

Microfluidics

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Determining Efficiency of On-Target CRISPR/Cas9 Genome Editing Using the NEB® EnGen™ Mutation Detection Kit on LabChip GX Touch Technology

Introduction

The simplicity and elegance of the CRISPR/Cas 9 system allows researchers to target and edit specific genes in a precise and directed fashion. Its function and biology was first discovered in select Bacteria and Archaea;

providing them adaptive immunity against foreign genetic material. This transformative technology has been adapted and engineered to enable researchers to cut genomic host DNA at targeted regions. The exacting precision enables knockout of existing genes, as well as the ability to add new DNA to the host genome.

After the editing experiment, researchers will need to verify that modification of the target locus has occurred. New England BioLabs®, Inc. (NEB®) has developed a T7 Endonuclease I – based mutation detection system to estimate targeted editing efficiency. In combination with this assay, the LabChip® GX Touch instrument was used to visualize the T7 Endonuclease I digestion products. The readout was used to estimate the on-target efficiency of the genome editing events.

Materials and Methods

The NEB® EnGen™ Mutation Detection kit (NEB# E3321) workflow is described in Figure 1. To demonstrate proof of concept, the supplied DNA positive control was used to provide a template for T7 Endonuclease I digestion. The control template consists of two similar plasmids which differ by a 10 bp insert. The resulting PCR products when denatured and allowed to re-anneal through a controlled slow temperature decline; will form heteroduplexes as a result of the two different amplicons annealing. These annealing products are subjected to digestion by T7E1. Only those that form heteroduplexes will be digested with the resulting fragments representing the edited fraction. Post digestion, the digestions were evaluated for editing efficiency on the LabChip GX Touch instrument where visualization of intact homoduplexes and heteroduplex digestion products can be easily distinguished.

Results

Amplified PCR products from the edited control template are shown in Figure 2. In this study, the expected base pair lengths for the control PCR reaction is 612 and 602 bp, with or without a 10 bp insertion respectively. The correct length denotes that the primer pairs properly annealed and amplified the edited region. The LabChip DNA 12K assay was utilized to visualize and quantitate the resulting PCR products because the heteroduplex fragment tends to run larger in size with higher resolution assays, perhaps due to unknown secondary structures. This result is not representative of a pool of amplicons from a gene editing experiment.

Table 1. Average Quantitation of parental fragments. Concentrations were reported within the LabChip assay results (n=3).

	Homoduplex 607 bp Fragment	Heteroduplex 754/819 bp Fragment
Average (ng/μL)	17.16	3.94 / 3.97

