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Authors
deBoer, Tara R
Wauford, Noreen
Chung, Jing-Yi
et al.

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A Cleavage-Responsive Stem-Loop Hairpin for Assaying Guide RNA Activity

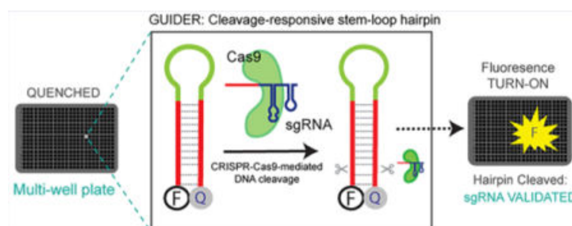
Tara R. deBoer[†], **Noreen Wauford**[†], **Jing-Yi Chung**[†], **Miguel Salvador Torres Perez**[†], and **Niren Murthy**[*,†]

[†]Berkeley Department of Bioengineering, University of California, Berkeley, California 94720, United States

Abstract

The scope of the CRISPR-Cas9 technology now reaches far beyond genomic engineering. While significant efforts are driving the evolution of this revolutionary biomedical tool, the *in vitro* cleavage assay remains the standard method implemented to validate the guide RNA that directs endonuclease Cas9 to a desired genomic target. Here, we report the development of an alternative guide RNA validation system called GUIDER. GUIDER features a hairpin loop structure with a proximal guanosine-rich unit, a distal fluorophore unit, and a gRNA-targeting stem component. Cleavage of GUIDER by its complementary RNA-guided Cas9 endonuclease complex yields a fluorescent emission at 525 nm, signaling effective cleavage of the hairpin structure. GUIDER was validated using the model gene target *mpcsk9*, and it was able to identify the gRNA that could most efficiently cleave the target *mpcsk9* gene. The modular design of GUIDER should allow it to have broad applicability in validating gRNAs, and its fluorescent signal output offers a rapid, simple, and quantitative measure of Cas9-mediated DNA cleavage.

Graphical abstract



[*]**Corresponding Author**, nmurthy@berkeley.edu.

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acschembio.7b00899. Experimental methods and Figures S1 and S2 (PDF)

ORCID

Tara R. deBoer: 0000-0002-1549-3157

Notes

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INTRODUCTION

The RNA-programmable gene-editing system CRISPR-Cas9 (clustered regularly interspaced palindromic repeats)-Cas9 (CRISPR-associated protein) has emerged as a powerful tool capable of directing site-specific genomic modifications.[1–3] More recently, the CRISPR-Cas9 technology has been used for applications beyond genome engineering. Reports highlighting innovative applications of CRISPR, such as chromosomal imaging,[4,5] diagnostics,[6] and genomic screening,[7] underscore the expanding scope of this technology.

The common thread among all CRISPR-Cas9 applications is the programmable guide RNA (gRNA) that directs endonuclease Cas9 to bind to a compliment DNA sequence, enabling highly selective gene targeting. Interestingly, while the evolution of the CRISPR technology proceeds at an exponential rate,[8] the cornerstone methods implemented to analyze and validate the activity of a designed gRNA have not advanced. *In vitro* cleavage and deep sequencing analysis remain the only methods for gRNA validation, and these can be tedious, time-consuming, expensive, instrument-dependent, and inaccessible to researchers outside the field of biology. Therefore, the development of innovative approaches to rapidly validate and analyze the efficacy of designed gRNAs would streamline workflow practices and make the CRISR-Cas9 technology more available to broader populations of scientists.

Here, we report the development of a rapid and quantitative gRNA validation tool named GUIDER. The GUIDER scaffold is a single-stranded DNA molecule comprised of a proximal guanosine-base (G-base) unit, a distal fluorophore unit consisting of fluorescein, a loop region, and a gRNA-complementary region (see Figure 1). When folded into its paired double-helix secondary hairpin structure, the G-base unit quenches fluorescein to afford the CRISPR-Cas9-responsive GUIDER hairpin. Cleavage of GUIDER by gRNA-associated Cas9 (ribonucleoprotein, RNP) results in an increase in fluorescence, because of separation of the G-bases unit from the fluorescein unit, and this enables the quantitation of gRNA activity. In contrast to *in vitro* cleavage assays, the GUIDER system utilizes fluorescence as the signaling modality, in contrast to tedious gel electrophoresis, and affords a simple readout that can be measured using a microplate reader or gel imager.

