A universal dark-to-bright GFP reporter for gene editing

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Abstract

Current fluorescent reporters for assessing activity of CRISPR-Cas system, such as Stoplight or split-GFP based on frameshift activation, often suffer from limitations including large vector size, low viral packaging efficiency, and insensitivity to in-frame insertions or deletions. Although specialized reporters for base editing (e.g., BEAR) and prime editing (e.g., PEAR and fluoPEER) have been developed, there remains a significant demand for a compact, sensitive, and universal single-fluorescent reporter compatible with diverse editing types.

We constructed a novel reporter system based on a single fluorescent protein employing a "dark-state quenching to bright-state activation" strategy, named CRISPR-Bright (CRB). Its core design involves fusing a green fluorescent protein with a 28-amino-acid quenching peptide derived from the influenza M2 protein. In unedited station, the fusion protein forms tetramers mediated by the quenching peptide, completely suppressing GFP fluorescence. When Cas9 or prime editor generates insertions or deletions (±1 or ±2 nt frameshift) or a designed premature stop codon at the target site, the coding sequence of the quenching peptide is disrupted or

terminated. Upon impaired expression of the quenching peptide, GFP folds, matures, and emits strong green fluorescence. We engineered this design into a lentiviral vector with a total size of only 5.8 kb, significantly improving viral packaging and delivery efficiency.

The result showed the CRB reporter efficiently reports Cas9 and prime editor mediated Indel events and enables sensitive, visual monitoring of prime editor activity. Its performance is notably superior to existing dual-fluorescent or large-vector-based reporter systems.

Keywords

CRISPR-Cas9, prime editor, GFP reporter

Introduction

CRISPR–Cas nucleases have emerged as transformative tools in gene therapy, cell engineering, and functional genomics. However, their editing efficiency is highly variable, influenced by factors such as cell type, chromatin accessibility, and guide RNA design. This variability underscores the critical need for reporter systems that enable real-time, quantitative, and direct coupling to editing outcomes. Such systems are essential for optimizing editing conditions and facilitating high-throughput screening.

Current methods for assessing CRISPR-Cas activity fall into two main categories. The first comprises endpoint assays, including T7E1 and Surveyor nuclease assays[1], which are cost-effective, lack real-time capability and offer limited throughput. The second, and more versatile category, utilizes fluorescent reporters, such as dual-fluorescence[2] or split-GFP systems [3-5], which allow for live-cell monitoring and are amenable to high-throughput analysis. Among these, a prevalent design strategy involves engineering a "dark-to-bright"

switch by embedding a CRISPR target sequence within the coding region of a fluorescent protein[6-10]. Successful editing via non-homologous end joining (NHEJ) can reconstruct the correct reading frame, thereby activating fluorescence. More advanced systems, like the "Stoplight[11]" reporter, employ dual fluorescent proteins to simultaneously distinguish between NHEJ and homology-directed repair (HDR) outcomes. Despite their utility, these reporters often rely on large, polycistronic plasmid constructs. This substantial size can hinder efficient viral packaging—a key consideration for therapeutic delivery—and pose challenges for consistent co-expression.

The advances of precision editing tools, notably base editors (BEs) and prime editors (PEs), have further driven the demand for specialized reporters. For instance, the Base Editor Activity Reporter (BEAR)[12] employs a defective splice site designed to be corrected by specific base conversions, providing a sensitive readout for BE activity. Similarly, dedicated prime editing reporters such as PEAR[13] and fluoPEER[8] have been developed to monitor and enrich for PE-mediated edits. While significant, these systems are often tailored to specific editors or sequence contexts, highlighting a persistent demand for a compact, universal reporter platform with broad applicability. A promising mechanism for constructing sensitive reporters was established by the Hardy laboratory[14]. They demonstrated that fusion of a 28-amino-acid tetramerization peptide from the influenza M2 protein to the C-terminus of GFP completely quenched fluorescence by preventing chromophore maturation. Cleavage of this peptide by caspase restored fluorescence with a 45-fold dynamic range, showcasing a high signal-to-noise "dark-to-bright" switch. However, this elegant quenching principle has not been exploited for reporting nuclease-mediated genome editing.

To address this gap and the limitations of existing tools, we developed CRISPR-Bright (CRB), a novel single-fluorescence reporter system. CRB reporter is built upon the M2 peptide quenching mechanism by inserting a CRISPR target sequence within the linker connecting GFP and the quencher. We hypothesized that any frameshift-inducing edit at this target—whether introduced by Cas9-NHEJ or by a precise prime editing event—would disrupt the production or function of the quenching peptide, thereby de-repressing GFP fluorescence (Fig. 1a). This design enables the detection of a wide range of editing events using a single fluorophore. Herein, we present the design, validation, and application of CRB reporter as a versatile and efficient platform for quantifying the activity of diverse genome editors.

