TECH NOTE

In Vitro Cleavage Efficiency of sgRNAs Correlates with Functional Genome Editing in Target Cells

A novel *in vitro* assay to test sgRNA cleavage efficiency
Screen various sgRNAs to determine the most effective sgRNAs prior to delivering to your cells >>

Accurate prediction of sgRNA cleavage efficiency
sgRNA cleavage efficiency predicted *in vitro* correlates with *in vivo* cleavage as assessed by both a nuclease assay and functional analysis >>

Overview

In CRISPR/Cas9 genome editing, targeting the Cas9 nuclease to a specific genomic locus is solely mediated by a user-defined sgRNA. Currently available web-based tools for sgRNA design will return a variety of candidate sgRNAs for a single gene target. Despite these *in silico* predictions, not every sgRNA will exhibit equivalent cleavage efficiency. Given this inconsistency, it is necessary to screen multiple sgRNAs to identify the most effective one.

An *In Vitro* Assay to Test sgRNA Cleavage Efficiency

The Guide-it sgRNA Screening Kit is a complete system for predicting the cleavage efficacy of sgRNAs *in vitro,* prior to use for genome editing in cells (Figure 1). With this kit, a template containing a sgRNA-target site is created by PCR; then the test sgRNA and recombinant Cas9 nuclease are added. The efficiency of Cas9-mediated cleavage can be measured by agarose gel electrophoresis.

