

Alt-R™ Genome Editing Detection Kit

Kit component*	25 rxn kit	100 rxn kit	1000 rxn kit
T7 Endonuclease I (T7EI, 1 U/μL)	50 μL	200 μL	2000 μL
T7EI Reaction Buffer (10X)	50 μL	200 μL	2000 μL
Alt-R™ Control A (template/primer mix)	20 μL	20 μL	20 μL
Alt-R™ Control B (template/primer mix)	20 μL	20 μL	20 μL

*PCR master mix and target-specific primers/probe sets are not included in the kits.

Kit storage conditions: –20°C.

Use the Alt-R Genome Editing Detection Kit to determine on-target genome editing and estimate editing efficiency using T7 endonuclease I (T7EI). In the T7EI assay, target genomic regions from CRISPR-modified cells are amplified by PCR. The PCR products are denatured and reannealed to allow heteroduplex formation between wild-type DNA and CRISPR–mutated DNA. Mutations are then detected using T7EI, which recognizes and cleaves mismatched DNA heteroduplexes. T7EI assay results are analyzed by visualizing cleavage products and full-length amplicons by gel or capillary electrophoresis.

For a quick, robust assessment of editing events in CRISPR experiments, we recommend using the T7EI assay, instead of alternative methods such as Surveyor® mismatch endonuclease assays or Sanger sequencing. The T7EI method is simple, fast, and provides clean electrophoresis results. The T7EI assay is also compatible with a broad range of PCR buffers and does not usually require purification of PCR products before digestion. Note that T7EI activity is sensitive to the DNA:enzyme ratio, as well as incubation temperature and time [1]. T7EI recognizes insertions and deletions of ≥2 bases that are generated by non-homologous end joining (NHEJ) activity in CRISPR experiments [2]. Because T7EI does not recognize 1 bp indels, T7EI underrepresents the total editing.

Design PCR primers that amplify your experimental target site and adjacent sequence. We recommend using a 600–1000 bp PCR amplicon with >100 bp flanking the CRISPR cut site and with the CRISPR cut site off-center to allow fragment resolution by gel analysis or capillary electrophoresis. Validate the PCR assay to determine the optimal annealing temperature to use with your samples and to verify that only the expected PCR product is synthesized. You can design the PCR assays using the PrimerQuest® Tool at www.idtdna.com/PrimerQuest. Calculate the T_m of your primers at www.idtdna.com/OligoAnalyzer.

Required materials	Ordering information*
Alt-R™ Genome Editing Detection Kit	IDT (Cat # 1075931, 1075932, 1075933)
KAPA HiFi HotStart PCR Kit	Kapa Biosystems (Cat # KK2501)
Nuclease-Free Water	IDT (Cat # 11-04-02-01)
PCR primers specific for your target	IDT Custom PrimeTime® PCR Primers (www.idtdna.com/PrimerQuest)
Phosphate buffered saline (PBS)	General laboratory supplier
QuickExtract™ DNA Extraction Solution	Epicentre (Cat # QE09050)
(Option 1) Agarose	General laboratory supplier
(Option 2) Mutation Discovery Kit†	Advanced Analytical Technologies, Inc. (Cat # DNF-910-K1000T)

* These are suggested sources for reagents used by the IDT R&D team when this protocol was written. Individual components (e.g., the polymerase and buffer from the PCR kit) may be substituted with some optimization.

† For use on a Fragment Analyzer™ system (Advanced Analytical Technologies, Inc.)

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A. Process CRISPR-Cas9–edited genomic DNA from cultured cells.

The volumes in **Steps A2** and **A5** are optimized for confluent samples in 96-well plates. Some cell types and samples in larger wells will require larger volumes.

