EnGen® Mutation Detection Kit

NEB #E3321S

25 reactions

Version 4.0_4/25

Table of Contents

The EnGen Mutation Detection Kit Includes:

All kit components should be stored at –20°C. Each kit contains sufficient reagents for 25 reactions. Additional PCR reagents are included to allow for optimization of the amplification reaction.

Q5® Hot Start High-Fidelity 2X Master Mix

NEBuffer 2

EnGen T7 Endonuclease I

Control Template and Primer Mix

Proteinase K

Quick-Load® Purple 1 kb Plus DNA Ladder

Gel Loading Dye, Purple (6X), no SDS

Required Materials Not Included:

Oligodeoxyribonucleotide primers for PCR

PCR reaction tubes or strips

Nuclease-free water

Thermocycler

Aerosol tips for PCR

Optional Materials:

Agarose gels for gel analysis

Fragment analyzer [i.e. Agilent Bioanalyzer®, Advanced Analytical Technologies, Inc. (AATI) Fragment Analyzer™]

Epicentre® QuickExtract™ DNA Extraction Solution (Epicentre Cat# QE09050)

DNAzol® Direct (Molecular Research Center, Inc. Cat# DN 131)

TE (10 mM Tris and 1 mM EDTA, adjusted to a pH of 8.0)

Gel image analysis software

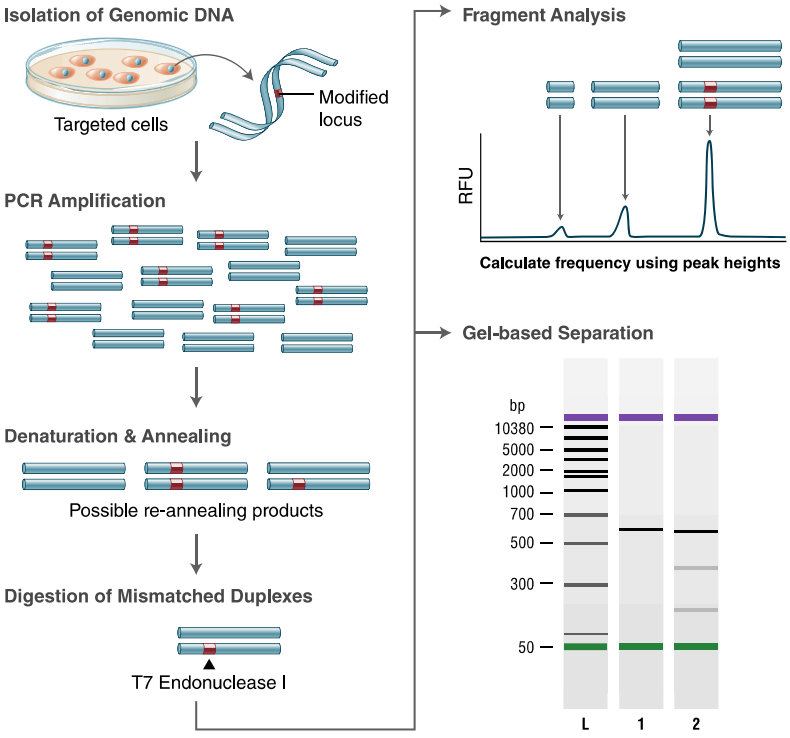
Introduction:

The EnGen Mutation Detection Kit provides reagents for detection of on-target genome editing events. In the first step, targeted regions from cells whose genomes were targeted (i.e., CRISPR/Cas9, TALENs, Zinc-finger Nucleases) are amplified using Q5 Hot Start High-Fidelity 2X Master Mix. Upon denaturation and re-annealing, heteroduplexes are formed when mutations from insertions and deletions (indels) are present in the amplicon pool. In the second step, annealed PCR products are digested with EnGen T7 Endonuclease I, a structure-specific enzyme that will recognize mismatches larger than 1 base. Both strands of the DNA are cut when a mismatch is present, which results in the formation of smaller fragments. Analysis of the resulting fragments provides an estimate of the efficiency of the genome editing experiments.

The EnGen Mutation Detection Kit includes a Control Template and Primer Mix that can be used as a control for the PCR reaction and T7 Endonuclease I digestion. The Control Template and Primer Mix provided contains two plasmids and primers that when amplified, denatured and re-annealed will form heteroduplexes that contain a 10-base insertion. This structure is a substrate for T7 Endonuclease I. The digestion of the 600 bp heteroduplex containing amplicon 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 or fragment analysis instrument.

The protocol has been optimized so that PCR products generated by the Q5 Hot Start High-Fidelity 2X Master Mix 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. Additional Q5 Hot Start High-Fidelity 2X Master Mix is also included to allow for optimization of target site amplification before digestion.

