TECH NOTE

Reduced off-target effects with delivery of active Cas9 protein complexed with sgRNA using gesicle technology

Guide-it CRISPR/Cas9 Gesicle Production System

Delivery of active ribonucleoprotein complexes via gesicles results in reduced off-target effects >>

Confirmation of reduction in off-target effects is seen in Sanger sequencing results >>

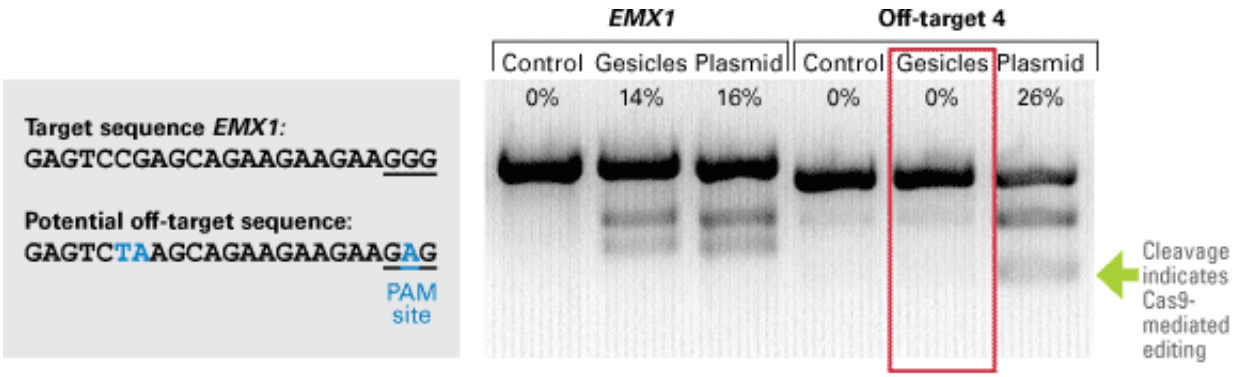
Overview

CRISPR/Cas9 gene editing is an RNA-programmable system which has democratized targeted genome modification by virtue of its simplicity and versatility. With this technology, gene editing is mediated by Cas9 nuclease and a single-guide RNA (sgRNA) which directs the Cas9 to a specific genomic locus. However, the utility of any genome modification system—for either basic research or therapeutic development—depends on its specificity (1). Early reports have warned of the frequent off-target effects of the CRISPR/Cas9 system (2–5), demonstrating the need for developing methodologies that reduce these events.

The Guide-it CRISPR/Cas9 Gesicle Production System is a novel methodology that uses cell-derived nanovesicles, called gesicles, for co-delivery of active Cas9 protein complexed with a gene-specific sgRNA. Gesicles are loaded with Cas9-sgRNA ribonucleoprotein (RNP) complexes, and can be added directly to target cells for CRISPR/Cas9 gene editing. Delivery of active Cas9 protein means no Cas9 coding gene is present in target cells, thus eliminating the problem of persistent and elevated of Cas9, which is common to plasmid-based delivery methods.

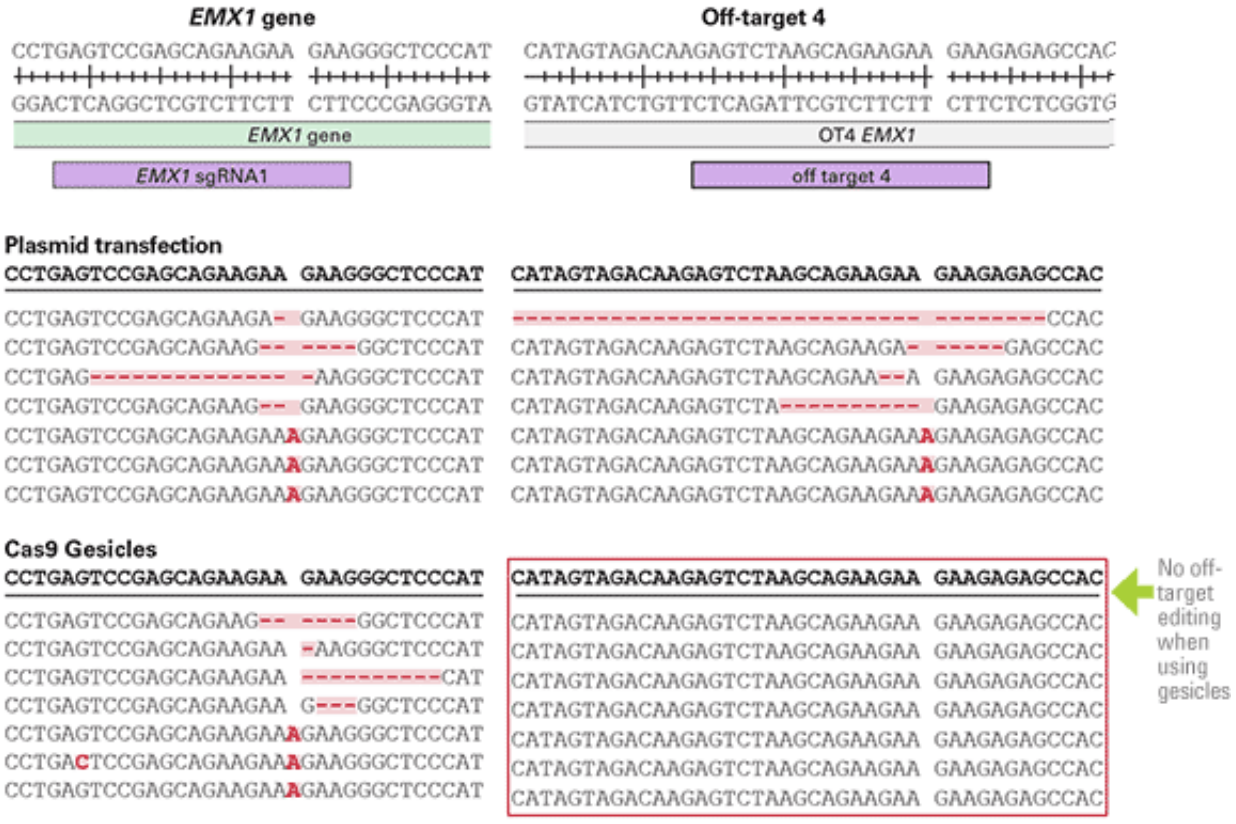
CRISPR/Cas9 gesicles reduce off-target effects compared to plasmid transfection

