Fluorescently labeled tracrRNA provides efficient genome editing while allowing cellular microscopy and FACS analysis

Abstract

For genome editing, the CRISPR-Cas9 system requires a ribonucleoprotein (RNP) that consists of a target-specific CRISPR RNA (crRNA), a transactivating crRNA (tracrRNA), and a Cas9 endonuclease. IDT scientists have previously found that transfection of a pre-formed RNP complex is an effective method of delivery of CRISPR reagents to the cells. Here, we show that addition of ATTO™ 550 fluorescent dye to the 5′ end of Alt-R™ CRISPR-Cas9 tracrRNA does not affect functional performance of the RNP. The fluorescent dye allows for a visual analysis of transfected cells and cell sorting by FACS, making it an important and convenient tool in research.

Useful applications of Alt-R™ CRISPR-Cas9 tracrRNA-ATTO™ 550	
Fluorescence microscopy	Fluorescence-activated cell sorting (FACS)
Monitor transfection during transfection optimization	Enrich successfully transfected cells by selecting positive cell fraction • Wash cells to minimize non-specific binding • Sort 24 hr after transfection
Monitor transfection during troubleshooting experiments	

Introduction

The recently discovered CRISPR-Cas9 system can be used to successfully alter genomic DNA in various model systems and organisms. Native to *S. pyogenes* bacteria, the system relies on a Cas9 endonuclease, whose activity leads to double-stranded breaks in DNA, and a guide RNA (gRNA) that directs the Cas9 protein to a specific, sequence-dependent location. Further, the gRNA itself may consist of a target-specific CRISPR RNA (crRNA) and a universal transactivating crRNA (tracrRNA), or a single guide RNA (sgRNA). For 2-part gRNA systems, all 3 components (Cas9, crRNA, and tracrRNA) must be present in a cell for DNA cleavage to occur; however, they can be introduced into the cell using a variety of approaches [**1–3**]. We have previously shown that delivery of the Cas9 and gRNA components as a Cas9:gRNA ribonucleoprotein (RNP) complex is an effective method of transfection (see **www.idtdna.com/CRISPR-Cas9**).

Fluorescent labeling allows detection and intracellular visualization of molecular components via fluorescent microscopy. For an RNP complex, such labeling could (in theory) be accomplished by fusing green fluorescent protein to Cas9, but it is also possible to fluorescently label the chemically synthesized gRNA components.

This application note explains how fluorescently labeled tracrRNA can be used to detect and visualize cells that have been successfully transfected with an RNP. Specifically, we show that 5′ addition of ATTO 550 fluorescent dye (ATTO-TEC GmbH) to Alt-R CRISPR-Cas9 tracrRNA does not interfere with RNP delivery or genome editing. We also demonstrate how the labeled tracrRNA can be visualized using fluorescence microscopy, and explain how optimized fluorescence-activated cell sorting (FACS) can be used to isolate and, thereby, enrich successfully transfected cell populations.

Genome editing performance

Coupling fluorescent dyes to crRNA or tracrRNA alters the structure of the gRNA complex, and therefore can influence its interaction with Cas9 protein. In addition, the hydrophobic (lipophilic) properties of many fluorescent dyes can lead to non-specific binding, which can prevent nuclear delivery of the gRNA complex and, ultimately, cause poor editing performance and false positives in fluorescence imaging [**4**]. This application note investigates how the addition of various fluorescent dyes to the gRNA affects the ability of Cas9 to correctly and efficiently alter genomic DNA. To test this, we generated gRNA complexes that possessed either unlabeled tracrRNA or tracrRNA with added ATTO 550 dye, and compared their respective editing efficiencies (see **Figure 1**).

We tested a variety of fluorescent dyes (data not shown), and found that Alt-R tracrRNA labeled at the 5′ end with ATTO 550 dye provided the best editing efficiencies and also avoided disruptive, non-specific binding observed with some other dyes. Additionally, we investigated the effectiveness of attaching the various fluorescent dyes to the crRNA component of the gRNA complex, rather than the tracrRNA. In these studies, fluorescently labeled crRNA resulted in reduced editing efficiencies and also demonstrated an extremely low rate of successful transfection (confirmed by FACS analysis).

