Alt-R™ crRNA:tracrRNA oligonucleotides optimized for CRISPR gene editing

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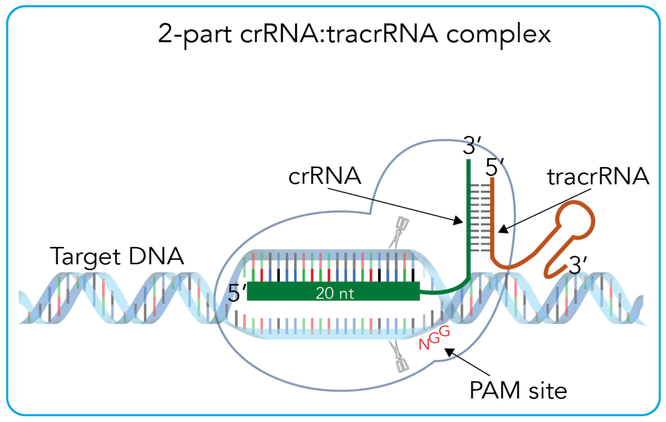
Introduction

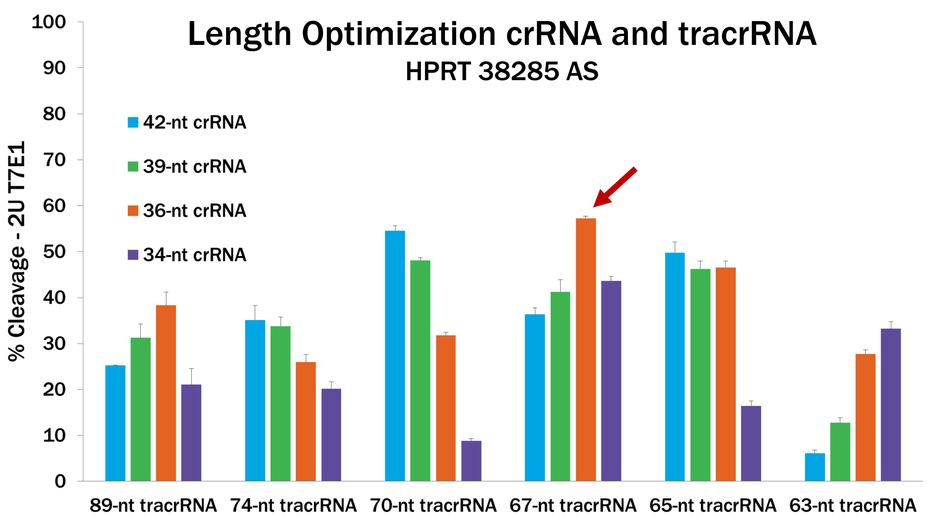
CRISPR/Cas9-mediated cleavage of double-stranded DNA is a versatile approach to gene editing. The strategies for delivering guide RNAs for sequence-specific cleavage of genomic DNA are many and varied, and include plasmid DNA, gBlocks® Gene Fragments, or *in vitro* transcribed RNA to introduce a sgRNA trigger. The native bacterial CRISPR system in *Strep. pyogenes* utilizes a 2-part crRNA:tracrRNA complex, as opposed to a single chimeric sgRNA. Having the guide RNA trigger in a 2 molecule format permits use of synthetic RNA oligonucleotides with lengths suitable for high-throughput manufacturing and QC (e.g., electrospray ionization mass spectrometry), offering researchers the convenience and ease of use they have come to expect from synthetic oligos. The Alt-R™ crRNA:tracrRNA are length-optimized for potent double-stranded DNA cleavage in mammalian cells when used in combination with the Cas9 protein from *Strep. pyogenes*.



In Vitro Gene Editing

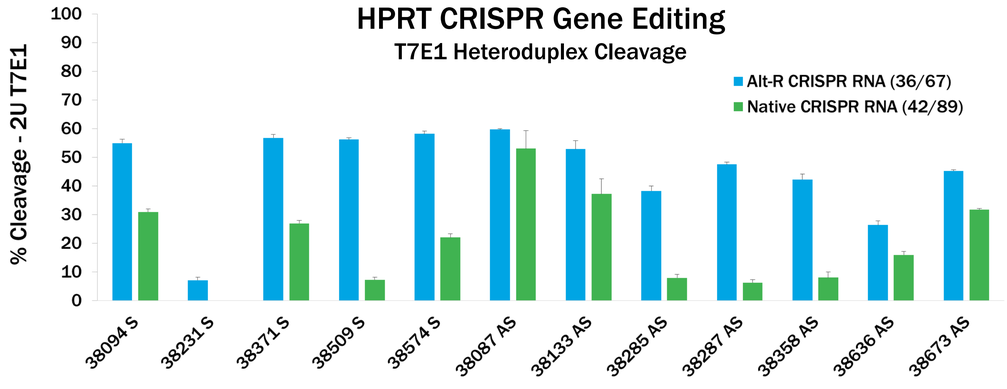
HEK293 cells stably expressing *S. pyogenes* Cas9 were transfected (48 hrs) with Alt-R™ CRISPR RNA oligonucleotides (30 nM) or gBlocks® Gene Fragments (3 nM) containing a U6 promoter-driven sgRNA targeting genomic DNA. Reverse transfections were done in 96-well plates using RNAiMAX (Life Technologies). Genomic DNA was isolated with QuickExtract (Epicentre), and regions containing the targeted sequence of genomic DNA were PCR-amplified using KAPA HiFi polymerase (Kapa Biosystems).