To demonstrate the potential of GUIDER as a gRNA validation tool, we selected the mus *pcsk9* gene (*mpcsk9*) as a model gene target. The *pcsk9* gene encodes for protein PCSK9 (protein convertase subtilisin/kexin type 9), which has been associated with cardiovascular disease by contributing to elevated low-density lipoprotein levels.[9] As such, interest in applying CRISPR-Cas9 to target and silence the *pcsk9* gene as a therapeutic tactic has been considered.[10] We designed and synthesized two single-guide RNAs (sgRNAs) that target unique loci within *mpcsk9* using the DNA 2.0 *crispr grna design tool* (sgRNA-1 and sgRNA-2) and synthesized a third sgRNA (sgRNA-3) reported in the literature by Ding et al.[10]

In this report, experiments were performed to validate GUIDER's ability to quantitatively analyze the cleavage capacity of three unique sgRNAs toward its complementary DNA targets within the *mpcsk9* gene. The results from these studies demonstrate the potential of GUIDER to act as a simple and quantitative gRNA-analyzing tool, free of tedious and

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laborious techniques. In addition, experiments were performed to compare the ribonuclease activity directed by each *mpcsk9*-targeting sgRNA using GUIDER, and GUIDER was able to identify the most effective sgRNA using its fluorescence-based signal output. The quantitative capacity and modular design of GUIDER makes it an attractive option for analyzing and validating the activity of gRNAs.

DESIGNING A CRISPR-CAS9-RESPONSIVE PROBE FOR GRNA ANALYSIS

GUIDER is a single-stranded DNA hairpin that mimics the cleavage template implemented in traditional *in vitro* cleavage assays. The modular DNA-hairpin in GUIDER is highlighted in Figure 1a and contains an interchangeable CRISPR-Cas9-responsive stem region comprised of a 20-nucleotide sequence that was first designed to validate a sgRNA targeting exon 5 of the *mpcsk9* gene target, termed sgRNA-1 (red dotted box in Figure 1a, sequence = ACAGTCAGCGGCACCCTCAT). The CRISPR-responsive stem region is flanked by a hairpin nucleation region (denoted in dark green in Figure 1a) at the base of the terminal loop structure (denoted in light green in Figure 1a), and a PAM region (denoted in pink in Figure 1a) that features the –NGG sequence specific to *S. pyogenes* Cas9. Finally, the terminal end of the hairpin is comprised of a 5-nucleotide spacer region (denoted in light blue in Figure 1a) that spatially buffers the signal elements from the RNP binding site.

The signal elements of GUIDER include the fluorescein-G-base fluorophore–quencher pair that enables a simple fluorescent signal output for GUIDER, where the intact GUIDER hairpin remains in a *quenched* nonfluorescing state and the RNP-cleaved product affords an *unquenched* fluorescing state. Based on the established literature,[11,12] we incorporated the guanine-5-carboxyfluoresceine (5-FAM) photoinduced electron transfer (PET) system into the GUIDER scaffold by labeling the 5′-terminus of the single-stranded CRISPR-Cas9-responsive DNA molecule with 5-FAM and four terminal guanine nucleobases at the proximal 3′-terminus (see Figure 1a). Heating of the single-stranded GUIDER DNA strand to 90°C and holding for 2 min with $-0.2\ \mathrm{min}^{-1}$ incremental cooling over a 2 min period afforded the quenched hairpin DNA structure that was confirmed by gel electrophoresis and fluorescence imaging (Figure 1). The PAGE gel image shown in the inset of Figure 1c displays several higher-order bands indicative of intermolecular double-stranded structures in the lane where unheated solutions of single-stranded GUIDER DNA were loaded. In contrast, a single band was visualized in the lane where folding of the single-stranded GUIDER DNA into its secondary hairpin structure was driven by heating.

