Characterization of synthetic guide RNAs for CRISPR/Cas9 genome editing: An extensive evaluation of guide RNA formats, delivery methods, and purity

Ashley Jacobi[1], Rolf Turk[1], Nathaniel Roberts[1], and Garrett Rettig[1]
[1] Integrated DNA Technologies, Coralville, IA, USA
[2] Genome Engineering and iPSC Center, Washington University, St. Louis, MO, USA

*** Corresponding author: ajacobi@idtdna.com**

Alt-R™ CRISPR Cas9 guide RNA formats

Guide RNAs	Cas9 guide RNAs		
Structure	Alt-R 2-part	Alt-R 2-part XT	Alt-R sgRNA
gRNA format	Alt-R CRISPR-Cas9 crRNA & tracrRNA	Alt-R CRISPR-Cas9 crRNA XT & tracrRNA	Alt-R CRISPR-Cas9 sgRNA
Components	crRNA tracrRNA	crRNA XT tracrRNA	sgRNA
Size (nt)	36 67	36 67	100
Annealing required	Yes	Yes	No
Stability	++	+++	++++
Applications	• Cas9-expressing cells • RNP in most cell types	• Co-delivery with Cas9 plasmid/Cas9 mRNA • RNP under diffcult expermental condiitions (e.g., high nuclease environments)	

Table 1. IDT has studied and optimized the lengths, chemical modifications, and manufacturing conditions of both the *S.p.* Cas9 2-part gRNA and single gRNA. All IDT chemically synthesized gRNA components are mass verified via ESI-MS. For the bipartite complex, the crRNA and tracrRNA components are annealed in an equimolar ratio to form the active gRNA complex. All versions contain chemically modified bases or linkages. Chemical modifications are known to stabilize oligos from serum and cellular nucleases (increasing editing efficiencies) and reduce risk of triggering the cell's innate immune response[1].

Activity comparison of 2-part vs. sgRNA – Cas9 RNP

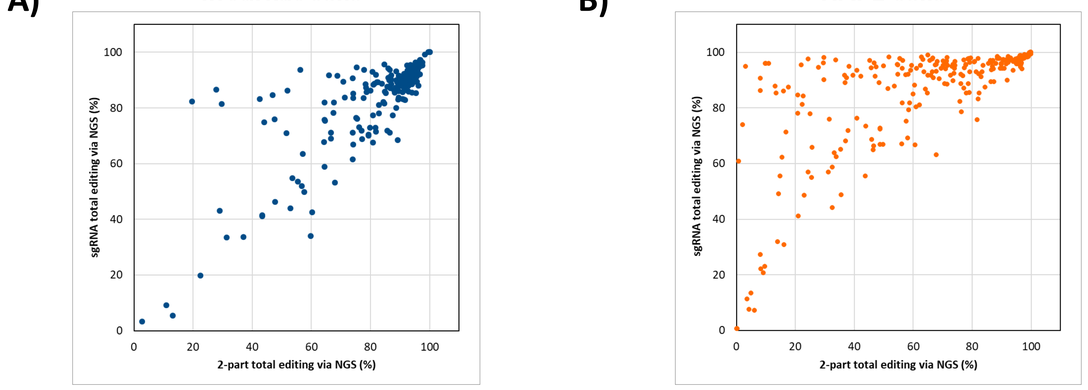

Figure 1. Cell line dependent preference for sgRNA vs. 2-part gRNAs with RNP delivery. 255 target sites, randomly selected across the human genome were synthesized as Alt-R 2-part gRNAs (crRNA:tracrRNA) or as Alt-R sgRNAs. RNP complexes were made by combining WT Alt-R Cas9 and gRNAs at a 1:1.2 protein to gRNA ratio and were electroporated into **A)** Jurkat cells and **B)** HAP1 cells at a final concentration of 4 µM with 3 µM Alt-R Electroporation Enhancer via Lonza Nucleofection. Genomic DNA was extracted after 72 hrs and total editing was assessed by NGS.

