Improved fluorescent CRISPR fusion proteins allow for enrichment of edited cells by fluorescence activated cell sorting

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Abstract

It is often desirable to visualize CRISPR enzymes in live cells as a means to verify efficient delivery of CRISPR reagents. The most common method to accomplish this goal with protein cargo is to make an in-frame fusion to fluorescent proteins such as GFP or RFP. Here we present an improved set of CRISPR reagents that have been carefully fine-tuned to allow visualization in live cells while still retain near-wildtype levels of on-target activity. We demonstrate that fluorescent CRISPR enzyme fusion proteins and fluorescently labeled tracrRNAs can be used in combination with fluorescence-activated cell sorting to achieve levels of editing greater than with unlabeled CRISPR enzymes. We expect that these reagents will be a valuable resource in research.

IDT fluorescent CRISPR proteins maintain high on-target activity

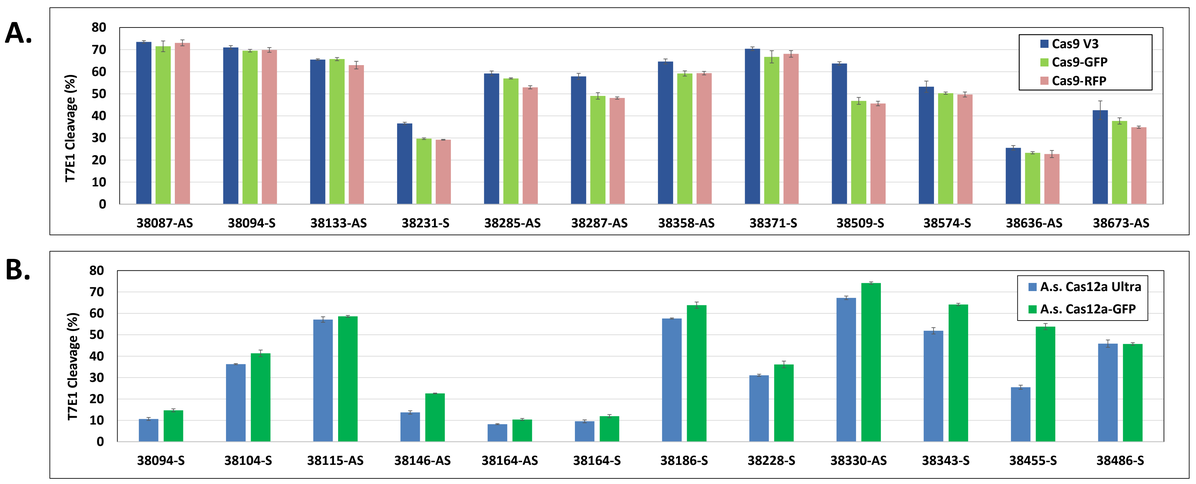

Figure 1. IDT fluorescent CRISPR proteins maintain high on-target activity across multiple guides. A. Alt-R™ CRISPR-Cas9 sgRNAs were designed to target NGG PAM sites within the human *HPRT* gene. Guides were complexed with Alt-R *S.p.* Cas9 Nuclease V3, Cas9-GFP, or Cas9-RFP to form ribonucleoproteins (RNPs). RNPs were then delivered into HEK293 cells using the Lonza 96 well shuttle Nucleofector™ at a concentration of 2.0 µM. After 48 hrs, genomic DNA was isolated (QuickExtract™ solution, Epicenter), and editing was assessed by T7EI mismatch endonuclease assay. **B.** Alt-R CRISPR-Cpf1 crRNAs were designed to target TTTV PAM sites within the human HPRT gene. Guides were complexed with either Alt-R *A.s.* Cas12a (Cpf1) *Ultra* or *A.s.* Cas12a-GFP to form RNPs. RNP delivery and editing analysis were conducted as in (A), with the exception that RNPs were delivered at a suboptimal dose of 50 nM to achieve a range of editing across the targeted sites. Error bars represent SD, N=3 replicates.

