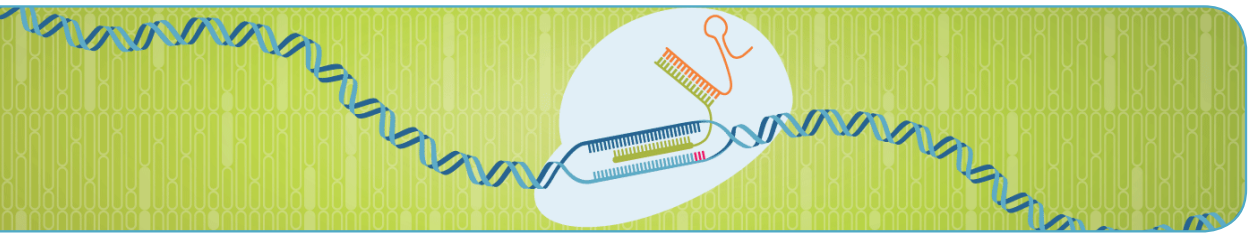
# Alt-R CRISPR-Cas9 System:

## Transfection of Cas9 plasmid and guide RNAs



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# Introduction

This protocol describes the delivery of the Alt-R S.p. Cas9 Expression Plasmid and Alt-R CRISPR-Cas9 guide RNA (crRNA:tracrRNA duplex or sgRNA) into cultured mammalian cells.

The Alt-R S.p. Cas9 Expression Plasmid, which is 7.3 kb, allows for easier transfection into mammalian cells compared to larger Cas9 expression plasmids or Cas9/sgRNA dual-expression plasmids. Go to www.idtdna.com/CRISPR-Cas9 (**Resources** section, **Application notes**), for tips on using fluorescently labeled tracrRNA (Alt-R Cas9 tracrRNA – 5' ATTO™ 550) to monitor transfection efficiency or to select for transfected cells via cell sorting [1].

Note that for most genome editing studies, we recommend delivery of CRISPR-Cas9 ribonucleoprotein (RNP) complexes, containing Alt-R CRISPR-Cas9 guide RNAs (gRNAs) and Cas9 enzymes. Alt-R CRISPR gRNAs are compatible with *S. pyogenes* Cas 9 from any source. Go to www.idtdna.com/CRISPR-Cas9 (**Resources** section, **User guides and protocols**) to find protocols for delivery of Alt-R RNP complexes by lipofection or electroporation. The **Alt-R CRISPR-Cas9 System: User guide for cationic lipid delivery of CRISPR-Cas9 ribonucleoprotein into mammalian cells** contains detailed information about genome editing experiments from experimental setup through mutation detection [2]

## Important considerations

Always include proper controls in your experiment. When using crRNA:tracrRNA duplexes, we recommend using the appropriate Alt-R CRISPR-Cas9 Control Kit for studies in human, mouse, or rat cells.

The control kits include an Alt-R CRISPR-Cas9 HPRT Positive Control crRNA targeting the *HPRT* gene and a computationally validated Alt-R CRISPR-Cas9 Negative Control crRNA. The kits also include the Alt-R CRISPR-Cas9 tracrRNA for complexing with the crRNA controls, Nuclease-Free Duplex Buffer, and validated PCR primers for amplifying the targeted *HPRT* region in the selected organism. The inclusion of the PCR assay makes the kits ideal for verification of *HPRT* gene editing using the Alt-R Genome Editing Detection Kit (T7 endonuclease I assay).

For assistance with control sgRNAs, contact applicationsupport@idtdna.com.

# Required materials

| General reagents | Ordering information* |
|---|---|
| Nuclease-Free Water | IDT (cat # 11-04-02-01) |
| Opti-MEM® Media | Thermo Fisher Scientific (cat # 51985091) |
| Trypsin | General laboratory supplier |
| **Reagents for plasmid delivery** | |
| Alt-R S.p. Cas9 Expression Plasmid (1 µg) | IDT (cat # 1072566) |
| Competent *E. coli* | General laboratory supplier |
| Plasmid preparation reagents | General laboratory supplier |
| TransIT-X2® Dynamic Delivery System | Mirus (cat # MIR 603 or MIR 604) |
| 100 mm, tissue culture–treated dish | General laboratory supplier |
| **Reagents for gRNA delivery** | |
| **Option 1, 2-part guide RNA** (crRNA + tracrRNA):<br>• Alt-R CRISPR-Cas9 crRNA<br>or Alt-R CRISPR-Cas9 crRNA XT<br>• Alt-R CRISPR-Cas9 tracrRNA<br>or Alt-R CRISPR-Cas9 tracrRNA – ATTO 550 | IDT predesigned and custom crRNA†:<br>www.idtdna.com/CRISPR-Cas9<br>IDT (cat # 1072532, 1072533, 1072534)<br>IDT (cat # 1075927, 1075928) |
| **Option 2, single guide RNA** (sgRNA):<br>Alt-R CRISPR-Cas9 sgRNA | IDT predesigned and custom sgRNA†:<br>www.idtdna.com/CRISPR-Cas9 |
| (Recommended for option 1, 2-part guide RNA)<br>Alt-R CRISPR-Cas9 Control Kit | IDT (cat # 1072554 [human], 1072555 [mouse], or 1072556 [rat]) |
| Nuclease-Free IDTE, pH 7.5 (1X TE solution) | IDT (cat # 11-01-02-02) |
| Nuclease-Free Duplex Buffer | IDT (cat # 11-01-03-01) |
| Lipofectamine® RNAiMAX Transfection Reagent | Thermo Fisher Scientific (cat # 13778100) |
| 1X Phosphate buffered saline (PBS) | General laboratory supplier |
| 96-well, tissue culture–treated plates | General laboratory supplier |

