Protocol

TD-P Revision 1.0

Creation Date: 2/15/2019
Revision Date: 5/2/2019

Determining Efficiency of Genome Targeting

Utilizing T7 Endonuclease I

Introduction

T7 Endonuclease I is a stable homodimer of identical 149 amino acid-subunits and is the product of a recombinant gene in *E. coli*. Double-stranded breaks (DSBs) generated by CRISPR/TALEN at desired target sites can be PCR-amplified, and the PCR products can be denatured and re-annealed to form mismatched DNA. If the mismatched DNA length position is more than 1 bp, T7 endonuclease I can recognize and cleave it. T7 Endonuclease is useful for quantitatively estimating the nuclease-induced mutation frequency of gene edited cells. Applications include resolving four-way junction or branched DNA, detection or cleaving of heteroduplex and nicked DNA and random cleaving of linear DNA for shot-gun cloning.

This protocol describes how to determine genome targeting efficiency by digesting annealed mismatched PCR products with T7 Endonuclease I. First, 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 re-annealed and then digested with T7 Endonuclease I. If two shorter fragments of the expected size are generated, then mutations, small insertions and deletions (indels typically 2-20 bp) were successfully introduced at the targeted chromosomal site. Fragments are then analyzed to determine the efficiency of genome targeting.

Process Overview:

Cell Lysis

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Genomic DNA Extraction (optional)

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Target Site PCR Amplification

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DNA Purification

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Denaturation and Re-Annealing

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T7 Endonuclease I Digestion

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Materials

- T7 Endonuclease I (GoldBio Catalog # T-970)
- 10x T7 Endonuclease reaction buffer

Note: The storage buffer contains 50mM Tris-HCl, 50mM KCl, 1mM DTT, 0.1mM EDTA, 50% glycerol, pH 7.5 at 25°C.

Note: 1x T7 Endonuclease I Reaction Buffer contains 10mM Tris-HCl, 50mM KCl, 10mM $MgCl_2$, 1mM DTT, pH 7.5 at 25°C.

Storage and Handling

- Store T7 Endonuclease I and the 10x T7 Endonuclease I reaction buffer at -20°C.
- These products may be shipped in blue ice and should be stored immediately upon arrival at -20°C. When stored under the recommended conditions and handled correctly, these products should be stable for at least 1 year from the date of receipt.

Method

Sample preparation

1. Samples can be prepared using two different methods.
    a. Genomic DNA extraction
        i. Harvest cells and extract genomic DNA according to the manufacturer's extraction protocol.
    b. Cell lysate preparation
        i. Collect cells and add 50-100 µl Lysis buffer and lyse cells at 95°C for 5-10 minutes.

PCR amplification

1. Thaw the kit components, then mix and pulse-spin each component in a microfuge prior to use.

2. Set up a 50 µl PCR reaction using up to 100-200 ng of genomic DNA as a template as follows:

Reagent	50 µl reaction
Genomic DNA	100-200 ng
Forward primer (10µM)	2.5 µl
Reverse primer (10µM)	2.5 µl
2x PCR Master Mix	25.0 µl
H_2O up to	50.0 µl

3. Mix the reaction and transfer the tubes to a PCR machine with the following thermocycling conditions:

PCR Cycling Conditions:			
Steps	**Temperature**	**Time**	**Cycles**
Initial Denaturation	95°C	5 min	1
Denaturation	95°C	30 sec	30
Annealing	Tm-3°C	30 sec	
Extension	72°C	1 min	
Final Extension	72°C	5 min	1

4. Run 3-5 µl of the PCR product on a 1-2% agarose gel. The final product should be within the range of 600-1000 bp.

Note: (Optional). If necessary, purify the DNA by using either a gel extraction kit or ampure XP beads according to the manufacturer's protocol.

Hybridization (Denaturation and reannealing)

1. Assemble the reaction as follows:

Component	50 µl reaction
DNA (wt)	15.0 µl
DNA (mutant)	15.0 µl
10x Endonuclease I buffer	5.0 µl
H_2O	15.0 µl
Total	50.0 µl

2. Mix the reaction gently.

3. Heat at 95°C for 5 minutes using a heat block. A thermocycler can also be used for hybridization.

4. Turn off the heat block and cool down gradually to room temperature.

T7 Endonuclease I digestion

1. Assemble the reaction as follows:

Component	10 µl reaction

Hybridized DNA	5.0 µl
10x Endonuclease I buffer	1.0 µl
T7 Endonuclease I (10 U)	1.0 µl
H_2O up to	10.0 µl

2. Incubate at 37°C for 15-30 minutes.

3. Stop the reaction by adding 1.0 µl of 0.5 M EDTA.

4. Run a 1-2% agarose gel to determine cleavage efficiency. A typical gel for Endonuclease I cleavage is shown below.

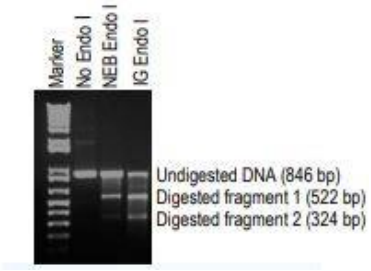

Figure 1. Cleavage efficiency of T7 Endonuclease I. T7 Endonuclease I activity testing: Double-stranded (ds) DNA fragments containing two types of sequences are PCR amplified, denatured and annealed to produce mismatched (heterologous) dsDNA. Purified mismatched dsDNA digested with Endonuclease I from NEB and Intact Genomics (IG) to compare the enzyme efficiency.

Troubleshooting

Problem	Possible Causes	Recommendations
Non-specific cleavage	Non-specific PCR amplification	• Purify DNA • Optimize PCR primers • Optimize PCR conditions
No cleavage	Low T7 Endonuclease I activity	Add more T7 Endonuclease I
No expected bands	Incomplete Lysis	• Adjust the volume of lysis buffer according to the cell number
	Poor PCR primer	• Increase lysis time • Optimize PCR primers

Associated Products

- 1 kb DNA Ladder (GoldBio Catalog # D010)
- 1 kb Plus™ DNA Ladder (GoldBio Catalog # D011)
- 100 bp DNA Ladder (GoldBio Catalog #D001)
- 100 bp PLUS™ DNA Ladder (GoldBio Catalog # D003)
- 50 bp DNA Ladder (GoldBio Catalog # D100)
- VersaLadder™ 100-10,000 bp (GoldBio Catalog # D012)
- dNTP mix (GoldBio Catalog # D-900)
- Agarose LE (GoldBio Catalog # A-201)
- Low Melt Agarose (GoldBio Catalog # A-204)
- Taq DNA Polymerase (GoldBio Catalog # T-514)
- Taq DNA Polymerase with Dye (GoldBio Catalog # T-516)
- Taq DNA Polymerase plus dNTP (GoldBio Catalog # T-515)
- Taq DNA Polymerase with dye plus dNTP (GoldBio Catalog # T-517)
- Taq DNA Polymerase 2x Premix with Dye (GoldBio Catalog # T-518)
- Hot Start Taq DNA Polymerase (GoldBio Catalog # T-510)
- Hot Start Taq DNA Polymerase plus dNTP (GoldBio Catalog # T-511)
- RT-PCR Kit (GoldBio Catalog # R-920)
- One Step RT-PCR Kit (GoldBio Catalog # R-910)
- First Strand cDNA Synthesis Kit (GoldBio Catalog # D-925)
- Reverse Transcriptase (GoldBio Catalog # R-900)