In a side-by-side comparison of Cas9-sgRNA delivery methods, the *EMX1* gene was edited in HEK 293T cells either by transfection with plasmids encoding Cas9 and a gene-specific sgRNA, or by treatment with gesicles loaded with Cas9-sgRNA RNP complexes. Following CRISPR/Cas9 modification, the *EMX1* locus and a potential off-target locus (off-target 4) were PCR-amplified from crude cell extracts. The presence of indels was detected using Guide-it Resolvase (a mismatch specific nuclease included in the Guide-it Mutation Detection Kit), followed by agarose gel electrophoresis. Densitometry (6) showed roughly equivalent indel formation at the *EMX1* target locus between the two methods. As expected, plasmid transfection resulted in significant indel formation at the off-target 4 locus, while gesicle delivery resulted in no observable indel formation at the off-target 4 locus.



Reduced off-target effects with CRISPR/Cas9 gesicles. HEK 293T cells were either transfected with plasmids encoding Cas9 and an sgRNA against *EMX1,* or treated with gesicles loaded with Cas9-sgRNA RNP complexes. After 72 hr, the *EMX1* gene and a potential off-target locus (off-target 4) were amplified from the treated cells by direct PCR. Using the Guide-it Mutation Detection Kit, the amplicons were melted and re-hybridized, and mismatched targets were cleaved using Guide-it Resolvase. A control sample that lacked Guide-it Resolvase was included for comparison (Control). The percentage of indel formation was determined by densitometry. No off-target effects were detected following the gesicle treatment.

To confirm the results of the resolvase assay, PCR amplicons of both the *EMX1* locus and off-target 4 were subcloned and sequenced. Corroborating previous results, plasmid delivery resulted in indel formation at both the *EMX1* and off-target 4 loci. Gesicle delivery resulted in indel formation only at the *EMX1* locus.



Sanger sequencing confirmed reduced off-target effects with CRISPR/Cas9 gesicles. *EMX1* and off-target 4 PCR amplicons were subcloned using the Guide-it Indel Identification Kit. Sequencing data for the different clones were aligned

with the wild-type sequence (underlined), revealing a range of deletions and insertions (highlighted in red) in both the *EMX1* and off-target 4 sites when cells were treated with plasmid transfection. For the cells treated with gesicles, indels were detected only at the *EMX1* target site, not at the off-target 4 site.

Conclusions

The Guide-it CRISPR/Cas9 Gesicle Production System is a novel methodology for the delivery of active Cas9-sgRNA RNP complexes to target cells for CRISPR/Cas9 gene editing. Delivery of these active RNP complexes in this manner prevents both the overexpression and genomic integration of Cas9 inherent to plasmid-based delivery. Therefore, Guide-it CRISPR/Cas9 Gesicles advance genome modification by enabling efficient editing of target loci while also reducing potential off-target effects.

Methods

Production of gesicles containing Cas9 protein and sgRNA

The target sgRNA against *EMX1* was cloned into the pre-linearized pGuide-it-sgRNA1 vector included in the Guide-it CRISPR/Cas9 Gesicle Production System. This cloned plasmid was added to the provided Guide-it Gesicle Packaging Mix. The Gesicle Packaging Mix contains lyophilized Xfect Transfection Reagent premixed with an optimized formulation of plasmids encoding Cas9 and all the other elements needed for gesicle production.

Modification of the EMX1 and off-target 4 genes in HEK 293T target cells

5.0 x10^5 HEK 293T cells were plated in 24-well plates. 24 hr later, cells were either cotransfected with 500 ng each of plasmids encoding Cas9 and an sgRNA targeting *EMX1* using Xfect Transfection Reagent, or treated with 30 µl of Cas9 gesicles (produced as described above).

Determination of indel formation

72 hr later, the level of indel formation was determined using the Guide-it Mutation Detection Kit. Crude DNA extracts were prepared from cells. The modified *EMX1* locus and potential off-target site (off-target 4) were amplified using direct PCR. The PCR amplicons were melted and re-hybridized, and analyzed using the mismatch-specific nuclease, Guide-it Resolvase. The cleavage reactions were run on an agarose gel, and the percentage of DNA cleavage was determined by densitometry.

Sanger sequencing for confirmation of indels

EMX1 and off-target 4 PCR amplicons were subcloned using the Guide-it Indel Identification Kitand submitted for Sanger Sequencing.

References:

1. Sternberg, *et al*. (2015) Expanding the Biologist's Toolkit with CRISPR-Cas9. *Molecular Cell***58**(4): 568–574.
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