Technical Bulletin

T7 Endonuclease Detection Assay Kit

Catalog Number T7E1001

Product Description

The T7 Endonuclease Detection Assay kit provides reagents for detection of on-target genome editing events. In the first step of the method, edited target regions from cells whose genomes were targeted (i.e., CRISPR/Cas9, TALENs, Zinc-finger Nucleases) are PCR-amplified. If the target contains insertions or deletions, denaturation and re-annealing of the PCR amplicon in the next step results in heteroduplexes in the amplicon pool. In the second step, re-annealed PCR product heteroduplexes are digested with T7 Endonuclease, a structure-specific enzyme that recognizes mismatches larger than 1 base. Both strands of the DNA are cut when a mismatch is present, resulting in the formation of smaller fragments. Analysis of the resulting fragments provides an estimate of the efficiency of the genome editing experiments.

The kit includes a Control Template and Primer Mix that can be used as a control for the PCR reaction and T7 Endonuclease digestion. The Control Template and Primer Mix provided contains two plasmids and primers that when amplified, denatured, and re-annealed, form heteroduplexes, which contain a 10-base insertion. The digestion of the 600 bp heteroduplex by T7 Endonuclease yields products of 200 bp and 400 bp. 600 bp Parental homoduplexes are uncleaved and are easily distinguished from cleaved heteroduplexes when separated and visualized by agarose gel electrophoresis.

Components

Each kit contains sufficient reagents for 25 reactions. Additional PCR reagents are included to allow for optimization of the amplification reaction.

- T7 Endonuclease — 25 µL
  Catalog Number T7E1001A
- Buffer — 120 µL
  Catalog Number T7E1001B
- Proteinase K — 25 µL
  Catalog Number T7E1001C
- Control Template and Primer Mix, Catalog Number T7E1001D — 1.25 mL
- DNA Ladder – 1KB — 200 µL
  Catalog Number T7E1001E
- Gel Loading Dye (6X) — 1 mL
  Catalog Number T7E1001F

Reagents and Equipment Required but Not Provided

- Oligodeoxyribonucleotide primers for PCR
- AccuTaq™ LA DNA Polymerase (Catalog Number D8045)
- Deoxynucleotide Mix, 10mM each dATP, dCTP, dGTP, and dTTP (Catalog Number D7295)
- PCR reaction tubes or strips
- Nuclease-free water

Precautions and Disclaimer

For R&D use only. Not for drug, household, or other uses. Please consult the Safety Data Sheet for information regarding hazards and safe handling practices.

Storage/Stability

The kit is shipped on wet ice. Store components at -20 °C.

Preparation Instructions

Briefly centrifuge small vials prior to opening.

DNA Template

Use of high quality, purified DNA templates greatly enhances PCR. Recommended amounts for a 25 µl reaction are 0.5–500 ng of genomic DNA (gDNA). If not using a purified gDNA sample, verify amplification of the PCR reaction before proceeding.

Primer Design

For best results, primers should be designed so PCR amplicons are in the range of 500-1000 bp. The target site should be offset from the center of the amplicon so that digestion results in easily resolvable fragments.

Reagent Preparation

Thaw the kit components on ice. Mix by vortexing each component prior to use.

Procedure

The protocol has been optimized so that PCR products generated amplified with AccuTaq DNA polymerase can be introduced directly into the T7 Endonuclease I digestion without the need for purification. Digestion of the heteroduplex is complete in only 15 minutes, and Proteinase K is included to stop the reaction efficiently.

Note: Users are encouraged to perform PCR and T7 Endonuclease digestion using the included Control Template and Primer Mix as a reaction control.

Note: For each amplicon, we recommend setting up two PCR reactions using the following templates:

a) gDNA from CRISPR or ZFN targeted cells
b) gDNA from negative control cells (non-specific DNA transfected cells)

PCR Setup

1. Set up 25 µl PCR reactions using up to 500 ng of gDNA as a template. Assemble the following reactions in PCR reaction tubes at room temperature according to Table 1.

Table 1.
Preparation of PCR Reactions

Reagent	Volume	Final Concentration
AccuTaq™ LA DNA Polymerase (2.5 Units/µl)	0.5 µl	0.05 units/ µl
10× Buffer for AccuTaq LA DNA Polymerase	2.5 µl	1×
dNTP Mix (10 mM)	0.5 µl	500 µM
10 µM Forward Primer	1.25 µl	0.5 µM
10 µM Reverse Primer	1.25 µl	0.5 µM
Template gDNA OR Control Template and Primer Mix (T7E1001D)	Variable 2.5 µl	125-250 ng 0.5 ng plasmid & 0.5 µM each primer
Nuclease-Free Water	q.s.to 25 µl	---

2. Gently mix the reactions. Collect all the liquid to the bottom of each tube by briefly centrifuging.

3. Transfer the tubes to a PCR machine and begin thermocycling using the following conditions as defined in Table 2.

Table 2.
Thermocycle Program for PCR Reactions

Cycle Step	Temp	Time	Number of Cycles
Initial Denaturation	98 °C	30 s	1
Denaturation	94 °C	15 s	35
Annealing	60 °C	30 s	
Extension	68 °C	45 s	
Final Extension	98 °C	5 m	1

4. Analyze a small amount of the PCR product on an agarose gel to verify amplification of a single product of the correct size. A DNA marker should also be run to help estimate amplicon concentration. The product of the control PCR reaction (using Control Template and Primer Mix) is ~600 bp.