Materials and methods

Cell line and strain

All strains, cell lines and plasmids used in this study are listed in Supplementary Table 1. The *E. coli* DH5α strain was used for plasmid construction. The HEK-293T cells were used to produce lentivirus and construct the CRB reporter stable cell line.

Plasmid Construction

The DNA fragment encoding the oxStayGold–quenching peptide protein was synthesized by Logenbio. This fragment was amplified by PCR using primers oLK-1 and oLK-15. The pLV3-CMV-CTGF (human)-EF1-TagRFP-Puro lentiviral vector (MiaoLingBio) was linearized using restriction enzymes *Bam*HI and *Eco*RI. The amplified fragment was then cloned into the linearized vector via Gibson assembly to generate the CRB reporter plasmid. The assembled plasmid was transformed into competent DH5α strain. The pU6-sgRNA (targeting seed1 or seed2) and pU6-pegRNA expression plasmids were constructed using the

same Gibson assembly strategy described above for the CRB reporter plasmid. All sequence of primer and gene used in this study are listed in Supplementary Table 2 and 3.

Lentiviral packaging and generation of monoclonal CRB reporter cell lines

HEK293T cells were seeded in 10-cm culture dishes at a density of 5×10^6 cells per dish one day prior to transfection. For lentiviral packaging, a total of 20 μg of plasmid DNA was transfected per 10-cm dish. The DNA mixture consisted of the CRB reporter transfer plasmid (10 μg), the packaging plasmid psPAX2 (7.5 μg), and the envelope plasmid pMD2.G (2.5 μg). The DNA was mixed with 60 μg of PEI in serum-free medium, incubated for 10 minutes at room temperature to form complexes, and then added dropwise to the cells. The culture medium was replaced with fresh complete medium 6 hours post-transfection. Viral supernatant was collected at 36 hours after transfection, pooled, and filtered through a 0.45-μm membrane for subsequent use or concentration.

HEK-293T cells were transduced with the CRB lentivirus. To obtain monoclonal stable cell lines, transduced cells were serially diluted 72 hours post-transduction and plated into 96-well plates at an average density of 0.5 cells per well in complete. Individual clones were expanded and screened for uniform and high expression of the TagRFP selection marker via fluorescence microscopy.

Gene editing

The pCMV-*Spy*Cas9, pU6-sgRNA, pCMV-PE6d, and pU6-pegRNA plasmids were purified using an endotoxin-free plasmid extraction kit (Tiangen, DP118-02). HEK-293T-CRB cells were seeded in 24-well plates at a density of 1×10^5 cells per well. Six hours later, cells

were transfected with total 800ng of plasmid DNA, transfected at a 1:1 mass ratio (e.g., pCMV-*Spy*Cas9:pU6-sgRNA and pCMV-PE6d:pU6-pegRNA), mixed with 1.5 μg polyethylenimine (PEI, Beyotime, C0537). The result was observed 24 h post-transfection.

T7 Endonuclease I (T7E1) Assay

Genomic DNA was extracted from edited cells using a commercial DNA purification kit according to the manufacturer's instructions. The target genomic region was amplified by PCR using high-fidelity DNA polymerase with gene-specific primers. The PCR products were purified and then subjected to a reannealing process to form heteroduplex DNA. The reannealed DNA was incubated with T7 Endonuclease I (Beyotime), which cleaves mismatched DNA at heteroduplex sites formed by indels. The digestion products were separated by agarose gel electrophoresis, and the band intensities were quantified using image analysis software. The indel frequency was calculated based on the relative intensities of the cleaved and uncleaved DNA bands.

Results

Construction and Characterization of the CRB Reporter

We constructed the CRB reporter system. The core design comprised three parts: the oxStayGold fluorescent protein, a quenching peptide, and a linker sequence inserted between them harboring two CRISPR target sites (seed1 and seed2; Fig. 1c). The oxStayGold protein was selected for its enhanced brightness compared to conventional EGFP[15]. The entire CRB reporter expression cassette was cloned into the pLV3 lentiviral vector (Fig. 1b), enabling either transient expression or the generation of stable cell lines. The resulting lentiviral vector has a

compact size of 5.8 kb, facilitating efficient viral packaging.

Validation of the CRB Reporter for Cas9 and Prime Editor Activity

The activity of both SpyCas9 and the PE6d prime editor was assessed using the CRB reporter system. Robust GFP fluorescence activation was observed upon delivery of *Spy*Cas9 and sgRNA plasmids, confirming successful editing (Fig. 1b). The formation of insertions or deletions (indels) at the target site was independently validated by T7 Endonuclease I (T7E1) assay (Fig. 2b). Although the editing efficiency mediated by PE6d was lower than that of *Spy*Cas9, a clear increase in GFP signal was detectable, demonstrating the system's capability to monitor prime editing activity (Fig. 2c).