We performed experiments to determine if the nucleotide composition of the loop sequence in GUIDER affects its fluorescence quenching capacity. Several studies have demonstrated that the sequence directly adjacent to the loop structure (the seed sequence) of a hairpin can influence the folding dynamics and, therefore, the quenching capacity of fluorophore-quencher pairs. In contrast to traditional fluorophore-quencher systems that harness Förster resonance energy transfer (FRET) pathways, the G-base quencher of GUIDER quenches the fluorescence of 5-FAM via a PET mechanism that requires that an electron from electron-rich guanine be transferred to an excited 5-FAM electron acceptor.[13] We designed two GUIDER probes comprised of identical CRISPR-Cas9 responsive sequence, PAM, and spacer sequences, but varied in their seed sequences with one containing a AA-TT (AA on

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one strand is paired with TT on the other strand) and the other containing a GG-CC. We compared the relative propensity of the two GUIDER DNA molecules to fold into the desired hairpin structure by determining the percent fluorescent quenching of the various GUIDER solutions after heating and cooling (Figure 1c).

The percent quenching was defined as the fluorescence emission intensity at 520 nm of the unheated, unfolded single-stranded GUIDER solutions (excitation = 490 nm) divided by the fluorescence emission intensity of the folded GUIDER hairpin solution at equivalent concentrations. GUIDER DNA molecules that display a lower percent quenching have a higher propensity toward hairpin formation, because the unheated stock hairpin solution has a higher abundance of folded DNA strands, in comparison to unfolded DNA strands. An additional single-stranded DNA molecule was analyzed as a control that featured an overhang sequence at the proximal quenching unit (overhang GUIDER) as a strategy to perturb the folding of the AA-TT GUIDER single-strand DNA. The results from this study confirmed that the adenosine–thymine nucleation sequence directs folding of the single-stranded GUIDER sequence into a hairpin secondary structure.

Similarly, the four-nucleobase sequence of the loop region of the GUIDER stem hairpin structure was varied to study its effect on hairpin folding and stability. In these experiments, TTTT, AAA, and GTAA loop sequences were examined by incorporating each unique loop sequence into a GUIDER comprised of the sgRNA-1 complementary CRISPR-Cas9 responsive sequence, –NGG PAM sequence, 5-bp spacer sequence, and GG-CC nucleation region. The GG-CC nucleation sequence was incorporated because it demonstrated a more-significant fluorescence range between folded and unfolded states. As described above, the percent quenching was calculated to measure the propensity of a GUIDER DNA strand toward hairpin formation and, therefore, hairpin stability. Interestingly, no significant difference in percent quenching was observed between the GUIDER loop variants (see Figure S1 in the Supporting Information). Therefore, we moved forward with the TTTT loop sequence to maintain symmetry and because thymine represents the smallest nucleobase and should therefore provide the least steric strain upon loop formation. Taken together, the core GUIDER sequence scaffold was defined to contain the AA-TT nucleation and TTTT loop sequences and was implemented in all future GUIDER hairpins, unless otherwise noted.

With the final GUIDER DNA sequence designed and optimized, we next investigated the potential of GUIDER to act as an effective gRNA validation assay. We chose sgRNA-1 (targeting exon 5 of *mpcsk9*) to verify the GUIDER system, because it featured a higher DNA 2.0 score, compared to sgRNA-2 (100% vs 67.13%). After validating sgRNA-1 by *in vitro* cleavage analysis, we ordered the single-stranded GUIDER molecule from Integrated DNA Technologies (IDT) that incorporated the sgRNA-1 complementary CRISPR-Cas9 responsive stem sequence. The active folded GUIDER hairpin, denoted hereafter as GUIDER-1, was prepared following the heating procedure described above.

GUIDER-1 mediated gRNA validation was investigated by incubating a 500 nM solution of GUIDER-1 (final concentration) with 300 ng of RNP-1 (300 ng of sgRNA-1 and 300 ng of Cas9), where RNP-1 was prepared by incubating sgRNA-1 with Cas9 for 15 min at 37°C. The samples were incubated at 37°C for 30 min, allowed to cool to 20°C for 10 min, and

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then were (i) transferred into the wells of a 384-well plate and analyzed by a microtiter plate reader or (ii) loaded into a polyacrylamide gel for PAGE analysis.

