Modified sgRNA Design Improves Results of CRISPR Knockout Screens

Introduction

The CRISPR single-guide RNA (sgRNA) design published by Jinek, et al. in 2012 has become the gold standard for CRISPR-mediated gene knockout. It was developed by fusing two oligonucleotide components of the native *Streptococcus pyogenes* CRISPR system—the tracrRNA and crRNA—into a single molecule where the first 40 bases contains the 20-base variable targeting region and the first part of the initial stem-loop. This first 5′ domain of the sgRNA corresponds to the crRNA in the native bacterial system. The rest of the sgRNA is derived from the tracr sequence in the bacterial system where it hybridizes with the crRNA. Thus, the sgRNA contains an initial variable region of about 20 bases followed by a constant sequence of about 80 nucleotides that contains all the key interactions with the Cas9 nuclease.

Several research groups have attempted to optimize the design of the initial variable region that defines the sequence the sgRNA targets, in order to ensure an effective knockout while minimizing off-target disruptions (Doench, et al., Fu, et al.) However, as described above, the 20-base targeting sequence makes up only a small portion—about one-fifth of the sgRNA sequence. The other 80 bases that are downstream of this targeting sequence interact primarily with the Cas9 endonuclease to catalyze gene knockout. If modifications to this constant 3′ region of the sgRNA molecule could improve knockout efficiency, any sgRNA could be made more effective and this might offer a general approach to improve our pooled genome-wide sgRNA libraries.

One study (Chen, et al.) with an inactive Cas9 nuclease has shown that sequence modifications to the constant region improved Cas9 binding significantly. However, since an inactive Cas9 mutant was used in these studies, it wasn't clear if the changes would actually increase the rate or the knockout efficiency of the active CRISPR system, and further, if they did, what effect they would have on the results of CRISPR-based pooled genetic screens. We initiated a study to address these points.

Experiment and Results

To investigate if changes in the 3′ Cas9-binding portion of the sgRNA did, in fact, increase the efficiency of CRISPR-mediated knockout, we first ran some initial experiments with a few sgRNA sequences targeting a GFP gene. We specifically looked at two modifications of the constant 3′ region of the guide sequence mentioned in the citation noted previously (Chen, et al., Figure 1). One modification swapped locations of adenine (A) and thymine (T) residues ("AT") to remove a transcription terminator site. Another alteration adds a 5′- nucleotide

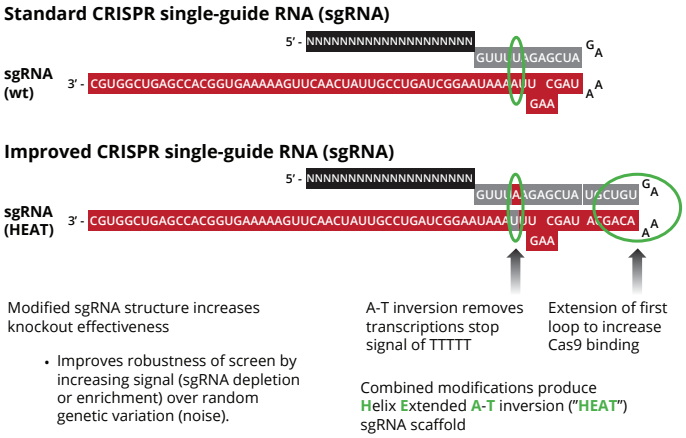

Figure 1

HEAT-modified sgRNA compared to Standard CRISPR sgRNA

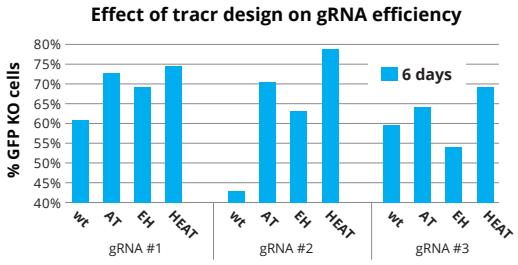

Figure 2

Lentiviral constructs with three different sgRNAs targeting a green fluorescent protein (GFP) sequence were transduced into cells stably expressing GFP. Four variants of each sgRNA were used: the "wild type" (wt) sequence, a variant with an AT inversion that eliminates a transcription termination site, a variant with an insertion (HE) that elongates and stabilizes a stem-loop structure, and a variant with both the HE and AT (HEAT) modifications. The change in GFP expression 6 days after transduction was assayed. In most cases, all three modified sgRNA designs reduced GFP fluorescence more quickly than the standard wt version.

extension ("HE") to the stem of a stem-loop structure which should make it more stable and accessible to the Cas9 protein. We found that these two substitutions did indeed increase the rate of target knockout—at least with the one target (Figure 2). Both the AT inversion and HE insertion significantly improved the knockout rate of GFP relative to the "wild type" (wt) sgRNA sequence. While the effects varied somewhat for each sequence, the overall positive impact of these changes on several targets in the GFP sequence was clear.

The knockout results of GFP with the modified sgRNAs indicated that including these changes to 3′ guide sequences in a pooled library might increase the knockout rate and