* Visit www.idtdna.com for additional sizes of IDT products, and for safety data sheets (SDSs) and certificates of analysis (COAs) for IDT products. These are suggested sources for reagents used by the IDT R&D team when this protocol was written. Individual components may be substituted with some optimization.

† We guarantee the performance of our predesigned gRNAs targeting human, mouse, rat, zebrafish, or nematode genes. For other species, use our proprietary algorithms to design custom gRNAs. If you have protospacer designs of your own or from publications, use our design checker tool to assess their on- and off-targeting potential before ordering gRNAs that are synthesized using our Alt-R gRNA modifications. For details about the predesigned gRNA guarantee, see www.idtdna.com/CRISPR-Cas9.

# Protocol

**Important:** When using any Cas9 expression construct, including mRNA or Alt-R S.p. Cas9 Expression Plasmid, we recommend that you transfect cells with the Cas9 construct at least 24 hr before introducing the gRNA. Co-transfection of Cas9 construct and gRNA may result in reduced efficiency as some of the RNA complexes may be degraded by endogenous nucleases before active RNPs form.

## Part 1: Delivery of Alt-R S.p. Cas9 Expression Plasmid

In addition to transfection with the Alt-R S.p. Cas9 Expression Plasmid, we recommend performing a separate transfection using a fluorescent reporter plasmid (not provided) of similar size to the Cas9 plasmid (7.3 kb) as a transfection control. The expressed fluorescent protein control can be used to quickly verify that the transfection is successful and to estimate and optimize transfection efficiency for your cells.

### A. Propagate the Cas9 expression plasmid in competent *E. coli*

1. Transform competent *E. coli* with the Alt-R S.p. Cas9 Expression Plasmid.

   **Note:** The Alt-R S.p. Cas9 Expression Plasmid expresses the beta-lactamase gene for ampicillin selection during standard subcloning protocols.

2. Prepare additional Cas9 plasmid from your transformed cells using your method of choice.

### B. Transfect cells with the Cas9 expression plasmid

1. Trypsinize and plate sufficient cells in a 100 mm dish to obtain 70–80% confluency after 24 hr incubation.

2. Incubate cells in a tissue culture incubator (37°C, 5% $CO_2$) for 24 hr.

3. After the 24 hr incubation, prepare the working stock of Alt-R S.p. Cas9 plasmid:

   a. Add 20 µL of Nuclease-Free Water to 20 µg of Alt-R S.p. Cas9 plasmid (from **step 1A2**) in a 1.5 mL microcentifuge tube (final concentration of 1 µg/µL).

   b. Mix well and centrifuge to collect the contents at the bottom of the tube.

4. Combine the following and incubate at room temperature (20–25°C) for 20 min to form transfection complexes:

| Component | Amount required per transfection |
|---|---|
| 1 µg/µL Alt-R S.p. Cas9 Expression Plasmid | 15 µL (15 µg) |
| Opti-MEM Media | 1440 µL |
| TransIT-X2 Dynamic Delivery System | 45 µL |
| **Total volume** | **1.5 mL** |

5. During incubation of the transfection complexes, replace the media in the plated cells (from **step 1B2**) with 15.5 mL of fresh complete media without antibiotics.

6. When incubation of the transfection complexes is complete, add 1.5 mL of transfection complex (from **step 1B4**) to the plate in **step 1B5**.

7. Remove media after 6 hr and replace with 20 mL of fresh complete media.

8. Incubate the plate containing the transfection complexes and cells in a tissue culture incubator (37°C, 5% $CO_2$) for 24 hr.