1. Wash CRISPR-Cas9–treated cells with 100 μL of PBS.
2. Lyse cells by adding 50 μL of QuickExtract™ DNA Extraction Solution.
3. Transfer cell lysate to appropriate PCR tubes or plate.
4. Vortex and heat in a thermal cycler at 65°C for 10 min, followed by 98°C for 5 min.
5. Add 100 μL of Nuclease-Free Water to dilute the genomic DNA.
6. Vortex and spin down.

B. Amplify genomic DNA and detect mutations.

1. Set up the PCR using template, primers, and components of the Alt-R Genome Editing Detection Kit and KAPA HiFi HotStart PCR Kit as follows:

Component	Amount: sample	Amount: Alt-R™ Control A	Amount: Alt-R™ Control B
Genomic DNA (Step A6)	4 μL (~40 ng)	—	—
Forward primer	300 nM	—	—
Reverse primer	300 nM	—	—
Alt-R™ Control A (template/primer mix)	—	2 μL	—
Alt-R™ Control B (template/primer mix)	—	—	2 μL
KAPA HiFi Fidelity Buffer (5X)*	5 μL (1X)	5 μL (1X)	5 μL (1X)
dNTPs	1.2 mM (0.3 mM each)	1.2 mM (0.3 mM each)	1.2 mM (0.3 mM each)
KAPA HiFi HotStart DNA Polymerase (1 U/ μL)*	0.5 U	0.5 U	0.5 U
Total volume	25 μL	25 μL	25 μL

* Note: the reagents in the Alt-R Genome Editing Detection Kit are also compatible with other polymerases and buffers.

2. Run the PCR using the following cycling conditions:

Step	Temperature (°C)	Time (min:sec)	Cycles
Denature	95	5:00	1
Denature	98	0:20	
Anneal	Variable (primer specific)* 64–67 for Alt-R™ Control A and B	0:15	30
Extend	72	0:30	
Extend	72	2:00	1

*Optimal annealing temperatures have been determined using KAPA HiFi HotStart DNA Polymerase. You may need to optimize for other polymerases. Annealing temperature for additional Alt-R controls: Alt-R CRISPR-Cas9 *HPRT* Primers, Human and Mouse = 67°C and Alt-R CRISPR-Cas9 *HPRT* Primers, Rat = 64°C.

C. Form heteroduplexes for T7EI digestion.

- Combine the following in an appropriate PCR tube:

Component	Amount: sample	Amount: Homoduplex control*	Amount: Heteroduplex control*
PCR (from Step B2)	10 μ L experimental target or Alt-R™ HPRT control	10 μ L Control A	5 μ L Control A 5 μ L Control B
T7EI Reaction Buffer (10X)	2 μ L	2 μ L	2 μ L
Nuclease-Free Water	6 μ L	6 μ L	6 μ L
Total volume	18 μL	18 μL	18 μL

* Alt-R Controls A and B are positive controls that each contain template and PCR primers for monitoring the function of the T7EI assay. Full length PCR products from Controls A and B contain the same sequences, except for a 6 bp deletion in Control B.

- Heat and cool PCR products in a thermal cycler as follows:

Step	Temperature (°C)	Time
Denature	95	10 min
Ramp 1	95–85	Ramp rate $-2^{\circ}\text{C}/\text{sec}$
Ramp 2	85–25	Ramp rate $-0.3^{\circ}\text{C}/\text{sec}$

- Combine the following in a microcentrifuge tube for the T7EI digestion:

Component	Amount (μ L)
PCR heteroduplexes (from Step C2)	18
T7 endonuclease I (1 U/ μ L)	2
Total volume	20

- Incubate the T7EI reaction at 37°C for 60 min.

D. Visualize T7EI mismatch detection results.

Visualize the digestion using one of the following methods:

- Use agarose gels.
- Dilute digestion with 150 μ L of 0.1X IDTE, and run on a Fragment Analyzer™ system with the Mutation Discovery Kit. See Figure 1 for representative results.

The expected amplicon and digested product sizes for Alt-R Controls A/B and the Alt-R CRISPR-Cas9 HPRT Positive Controls are listed in Figures 1 and 2, respectively.