Enrichment of edited cells by FACS

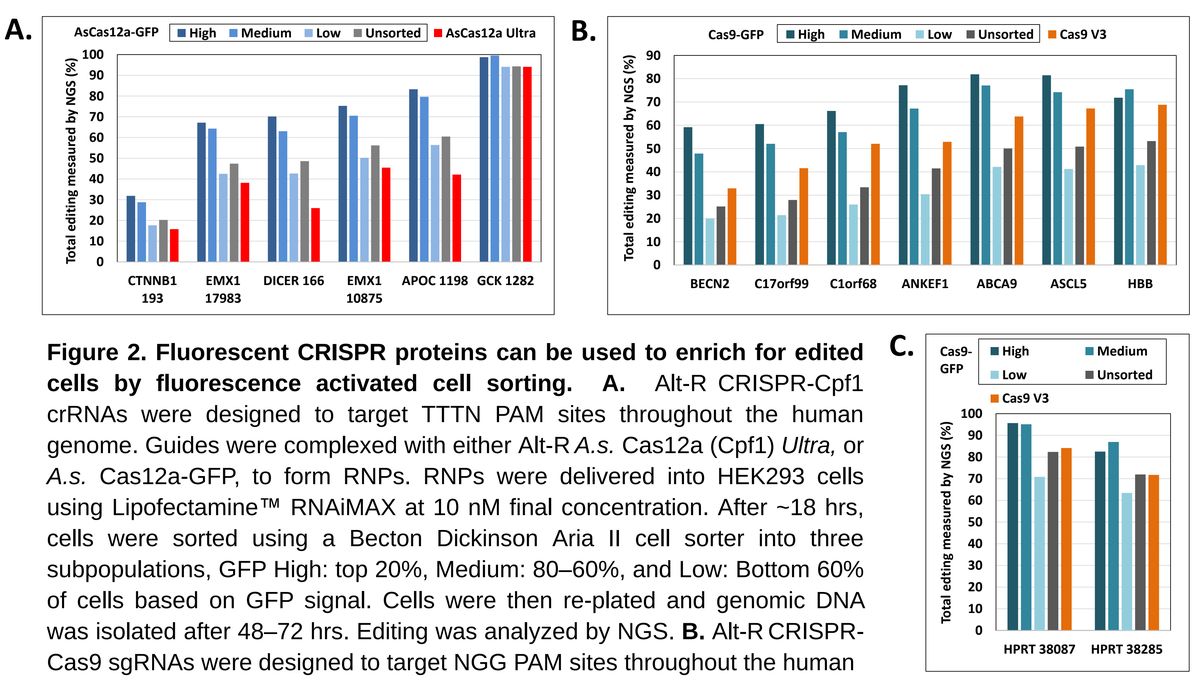

Figure 2. Fluorescent CRISPR proteins can be used to enrich for edited cells by fluorescence activated cell sorting. A. Alt-R CRISPR-Cpf1 crRNAs were designed to target TTTN PAM sites throughout the human genome. Guides were complexed with either Alt-R *A.s.* Cas12a (Cpf1) *Ultra,* or *A.s.* Cas12a-GFP, to form RNPs. RNPs were delivered into HEK293 cells using Lipofectamine™ RNAiMAX at 10 nM final concentration. After ~18 hrs, cells were sorted using a Becton Dickinson Aria II cell sorter into three subpopulations, GFP High: top 20%, Medium: 80–60%, and Low: Bottom 60% of cells based on GFP signal. Cells were then re-plated and genomic DNA was isolated after 48–72 hrs. Editing was analyzed by NGS. **B.** Alt-R CRISPR-Cas9 sgRNAs were designed to target NGG PAM sites throughout the human genome. Guides were complexed with either Cas9-GFP or Alt-R Cas9 V3. RNP delivery, cell sorting and editing analysis were conducted as in (A). **C.** Cas9 RNP was prepared as in (B) and delivered into HEK293 cells using a Lonza 96 well shuttle Nucleofector™ at 2.0 µM concentration. Cells were sorted and analyzed as in (A). N=1 run/sample.