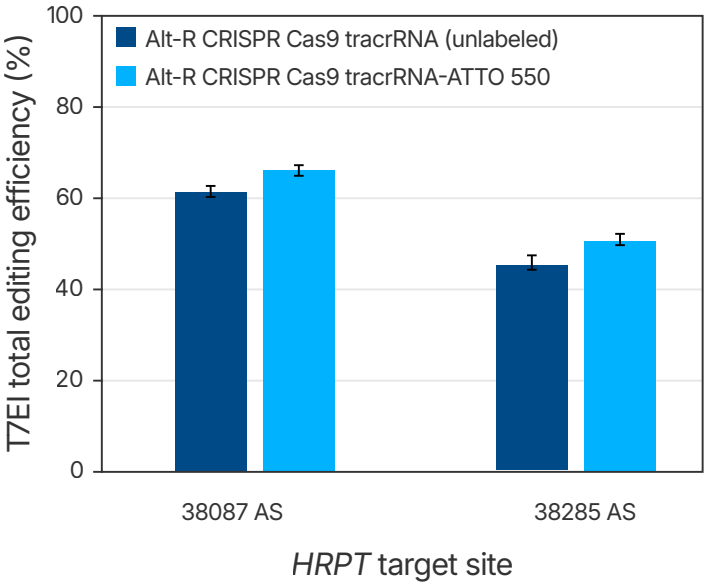

Figure 1. Addition of ATTO 550 fluorescent dye to Alt-R CRISPR-Cas9 tracrRNA does not affect genome editing performance. Guide RNA complexes (30 nM of crRNA complexed to either unlabeled or fluorescently labeled tracrRNA) targeting 2 sites in the *HRPT* gene were reverse-transfected into Cas9-expressing HEK-293 cells using RNAiMAX™ reagent (Thermo Fisher Scientific). 48 hr after transfection, genomic DNA was isolated from cells using QuickExtract™ solution (Epicentre), and total editing efficiency was measured using the Alt-R Genome Editing Detection Kit (T7 endonuclease I assay) (*n* = 1).

A marker of transfection efficiency

An important benefit of coupling a fluorescent dye to the tracrRNA is the ability to detect and visualize the gRNA complex intracellularly to monitor successful uptake of the gRNA. **Figure 2** shows how tracrRNA labeled with ATTO 550 dye can be used as a reliable marker of transfection efficiency.

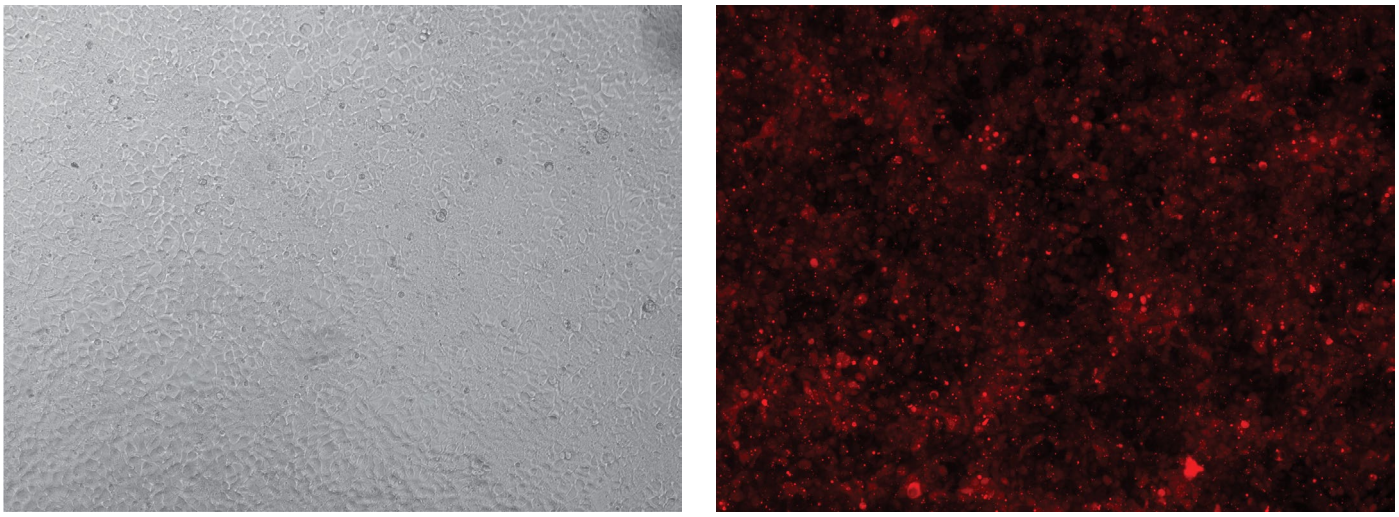

Figure 2. Detection of fluorescently labeled tracrRNA by fluorescence microscopy. HEK-293 cells stably expressing Cas9 nuclease were reverse transfected (RNAiMAX reagent, Thermo Fisher Scientific) with Alt-R CRISPR-Cas9 crRNA (unlabeled) complexed with Alt-R CRISPR-Cas9 tracrRNA-ATTO 550 (final concentration of 10 nM). Images were taken 48 hours after transfection. Magnification: 10X.

