

Alt-R CRISPR-Cas9 System:

In vitro cleavage of target DNA with ribonucleoprotein complex



See what more we can do for you at www.idtdna.com.





Introduction

This protocol describes how to use a Cas9 ribonucleoprotein (RNP) complex to enable *in vitro* cleavage of double-stranded, targeted DNA. The Cas9 RNP complex contains both an Alt-R CRISPR-Cas9 guide RNA (crRNA:tracrRNA duplex or sgRNA) and an *S. pyogenes* Cas9 endonuclease. This protocol demonstrates a method to experimentally validate the activity of CRISPR guide RNA before practical application.



Required materials

Reagents	Ordering information
Option 1, 2-part guide RNA (crRNA + tracrRNA): <ul style="list-style-type: none"> Alt-R CRISPR-Cas9 crRNA or Alt-R CRISPR-Cas9 crRNA XT Alt-R CRISPR-Cas9 tracrRNA or Alt-R CRISPR-Cas9 tracrRNA – ATTO™ 550 	IDT predesigned and custom crRNA: www.idtdna.com/CRISPR-Cas9 IDT (cat # 1072532, 1072533, 1072534) IDT (cat # 1075927, 1075928)
Option 2, single guide RNA (sgRNA): Alt-R CRISPR-Cas9 sgRNA	IDT predesigned and custom sgRNA: www.idtdna.com/CRISPR-Cas9
Alt-R <i>S.p.</i> Cas9 Nuclease V3†	IDT (cat # 1081058, 1081059)
Alternative: Alt-R <i>S.p.</i> HiFi Cas9 Nuclease V3	IDT (cat # 1081060, 1081061)
Nuclease-Free Duplex Buffer	IDT (cat # 11-05-01-12, various sizes available)
Nuclease-Free IDTE, pH 7.5 (1X TE solution)	IDT (cat # 11-01-02-02)
Nuclease-Free Water	IDT (cat # 11-04-02-01)
DNA substrate containing the target sequence	gBlocks Gene Fragments: www.idtdna.com/gBlocks , or similar
For 10X Cas9 Nuclease Reaction Buffer combine: <ul style="list-style-type: none"> 200 mM HEPES 1 M NaCl 50 mM MgCl₂ 1 mM EDTA, pH 6.5 at 25°C 	General laboratory supplier
PBS	
Alternative: For Cas9 Dilution Buffer combine: <ul style="list-style-type: none"> 30 mM HEPES 150 mM KCl, pH 7.5 	General laboratory supplier
Proteinase K (molecular biology grade)	General laboratory supplier



Protocol

A. Prepare the double-stranded DNA template as cleavage substrate

Design your template, considering the following:

- Multiple types of double-stranded DNA can be used as substrates for Cas9 cleavage. Three common examples:
 - linearized plasmid
 - purified PCR products
 - duplexed synthetic oligos
- Your template must contain a 20 nt guide sequence, followed by the Cas9 PAM site (NGG).
- The guide sequence should match the target-specific guide RNA that will be used in the digestion (**step D**).
- The amount of template needed for the digestion may vary depending on the detection method (**step E**) and the template size, as shown in this table:

DNA template	Length (bp)	Final concentration	Visualization method
DNA oligo duplex	30–100	2–5 μ M	PAGE
gBlocks fragment or PCR product	100–500	5–50 nM	Fragment Analyzer, agarose gel
gBlocks fragment or PCR product	500–2000	2–5 nM	Fragment Analyzer, agarose gel
Linearized plasmid	\geq 2000	1–2 nM	Fragment Analyzer, agarose gel

1. Ensure you are using a 10:1 molar ratio of Cas9 RNP:DNA substrate to obtain the best cleavage efficiency.
2. Resuspend or dilute the DNA substrate in Nuclease-Free Water to the required concentration.

Note: You can use the IDT resuspension calculator at www.idtdna.com/SciTools.

B. Prepare the guide RNA

1. Resuspend each RNA oligo (Alt-R CRISPR-Cas9 crRNA, tracrRNA, sgRNA) in IDTE buffer to a final concentration of 100 μM .
2. If you are using sgRNA, go to **step C** (Create the RNP complex).
3. Mix the crRNA and tracrRNA oligos in equimolar concentrations in a sterile microcentrifuge tube to a final duplex concentration of 10 μM . The following table shows an example of a 10 μL final volume duplex:

Component	Amount (μL)
100 μM Alt-R CRISPR-Cas9 crRNA	1
100 μM Alt-R CRISPR-Cas9 tracrRNA	1
Nuclease-Free Duplex Buffer	8
Total volume	10

4. Heat the duplex at 95°C for 5 min.
5. Remove from heat and allow to cool to room temperature (15–25°C).

C. Create the RNP complex

1. Combine the guide RNA and Cas9 enzyme in equimolar amounts.

Component	Amount (μL)
10 μM Alt-R guide RNA (crRNA:tracrRNA duplex from step B5 or sgRNA from step B2)	10
Alt-R S.p. Cas9 enzyme (62 μM stock)*	1.6
PBS†	88.4
Total volume	100

* All Alt-R S.p. Cas9 enzymes are provided at a stock concentration of 62 μM .