We collected the fluorescence emission intensity at 520 nm (excitation 490 nm) of each control and sample solution using a microtiter plate reader and observed a 6.25-fold emission enhancement in fluorescence when GUIDER-1 was incubated with RNP-1. No appreciable fluorescence emission enhancement was observed in control samples where GUIDER-1 was incubated with Cas9 or sgRNA-1. Results reported in Figure 2d display the difference in emission intensity of each condition collected at time zero and time = 30 min. Supporting PAGE analysis of the GUIDER experimental samples was performed and showed two new bands corresponding to RNP-GUIDER complex formation (greater than 200 bp marker, Figure 2d) and 1 cleavage product band below the 50 bp marker that corresponds to the 31 bp hairpin cleavage product (Figure 2e).

Further control experiments were conducted to establish the selective responsiveness of GUIDER by analyzing the fluorescence output triggered upon incubation of GUIDER-1 with nonhomologous RNPs. To do so, we synthesized sgRNA-2 and sgRNA-3 that target alternative loci in the *mpcsk9* gene. GUIDER-1 was therefore incubated with RNP-2 or RNP-3 that were prepared by incubating sgRNA-2 or sgRNA-3 with Cas9 as described above. In addition, RNP-1 was analyzed by GUIDER-2 and GUIDER-3 to confirm that the fluorescence observed in the presence of GUIDER-1 does not correspond to nonspecific interactions. When GUIDER-1 was incubated with RNP-1, RNP-2, or RNP-3, an appreciable turn-on in fluorescence was only observed in the presence of RNP-1 (Figure 3b). Similarly, RNP-1 only elicited a signal response in the presence of GUIDER-1 and not in the presence of GUIDER-2 or GUIDER-3. Taken together, these results verified the selective cleavage of the GUIDER hairpin system by sgRNA-directed Cas9 (Figure 3c).

Lastly, we explored the ability of GUIDER to quantitatively compare the activity of various gRNAs by comparing the fluorescence output afforded by RNP-1, RNP-2, and RNP-3 when analyzed by their corresponding GUIDER probe (GUIDER-1, GUIDER2, and GUIDER-3, respectively). The observed fluorescence enhancements are reported in Figure 4a, and they indicate that sgRNA-1 was the most efficient at directing Cas9-mediated cleavage of its target DNA. These results were corroborated using an *in vitro* DNA cleavage assay. RNP-1, RNP-2, and RNP-3 were serially diluted and incubated with 300 ng target *mpsk9* gene target (see Figure 4b, as well as Figure 2S in the Supporting Information). After 1 h of incubation at 37°C, each sample was studied by PAGE gel analysis. The gel analysis confirmed that RNP-1 was the most efficient ribonuclease. Only 400 ng of RNP-1 was required to completely cleave its target DNA template, whereas RNP-2 and RNP-3 required 1000 and 900 ng.

In conclusion, this work supports the potential of GUIDER as an alternative CRISPR guide RNA analysis tool. The advantages of GUIDER are 3-fold. First, the tool is modular, and the CRISPR-Cas9 responsive region of GUIDER can be easily replaced to match the complementary sequence of any gRNA. Second, the 5-FAM-G-base fluorophore–quencher system of GUIDER offers a simple signal output and introduces a feature of quantification into gRNA analysis that has not been previously available. Lastly, GUIDER-mediated gRNA

