Determining Genome Targeting Efficiency using T7 Endonuclease I  
使用T7内切酶I确定基因组靶向效率

**an**[**interactive version of this protocol**](https://www.protocols.io/view/Determining-Genome-Targeting-Efficiency-using-T7-E-imsvvm)

Overview: 概述：

[T7 Endonuclease I](https://www.neb.cn/zh-cn/products/m0302-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.

T7核酸内切酶I识别并切割非完全匹配的DNA。该方案描述了如何通过T7核酸内切酶i消化退火后的PCR产物来确定基因组靶向效率。第一步，PCR产物来自使用Cas9，TALEN，ZFN等基因组靶向的细胞的基因组DNA。第二步，PCR产物退火后用T7核酸内切酶i消化，分析片段以确定基因组靶向效率。

Required Materials:必需材料：

Q5® Hot Start High-Fidelity 2X Master Mix (M0494)

T7 Endonuclease I (M0302)

0.25 M EDTA

Purified genomic DNA from targeted cells

PCR primers to amplify a ~1 kb region containing the target site

PCR 引物以扩增包含靶位点的约 1 kb 区域

The target site should be offset from the center of the amplicon so that digestion produces easily resolvable DNA fragments

目标位点应与扩增子中心偏移，以便消化产生易于分解的DNA片段PCR

primer design is critical. Please visit NEB's [Tools and Resources](https://www.neb.cn/zh-cn/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:

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

设置50 µl的PCR反应，使用约100 ng的基因组DNA作为模板。对于每个扩增子设置3个PCR反应，使用以下模板

1. gDNA from targeted cells (e.g. Cas9, or TALEN transfected cells)
2. gDNA from negative control cells (e.g. non-specific DNA transfected cells)
3. water (i.e. no template control)

**PCR using Q5 High-Fidelity DNA Polymerase**

|  |  |  |
| --- | --- | --- |
| COMPONENT | 50 µl REACTION | FINAL CONCENTRATION |
| Q5 Hot Start High-Fidelity 2X Master Mix (M0494) | 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 |  |

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.

**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.  
注意：Q5 Hot Start 高保真 2X Master Mix 不需要单独激活步骤。建议使用标准 Q5 循环条件。*

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

分析少量 PCR 产物以验证大小和适当的扩增。

Purify the PCR reaction using 90 µl of Ampure XP beads following the manufacturer’s recommendations. Other PCR purification systems (e.g. Monarch PCR & DNA Clean Up Kit, or Zymo DNA Clean and Concentrator™) are acceptable.  
使用 90 µl 的 Ampure XP 磁珠，按照制造商的建议纯化 PCR 反应。其他 PCR 纯化系统（例如 Monarch PCR & DNA Clean Up Kit 或 Zymo DNA Clean and Concentrator™）均可接受。

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

从30µl水中洗脱PCR产物，回收25µl。

Measure the concentration of the purified PCR products.

测量纯化PCR产物的浓度。

T7 Endonuclease I digestion:  
T7 内切酶 I 消化：

1. Assemble reactions as follows  
   按照以下步骤组装反应

|  |  |
| --- | --- |
| COMPONENT | 19 µl ANNEALING REACTION |
| DNA | 200 ng |
| 10X NEBuffer 2 | 2 µl |
| Nuclease-free Water | To 19 µl |

1. Anneal the PCR products in a thermocycler using the following conditions:  
   在热循环仪中按照以下条件退火 PCR 产物：

**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 |

1. Add T7 Endonuclease I to the annealed PCR products

将 T7 内切酶 I 添加到退火后的 PCR 产物中

|  |  |
| --- | --- |
| COMPONENT | 20 µl REACTION |
| Annealed PCR product | 19 µl |
| T7 Endonuclease I (M0302) | 1 µl |
|  | |
| Incubation Time | 15 minutes |
| Incubation Temperature | 37°C |

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

停止反应，加入 1.5 µl 的 0.25 M EDTA。

1. Purify the reaction using 36 µl of Ampure XP beads according to the manufacturer’s suggestion. This step is optional since 1 µl of the reaction will not interfere with analysis on an Agilent Bioanalyzer using DNA1000 reagents.

使用制造商的建议，用 36 µl 的 Ampure XP 磁珠纯化反应。此步骤为可选，因为1 µl的反应不会干扰使用DNA1000试剂在Agilent Bioanalyzer 上的分析。

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

从20 µl水中洗脱DNA片段，回收15µl。

Analysis: 分析：

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

分析断裂的 PCR 产物并确定核酸酶特异性切割产物的百分比（切割分数）

* Calculate the estimated gene modification using the following formula:  
  计算以下公式的估计基因修改：

% gene modification = 100 x (1 – (1- fraction cleaved)1/2)

基因修改率 = 100 x (1 – (1-切割分数)1/2