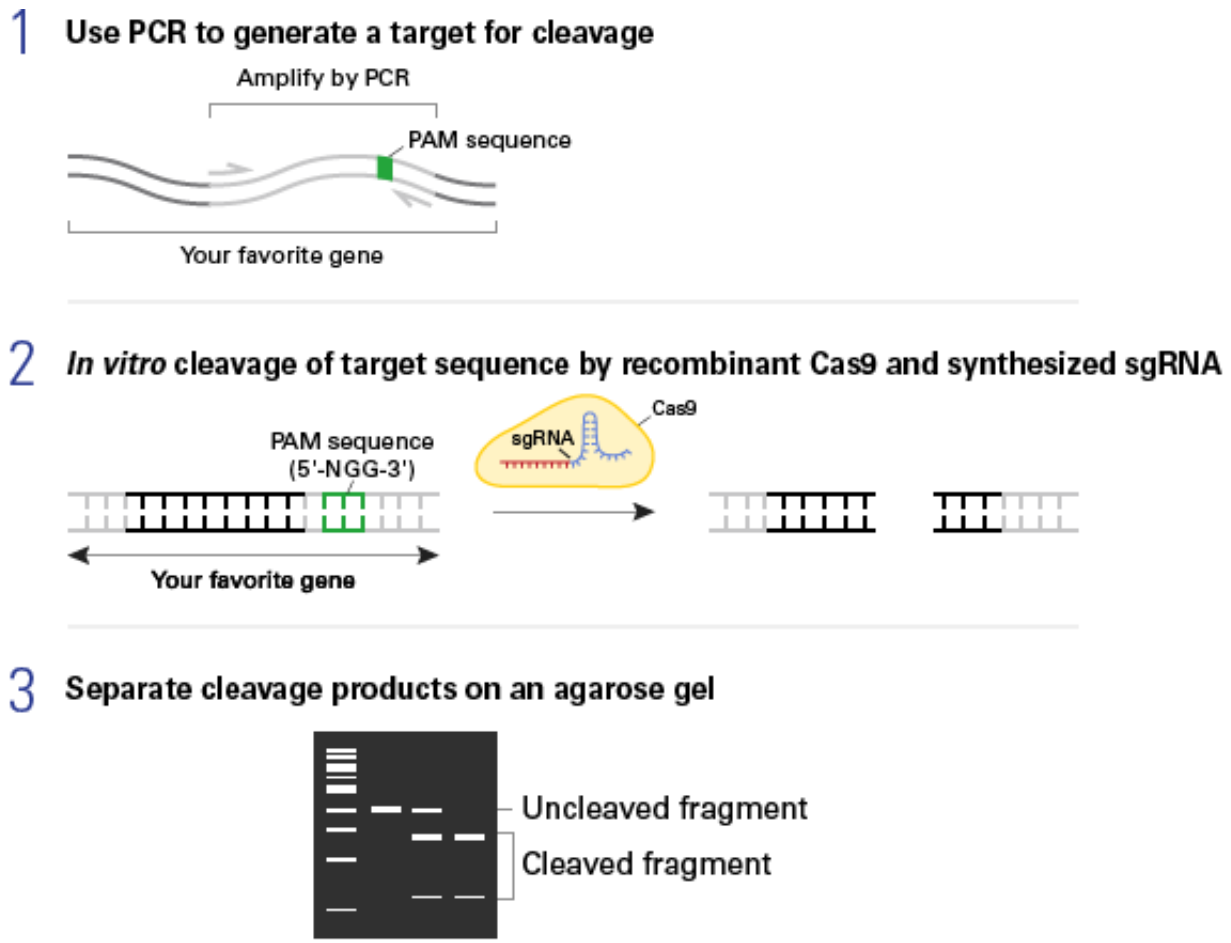

Figure 1. Overview of the Guide-it sgRNA Screening Kit protocol. A PCR amplicon containing a sgRNA target site is synthesized from genomic DNA (Step 1). The PCR fragment is then combined with a candidate sgRNA and recombinant Cas9 (Step 2). The entire reaction is separated by agarose gel electrophoresis (Step 3). Since the sgRNA-target sequence is located asymmetrically within the amplicon, cleavage by the Cas9-sgRNA complex results in two bands of unequal length that can be easily distinguished on an agarose gel.

sgRNAs Exhibit Different Cleavage Efficiencies

CRISPR/Cas9 genome editing was used to disrupt the *CXCR4* locus in HeLa cells. *CXCR4*encodes a cell surface chemokine receptor that interacts with the CXCL12 chemokine and plays an important role in the immune system.

In this experiment, four different sgRNAs targeting the *CXCR4* locus were tested using the Guide-it sgRNA Screening Kit. Briefly, sgRNAs targeting the *CXCR4* gene were synthesized using the Guide-it sgRNA *In Vitro* Transcription Kit. A PCR fragment containing the sgRNA target sequence was mixed with recombinant Cas9 protein and each sgRNA. The cleavage reaction was analyzed by agarose gel electrophoresis. Densitometry (Cong *et al.,* 2013) showed that sgRNA3 had the lowest cleavage efficiency (Figure 2).

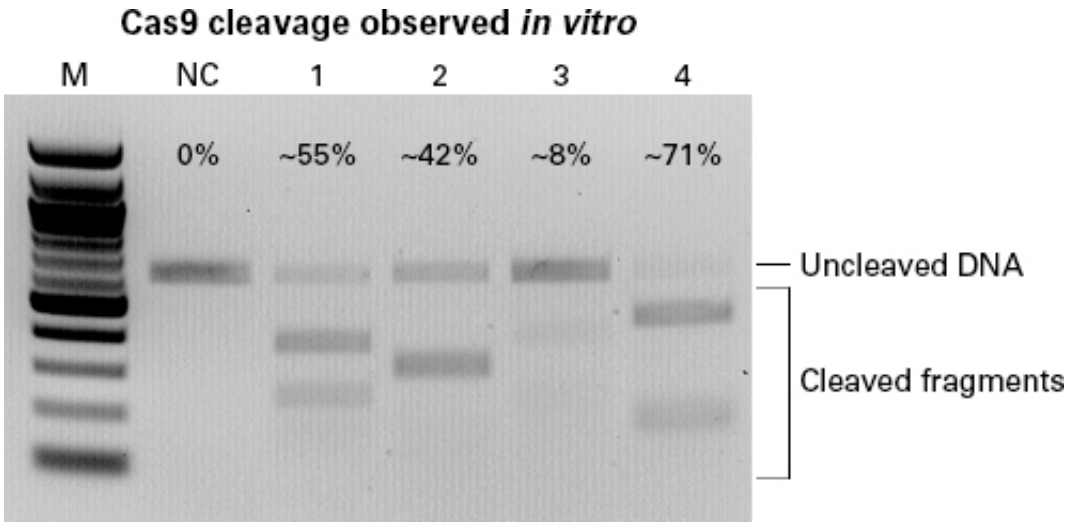

Figure 2. Differences in *in vitro* cleavage efficiency as determined by the Guide-it sgRNA Screening Kit. The cleavage efficiency of four different sgRNAs targeting the *CXCR4* locus were tested. A PCR fragment containing the *CXCR4* target sequence was synthesized and mixed with Cas9 and each sgRNA. A negative control that lacked sgRNA was included for comparison (NC). Cleavage efficiency was assessed by agarose gel electrophoresis and measured using densitometry (%).

In Vitro Cleavage Efficiency Predicts *In Vivo* Cleavage

HeLa cells were co-transfected with plasmids encoding Cas9 and one of the four different sgRNAs tested above. The presence of mutations in the *CXCR4* locus as was assayed using the Guide-it Mutation Detection Kit. This assay uses a mismatch-specific nuclease, Guide-it Resolvase, to identify insertions or deletions in specific loci in cells treated with engineered nucleases. Mismatches were detected with high efficiency in cells treated with sgRNA1, 2, and 4 (Figure 3). However, cells treated with sgRNA3 exhibited a very low efficiency of mismatches, consistent with the efficiency predicted by the Guide-it sgRNA Screening Kit (Figure 2).