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analysis is inexpensive at only $0.40 U.S. dollars per validation study. At this cost, the GUIDER technology has the potential to translate into a high-throughput tool to perform rapid sgRNA or Cas9 activator or inhibitor screens. Taken together, GUIDER represents a simple approach to streamline and innovate CRISPRS-Cas9 analysis practices.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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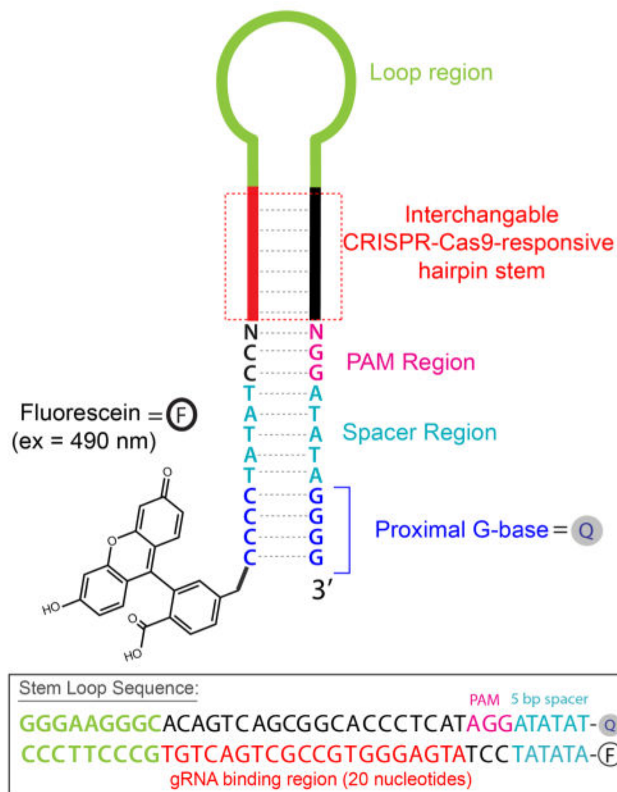

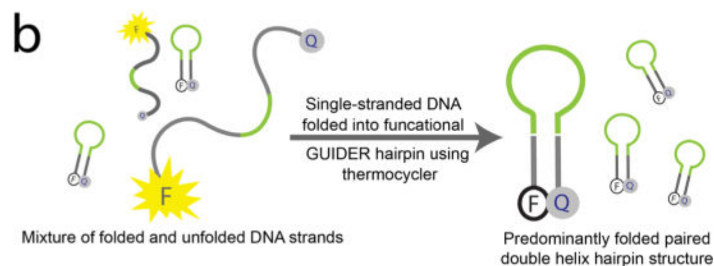

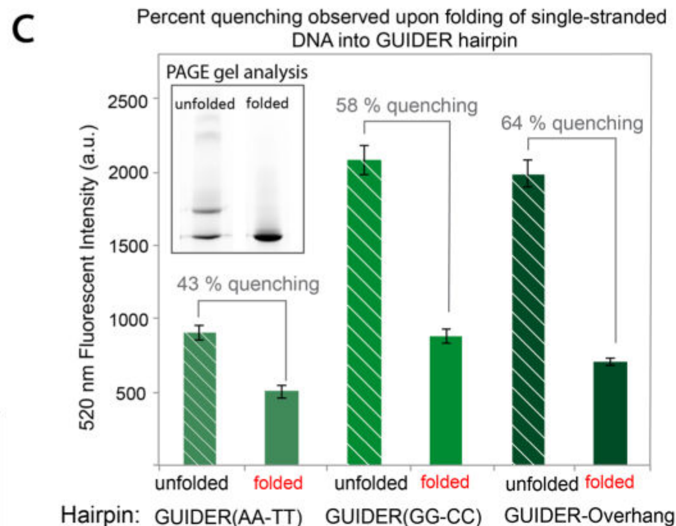

Figure 1.
CRISPR-Cas9-responsive GUIDER system is a modular system. (a) The folded GUIDER hairpin is comprised of a loop region, an interchangeable CRISPR-Cas9-responsive stem region and terminal fluorophore and quenching units. (b) Cas9-responsive GUIDER is prepared by heating single-stranded GUIDER DNA to direct hairpin formation. (c) The calculated percent quenching of GUIDER(AA-TT), GUIDER(GG-CC) and GUIDER-overhang are shown in the plot, where percent quenching is calculated as a percentage difference between the fluorescence emission at 520 nm of the single-stranded GUIDER DNA molecule (denoted as "unfolded") and the final folded GUIDER hairpin (denoted as "folded") afforded after heating. A pictorial representation of each GUIDER hairpin is represented under the corresponding percent quenching values of the plot. Error bars in panel (c) represent the standard deviation (s.d.) where $n = 3$.