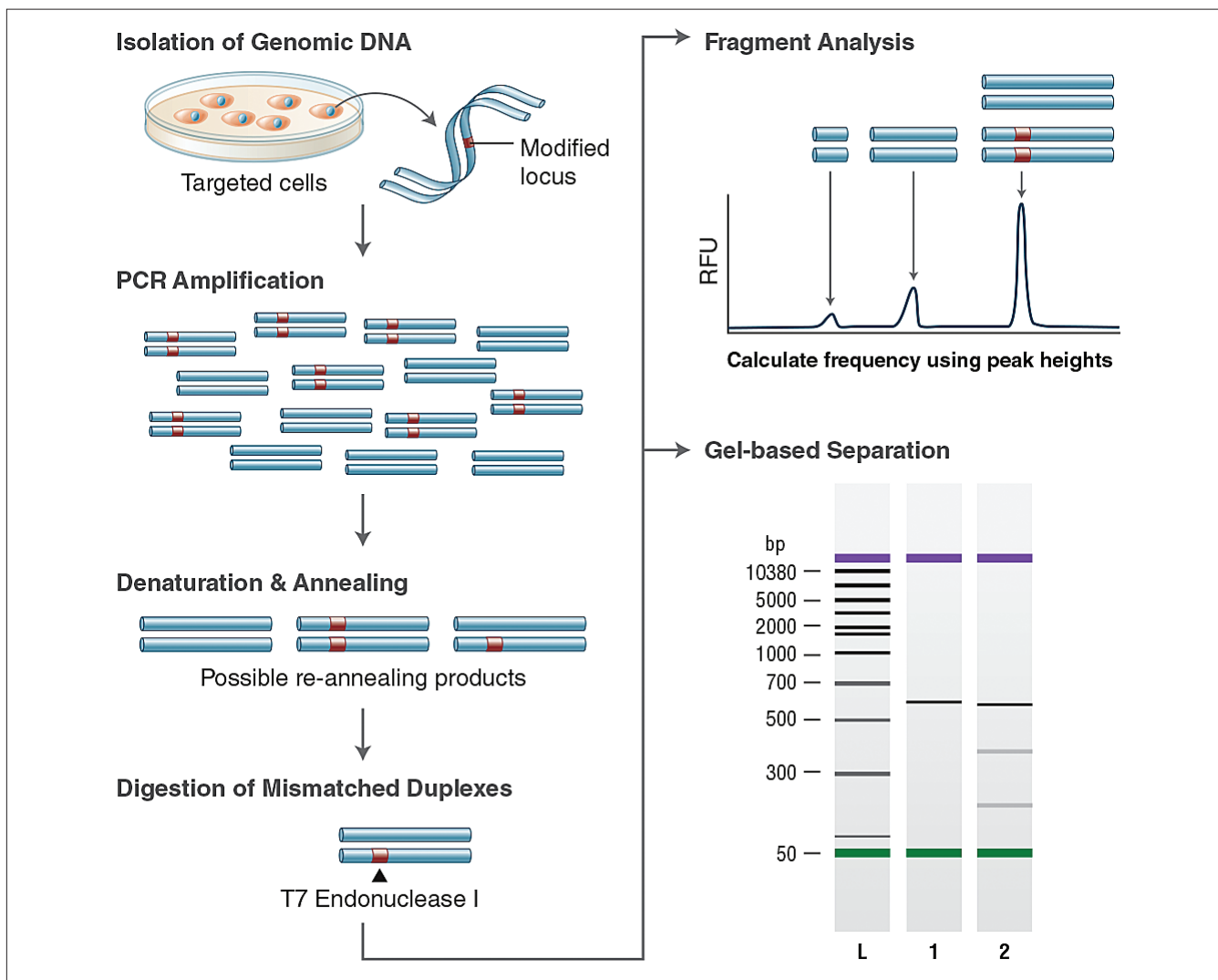


Figure 1. Genomic DNA is amplified with primers bracketing the modified locus. PCR products are then denatured and re-annealed yielding three classes of possible structures. Duplexes containing a mismatch greater than one base are digested by T7 Endonuclease I. The DNA is then electrophoretically separated and fragment analysis is used to estimate targeting efficiency. "Reprinted from www.neb.com (2017) with permission from New England Biolabs, Inc."

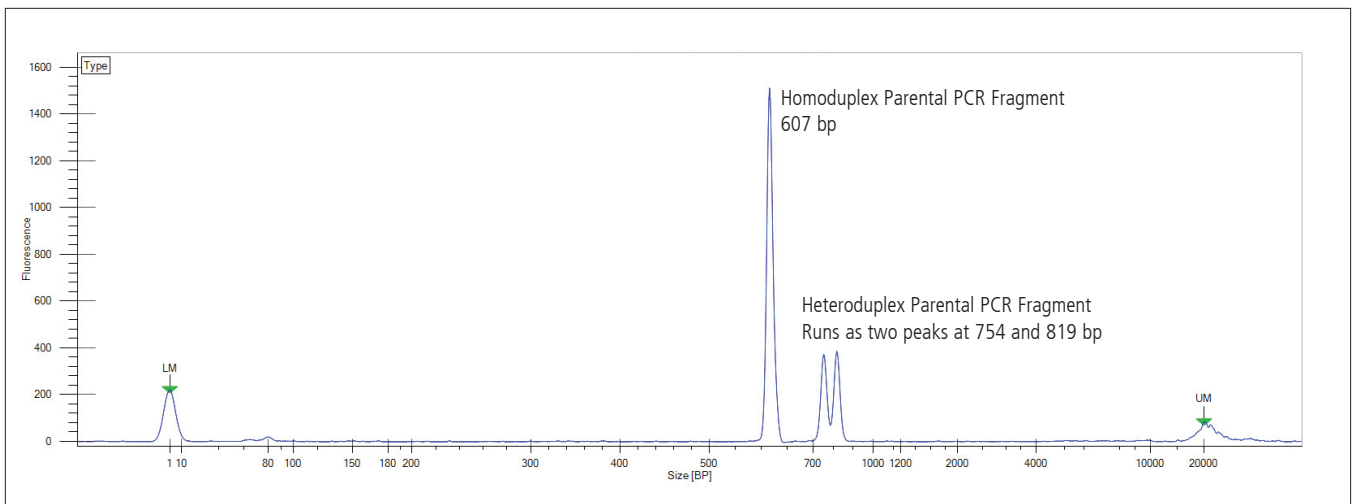


Figure 2. Electropherogram of the amplified control template on the LabChip GX Touch system using the LabChip 12K DNA Assay. The homoduplex wildtype and heteroduplex PCR products verifies proper size amplification based on the NEB protocol. LM = Lower marker. UM = Upper marker.

Verification of on-target gene editing is determined by allowing the PCR products to form heteroduplex complexes through controlled denaturation and re-annealing PCR cycles. Heteroduplex products are formed when mutations from insertion and deletion events are present in the amplicon pool. Subsequent digestion with T7E1 (provided within the kit) is a structure-specific enzyme that will recognize mismatched nucleotide sequences greater than one base. Both strands of the DNA are cut by the enzyme when a heteroduplex is present, resulting in the formation of smaller fragments. Analysis of the resulting fragments on the LabChip GX system (Figure 3 and Table 2) provides an estimate for the efficiency of the genome editing experiments, as well as fragment quantitation. The digestion of this heteroduplex-containing

amplicon yields two bifurcated fragment products of ~200 and ~400 bp, while the 607 bp parental homoduplexes are left uncleaved. The calculated target efficiency ($100 \times \frac{\text{digested [conc.]}}{\text{digested [conc.] + \text{undigested [conc.]}}$) for the control reaction was 19%.