Cas9-GFP allows visualization of delivery

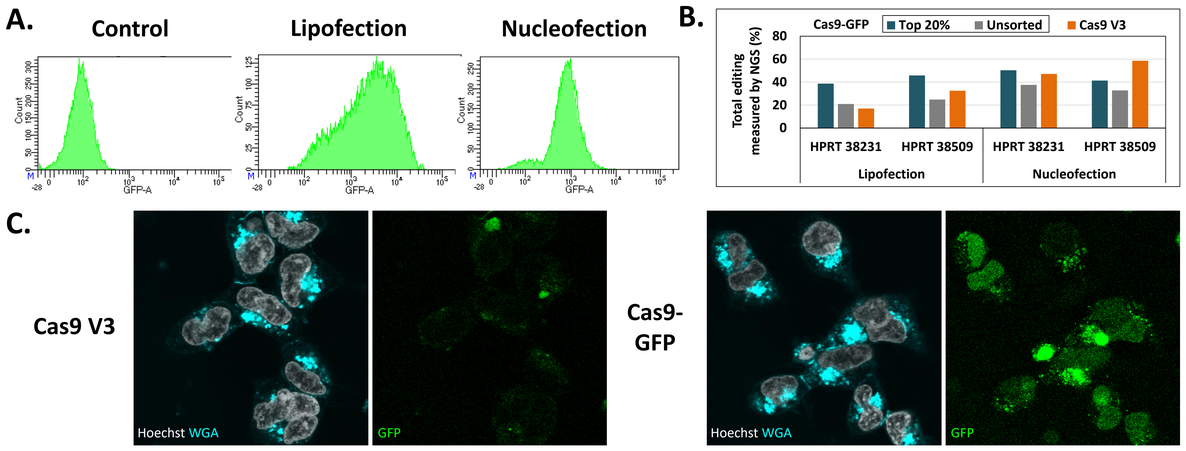

Figure 3. Use of Cas9-GFP is most beneficial when the delivery of RNP is uneven across cells. A. RNPs consisting of either Cas9-GFP or Alt-R Cas9 V3 complexed with Alt-R CRISPR-Cas9 sgRNAs targeting two sites in the *HPRT* gene were delivered into HEK293 cells using either Lipofectamine™ RNAiMAX at 10 nM RNP or using the Lonza Nucleofector™ system at 2 µM RNP. The graphs show the GFP signal versus cell count when sorting cells that had Cas9-GFP delivered using either lipofection or Nucleofection™ compared to Cas9 V3 delivered by Nucleofection™ as a control. **B.** The graph shows the total editing measured by NGS in either unsorted cells or the top 20% of cells based on GFP signal from (A). Cells were sorted and editing was analyzed as in Figure 2. **C.** Confocal images of HEK293 cells taken ~18 hours after delivery of either Cas9-GFP or Alt-R Cas9 V3 protein complexed with Alt-R CRISPR-Cas9 sgRNA delivered by Nucleofection™ at 2 µM RNP. Prior to imaging, live cells were incubated with Hoechst 33342 and wheat germ agglutinin (WGA)–Alexa Fluor™ 647 with prior and subsequent PBS wash steps and imaged in complete media in a Lab-Tek II #1.5 Chambered Coverglass using a Leica SP8 confocal microscope. N=1.

IDT fluorescent Cas9 is comparable to that of other vendors

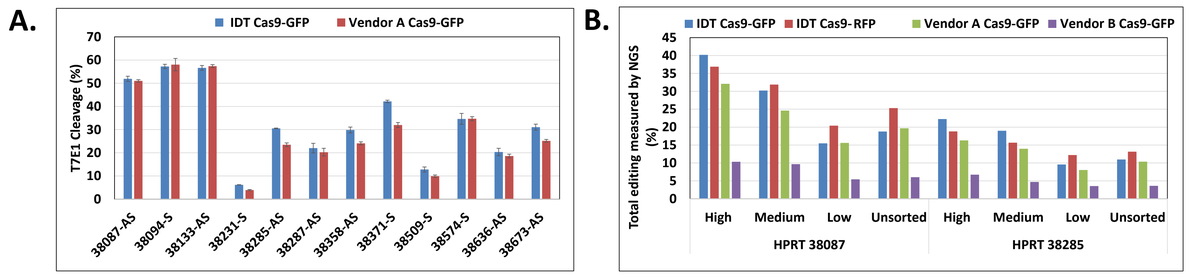

Figure 4. Editing activity of IDT Cas9-GFP is comparable to that of other vendors. A. Alt-R CRISPR-Cas9 sgRNAs targeting sites within the *HPRT* gene were complexed with IDT or Vendor A Cas9-GFP to form RNPs. RNPs were then delivered into HEK293 cells using the Lonza 96 well shuttle Nucleofector™ at a suboptimal concentration of 0.25 µM RNP. After 48 hrs, genomic DNA was isolated and editing was assessed by T7EI mismatch endonuclease assay. Error bars represent SD, N=3 replicates. **B.** Alt-R CRISPR-Cas9 sgRNAs targeting two sites with the human *HPRT* gene were complexed with IDT, Vendor A, or Vendor B Cas9-GFP to form RNPs. RNPs were delivered into HEK293 cells using Lipofectamine™ RNAiMAX at a suboptimal dose of 1 nM RNP. Cell sorting and editing were analyzed as in Figure 2 (N=1).

IDT offers a variety of labelled CRISPR products

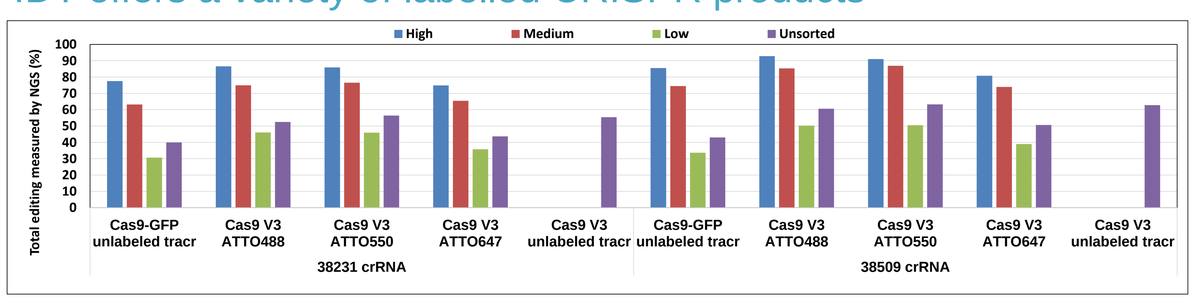

Figure 5. IDT Cas9-GFP and fluorescent-labeled CRISPR-Cas9 tracrRNAs offer a variety of options that allow for enrichment of edited cells by fluorescence signal. Alt-R crRNA XT was duplexed with either unlabeled, or ATTO™ labeled, tracrRNA and complexed with either Cas9-GFP or Alt-R Cas9 V3 and delivered into HEK293 cells using Lipofectamine™ RNAiMAX at 10 nM RNP. The graph shows total editing measured by NGS in cells that were sorted and analyzed as in Figure 2. N=1 run/sample.