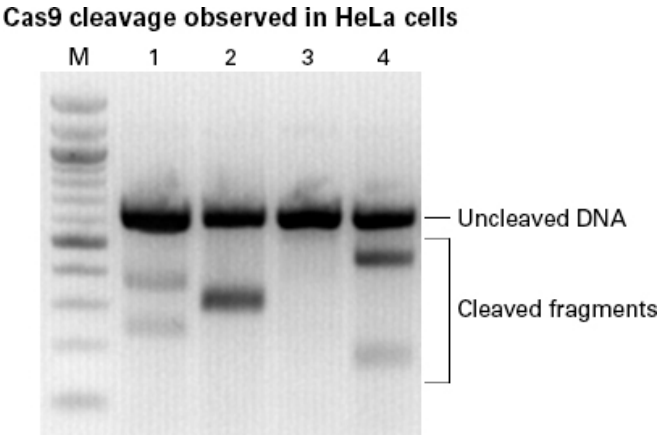

Figure 3. sgRNA-mediated cleavage in HeLa cells as determined by the Mutation Detection Kit. HeLa cells were co-transfected with plasmids encoding Cas9 and one of the four different sgRNAs using Xfect Transfection Reagent. Six days after transfection, cells were assayed for the presence of mutations using Guide-it Resolvase, a mismatch-specific nuclease. Cleavage fragments were present for all sgRNAs except sgRNA3, indicating low Cas9 guiding efficiency for this particular sgRNA.

CXCR4 gene disruption was also assessed by flow cytometry; since CXCR4 is a cell surface receptor, it can be detected by flow cytometry using a FITC-labeled CXCR4 antibody. Disruption in CXCR4 expression could be detected in cells transfected with Cas9 and sgRNA1, 2, and 4 (Figure 4). In contrast, for cells transfected with Cas9 and sgRNA3, a much smaller proportion of the cells had disruption of CXCR4 expression. These functional data confirm the results obtained by both the Guide-it sgRNA Screening Kit and Mutation Detection Kit.