FACS enrichment of successfully transfected cells

Another benefit of using fluorescently labeled tracrRNA is that it supports FACS analysis. With this technology, cells containing the transfected RNP can be isolated, and thereby enriched, allowing researchers to confidently obtain and work with higher levels of successfully edited cells. The effects of such enrichment can be clearly seen when suboptimal concentrations of RNP are used, as shown in **Figure 3**. Here, FACS was used to select cells that contained fluorescently labeled tracrRNA (i.e., underwent successful uptake of the RNP), and their total editing efficiency was compared to cells which had not been previously sorted by FACS.

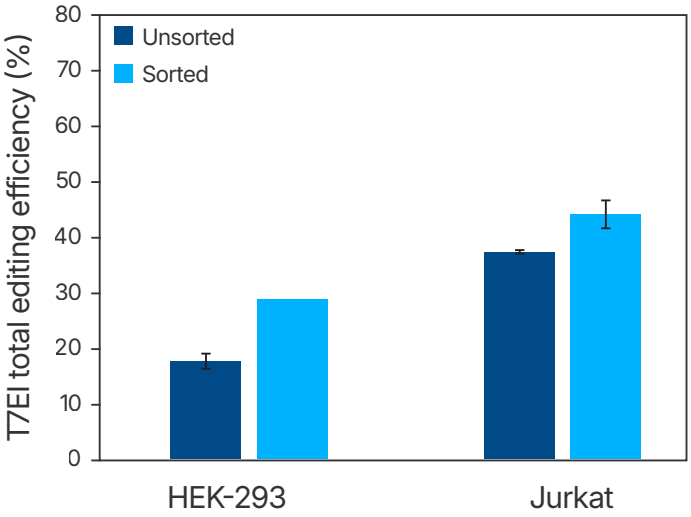

Figure 3. Enrichment of sorted cells leads to higher editing efficiencies. HEK-293 and Jurkat cells were transfected (Neon® electroporation system, Thermo Fisher Scientific) with 0.5 µM ribonucleoprotein (RNP: Alt-R S.p. Nuclease 3NLS complexed with Alt-R CRISPR-Cas9 crRNA and Alt-R CRISPR-Cas9 tracrRNA-ATTO 550) and carrier DNA (Alt-R Cas9 Electroporation Enhancer). Cells subjected to RNP, but without electroporation, were used as background controls and were used to set the gates during FACS. Cells were sorted 24 hr post-transfection, and positive cells were re-plated and grown for an additional 48 hr. A population of the cells was not sorted, but simply re-plated, to serve as the unsorted control. Genomic DNA was isolated using QuickExtract solution (Epicentre) after cell incubation for 72 hr. Total editing efficiency was measured using the Alt-R Genome Editing Detection Kit (T7 endonuclease I assay) (n = 1).

Notably, the data in **Figure 3** show that increases in genome editing levels are observed when the RNP is administered at suboptimal concentrations (0.5 µM). This suggests that enrichment by FACS analysis can be an especially valuable tool when transfection efficiency is expected to be low, or when the gRNA has suboptimal activity.

How to optimize FACS analysis

In performing FACS analysis, we observed that flow cytometric resolution was time dependent. As **Figure 4** shows, this was evident when using 2 RNP concentrations (0.15 µM and 1.5 µM) in 2 different cell types (Jurkat and HEK-293). At both RNP concentrations, fluorescence intensity decreased with time, resulting in a decrease of positive (sortable) Jurkat cells. Similar results were seen in HEK-293 cells, suggesting that sorting cells 24 hr post-transfection is optimal, regardless of cell type.

Note: Genomic DNA does not need to be isolated 24 hr post-transfection. If you are concerned that genome editing requires >24 hr, the positive cell fraction can be re-plated for downstream studies at a later time point.