† Cas9 RNP complexes can be made in PBS or in Cas9 dilution buffer (30 mM HEPES, 150 mM KCl, pH 7.5).

2. Incubate at room temperature for 5–10 min for optimal formation of the RNP complex.

D. Perform the *in vitro* digestion reaction

1. Assemble the reaction at room temperature (15–25°C).

Component	Amount (μL)
10X Cas9 Nuclease Reaction Buffer	1
1 μM Cas9 RNP	1
100 nM DNA substrate	1
Nuclease-Free Water	7
Total volume	10

2. Incubate the reaction at 37°C for 60 min.
3. Add 1 μL Proteinase K (20 mg/mL) to the reaction, then incubate the mixture at 56°C for 10 min to release the DNA substrate from the Cas9 endonuclease.

E. Visualize cleaved products

Analyze the digestion by using one of the following methods:

- Agarose gel electrophoresis
- Fragment Analyzer™ System (Advanced Analytical), or similar

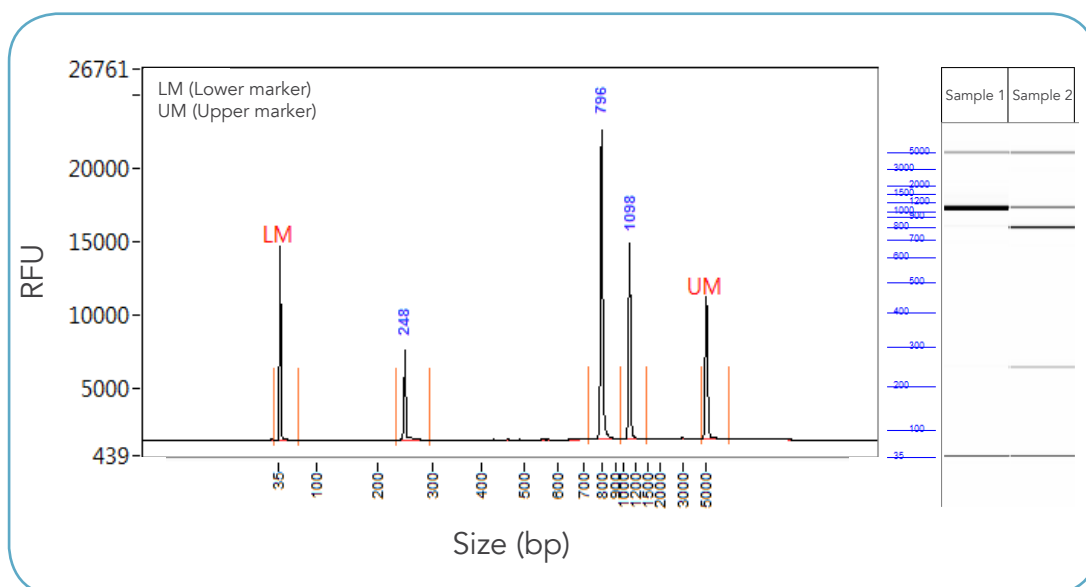


Figure 1. Sample data showing *in vitro* digestion reaction using Alt-R tracrRNA with Alt-R CRISPR-Cas9 Positive Control crRNA (HPRT). Column-purified PCR product consisting of the Hs *HPRT* crRNA positive control sequence was used as a template in a 10 μL *in vitro* Cas9 digestion reaction. Sample 1 contains template without RNP. Sample 2 contains template and RNP. Digestion reactions were analyzed on a Fragment Analyzer system and a gel imaging system. Trace (left) shows results from Sample 2. Gel image (right) shows results for Samples 1 and 2.



Revision history

Version	Date released	Description of changes
2	July 2018	Added instructions for using Alt-R CRISPR-Cas9 sgRNA. Updated names and catalog numbers for Alt-R enzymes (V3).
1.1	May 2018	Added note about use of improved Alt-R enzymes (V3): direct substitution in protocol of V3 enzymes for original enzymes (3NLS).
1	November 2017	Original protocol.

Alt-R CRISPR-Cas9 System:

In vitro cleavage of target DNA with RNP complex

Technical support:

applicationsupport@idtdna.com

For Research Use Only. Not for use in diagnostic procedures.

© 2018 Integrated DNA Technologies, Inc. All rights reserved. Alt-R and gBlocks are trademarks of Integrated DNA Technologies, Inc., and registered in the USA. ATTO is a trademark of ATTO-TEC GmbH. All other marks are the property of their respective owners. For specific trademark and licensing information, see www.idtdna.com/trademarks. CRS-10096-PR 07/2018