Heteroduplex Formation

The products of the PCR reaction must be denatured and annealed to allow formation of heteroduplex between PCR products with and without mutations. T7 Endonuclease digestion has been optimized for use with 5 µl of the PCR reaction product, containing up to 250 ng of amplified DNA.

The following protocol applies to both experimental and control reactions:

1. Assemble each 19 µL reaction mixture in fresh PCR tubes according to Table 3.

Table 3.
Preparation of Annealing Reactions

Reagent	Annealing Reaction
PCR Reaction Product	5 µl
Buffer (T7E1001B)	2 µl
Nuclease-free water	12 µl

2. Denature and then anneal the products in a thermocycler using the following program according to Table 4.

Table 4.
Thermocycle Program for Heteroduplex Formation

Cycle Step	Temp	Rate of Change
Initial Denaturation	98 °C	Heat for 5 m
Annealing	95 °C to 85 °C	-2 °C/s
	85 °C to 25 °C	-0.1 °C/s
Hold	4 °C	---

3. Proceed to Heteroduplex Digestion.

Heteroduplex Digestion

The digestion reaction conditions have been optimized for 5 µl of the unpurified Heteroduplex Formation reaction containing up to 250 ng of amplified DNA. Increased amounts of PCR reaction and/or DNA may lead to inaccurate estimates of editing efficiencies.

1. In the same reaction tubes as used for Heteroduplex Formation, set up each Digestion reaction according to Table 5.

Table 5.
Preparation of T7E1 Digestion Reactions

Reagent	**T7E1 Digestion Reaction**
Annealed PCR Product	19 µl
T7 Endonuclease	1 µl

2. Mix well and briefly centrifuge. Incubate each reaction at 37°C for 15 minutes.
3. Following digestion, add 1 µl of Proteinase K to each reaction well and mix thoroughly.
4. Incubate for 5 minutes at 37°C to inactivate the T7 Endonuclease.
5. Proceed with Electrophoresis Analysis of DNA Fragments. Alternatively, store reaction solutions at -20°C until ready for analysis.

Electrophoresis Analysis of DNA Fragments

1. Add 4 µl of Gel Loading Dye (6×) to each reaction.
2. Run electrophoresis of all reactions on a 2% agarose gel stained with ethidium bromide. Run one lane with the included DNA ladder next to the sample on the gel for reference.
3. Digestion of the Control amplicon (from the Control Template and Primer Mix) yields fragments of ~200 bp and ~400 bp in addition to the parental band.
4. Analyze the banding pattern in each sample by standard imaging software. Measure the intensity of each band and plug the values into the equation below to estimate fraction cleaved. Alternatively, samples can be analyzed using a fragment analyzer.
5. Estimate the fraction of DNA Fragments cleaved:

$$\text{Fraction cleaved} = \frac{\text{Concentration of digested products}}{\text{Concentration of digested products} + \text{Concentration of undigested band}}$$

6. Calculate the estimated % modification using the following formula:

$$\% \text{ Modification} = 100\% \times [1 - (1 - \text{Fraction Cleaved})^{0.5}]$$

When calculating % modification for reactions with the control template where the starting material is known, use the following equation:

$$100\% \times \text{Fraction Cleaved}$$

Troubleshooting Guide

Part of the Protocol	Issues	Solutions
PCR	No PCR product of desired size detected	Perform the reaction using the Control Template and Primer Mix included with the kit. May be necessary to re-design primers and optimize PCR conditions.
Cleavage: No cleavage products observed	Amplicon may be too small to observe edits	May be necessary to re-design primers so that the target site is further from the end of the amplicon.
	Enzymes are non-functional	Perform control reaction to verify performance of the enzymes/
	Amount of PCR product is too high for complete enzyme digestion	Quantify the PCR product. Add no more than 250 ng to the heteroduplex digestion reaction.
Cleavage: Low cleavage efficiencies	Amount of PCR product is too high for complete enzyme digestion	Quantify the PCR product. Add no more than 250 ng to the heteroduplex digestion reaction.
	Editing reagents used do not adequately edit the DNA target sequence	Re-design gRNA or ZFN target sequences or optimize editing experiment to maximize editing results.

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