Activity comparison of 2-part vs. sgRNA – Cas9 mRNA

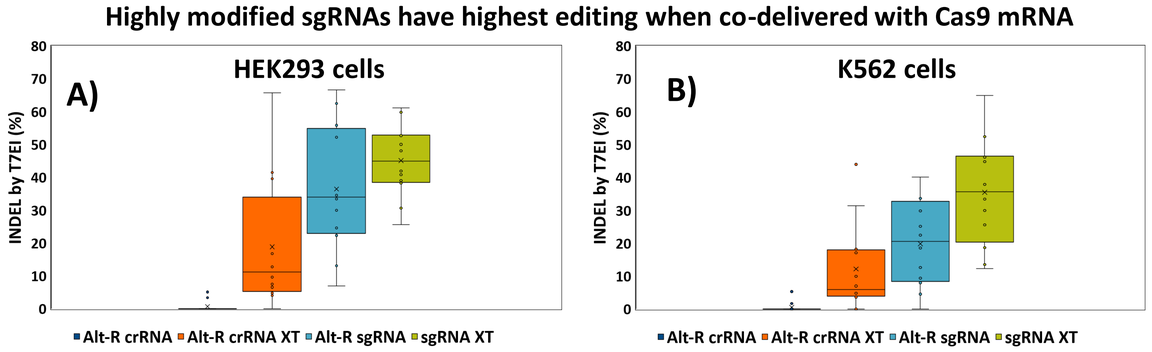

Figure 2. sgRNAs give optimal editing when delivering with Cas9 mRNA. 2-part gRNAs or sgRNAs (5 µM) targeting 12 regions in *HPRT1* were electroporated with 1 µg Cas9 mRNA (TriLink) into **A)** HEK293 and **B)** K562 cells. Total editing was assessed by the T7EI assay (IDT). The standard Alt-R sgRNAs contain three 2'OMe modified bases and three phosphorothioate linkages on each end of the gRNA. Additional chemical modification patterns were studied to add greater nuclease protection while maintaining full potency (data not shown). sgRNA XT, shown here, has approximately 50% of the bases chemically modified with 2'OMe bases and provides the highest level of editing when co-delivered with Cas9 mRNA.

References:
1. Schubert et. al. (2018) Chemical Modification of CRISPR *gRNAs* Eliminate type I Interferon Responses in Human Peripheral Blood Mononuclear Cells. J Cytokine Biol, 3:120.
2. Anzalone. et. al (2019) Search and replace genome editing without double-stranded breaks or donor DNA. Nature, 576:149-157.

Alt-R sgRNA purification options – standard desalt vs. HPLC

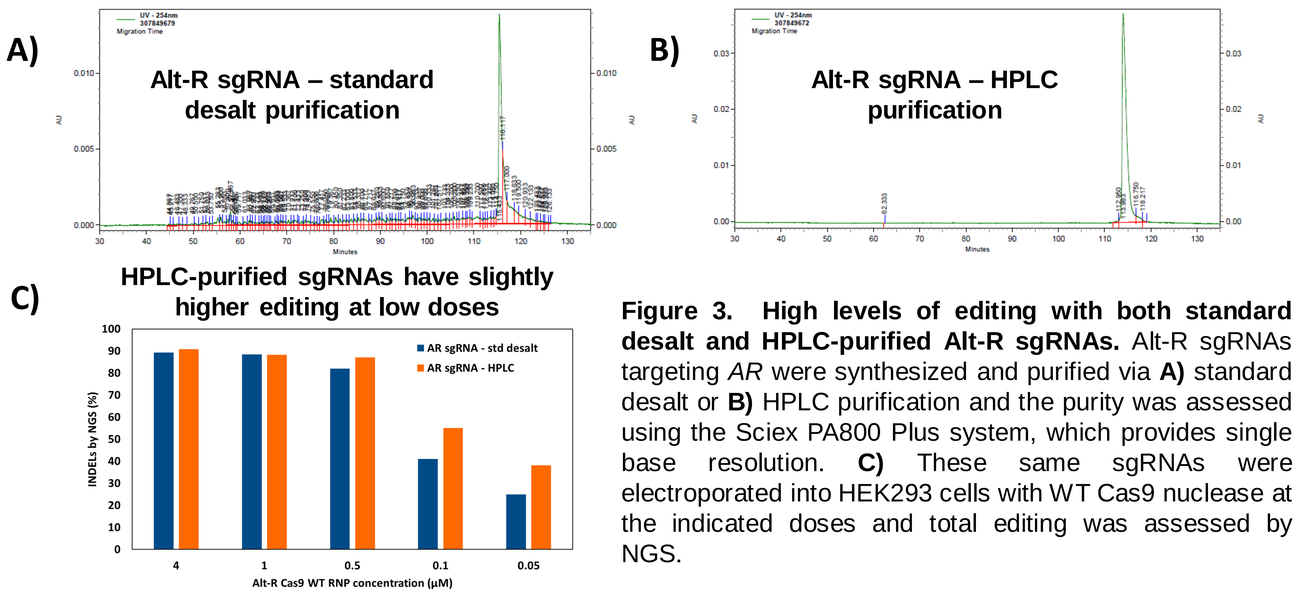

Figure 3. High levels of editing with both standard desalt and HPLC-purified Alt-R sgRNAs. Alt-R sgRNAs targeting *AR* were synthesized and purified via **A)** standard desalt or **B)** HPLC purification and the purity was assessed using the Sciex PA800 Plus system, which provides single base resolution. **C)** These same sgRNAs were electroporated into HEK293 cells with WT Cas9 nuclease at the indicated doses and total editing was assessed by NGS.

