



EnGen Mutation Detection Kit (NEB E3321S)

Materials:

- gDNA from Gene-edited cells (Cas-CLOVER)
- gDNA from negative control cells (NTC or eGFP)
- NEB EnGen Mutation Detection Kit (E3321S)
 - Q5 Hot Start High-Fidelity 2x Master Mix
 - Positive Control Template and Primer Mix
 - Proteinase K, Molecular Grade
 - Quick-Load Purple 1 kb Plus DNA Ladder
 - Gel Loading Dye, Purple (6x), no SDS
 - EnGen T7 Endonuclease I
 - 10X NEBuffer 2

Sterile PCR tubes

PCR machine → Programs in method description

Axygen AxyPrep Mag PCR Clean-Up Beads (MAG-PCR-CL-5) or equivalent PCR clean up method

Magnetic PCR tube rack

70% Ethanol

Nuclease-free Water

Novex™ TBE Gels, 4-20%, 12-well (ThermoFisher, EC62252BOX)

TBE 10X Solution (RPI, T32020-1000.0)

Ethidium Bromide or SYBR Safe DNA gel stain

Agarose Gel, 2%

NEB Low Molecular Weight DNA Ladder (NEB N3233S)

Prior to target amplification, design primers across the DNA target site.

- 650bp to 750bp amplicon size
- Recommend staggering primers so that bands will be distinguishable from one another (i.e. 450bp and 300bp)
- Primers should always be at least 150bp away from the target site
- Tm of primers should be 55°C to 65°C
- Standard GC requirements

1. Thaw kit components, mix and pulse-spin each component prior to use and keep on ice
2. Set up a 25 uL PCR reaction using 200 ng of genomic DNA as a template for each sample. Assemble as follows:

Reagent	25 uL Total Volume	Reaction Final Concentration
Q5 Hot Start High-Fidelity 2x Master Mix	12.5 uL	1X
10 uM Forward Primer	1.25 uL	0.5 uM
10 uM Reverse Primer	1.25 uL	0.5 uM
Template DNA	variable	200 ng genomic DNA
Nuclease-Free Water	Up to 25 uL	

*** Q5 should be the last component added**

3. Gently mix the reaction and briefly spin reaction. Transfer PCR tubes to a PCR machine and begin the following thermocycler program:

Cycle Step	Temperature	Time	Cycles
	98°C	2 minutes	1



Denaturation	98°C	5 seconds	35
Annealing	50-72°C	15 seconds	
Extension	72°C	20 seconds	
Final Extension	72°C	2 minutes	1
Hold	4°C		

Control reaction:

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

Reagent	25 uL Total Volume	Reaction Final Concentration
Q5 Hot Start High-Fidelity 2x Master Mix	12.5 uL	1X
Control Template and Primer Mix	2.5 uL	0.5 ng plasmid and 0.5 uM each primer
Nuclease-free water	10 uL	

*** Q5 should be the last component added**

2. Gently mix the reaction and briefly spin reaction. Transfer PCR tubes to a PCR machine and begin the thermocycler program as described above with the exception of the annealing temperature set to 63°C.

Analysis of amplicons:

1. Analyze 5 uL of the PCR reaction on a 2% agarose gel to verify amplification of a single product of the correct size. The control PCR should yield a 600 bp amplicon.

PCR Clean Up

1. Axyprep Mag beads should be mixed by vortexing briefly. With the remaining 20 uL of each PCR reaction, add 40 uL of Axyprep Beads.
2. Mix reaction by pulse vortexing and briefly spin components (2-3 seconds)
3. Incubate at room temperature for 5 minutes in a PCR tube rack
4. Place tubes on a magnetic PCR rack and incubate for 5 minutes
5. Aspirate supernatant without disturbing pellet
6. Wash pellet with 100 uL 70% ethanol and incubate for 3 minutes – still on magnetic rack
7. Aspirate supernatant and repeat wash step
8. Aspirate supernatant very thoroughly and dry pellet for 3 minutes
 - a. This step is important so that no residual ethanol remains
 - b. Do not over dry the beads as this will result in loss of DNA recovery
9. Elute DNA with 12 uL nuclease-free water, mix by vortexing PCR tubes and briefly spin components (2-3 seconds)
10. Incubate at room temperature for 5 minutes
11. Place tubes on magnetic rack for 5 minutes
12. Transfer supernatant to new PCR tubes and take the concentration of cleaned PCR product

Heteroduplex Formation



* For the heteroduplex formation, DNA is normalized to 200 ng. 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. The T7 Endonuclease I digestion as been optimized for reactions containing up to 250 ng of amplified DNA.

1. Assemble the reaction as follows:

Reagent	19 uL Annealing Reaction
Cleaned PCR product	200 ng (variable)
10X NEBuffer 2	2 uL
Nuclease-free water	Up to 19 uL

*Recommend adding 200 ng Cleaned DNA and up to 17 uL nuclease-free water, then adding 2 uL 10X NEBuffer 2 lastly making a total of 19 uL reaction.

