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
Overview: 概述：
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 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

2. 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

3. 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

4. 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。
5. 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。
6. 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)$^{1/2}$