Figure 1: Workflow for Mutation Detection Kit



Genomic DNA is amplified with primers bracketing the modified locus. PCR products are then denatured and re-annealed yielding three classes of possible structures. Duplexes containing a mismatch greater than one base are digested by T7 Endonuclease I. The DNA is then electrophoretically separated and fragment analysis is used to estimate targeting efficiency.

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 it is important to test the PCR reaction and verify amplification before proceeding. Additional amplification reagents are provided to allow for optimization before proceeding. Crude extract prepared using QuickExtract (Epicentre Cat# QE09050) and DNAzol Direct (Molecular Research Center, Inc., Cat# DN 131) have been successfully tested with this kit.

Primer Design:

For best results, primers should be designed so that amplification products 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. For optimization of primer design, we recommend using the NEB Tm calculator at **Tmcalculator.neb.com**.

Protocols:

PCR – 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 REACTION	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	98°C	10 seconds	
Annealing	50-72°C*	10-30 seconds	35
Extension	72°C	20-30 seconds/kb	
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 REACTION	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	98°C	5 seconds	
Annealing	65°C	10 seconds	35
Extension	72°C	20 seconds/kb	
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	TIME	CYCLES
Initial Denaturation	98°C		5 minutes
Annealing	95–85°C	-2°C/ second	
	85–25°C	-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.

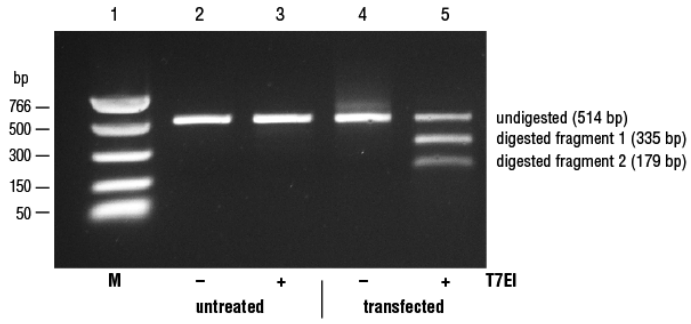
1. Set up each reaction as follows:

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)

Troubleshooting

PCR

- If no PCR product of the desired size is detected, perform the reaction using the Control Template and Primer Mix included with the kit. It may be necessary to redesign your primers and optimize the PCR reaction. Check the annealing temperature of your primers using the NEB Tm calculator at **Tmcalculator.neb.com**.
- Find more amplification troubleshooting information at the NEB tools and resources – PCR troubleshooting guide **www.neb.com/tools-and-resources/troubleshooting-guides/pcr-troubleshooting-guide**.

Control Reaction

- The Control Template and Primer Mix included with the kit contains two plasmids that will produce 600 bp amplicons with and without a 10 bp insertion. Denaturation and re-annealing of these PCR products will result in a mixture of products, including some heteroduplexes. T7 Endonuclease I will digest the heteroduplexes yielding fragments of ~200 bp and ~400 bp.

Cleavage Reaction

- If there are no cleavage products observed, it may be necessary to redesign primers such that the target site is further from the end of the amplicon. Be sure to perform the control reaction to verify performance of the enzymes. If too much PCR product was made during the PCR reaction, the resulting cleavage could be incomplete. Quantify the PCR product and be sure that no more than 250 ng is added to the heteroduplex digestion reaction.
- If low cleavage efficiencies are observed it may be necessary to redesign the conditions of your genome editing experiment to obtain the desired results.

Ordering Information

NEB #	PRODUCT	SIZE
E3321S	EnGen Mutation Detection Kit	25 rxns

COMPANION PRODUCTS

NEB #	PRODUCT	SIZE
M0386S	Cas9 Nuclease, *S. pyogenes*	70 pmol
M0386T/M	Cas9 Nuclease, *S. pyogenes*	400/2,000 pmol
B7025S	Gel Loading Dye, Purple (6X) no SDS	4 ml
N0550S/L	Quick-Load Purple 1 kb Plus DNA Ladder	1.25/3.75 ml
M0494S/L/X	Q5 Hot Start High-Fidelity 2X Master Mix	100/500/500 rxns
T1030S/L	Monarch PCR & DNA Cleanup Kit (5 µg)	50/250 preps
E2050S	HiScribe® T7 Quick High Yield RNA Synthesis Kit	50 rxns
E5520S	NEBuilder® HiFi DNA Assembly Cloning Kit	10 rxns
E2621S/L/X	NEBuilder HiFi DNA Assembly Master Mix	10/50/250 rxns

Revision History

REVISION #	DESCRIPTION	DATE
1.0	N/A	3/16
2.0		6/18
3.0	New Format applied	7/20
4.0	Update protocol. Update formatting and legal text.	4/25