Comparison of Different Fluorescent Reporter Systems

We compared four fluorescent reporter systems—Stoplight[11], SplitAX[4], PEAR[13], and CRB (this study)—based on their scope of application, reporter size, suitability for transient expression, compatibility with generating stable cell lines, and the number of targetable sites . As summarized in Table 1, CRB offers distinct advantages. It can report the activity of both CRISPR-Cas9 and prime editors and is responsive to a broad range of editing outcomes, including insertions, deletions, and substitutions. The system detects approximately 66% of frameshift-inducing mutations. With a compact gene size of only 808 bp, it can be delivered either transiently or as part of a stable cell line. Furthermore, its design supports multiplexed targeting and, in principle, allows flexible sequence selection, significantly enhancing experimental versatility.

Discussion

The development of the CRB reporter addresses several key limitations in the current toolkit for monitoring genome editing activity. By repurposing the well-characterized M2 peptide-mediated fluorescence quenching mechanism, we have engineered a compact, single-fluorescence protein reporter that demonstrates remarkable versatility. Unlike specialized reporters such as BEAR for base editing or PEAR/fluoPEER for prime editing, CRB is designed around a universal output—the disruption of a translational reading frame. This allows it to respond to a broad spectrum of editing events, from Cas9-induced indels to precise, PE-mediated frameshift mutations, fulfilling the need for a more generalized reporting platform.

The advantage of CRB reporter lies in its elegant simplicity and small footprint. The entire reporter cassette, at only 808 bp, is significantly smaller than dual-fluorescence or polycistronic constructs like the Stoplight reporter. Furthermore, the "dark-to-bright" switching mechanism, with fluorescence suppressed in the unedited state, provides a high signal-to-noise ratio essential for sensitive detection, particularly when editing efficiencies are low, as often observed with prime editors.

Looking forward, the primary direction for improving the CRB reporter lies in further exploiting its flexible design to create a suite of reporters tailored for specific editing outcomes. Future work will focus on systematically characterizing and optimizing target sequences that are engineered to be converted into stop codons by base editors, thereby formally establishing CRB reporter as a quantitative tool for both adenine and cytosine base editor activity. Additionally, developing dual-color versions by pairing GFP-M2 with spectrally distinct fluorescent protein-M2 fusions could enable simultaneous, multiplexed monitoring of different editing events within a single cell. Extending the principle to report on the activity of other

CRISPR systems, such as Cas12 or Cas13, also represents a promising avenue for creating a unified reporting toolbox.

Conclusion

In summary, we have developed CRB, a novel fluorescent reporter system that leverages a quenching peptide to create a sensitive, dark-to-bright switch for genome editing activity. CRB effectively reports the activity of both CRISPR-Cas9 nucleases and prime editors, bridging a gap between specialized tools. This versatile platform is poised to facilitate the optimization of editing conditions, high-throughput screening of guide RNAs and editor variants, and the efficient enrichment of edited cells, thereby accelerating basic research and translational applications in genome engineering.

Declaration of Competing Interest

The authors have no conflict of interests to declare.

Data availability

All data supporting the findings of this study are available within the article and its supplementary files. Any additional requests for information can be directed to and will be fulfilled by the corresponding authors.