Custom Alt-R CRISPR libraries for 2-part gRNAs and sgRNAs

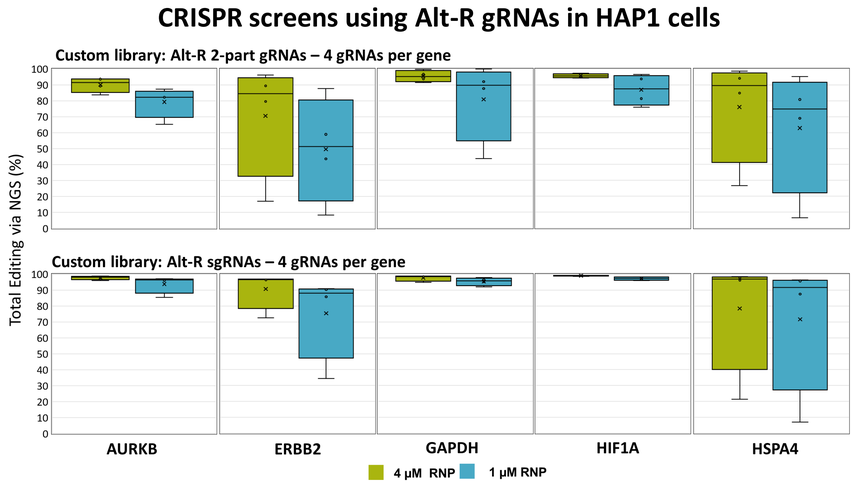

Figure 4. A subset of an arrayed custom library provides high levels of editing using both 2-part gRNAs and sgRNAs. 4 gRNAs targeting 5 different human genes were designed using the Alt-R Cas9 gRNA Design Tool. All gRNAs were chemically synthesized as Alt-R 2-part gRNAs or Alt-R sgRNAs. RNP complexes were made by combining WT Alt-R Cas9 to each gRNA at a 1:1.2 protein to gRNA ratio and were individually electroporated into HAP1 cells at a final concentration of 4 and 1 µM with 3 µM Alt-R Electroporation Enhancer via Lonza Nucleofection. Total editing was assessed by NGS.

Prime Editing with chemically synthesized pegRNAs

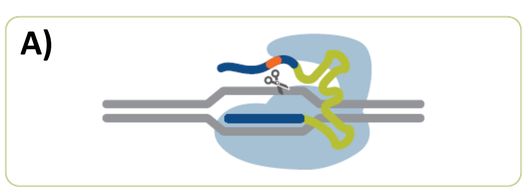

Figure 5A. Schematic of pegRNA used for CRISPR prime editing. Prime editing[2] utilizes a fusion protein of Cas9 H840A nickase and a reverse transcriptase (light blue), and a long guide RNA, called a pegRNA. pegRNA is composed of targeting RNA (lower dark blue), enzyme-binding region (green), and a region pairing to the cut strand of DNA (upper dark blue).The orange region represents the new sequence.

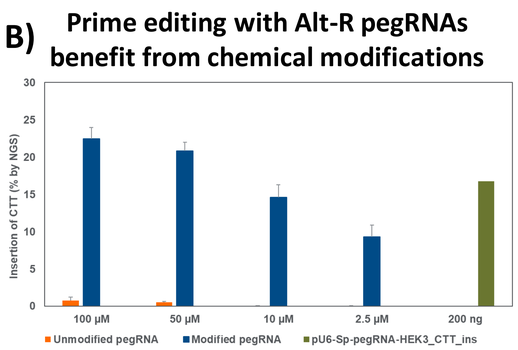

Figure 5B. Prime editing with chemically synthesized pegRNAs. 200 ng of PE2 plasmid was electroporated into K562 cells along with a 122 nt pegRNA inserting a CTT at site HEK3. pegRNAs were chemically synthesized as all RNA bases or with three 2'OMe modified bases and three phosphorothioate linkages on each end. A plasmid containing the PE2 construct and HEK3 +CTT pegRNA (Addgene) was electroporated as a control. Modified pegRNAs had similar levels of PE as the plasmid, but the unmodified pegRNA had no detectable levels of PE. Studies with alternate chemical modifications, sequences and RNP delivery are underway.

Alt-R guide RNAs can support any type of experiment

- Screening, research grade gRNAs (standard desalt)
  - 2, 10, 50, 100 nmol scales, ship in 3-5 business days
  - Custom CRISPR libraries
- Manufacturing processes developed to avoid oligo cross contamination
- Highest quality HPLC-purified sgRNAs
- Large scale synthesis
  - HPLC purified, up to 2 gram scale
  - Sterile filtration
  - Optional: endotoxin analysis, purity assessment
- Custom chemical modification patterns available
- Custom gRNAs for Cas9, Cas12a, Cas13, pegRNA or any RNA-guided nuclease up to 160 nt – **online ordering tool coming soon**