Heteroduplex formation Continued:

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

Cycle Step	Temp	Ramp Rate and Cycles	Time
Initial Denaturation	95°C		10 minutes
Annealing	95°C	-2°C/cycle, 5 cycles	
	85°C	1 minute then -0.3°C/cycle, 34 cycles	
	75°C	1 minute then -0.3°C/cycle, 34 cycles	
	65°C	1 minute then -0.3°C/cycle, 34 cycles	
	55°C	1 minute then -0.3°C/cycle, 34 cycles	
	45°C	1 minute then -0.3°C/cycle, 34 cycles	
	35°C	1 minute then -0.3°C/cycle, 34 cycles	
	25°C	1 minute then -0.3°C/cycle, 34 cycles	
Hold	4°C		

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

3. Proceed to heteroduplex digestion.

Heteroduplex Digestion:



1. Add 1 uL EnGen T7 Endonuclease I directly to 19 uL heteroduplex reaction from previous step
2. Mix well and briefly spin. Incubate at 37°C for 15 minutes
3. Following digestion, add 1 uL 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.

Fragment Analysis:

1. Add 4 uL Gel Loading Dye, Purple (6X), no SDS) to the reaction and mix well.
2. Briefly spin reaction (~25 uL)
3. We recommend running these samples on a Novex™ 4-20% TBE Gel for the most definitive analysis of gene editing efficiencies.
 - Prepare pre-made gel by removing the gel from the packaging, remove white tape from bottom of gel cassette, and carefully remove the well comb from cassette.
 - There may be residual TBE gel material after the removal of the comb which may impact loading and running of the samples. Pay close attention and remove any leftover gel material.
 - Rinse wells with ddH₂O
4. Load 4 uL NEB Low Molecular DNA ladder into the first well
5. Proceed by loading 12 uL (half of the sample) of each sample into each well
 - a. Recommend separating the non-transfected and transfected samples from the positive control of the EnGen Mutation Detection Kit with another lane for the Low Molecular weight DNA ladder (see example below).
6. Run TBE gel at 120V until dye front has reached the bottom of the gel (~90 minutes)
7. Fill two small glass dishes or trays with water or enough to cover the entire surface of the container.
8. Add 10uL Ethidium Bromide to the first container which will be used to stain the TBE gel.
9. Open the cassette with a gel prying tool and cut the top wells off the gel as well as the lowest portion of the TBE gel with a razor blade (See figure below).

Fragment Analysis Continued:

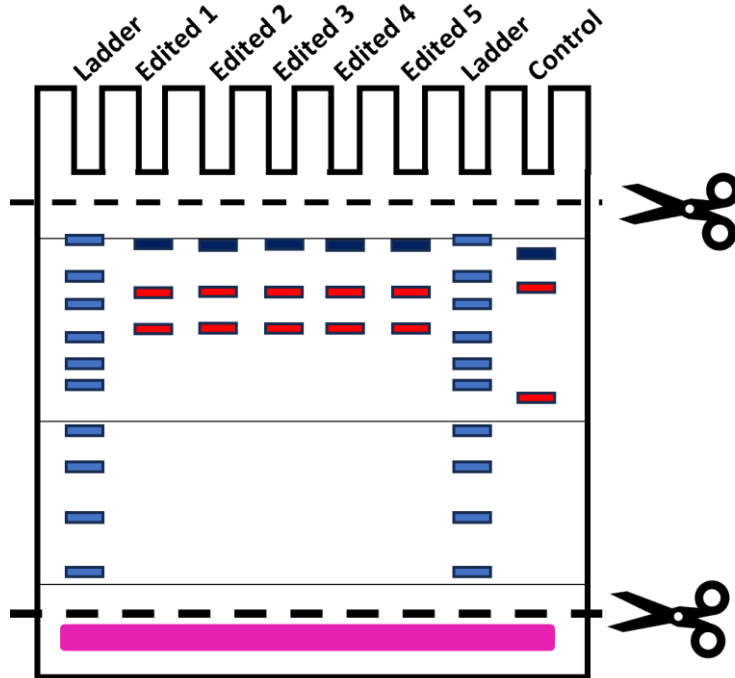
10. Carefully transfer the gel to the first container and agitate the gel in the staining solution for 1 minute MAX.
11. Carefully transfer the stained gel to the second container to wash the gel for 1 minute
12. Image the gel with a standard UV imager and save the image
13. Perform densitometry to calculate the % editing of each sample

Alternatively, samples can be run on a 2% agarose gel with ethidium bromide or SYBR safe. The digestion of the control amplicon yields fragments of ~200 bp and ~400 bp in addition to the 600 bp parental band.

To calculate the estimated % editing use the formula:

$$\% \text{ Modification} = 100 \times [1 - (1 - \text{fraction cleaved})^{1/2}]$$

Demeetra



Dark blue band is the non-edited amplicon. Red bands are the edited portion produced by T7 digestion. Pink area is the dye front.