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Determining Genome Targeting Efficiency using T7 Endonuclease I (M0302) V.3

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Abstract

T7 Endonuclease I recognizes and cleaves non-perfectly matched DNA. This protocol describes how to determine genome targeting efficiency by digesting annealed PCR products with T7 Endonuclease I. In the first step PCR products are produced from the genomic DNA of cells whose genomes were targeted using Cas9, TALEN, ZFN etc. In the second step, the PCR products are annealed and digested with T7 Endonuclease I. Fragments are analyzed to determine the efficiency of genome targeting.

Guidelines

Overview:

T7 Endonuclease I recognizes and cleaves non-perfectly matched DNA. This protocol describes how to determine genome targeting efficiency by digesting annealed PCR products with T7 Endonuclease I. In the first step PCR products are produced from the genomic DNA of cells whose genomes were targeted using Cas9, TALEN, ZFN etc. In the second step, the PCR products are annealed and digested with T7 Endonuclease I. Fragments are analyzed to determine the efficiency of genome targeting.

Required Materials:

- Q5® Hot Start High-Fidelity 2X Master Mix (M0494S)
- T7 Endonuclease I (M0302S)
- 0.25 mM EDTA
- Purified genomic DNA from targeted cells
- PCR primers to amplify a ~1kb region containing the target site
- 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 visit NEB's Tools and Resources page to optimize your primer design using the NEB Tm Calculator
- A PCR thermocycler with programmable temperature ramp rate
- DNA purification system - we recommend Ampure XP beads
- Apparatus to quantitate DNA - spectrophotometer or fluorometer
- Apparatus to analyze DNA fragments – e.g. Agilent Bioanalyzer, Qiagen Qiaxel, or standard agarose gel electrophoresis

PCR

Cycling Conditions

	Step	Temperature	Time
	Initial Denaturation	98°C	30 seconds
	35 cycles	98°C	5 seconds
	*50-72°C	10 seconds	
	72°C	20 seconds	
	Final Extension	72°C	2 minutes
	Hold	4-10°C	

***Use of the NEB Tm Calculator is highly recommended.**
Note: Q5 Hot Start High-Fidelity 2X Master Mix does not require a separate activation step. Standard Q5 cycling conditions are recommended.

T7 Endonuclease I digestion:

Anneal the PCR products in a thermocycler using the following conditions:
Hybridization Conditions

	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		Hold

REFERENCES:

Guschin, D.Y., et. al.(2010) **A rapid and general assay for monitoring endogenous gene modification**. Methods Mol Biol, 649, 247–256.

Materials

MATERIALS

Q5 Hot Start High-Fidelity 2X Master Mix - 100 rxns **New England Biolabs Catalog #**M0494S

T7 Endonuclease I - 250 units **New England Biolabs Catalog #**M0302S

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 (positive, negative, no-template control)

	Component	50 µl reaction	Final Concentration
	Q5® Hot Start High-Fidelity 2X Master Mix (M0494S)	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 total
	Nuclease-free water	To 50 µl	

Note

For each amplicon set up 3 PCR reactions using the following templates:
- gDNA from targeted cells (e.g. Cas9, or TALEN transfected cells)
- gDNA from negative control cells (e.g. non-specific DNA transfected cells)
- water (i.e. no template control)

Protocol

NAME

Q5 PCR Mixture for M0302

CREATED BY

New England Biolabs

PREVIEW

1.1 Q5® Hot Start High-Fidelity 2X Master Mix (M0494S)

25 µL

Q5 Hot Start High-Fidelity 2X Master Mix - 100 rxns **New England Biolabs Catalog #**M0494S

1.2 10 µM Forward Primer, **2.5 µl**

3 µL

1.3 10 µM Reverse Primer, 2.5 µl
3 µL

1.4 Template DNA

1.5 Nuclease-free water to **50 µl**

2 Gently mix the reaction.

3 Collect all liquid to the bottom of the tube by a quick spin if necessary.

4 Transfer PCR tubes to a PCR machine and begin thermocycling.

Note

Hot Start High-Fidelity 2X Master Mix does not require a separate activation step. Standard Q5 cycling conditions are recommended.

5 Analyze a small amount of the of the PCR product to verify size and appropriate amplification.

6 Purify the PCR reaction using 90 µl of Ampure XP beads following the manufacturer's recommendations.

Note

Other PCR purification systems (e.g. Qiagen MinElute PCR Purification Kit PCR purification columns, or Zymo DNA Clean and Concentrator™) are acceptable.

7 Elute PCR products in **30 µl** of water, recovering **25 µl**.

8 Measure the concentration of the purified PCR products.

T7 Endonuclease I digestion

9 Assemble reactions as follows:

Protocol

NAME

Digestion Mixture for M0302 (v2)

CREATED BY

New England Biolabs

PREVIEW

9.1 DNA, **200 ng**

9.2 10X NEBuffer 2, **2 µl**

2 µL

9.3 Nuclease-free Water to **19 µl**

10 Anneal the PCR products in a thermocycler (see **guidelines** for conditions)

11 Add **1 µl** of the T7 Endonuclease I to the annealed PCR products

1 µL

12 Incubate at 37°C for 15 minutes

00:15:00

13 Stop the reaction by adding **1.5 µl** of 0.25 M EDTA.

2 µL

14 Purify the reaction using 36 µl of Ampure XP beads according to the manufacturer's suggestion.

Note

This step is optional since 1 µl of the reaction will not interfere with analysis on an Agilent Bioanalyzer using DNA1000 reagents.

15 Elute the DNA fragments in **20 µl** of water, recovering **15 µl.**

Analysis

16 Analyze the fragmented PCR products and determine the percent of nuclease-specific cleavage products (fraction cleaved)

17 Calculate the estimated gene modification using the following formula:
% gene modification = 100 x (1 – (1- fraction cleaved)$^{1/2}$)