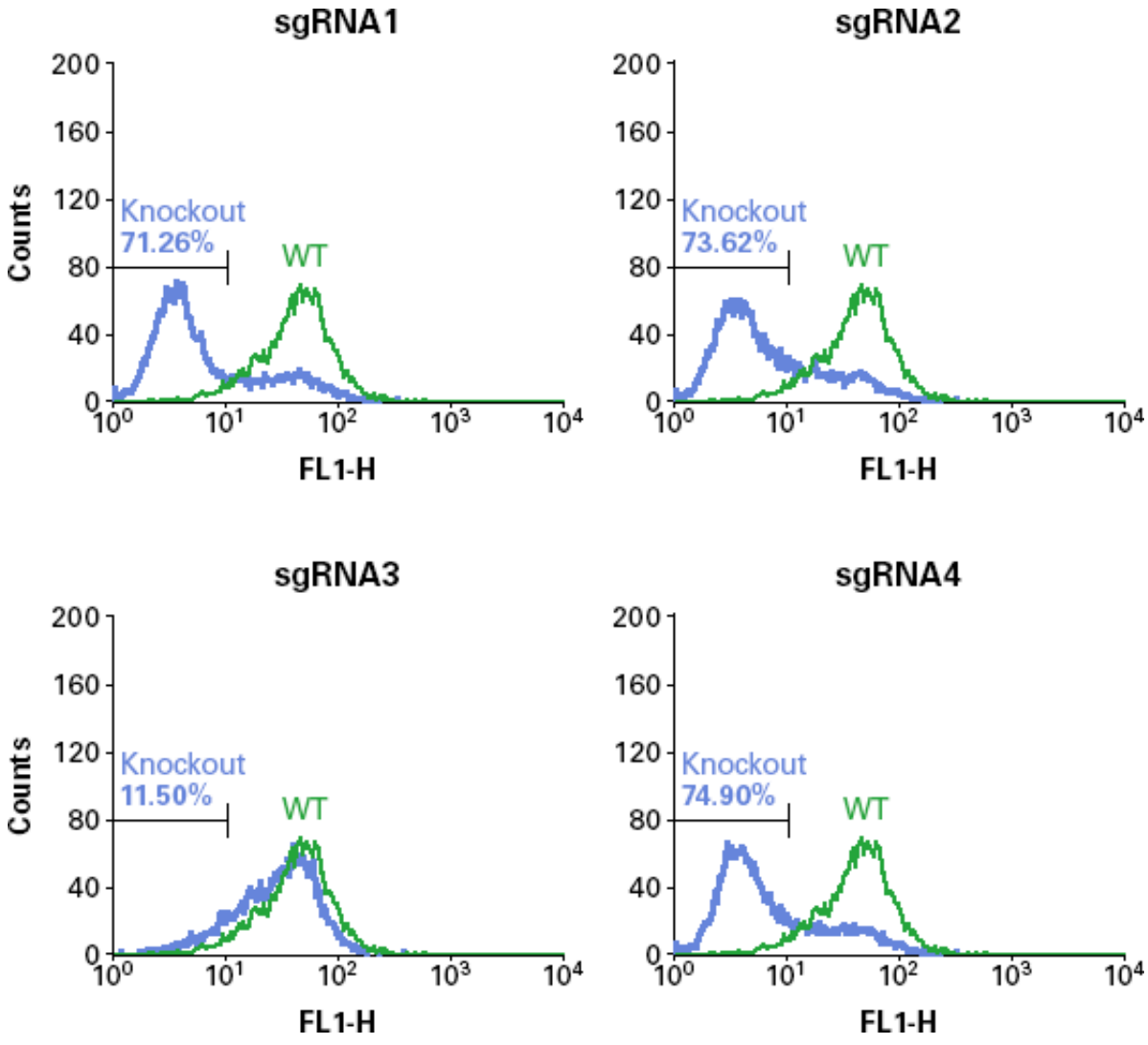

Figure 4. Flow cytometric analysis detects sgRNA-mediated loss of CXCR4 function. HeLa cells cotransfected with plasmids encoding Cas9 and one of four sgRNAs, were stained with a FITC-labeled antibody against CXCR4. Knockout of *CXCR4* gene by CRISPR/Cas9 editing will result in in reduced protein expression. Therefore, FITC staining is inversely correlated with efficient genome editing. The percentage (%) of the cell population that were not labeled with FITC is shown in blue. Cells treated with Cas9 and sgRNA3 exhibit the greatest percentage of FITC+ cells and the least efficient genome editing.

Conclusions: Accurate Prediction of sgRNA Cleavage Efficiency

There is a clear correlation between *in vitro* sgRNA cleavage efficiency as predicted by the Guide-it sgRNA Screening Kit, and *in vivo* sgRNA-mediated cleavage as assessed by the presence of indels and functional gene knockout (Figure 5). These results indicate that the Guide-it sgRNA Screening Kit is ideal method for screening for ineffective sgRNAs during CRISPR/Cas9 genome editing projects.

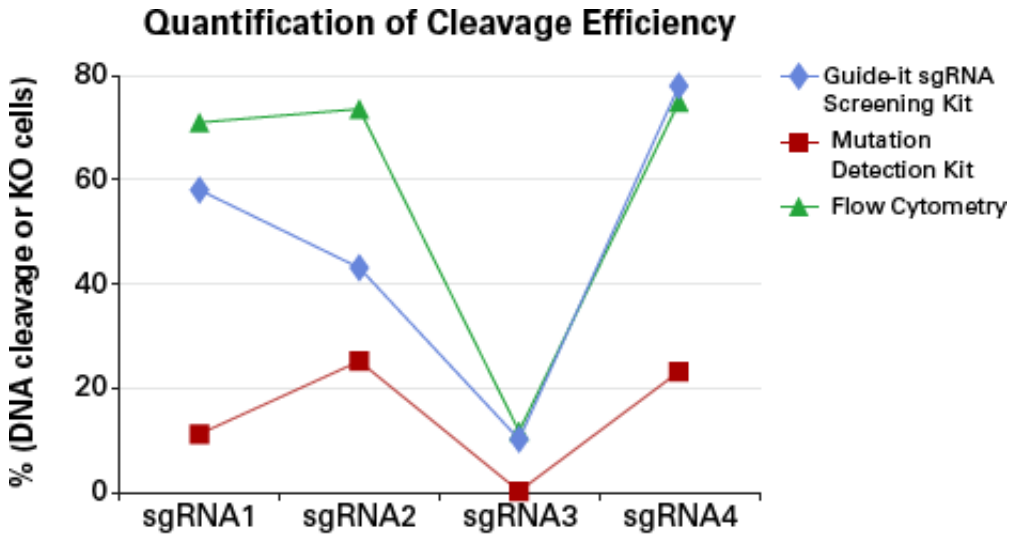

Figure 5. Guide-it sgRNA Screening Kit accurately predicts *in vivo* sgRNA efficacy. Cleavage efficiency was assessed by *in vitro* cleavage (Figure 2) and the Guide-it Mutation Detection Kit (Figure 3); functional knockout was assessed by flow cytometry (Figure 4, % of CXCR4- cells). There is a clear correlation between the efficiency predicted by the Guide-it sgRNA Screening Kit, and estimation of *in vivo* cleavage (Mutation Detection Kit) and functional knockout (Flow Cytometry).

References

Cong, L. *et al.* (2013) Multiplex genome engineering using CRISPR/Cas9 systems. *Science* **339**(6121):819–23.

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http://www.clontech.com/US/Products/Genome_Editing/CRISPR_Cas9/Technical_Notes/Screening_for_effective_guideRNAs