells and from negative control cells (e.g., non-transfected cells).

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- Gently aspirate the media from the cells and wash once with 100 µl 1X PBS.
- Harvest the cells and extract genomic DNA using your method of choice.
- Determine DNA concentration.
  Note: Store genomic DNA at 4°C (do not freeze).

Step 1: Amplification reactions

1. Set up and run the PCR using template, primers and components of the MethylTaq polymerase Hot Start kit as follows:

   For a 50 µl reaction

Components	Amount
MethylTaq 10x buffer	5 µl
dNTPs (5 mM stock solution):	2 µl
Genomic DNA template	100 ng
Primers	0.1 nmol
$MgCl_2$ (25 mM stock solution)	4 µl
MethylTaq DNA polymerase (5U/µl)	1 to 2.5 units

   $MgCl_2$ (25 mM): MethylTaq is a magnesium-dependent enzyme. The recommended magnesium concentration varies from 1 to 4 mM.

   Nuclease-free H_2O: up to 50 µl

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Cycling conditions

Step	Conditions	Cycles
MethylTaq DNA polymerase activation	10 min at 95°C	1
Denaturation	30 sec at 95°C	35
Annealing	1 min, temperature depends on Primers Tm	
Elongation	1 min at 72°C	
Final extension	5 min at 72°C	1
	hold at 4°C	1

Note: No amplification will be observed without the 10 minute activation step at 95°C. Time and temperature for denaturation and annealing steps depend on the type of machine and primers. We advise that you check primer design using primer design software. This data is intended for use as a guide only; conditions will vary from reaction to reaction and may need optimization.

Step 2: Heteroduplexes formation

The products of the PCR reaction must be denatured and annealed in order to allow formation of heteroduplex between PCR products with and without mutations. T7 Endonuclease I digestions has been optimized for use with 10 µl of the PCR reaction, containing up to 200 ng of the amplified DNA. Heat and cool PCR products in a thermal cycler to form heteroduplexes as follows:

1. Assemble the reactions as follows:

Components	Amount
PCR (from step 1)	10 µl or 200 ng
10X NEBuffer 2*	2 µl
Nuclease free H2O	To 19 µl

* Provided with T7 Endonuclease I from NEB

2. Denature and then anneal the products in a thermocycler using the following program:

Cycle step	Temperature	Ramp rate	Time
Initial denaturation	95°C		5 minutes
Annealing	95-85°C	-2°C/ second	
	85-25°C	-0.1°C/second	
Hold	4°C		

Note: If a thermocycler is not available with these ramps rates, the samples can be heated to 95°C for 10 minutes and then allowed to cool slowly to room temperature.

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Step 3. Heteroduplexes digestion

The digestion reaction have been optimized for 10 µl of the unpurified PCR containing up to 250 ng of amplified DNA. Increased amounts of PCR reaction and/or DNA may lead to inaccurate estimates of gene editing efficiencies.

1. Set-up each reaction as follows

Components	Amount
Annealed PCR product	19 µl
T7 Endonuclease I (5 units/µl)	1 µl

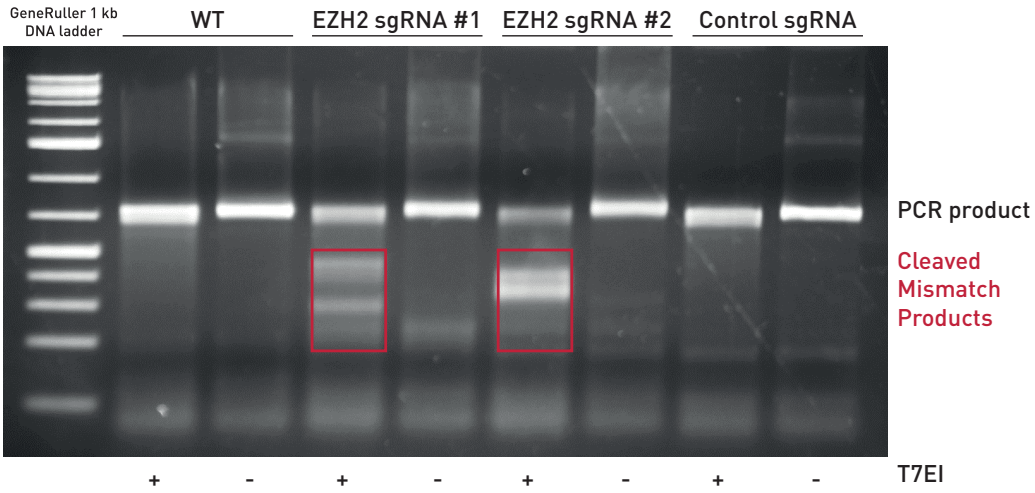
2. Mix well and briefly spin. Incubate each reaction at 37°C for 15 minutes.

   Note: Prolonged incubation may lead to unspecific digestion. Do not incubate for more than 30 minutes.

3. Stop the reaction by adding 1 µl of EDTA 0.5 M.

4. Proceed with fragment analysis or store at -20°C until ready.

Figure 1. Example of Mutation detection on targeted 293 cells.



HEK293T cells were transfected by Lipofectamine (Lipofectamine 2000) with Cas9 protein NLS (Diagenode, Cat. No. C29010001) and sgRNA targeting EZH2 (NG_032043). We designed two different sgRNAs against EZH2 and a non targeting (control) sgRNA. Untransfected cells (WT) and non-targeting sgRNA (control sgRNA) were used as controls. Genomic PCR was prepared to amplify the region flanking the CRISPR site (700 bp amplicon) using MethylTaq DNA polymerase (Diagenode, Cat. No. C09010010) and tested for CRISPR/Cas9 induced mutations by T7 Endonuclease I assay. Cleavage at heteroduplex mismatch sites (in red) was assessed by agarose gel electrophoresis. As can be seen, Cas9 Nuclease Protein NLS, when combined with specific sgRNAs (#1 or #2) provides consistent and effective gene editing.

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