References

- Mean RJ, Pierides A, Pollet N. (2004) Modification of the enzyme mismatch cleavage method using T7 endonuclease I and silver staining. *Biotechniques*, 36(5):758–760.
- Vouillot L, Thélie A, et al. (2015) Comparison of T7EI and Surveyor mismatch cleavage assays to detect mutations triggered by engineered nucleases. *G3: Genes|Genomes|Genetics*, 5(3):407–415.

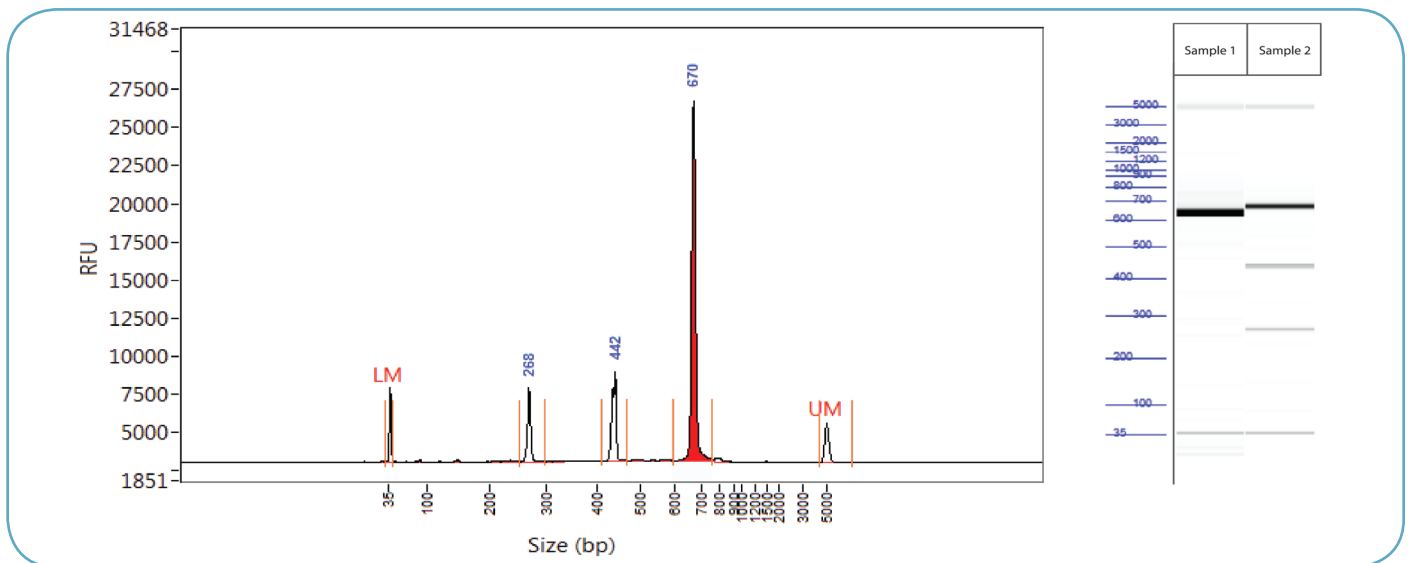


Figure 1. Sample data from Fragment Analyzer™ system—T7EI digestion containing PCR products from Alt-R™ Controls A and B. PCR using template and primers in Controls A and B (Alt-R Genome Editing Detection Kit) were run using KAPA HiFi HotStart DNA Polymerase (Kapa Biosystems). PCR cycling conditions (annealing temperature, 64°C), denaturation/reannealing conditions, and T7EI digestion conditions were as described in this protocol. Digestion reactions were analyzed on a Fragment Analyzer system (Advanced Analytical Technologies, Inc.). Sample 1 contains Control A PCR products, while Sample 2 contains Control A and B PCR products. Trace (left) shows results from Sample 2. Gel image (right) shows results for Samples 1 and 2. LM = lower marker; UM = upper marker