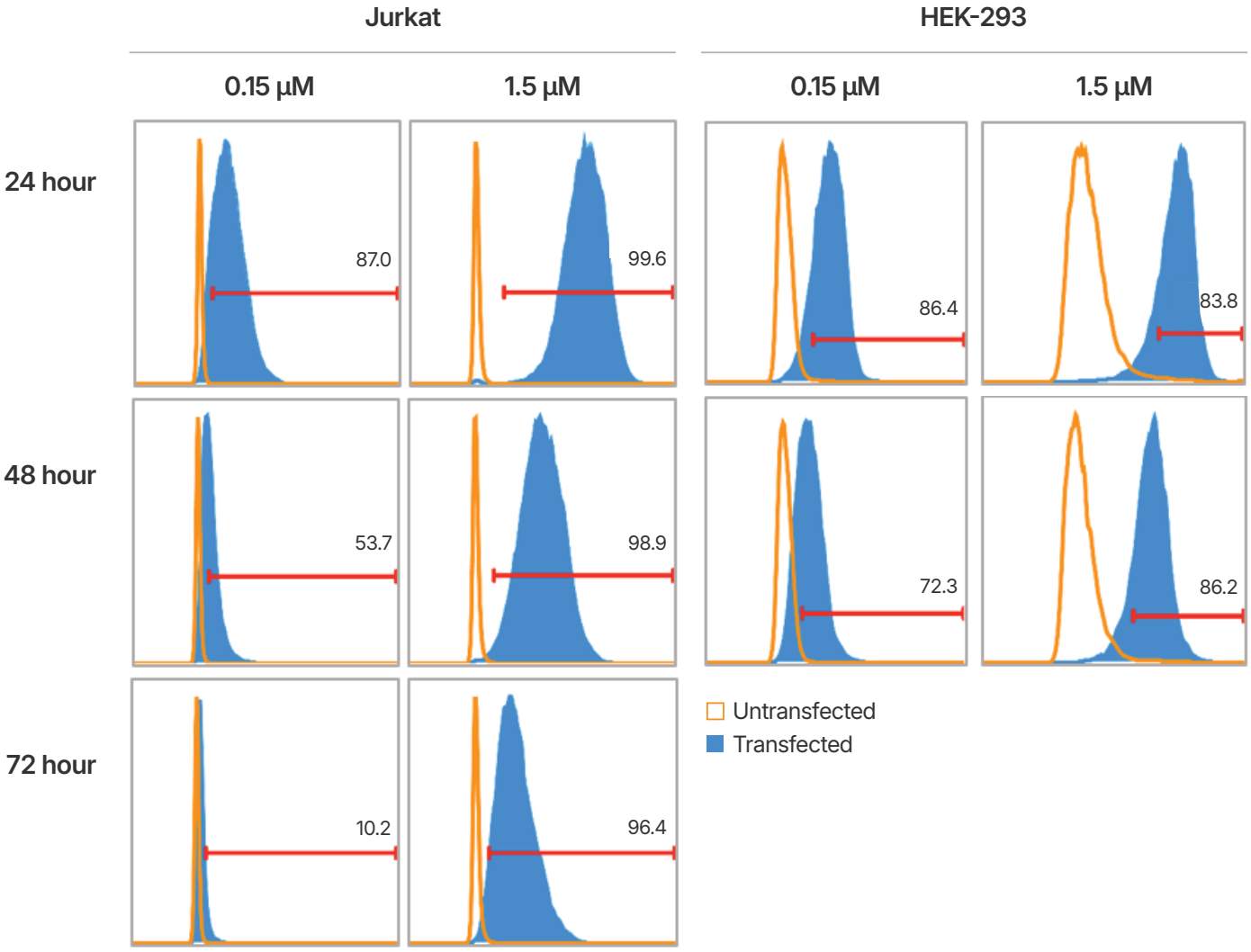

Figure 4. Flow cytometric resolution decreases over time after transfection. Jurkat and HEK-293 cells were transfected (Neon electroporation system, Thermo Fisher Scientific) with 0.15 or 1.5 µM ribonucleoprotein (RNP: Alt-R *S.p.* Nuclease 3NLS complexed with Alt-R CRISPR-Cas9 crRNA and Alt-R CRISPR-Cas9 tracrRNA-ATTO 550) and carrier DNA (Alt-R Cas9 Electroporation Enhancer). Cells were sorted 24, 48, or 72 hr after transfection. Prior to sorting, cells were washed once with PBS containing 1% FBS. Histogram plots show fluorescence intensities and percent positive cells (n = 1).

We also investigated the effects of washing cells before FACS analysis (data not shown). In these studies, we examined multiple cell types, as well as various fluorescent dyes. While the relative effectiveness was shown to be both cell- and dye-type dependent, we found that washing cells once with PBS containing 1% FBS before FACS analysis consistently decreased non-specific binding and led to more positive (sortable) cells. Therefore, regardless of the cell type used, we recommend performing this step before FACS analysis to avoid non-specific binding.

Conclusions

- Labeling Alt-R CRISPR-Cas9 tracrRNA with ATTO 550 fluorescent dye does not affect genome editing performance of the RNP.
- When using Alt-R CRISPR-Cas9 tracrRNA-ATTO 550, transfection efficiency of the RNP can be visualized by fluorescence microscopy during transfection optimization or troubleshooting.
- FACS analysis allows for enrichment of successfully transfected cells.
- For optimal results, FACS analysis should be preceded by 1 wash step and performed 24 hr post-transfection.

References

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Additional information and related protocols can be found at **www.idtdna.com/CRISPR-Cas9.**

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