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Figure

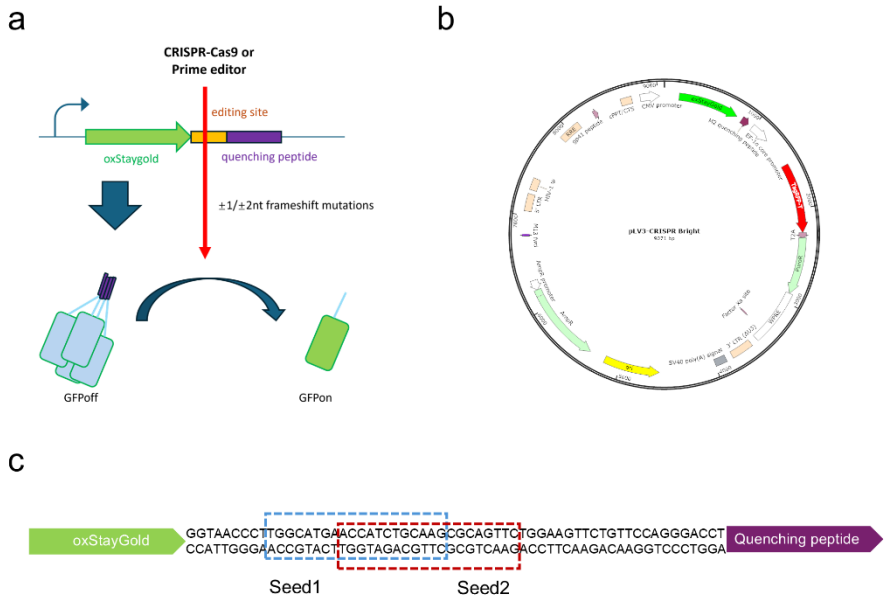

Figure 1. **Schematic of the CRB reporter. a**, Working principle. The CRB reporter gene encodes a fusion protein consisting of a green fluorescent protein (oxStayGold) and a quenching peptide derived from the influenza M2 protein, connected by a linker containing the CRISPR target sequence. In the unedited state, QP-mediated tetramerization prevents chromophore maturation of GFP, resulting in a dark state. Upon CRISPR-mediated editing that introduces a frameshift-inducing insertion or deletion (±1 or 2 nt) within the target sequence, translation of the functional QP is disrupted. This allows GFP to fold into its fluorescent monomeric form, restoring fluorescence. **b**, Lentiviral vector design. The CRB reporter expression cassette was cloned downstream of the CMV promoter in the pLV3 lentiviral vector. A TagRFP gene, expressed via the EF1α promoter, serves as a visual marker for transduction and enables the generation of stable reporter cell lines. **c**, Linker sequence design. A 60-bp linker was inserted between the genes encoding oxStayGold and the QP. This linker harbors two distinct CRISPR-Cas9 target sites for validation.

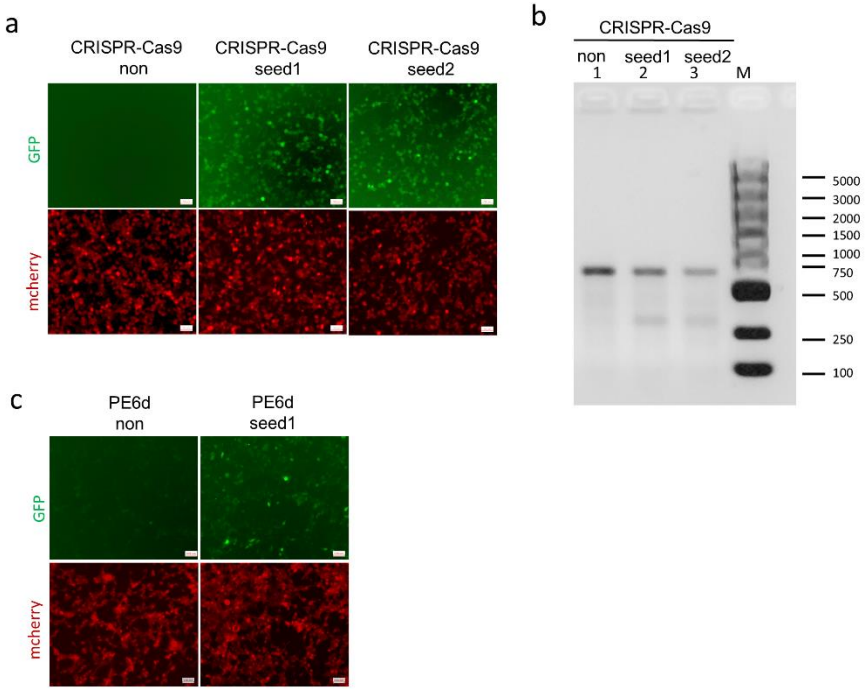

Figure 2. **Validation of the CRB reporter. a**, Detection of Cas9 activity. Co-delivery of *Spy*Cas9 and sgRNA plasmids targeting the linker sequence showed strong GFP fluorescence activation, indicating successful genome editing. b, Validation by T7E1 assay. The expected insertions or deletions resulting from Cas9 cleavage of the CRB reporter gene were confirmed using the T7 Endonuclease I assay. c, Detection of Prime Editor activity. The CRB reporter also reported successful editing by PE6d, as shown by increased GFP fluorescence, demonstrating its utility for monitoring Prime Editor activity.

273 **Table 1**. Comparison of four fluorescent reporters designed to assay gene-editor.

Method	**Editor used**		**Gene size**	**Detection ratio of NHEJ**	**Is the reporter transient?**	**A stable cell line?**	**Numbers of targets**
	CRISPR cas9	Prime editor					
Stoplight	Yes	No data	2368bp	66%	Yes	Yes	Mult
SplitAx	Yes	No data	800bp	33%	Yes	Yes	Mult
PEBR	No	Yes	1500bp	no	Yes	No	One
CRISPR Bright (this study)	Yes	Yes	909bp	66%	Yes	Yes	Mult

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