Determining Genome Targeting Efficiency using T7 Endonuclease I (M0302)

New England Biolabs

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.

Citation: New England Biolabs Determining Genome Targeting Efficiency using T7 Endonuclease I (M0302). **protocols.io**
dx.doi.org/10.17504/protocols.io.cqpvvm
Published: 05 Feb 2015

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
	98°C	5 seconds
35 cycles	*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

Materials

- Q5 Hot Start High-Fidelity 2X Master Mix - 100 rxns M0494S by New England Biolabs
- T7 Endonuclease I - 250 units M0302S by New England Biolabs

Protocol

PCR

Step 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

PROTOCOL

. Q5 PCR Mixture for M0302

CONTACT: New England Biolabs

NOTES

New England Biolabs 29 Jan 2015
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)

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

AMOUNT
25 µl Additional info:

REAGENTS
Q5 Hot Start High-Fidelity 2X Master Mix - 100 rxns M0494S by New England Biolabs

Step 1.2.
10 µM Forward Primer, **2.5 µl**

AMOUNT
3 µl Additional info:

Step 1.3.
10 µM Reverse Primer, 2.5 µl

AMOUNT
3 µl Additional info:

Step 1.4.
Template DNA

Step 1.5.
Nuclease-free water to **50 µl**

PCR

Step 2.
Gently mix the reaction.

PCR

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

PCR

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

NOTES

New England Biolabs 29 Jan 2015
Hot Start High-Fidelity 2X Master Mix does not require a separate activation step. Standard Q5

cycling conditions are recommended.

PCR

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

PCR

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

NOTES

New England Biolabs 29 Jan 2015
Other PCR purification systems (e.g. Qiagen MinElute PCR Purification Kit PCR purification columns, or Zymo DNA Clean and Concentrator™) are acceptable.

PCR

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

PCR

Step 8.
Measure the concentration of the purified PCR products.

T7 Endonuclease I digestion

Step 9.
Assemble reactions as follows:

PROTOCOL

. Digestion Mixture for M0302

CONTACT: New England Biolabs
Step 9.1.
DNA, **200 ng**

Step 9.2.
10X NEBuffer 2, **2 µl** (1X)

AMOUNT

2 µl Additional info:

Step 9.3.
Nuclease-free Water to **19 µl**

T7 Endonuclease I digestion

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

T7 Endonuclease I digestion

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

AMOUNT

1 µl Additional info:

T7 Endonuclease I digestion

Step 12.
Incubate at 37°C for 15 minutes

DURATION

00:15:00

T7 Endonuclease I digestion

Step 13.

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

AMOUNT

2 µl Additional info:

T7 Endonuclease I digestion

Step 14.

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

NOTES

New England Biolabs 29 Jan 2015

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

T7 Endonuclease I digestion

Step 15.

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

Analysis

Step 16.

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

Analysis

Step 17.

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