Alt-R™ Genome Editing Detection

REVISION HISTORY

Version	Release date	Description of changes
1.1	August 2023	Updated volume of control A for PCR amplification
1	November 2022	Original version

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Alt-R GENOME EDITING DETECTION KIT—CONSUMABLES

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.

Note: Store the Alt-R Genome Editing Kit at –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. Test 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.).

Go to www.idtdna.com for safety data sheets (SDSs) and certificates of analysis (COAs) for IDT products. Visit www.idtdna.com/protocols to verify that you are using the most current version of this protocol.

PROCESS CRISPR-Cas9-EDITED GENOMIC DNA FROM CULTURED CELLS

The volumes in Steps 2 and 5 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.

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 (Process CRISPR-Cas9–edited genomic DNA from cultured cells, step 6)	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**

* 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	30
Anneal	Variable (primer specific)* 64–67 for Alt-R™ Control A and B	0:15	
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.

FORM HETERODUPLEXES FOR T7EI DIGESTION

1. Combine the following in an appropriate PCR tube:

Component	Amount: Sample	Amount: Homoduplex control*	Amount: Heteroduplex control*
PCR (Amplify genomic DNA and Detect Mutations, step 2)	10 µL experimental target or Alt-R HPRT control	10 µL Control A	5 µL Control A 5 µL Control B
T7EI Reaction Buffer (10X)	2 µL	2 µL	2 µL
Nuclease-Free Water	6 µL	6 µL	6 µL
Total volume	**18 µL**	**18 µL**	**18 µL**

* Alt-R Controls A and B are positive controls for the T7E1 assay that each contain template and PCR primers. Full length PCR products from Controls A and B contain the same sequences, except for a 6 bp deletion in Control B.

2. 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°C/sec
Ramp 2	85–25	Ramp rate –0.3°C/sec

3. Combine the following in a microcentrifuge tube for the T7EI digestion:

Component	Amount (µL)
PCR heteroduplexes (Form heteroduplexes for T7EI digestion, step 2)	18
T7 endonuclease I (1 U/µL)	2
Total volume	**20**

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

VISUALIZE T7EI MISMATCH DETECTION RESULTS

Visualize the digestion using one of the following methods:

- Use agarose gels.
- Dilute digestion with 150 µL 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.

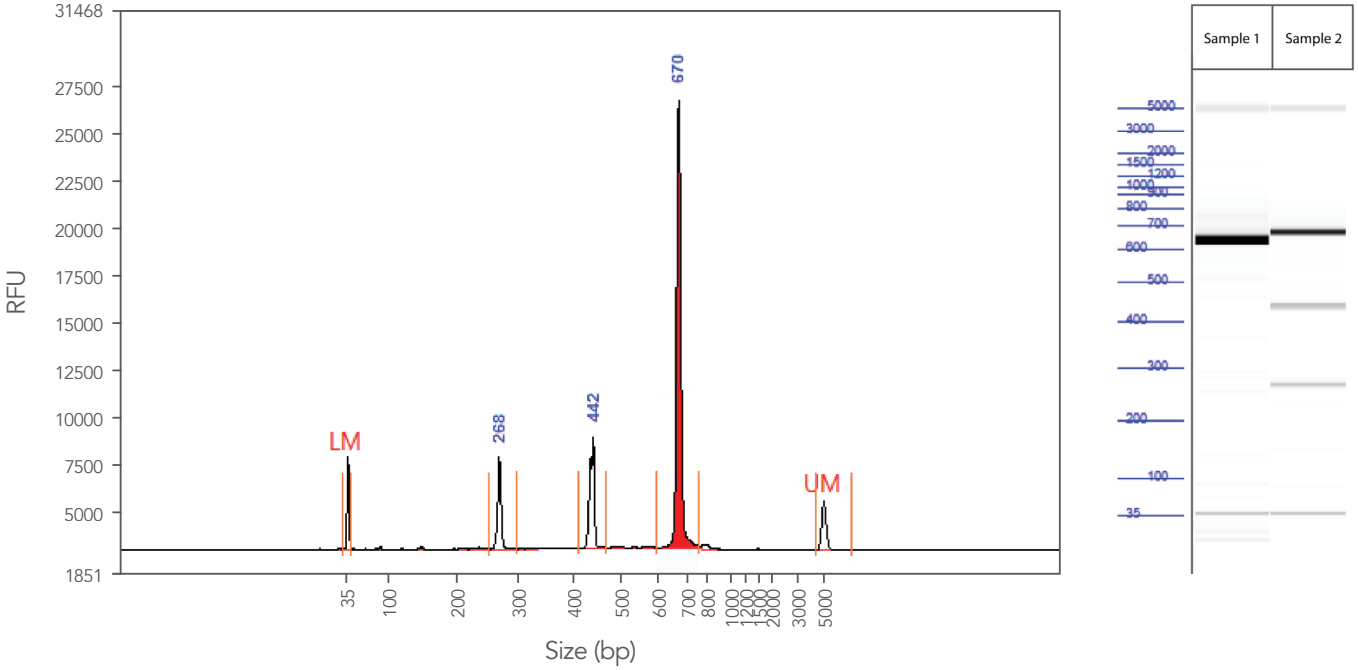
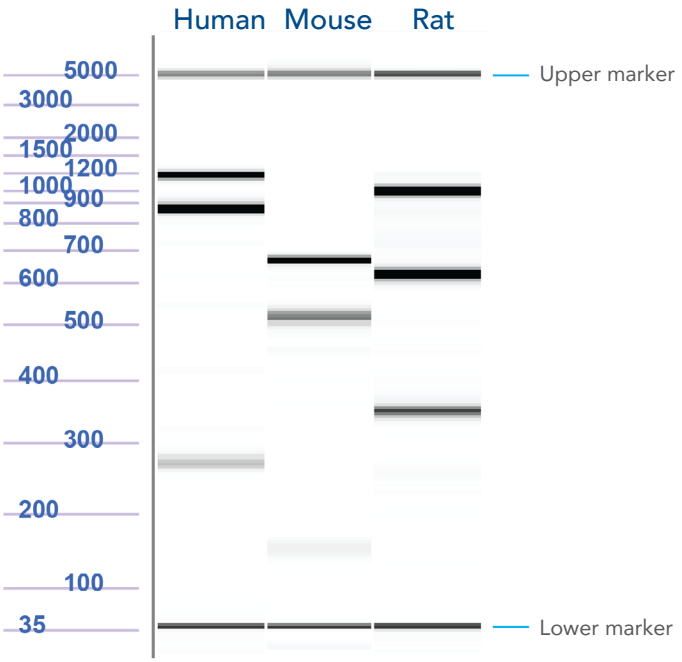