The degree of editing mediated by RNA triggers was evaluated by heteroduplex cleavage with the mismatch endonuclease T7E1 (New England Biolabs). Separation and quantification of cleavage products was done using the Fragment Analyzer™ – a 96-well capillary electrophoresis system from Advanced Analytical.

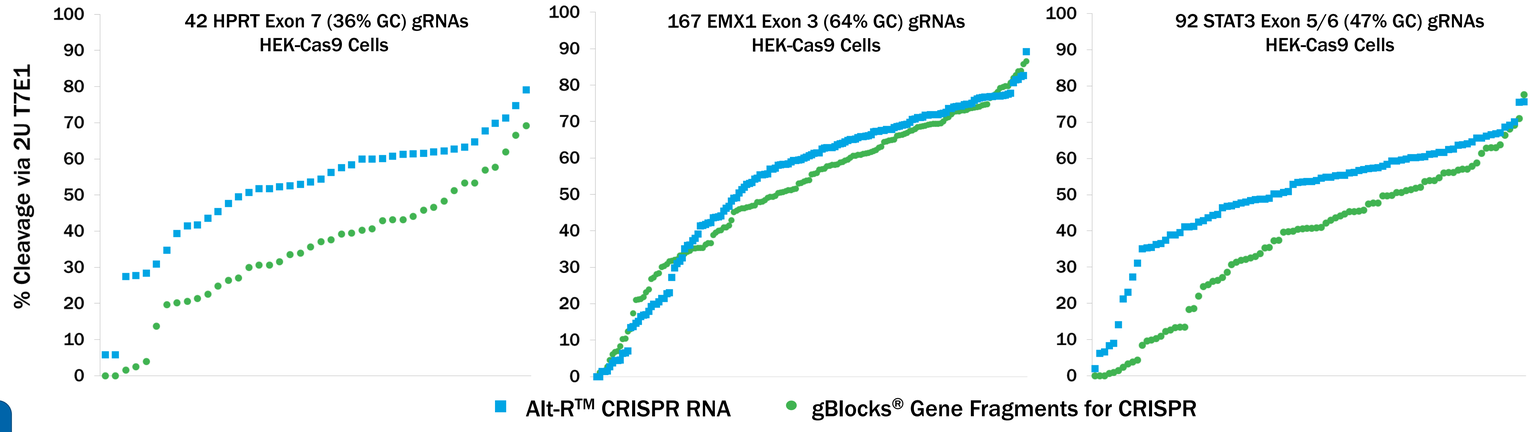


Comparing Alt-R™ and native crRNA:tracrRNA

The 36-nt crRNA paired with the 67-nt tracrRNA is a more potent trigger for CRISPR/Cas9 gene editing than the native 42-nt crRNA/89-nt tracrRNA at 12 sites tested in exon 7 of the HPRT1 gene. This was observed in biochemical assays as well as in HEK293 cells expressing Cas9.

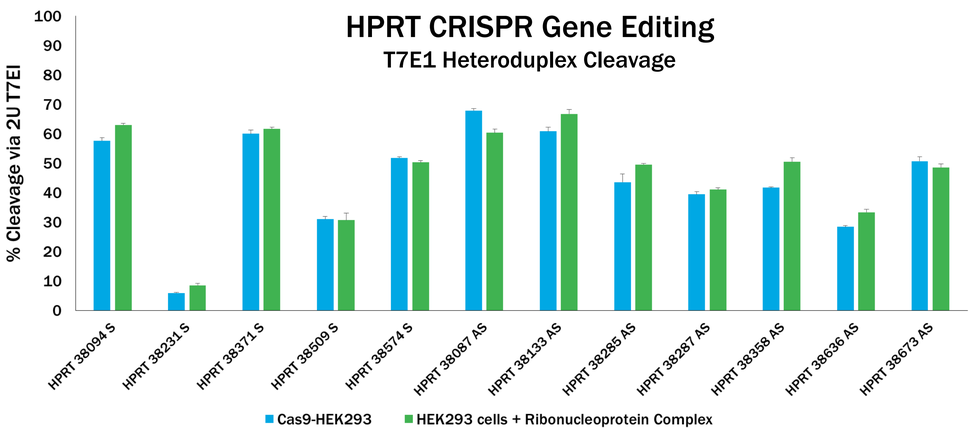


CRISPR Screen: Alt-R™ and gBlocks® Gene Fragments



RNP transfections

Alt-R™ CRISPR RNAs were lipofected with a recombinant Cas9-NLS modified protein as an RNP (ribonucleoprotein) complex. These transfections were done at a final concentration of 10 nM Alt-R™ CRISPR RNAs at a 1:1 molar ratio of RNA to Cas9. This approach gave gene editing in native HEK293 cells identical to the potency observed in the Cas9-stable transgenic cell line



Conclusions

Systematic variation of the crRNA and tracrRNA length led to development of a crRNA:tracrRNA complex that shows improved gene editing in mammalian tissue culture with *S. pyogenes* Cas9. The Alt-R™ crRNA:tracrRNA system pairs a 67-nt universal tracrRNA with a custom-synthesized target-specific 36-nt crRNA. In addition to improved activity, the shorter size of the new Alt-R™ CRISPR RNAs makes them more amenable to high throughput synthetic manufacturing, including the option for chemical modification. CRISPR screens comparing gene editing by Alt-R™ CRISPR RNAs to gBlocks® Gene Fragments containing a U6 promoter driving the transcription of the corresponding sgRNA show that both routes are useful strategies; the Alt-R™ CRISPR RNAs give similar or improved gene editing at >85% of sites. Delivery of a crRNA:tracrRNA-Cas9 RNP complex offers a highly efficient approach to edit genomic DNA in a cell line of interest without the need to co-transfect large expression cassettes to produce Cas9 protein.

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