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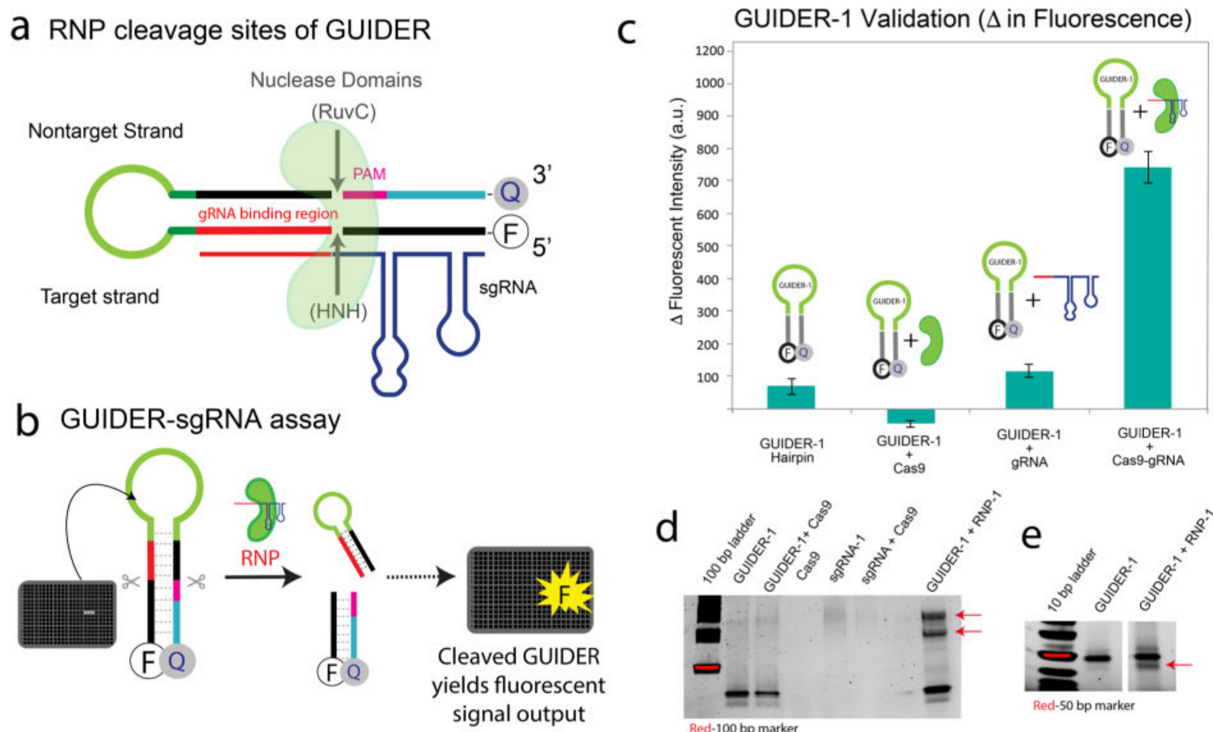

Figure 2.

GUIDER is a CRISPR-Cas9-responsive tool. (a) Schematic representation of the proposed CRISPR-Cas9 cleavage sites, where the RuvC and HNH nuclease target regions are denoted by gray arrows. The gRNA-binding site, PAM region, and 5-bp spacer are represented in red, pink, and light blue, respectively. The corresponding sgRNA is shown with the target sequence denoted in red (the targeted complement RNA-binding site of GUIDER is also denoted in red). (b) An overview of the GUIDER assay where the GUIDER probe is incubated with an analogous gRNA-associated Cas9 (RNP) that cleaves the hairpin and affords a fluorescent signal output. (c) The results from the sgRNA-1 validation studying using probe GUIDER-1 are shown in the plot. The fluorescence values are reported as a change in the fluorescence emission intensity at 520 nm, relative to a time zero reading. Error bars denoted in the plot represent the s.d. where $n = 2$. (d) PAGE gel image of the samples corresponding wells completed in parallel with the GUIDER assay, where GUIDER was incubated with Cas9 only, sgRNA-1 only, and the active RNP-1 that was prepared by incubating sgRNA-1 with Cas9, as described previously. Each sample is identified with the associated label above the corresponding sample lane and a 100 base-pair (bp) ladder was used as a standard. The red arrows indicate GUIDER-RNP complexes that are likely associated with intact and cleaved GUIDER hairpins. (e) A secondary PAGE gel image highlighting the cleavage product afforded upon incubation of GUIDER-1 with RNP-1, with a 10 bp ladder incorporated as the standard. The 31 bp cleavage product is indicated by the red arrow.