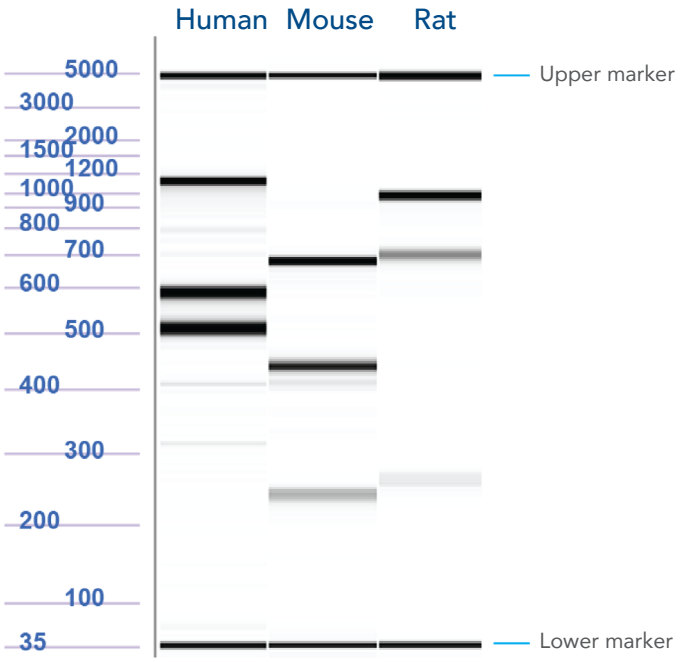
Figure 1. Sample data from Fragment Analyzer system—T7EI digestion containing PCR products from Alt-R Controls A and B. PCR using template and primers in Controls A and B (Alt-R Genome Editing Detection Kit) were run using KAPA HiFi HotStart DNA Polymerase (Kapa Biosystems). PCR cycling conditions (annealing 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. The trace (left) shows results from Sample 2. The gel image (right) shows results for Samples 1 and 2. RFU = relative fluorescence unit; LM = lower marker; UM = upper marker.

A. T7EI digestion—CRISPR-Cas9 editing (HPRT crRNA)



Cas9	Human	Mouse	Rat
Full-length (bp)	1083	647	930
Fragment 1 (bp)	827	512	599
Fragment 2 (bp)	256	135	331

B. T7EI digestion—CRISPR-Cas12a editing (HPRT crRNA)



Cas12a	Human	Mouse	Rat
Full-length (bp)	1083	647	930
Fragment 1 (bp)	578	400	657
Fragment 2 (bp)	505	147	273

Figure 2. Sample data from T7EI digestion of Alt-R CRISPR HPRT control samples. Genomic DNA from CRISPR-Cas9 (**A**) or Cas12a (**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).

REFERENCES

1. Mean RJ, Pierides A, Deltas CC, *et al*. Modification of the enzyme mismatch cleavage method using T7 endonuclease I and silver staining. *Biotechniques*. 2004;36(5):758-760.

2. Vouillot L, Thelie A, Pollet N. Comparison of T7EI and Surveyor mismatch cleavage assays to detect mutations triggered by engineered nucleases. *G3 (Bethesda)*. 2015;5(3):407-415.

Alt-R™ Genome Editing Detection Kit

Technical support: www.idtdna.com/ContactUs

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