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Determining Genome Targeting Efficiency using T7 Endonuclease I (M0302) V.4

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Protocol status: Working

We use this protocol and it's working

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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

REFERENCES:

CITATION

Guschin DY, Waite AJ, Katibah GE, Miller JC, Holmes MC, Rebar EJ (2010). A rapid and general assay for monitoring endogenous gene modification.. Methods in molecular biology (Clifton, N.J.).

LINK

https://doi.org/10.1007/978-1-60761-753-2_15

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**



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 T_m 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 Qiaxcel, or standard agarose gel electrophoresis

Safety warnings

 Please refer to the Safety Data Sheets (SDS) for health and environmental hazards.

PCR

- Set up a  50 µL PCR reaction using ~  100 ng genomic DNA as a template. 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)

PCR using Q5 High-Fidelity DNA Polymerase

A	B	C
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

A	B	C
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 T_m Calculator is highly recommended.

Note

Note: Q5 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 Ampure XP beads following the manufacturer's recommendations.

Note

Other PCR purification systems (e.g. **Monarch PCR & DNA Clean Up Kit**, or Zymo DNA Clean and Concentrator™) are acceptable.

7 Elute PCR products in  30 µL water , recovering  25 µL .

8 Measure the concentration of the purified PCR products.



T7 Endonuclease I digestion

9 Assemble reactions as follows:



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

10 Anneal the PCR products in a thermocycler using the following conditions:



Hybridization Conditions

A	B	C	D
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	



A	B	C	D
Hold	4°C		Hold

11 Add 1 µL T7 Endonuclease I (M0302) to the annealed PCR products for a final volume of 20 µL .



12 Incubate at 37 °C for 00:15:00 .



13 Stop the reaction by adding 1.5 µL 0.25 M EDTA .



14 Purify the reaction using 36 µL 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 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 = $100 \times (1 - (1 - \text{fraction cleaved})^{1/2})$



Citations

Guschin DY, Waite AJ, Katibah GE, Miller JC, Holmes MC, Rebar EJ. A rapid and general assay for monitoring endogenous gene modification.

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