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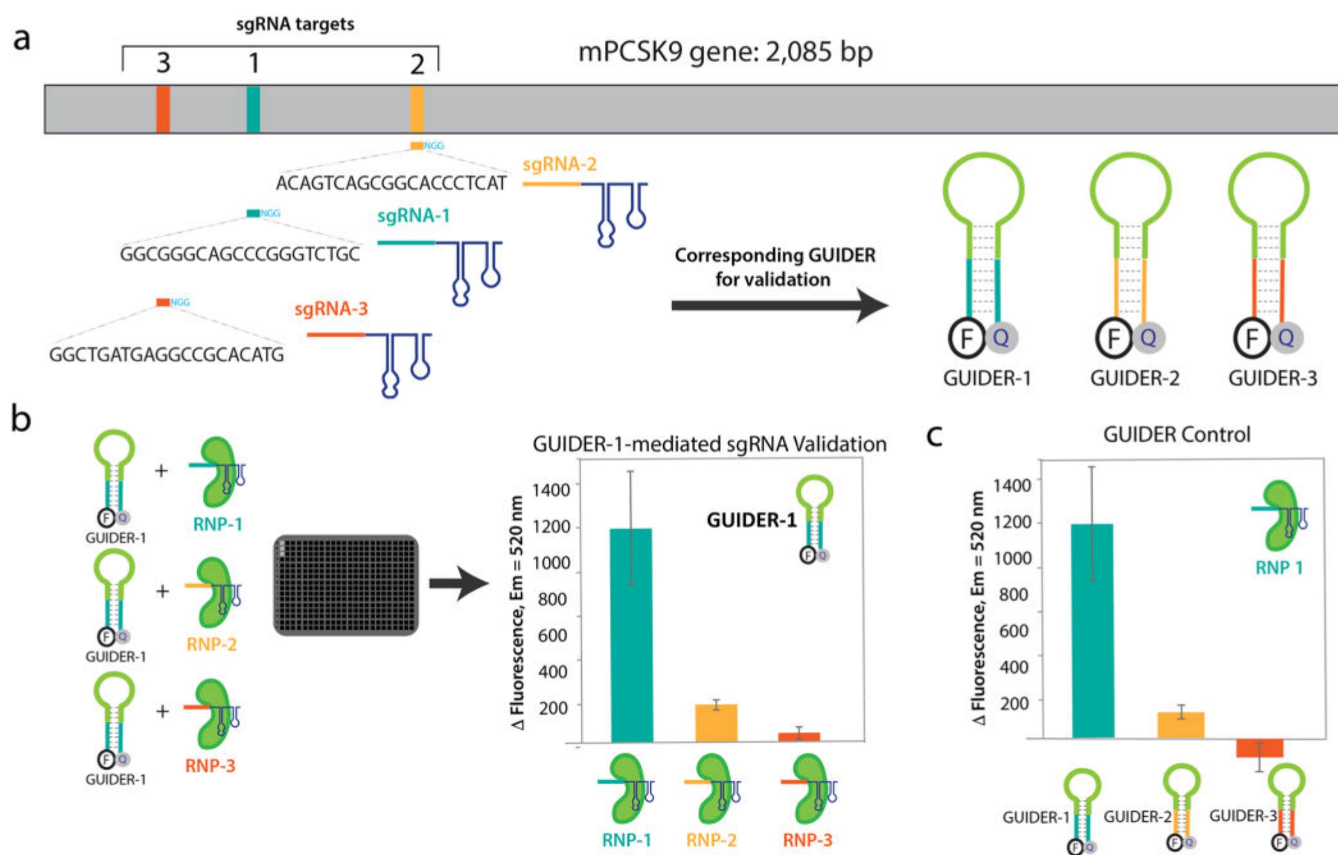

Figure 3.