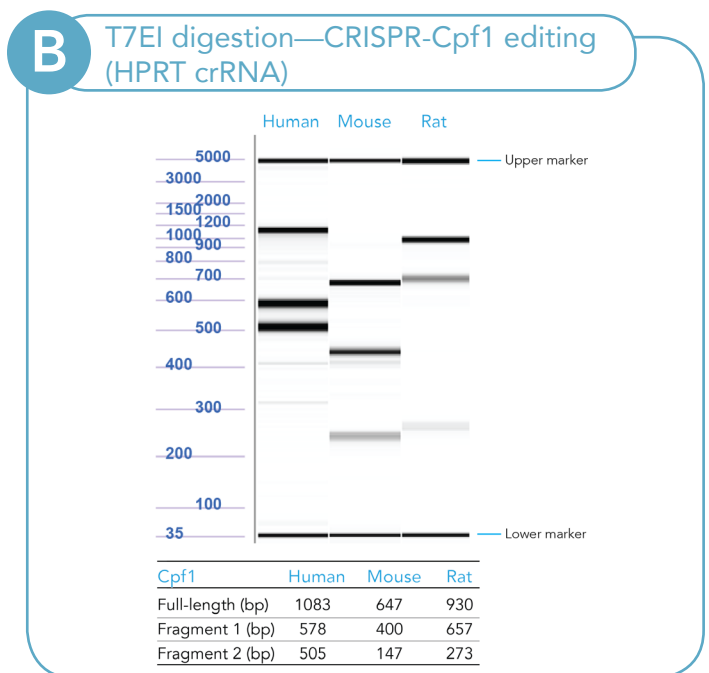
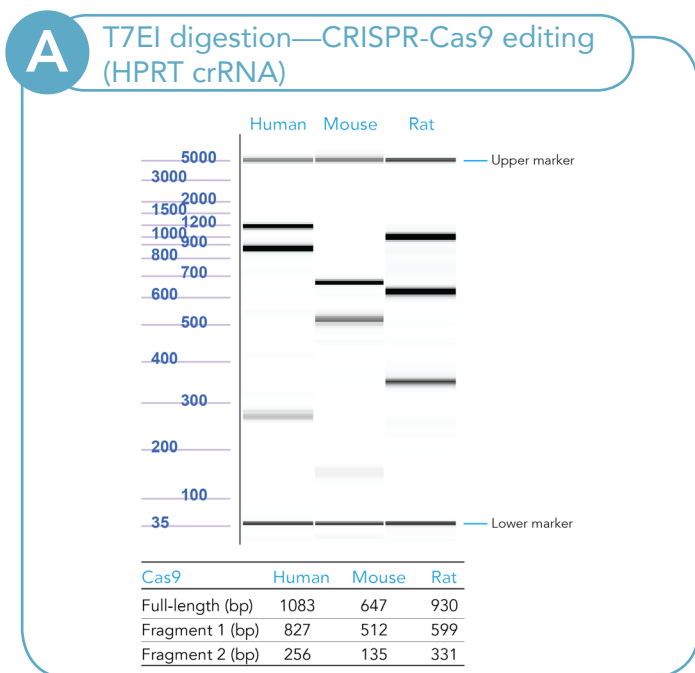


Figure 2. Sample data from T7EI digestion of Alt-R™ CRISPR HPRT control samples. Genomic DNA from CRISPR-Cas9 (A) or -Cpf1 (B) edited human, mouse, and rat HPRT controls were PCR amplified, digested using T7 endonuclease I, and run on the Fragment Analyzer™ system. Reference standard bands at 5000 bp (upper marker) and 35 bp (lower marker) are used to align the gel and analyze the results. Estimated band sizes for the cut and uncut positive control amplicons are listed in the tables. Cell lines: HEK-293 (human), Hepa1-6 (mouse), and RG2 (rat).

Technical support:

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