T7 Endonuclease I-based Mutation Detection with the EnGen® Mutation Detection Kit (NEB #E3321)

Materials Required but not Supplied

EnGen® Mutation Detection KitOligodeoxyribonucleotide primers for PCR
PCR reaction tubes or strips
Nuclease-free water
Thermocycler
Aerosol tips for PCR

Overview

New users are encouraged to perform PCR and T7 Endonuclease I digestion using the included control template and primer mix. For each amplicon we recommend setting up three PCR reactions using the following templates:

a. gDNA from targeted cells (e.g., Cas9, TALEN or ZFN transfected cells)
b. gDNA from negative control cells (e.g., non-specific DNA transfected cells)
c. Water (i.e., no template control)

Amplification reactions for experimental samples

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

2. Set up a 25 µl PCR reaction using up to 500 ng of genomic DNA as a template. Assemble the following reaction at room temperature:

REAGENT	25 µl	RXN FINAL CONCENTRATION
Q5 Hot Start High-Fidelity 2X Master Mix	12.5 µl	1X
10 µM Forward Primer	1.25 µl	0.5 µM
10 µM Reverse Primer	1.25 µl	0.5 µM
Template DNA	variable	0.5–500 ng genomic DNA*
Nuclease-free water	to 25 µl	

* To use cell lysate directly in PCR, lyse cells in QuickExtract or DNAzol Direct using 50 µl cells in each well of a 96-well plate (~40,000 cells) according to the manufacturers' recommendation. Dilute the lysate 1:5 in TE (10 mM Tris, 1 mM EDTA, pH 8.0) and use 2.5 µl of the diluted lysate.

3. Gently mix the reaction. Collect all the liquid to the bottom of the tube with a brief spin. Transfer the tubes to a PCR machine and begin thermocycling using the following conditions:

CYCLE STEP	TEMP	TIME	CYCLES;
Initial Denaturation	98°C	30 seconds	1

Denaturation Annealing Extension	98°C 50–72°C* 72°C	5 seconds 10 seconds 20 seconds	35
Final Extension	72°C	2 minutes	1
Hold	4–10°C		

* Please visit Tmcalculator.neb.com to determine correct annealing temperature.

Control reaction using included Control Template and Primer Mix.

1. Set up a 25 µl PCR reaction as follows:

REAGENT	25 µl RXN	FINAL CONCENTRATION
Q5 Hot Start High-Fidelity 2X Master Mix	12.5 µl	1X
Control Template and Primer Mix	2.5 µl	0.5 ng plasmid and 0.5 µM each primer
Nuclease-free water	10 µl	

2. Gently mix the reaction. Collect all the liquid to the bottom of the tube with a brief spin. Transfer the tubes to a PCR machine and begin thermocycling using the following conditions:

CYCLE STEP	TEMP	TIME	CYCLES;
Initial Denaturation	98°C	30 seconds	1
Denaturation Annealing Extension	98°C 65°C 72°C	5 seconds 10 seconds 20 seconds	35
Final Extension	72°C	2 minutes	1
Hold	4–10°C		

3. 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 is ~600 bp.

Heteroduplex formation:

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

The following protocol applies to both experimental and control reactions:

1. Assemble the reaction as follows:

REAGENT	19 µl ANNEALING REACTION

PCR Reaction	5 µl
10X NEBuffer 2	2 µl
Nuclease-free water	12 µl

2. Denature and then anneal the products in a thermocycler using the following program*:

CYCLE STEP	TEMP	RAMP RATE	TIME
Initial Denaturation	95°C		5 minutes
Annealing	95–85°C 85–25°C	-2°C/second -0.1°C/second	
Hold	4°C		

* Alternatively, if a thermocycler is not available with these ramp speeds, the sample can be heated to 95ºC for 10 minutes and then allowed to cool slowly to room temperature.

3. Proceed to heteroduplex digestion.

Heteroduplex digestion:

The digestion reaction conditions have been optimized for 5 µl of the unpurified PCR 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. Set up each reaction as follows:

REAGENT	20 µl T7E1 REACTION
Annealed PCR Product	19 µl
EnGen T7 Endonuclease I	1 µl

2. Mix well and briefly spin. Incubate each reaction at 37°C for 15 minutes.

3. Following digestion, add 1 µl of Proteinase K and mix well.

4. Incubate for 5 minutes at 37°C to inactivate the T7 Endonuclease I.

5. Proceed with fragment analysis or store at –20°C until ready.

Optional: If needed, reactions can be purified prior to fragment analysis. For this we recommend the Monarch® PCR & DNA Cleanup Kit (5 µg) (NEB #T1030) .

Figure 2: Example of Mutation Detection on targeted 293 cells.

Genomic DNA was isolated from HEK 293 cells using Epicentre QuickExtract DNA extraction solution. Cells were either untreated (neg control) or transfected with Cas9 and guide RNA. Genomic DNA was amplified using Q5 Hot Start High-Fidelity Master Mix and denatured/annealed and digested with T7 Endonuclease I (T7E1) according to the recommended protocol. Lane 1: NEB PCR Marker (NEB #N3234), Lane 2: untreated genomic DNA, Lane 3: untreated genomic DNA digested with T7E1, Lane 4: DNA transfected with Cas9 and guide RNA, Lane 5: DNA transfected with

Cas9 and guide RNA and digested with T7E1. Note that heteroduplexes can sometimes be seen running above the parental band, as seen in undigested test sample (lane 4).

Analysis of DNA Fragments:

1. Gel Analysis

Add 4 µl of Gel Loading Dye, Purple (6X), no SDS (NEB #B7025) to the reaction and run on a 2% agarose gel stained with ethidium bromide. Run the included DNA ladder or an appropriate DNA size marker along side the sample for reference.

Alternatively, samples can be analyzed using a fragment analyzer (e.g.,Agilent Bioanalyzer or Advanced Analytical Technologies, Inc (AATI) Fragment Analyzer). For the Agilent Bioanalyzer, 1 µl of the reaction will not interfere when using the standard sensitivity Agilent DNA analysis kits. For the AATI Fragment Analyzer, 2 µl of the reaction can be used with the Standard Sensitivity NGS Fragment Analysis Kit (AATI Cat# DNF-473).

Digestion of the control amplicon yields fragments of ~200 bp and~400 bp in addition to the parental band.

2. Calculate the estimated % modification using the following formula:
% Modification = 100 x [1-(1-fraction cleaved)$^{1/2}$]

When calculating % modification for reactions with the control template where the starting material is known, the equation (100 x fraction cleaved) can be used.

Where fraction cleaved = concentration of digested products/
(concentration of digested products + concentration of undigested band)