# Part 2: Delivery of Alt-R CRISPR RNAs

## A. Prepare RNA oligos

1. Resuspend each RNA oligo (Alt-R CRISPR-Cas9 crRNA, tracrRNA, sgRNA) in Nuclease-Free IDTE Buffer or Nuclease-Free Duplex Buffer.
   - We suggest resuspending the RNA oligos to 100 µM stock concentrations, using the volumes in the following table:

| Normalized amount delivered (nmol)* | Volume of resuspension buffer (µL) |
|---|---|
| 2 | 20 |
| 5 | 50 |
| 10 | 100 |
| 20 | 200 |
| 100 | 1000 |

* Prepare positive and negative controls using the same methods as the experimental complexes—ideally using the same lots of buffers. Alt-R CRISPR-Cas9 HPRT Positive Control crRNAs and Alt-R CRISPR-Cas9 Negative Control crRNAs are available at 2 nmol scale. Custom Alt-R CRISPR-Cas9 crRNAs and sgRNA are available at 2 and 10 nmol scales. Alt-R CRISPR-Cas9 tracrRNA is available at 5, 20, and 100 nmol scales.

   - To calculate your own dilutions, use the IDT Resuspension Calculator at www.idtdna.com/SciTools.

   **Store resuspended RNA oligos at –20°C.**

2. If using sgRNA, proceed to **step 2A6**.

3. Mix the crRNA and tracrRNA oligos in equimolar concentrations in a sterile microcentrifuge tube. For example, create a final complex concentration of 3 µM using the following table:

| Component | Amount |
|---|---|
| 100 µM Alt-R crRNA | 3 µL |
| 100 µM Alt-R tracrRNA | 3 µL |
| Nuclease-Free Duplex Buffer | 94 µL |
| **Total volume** | **100 µL** |

4. Heat at 95°C for 5 min.

5. Remove from heat and allow to cool to room temperature (20–25°C) .

6. If needed, dilute the crRNA:tracrRNA duplex or sgRNA to a working concentration (for example, 3 µM) in Nuclease-Free Duplex Buffer or IDTE Buffer.

> **Note:** crRNA:tracrRNA duplexes are stable for at least 6 months with no loss in activity when stored at –20°C at a concentration of ≥1 µM.

## B. Reverse transfect gRNA in a 96-well plate

1. Incubate the following at room temperature (20–25°C) for 20 min to form transfection complexes:

| Component | Amount |
|---|---|
| 3 µM gRNA (from **step 2A6**) | 1.5 µL |
| Lipofectamine RNAiMAX Transfection Reagent | 0.75 µL |
| Opti-MEM Media | 47.75 µL |
| **Total volume** | **50 µL** |

2. During incubation (**step 2B1**), prepare cells.
    a. Wash cells expressing Cas9 (from **step 1B8**) with PBS and trypsinize.
    b. Dilute using complete media without antibiotics to obtain 75% confluency in a 96-well plate.

3. When incubation of transfection complexes is complete, add 50 µL of transfection complexes (from **step 2B1**) to a 96-well tissue culture plate.

4. Add 100 µL of diluted cells (from **step 2B2**) to the 96-well tissue culture plate (final concentration of Alt-R CRISPR RNAs will be 30 nM).

5. Incubate the plate containing the transfection complexes and cells in a tissue culture incubator (37°C, 5% $CO_2$) for 48 hr.

To detect on-target mutations with the mismatch endonuclease T7EI, use the Alt-R Genome Editing Detection Kit (cat # 1075931, 1075932, 1075933) [3].

# References


1. Schubert M, Turk R, et al. (2017) **Fluorescently labeled tracrRNA provides efficient genome editing while allowing cellular microscopy and FACS analysis**. [Online] Coralville, IA, Integrated DNA Technologies, Inc. [Accessed 26 Jun 2018]
2. Integrated DNA Technologies. (2017) **Alt-R CRISPR-Cas9 System: User Guide for cationic lipid delivery of CRISPR-Cas9 ribonucleoprotein into mammalian cells**. [Online] Coralville, IA, Integrated DNA Technologies, Inc. [Accessed 26 Jun 2018]
3. Integrated DNA Technologies. (2017) **Alt-R Genome Editing Detection Kit protocol**. [Online] Coralville, IA, Integrated DNA Technologies, Inc. [Accessed 26 Jun 2018]


# Revision history

| Version | Date released | Description of changes |
|---|---|---|
| 4 | July 2018 | Added instructions for using Alt-R CRISPR-Cas9 sgRNA.<br>Added information about new IDT crRNA design tools.<br>Simplified protocol (deleted sections are available in our main Alt-R CRISPR-Cas9 user guide [2]).<br>Updated to current IDT styles and formatting. |
| 3 | April 2016 | Updated molecular weights of Alt-R CRISPR Controls.<br>Updated Figure 6 to reflect changes in amplicons and cleavage products for Alt-R HPRT PCR assays. |
| 2 | November 2015 | Added detail for experimental controls throughout protocol.<br>Added appendix A and B relating amount of material supplied to number of transfection reactions. |
| 1 | October 2015 | Original protocol. |

# Alt-R CRISPR-Cas9 system: Transfection of Cas9 plasmid and guide RNAs

## Technical support:

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