Alt-R™ CRISPR-Cas9 System

In vitro cleavage of target DNA with ribonucleoprotein complex

REVISION HISTORY

Version	Release date	Description of changes
4	April 2022	Updated to include new products, updated text.
3	October 2021	Corrected concentrations of reagents, updated text.
2.2	August 2019	Corrected a component amount needed to create the RNP complex.
2.1	April 2019	Adjusted component amount needed to perform the *in vitro* digestion reaction from 100 nM to 50 nM DNA substrate.
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 publication

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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 confirm the activity of CRISPR guide RNA before practical application.

CONSUMABLES

Consumables—IDT

Item	Catalog #
Option 1, 2-part guide RNA (crRNA + tracrRNA):	
• Alt-R CRISPR-Cas9 crRNA or Alt-R CRISPR-Cas9 crRNA XT	IDT predesigned and custom crRNA (www.idtdna.com/CRISPR-Cas9)
• Alt-R CRISPR-Cas9 tracrRNA or Alt -R CRISPR-Cas9 tracrRNA – ATTO™ 550 or Alt-R CRISPR-Cas9 tracrRNA - ATTO 488 or Alt-R CRISPR-Cas9 tracrRNA - ATTO 647	1072532, 1072533, 1072534 cat # 1075927, 1075928 cat # 10007810 cat # 10007853
Option 2, single guide RNA (sgRNA): Alt-R CRISPR-Cas9 sgRNA	IDT predesigned and custom sgRNA: (www.idtdna.com/CRISPR-Cas9)
Alt-R *S.p.* Cas9 Nuclease V3	1081058, 1081059, 10000735
Alternatives: Alt-R *S.p.* HiFi Cas9 Nuclease V3 glycerol-free and GFP/RFP	1081060, 1081061, 10007803
Alt-R *S.p.* Cas9 V3, glycerol-free Alt-R *S.p.*Cas9-GFP V3 Alt-R *S.p.*Cas9-RFP V3	10007806, 10007807, 10007808 10008100, 10008161 10008162, 10008163
Nuclease-Free Duplex Buffer	11050112, various sizes available
Nuclease-Free IDTE, pH 7.5 (1X TE solution)	11010202
Nuclease-Free Water	11040201
DNA substrate containing the target sequence	gBlocks Gene Fragments (www.idtdna.com/gBlocks), or similar

Consumables—Other suppliers

Item	Supplier
For 10X Cas9 Nuclease Reaction Buffer combine: • 200 mM HEPES • 1 M NaCl • 50 mM MgCl2 • 1 mM EDTA, pH 6.5 at 25°C	Varies
PBS Alternative: For Cas9 Dilution Buffer, combine: • 30 mM HEPES • 150 mM KCl, pH 7.5	Varies
Proteinase K (Molecular biology grade)	Varies

PROTOCOL

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 are:
  - 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 Perform the *in vitro* digestion reaction.
- The amount of template needed for the digestion may vary depending on the detection method used to Visualize cleaved products and the template size, as shown in this table:

DNA template	Length (bp)	Final concentration	Visualization method
DNA oligo duplex	30–100	2–5 µM	PAGE
gBlocks™ Gene Fragment or PCR product	100–500	5–50 nM	Fragment Analyzer™ (Agilent), agarose gel
gBlocks™ Gene Fragment or PCR product	500–2000	2–5 nM	
Linearized plasmid	≥2000	1–2 nM	

1. Make sure 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: IDT provides a resuspension calculator at www.idtdna.com/SciTools.

Prepare the guide RNA

1. Resuspend each RNA oligo (Alt-R CRISPR-Cas9 crRNA, tracrRNA, sgRNA) in IDTE buffer to a stock concentration of 100 µM.
2. If you are using sgRNA, dilute the **100 µM** stock to a working concentration of 10 µM (1:10 dilution) in IDTE Buffer before proceeding to the next section: 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).