Table 2. Average Quantitation of digested and undigested fragments post T7E1 digestion. Concentrations were reported within the LabChip GX assay results (n=3).

	Heteroduplex Fragment 1	Heteroduplex Fragment 2	Homoduplex 607 bp Fragment
Average (ng/μL)	0.40	1.96	10.16

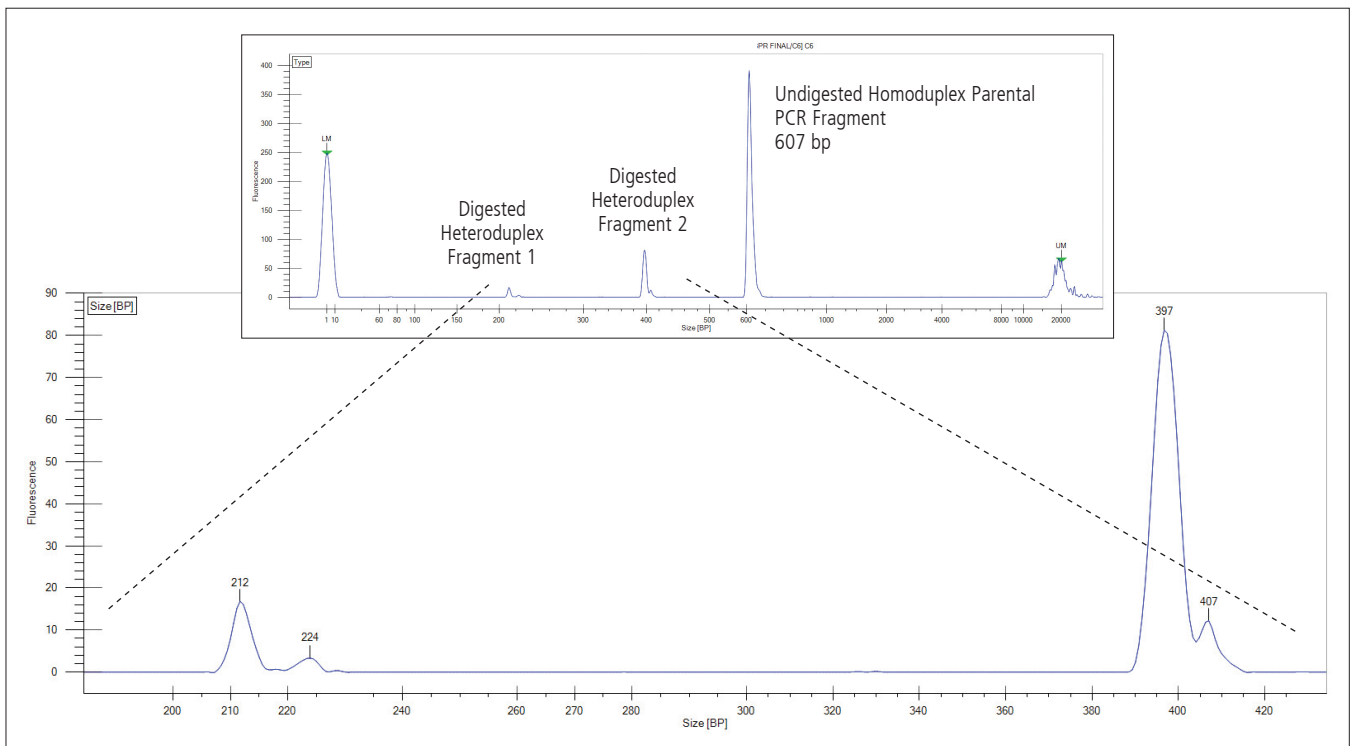


Figure 3. Electropherogram analysis of heteroduplex PCR digestion with T7E1. The digestion of the heteroduplex containing amplicons yield fragment combinations of 212 and 397 bp and 224 bp and 407 bp, while the un-augmented homoduplexes PCR parental products (607 bp) are left uncleaved. Results were visualized using the LabChip 12K DNA assay.

Conclusion

Here, we demonstrated the accurate sizing and quantitation of the control DNA reaction provided with the NEB® EnGen™ Mutation Detection Kit, illustrating that this method can be used for interpretation of genome editing experiments. The LabChip instrument provides both visual inspection through digitally represented electropherograms and tabular quantitative data to effectively determine on-target efficiencies from the targeted editing events. The diverse application versatility and experimental simplicity of the CRISPR/Cas9 system continues to help drive its acceptance within research, prompting new questions and widening the door for discovery.

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