RNP-1 selectively triggers GUIDER-1. (a) Schematic representation of the *mpcsk9* gene with targeted cleavage sites denoted by teal (site 1), yellow (site 2), and orange (site 3) bands. The corresponding target sequences of the sgRNAs synthesized to target the gene sites are listed below each site, where sgRNA-1, sgRNA-2, and sgRNA-3 target sites 1, 2, and 3, respectively. The target sequences were then incorporated into individual GUIDER hairpin sequences to afford the final CRISPR-Cas9 responsive GUIDER hairpin. (b) Experimental workflow of the sgRNA screen using GUIDER-1, containing the CRISPR-Responsive hairpin stem sequence complement to target sgRNA-1. GUIDER-1 was incubated with RNP-1, RNP-2, and RNP-3, respectively, in the wells of a 384-well plate for 30 min at 37°C. After incubation, the plate was allowed to cool to room temperature (RT) for 10 min and then the samples wells were analyzed using a microtiter plate reader. Emission intensities at 520 nm were collected (excitation = 490 nm), and the change in fluorescence was calculated and plotted. Results from this study demonstrated that GUIDER-1 is selectively triggered by RNP-1 that contains complement sgRNA-1. Error bars denoted in the plot represent the standard deviation (s.d.) error, where $n = 2$. (c) Similarly, RNP-1 was incubated with GUIDER-1, GUIDER-2, and GUIDER-3 in control wells (30 min at 37°C) to confirm selective turn-on of GUIDER-1 by RNP-1.

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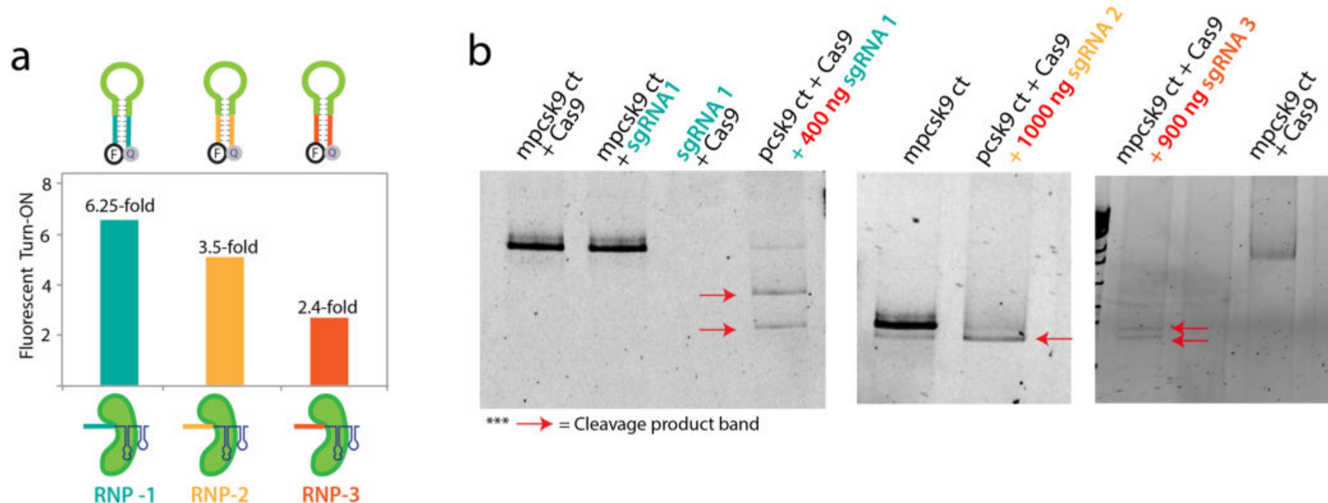

Figure 4.

GUIDER introduces the possibility of quantitation to gRNA analysis and validation. (a) By measuring and calculating the fluorescence enhancement observed upon incubation of each GUIDER with its respective RNP (i.e., GUIDER-1 with RNP-1), the corresponding sgRNAs were quantitatively compared. These comparisons are shown in the plot, where RNP-1 was observed to elicit the strongest turn-on of the GUIDER system with a 6.25-fold enhancement in fluorescence. Error bars denoted in the plot represent the standard error, where $n = 2$. (b) Validation of sgRNA-1, sgRNA-2, and sgRNA-3 using traditional *in vitro* cleavage assay, where each sgRNA and corresponding RNP were incubated with *mpcsk9* cleavage template for 1 h at 37°C and then analyzed by PAGE. Red arrows highlight the resulting cleavage bands.