Create the RNP complex

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

Component	Amount (µL)
10 µM Alt-R guide RNA [From **Prepare the guide RNA**, step 2 (sgRNA), or step 5 (crRNA:tracrRNA)][1]	10
Alt-R *S.p.* Cas9 enzyme (62 µM stock)[2]	1.6
PBS[3]	88.4
Total volume	**100**

[1] If working with Cas9-GFP or Cas9-RFP, we recommend using a 1:1.2 ratio of Cas9:gRNA, instead of a 1:1 ratio. See the **Bio-Rad Gene Pulser® Xcell™ Electroporation System protocol** for reference.

[2] The Alt-R *S.p.* Cas9 enzyme is provided at a stock concentration of 62 µM (10 mg/mL). The Alt-R Cas9-GFP and Cas9-RFP enzymes are provided at 52 µM (10 mg/mL). Cas9 RNP complexes can be made in PBS or in Cas9 dilution buffer (30 mM HEPES, 150 mM KCl, pH 7.5).

[3] 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.

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.

Visualize cleaved products

Analyze the digestion by using one of the following methods:

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

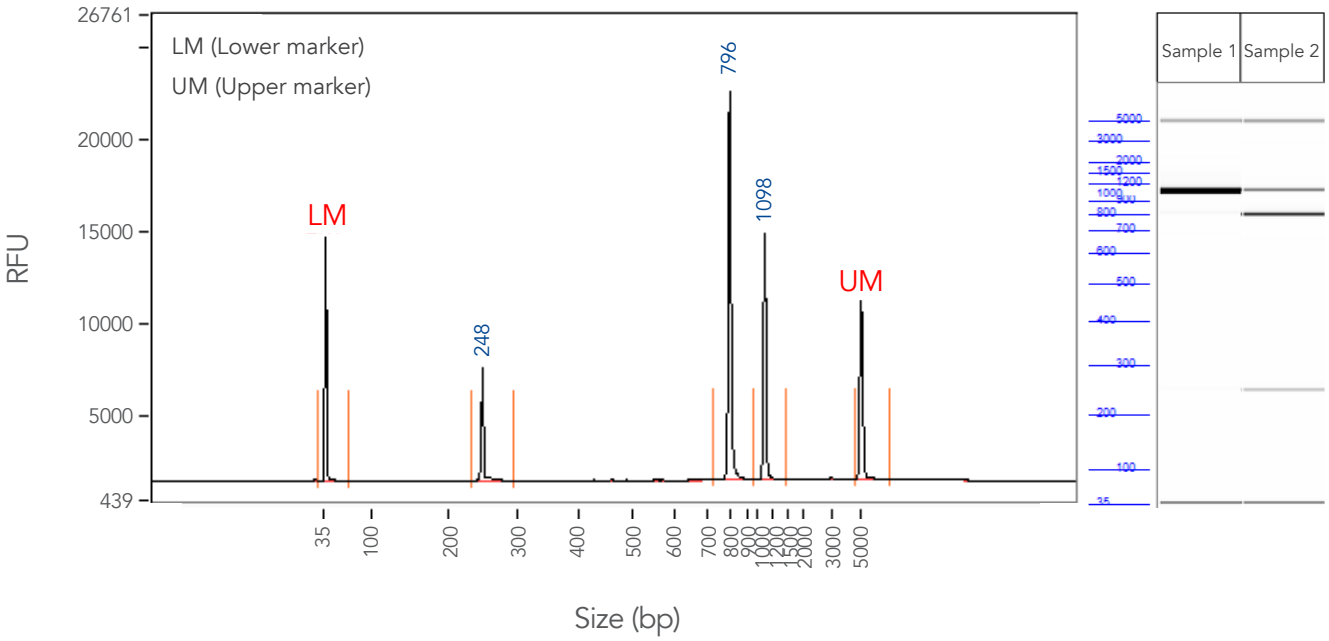

Figure 1. Sample data showing *in vitro* digestion reaction using Alt-R tracrRNA with Alt-R CRISPR-Cas9 Positive Control crRNA (HPRT). Column-purified PCR product consisting of the Hs HPRT crRNA positive control sequence was used as a template in a 10 µL *in vitro* Cas9 digestion reaction. Sample 1 contains 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.

Alt-R CRISPR-Cas9 System: *In vitro* cleavage of target DNA with RNP complex

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