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Development and testing of a versatile genome editing application reporter (V-GEAR) system

Evan W. Kleinboehl,[1,2,3] Kanut Laoharawee,[1,2,3] Walker S. Lahr,[1,2,3] Jacob D. Jensen,[1,2,3] Joseph J. Peterson,[1,2,3] Jason B. Bell,[1,2,3] Beau R. Webber,[1,2,3,4] and Branden S. Moriarity[1,2,3,4]

[1]Department of Pediatrics, University of Minnesota, Minneapolis, MN 55455, USA; [2]Masonic Cancer Center, University of Minnesota, Minneapolis, MN 55455, USA; [3]Center for Genome Engineering, University of Minnesota, Minneapolis, MN 55455, USA; [4]Stem Cell Institute, University of Minnesota, Minneapolis, MN 55455, USA

CRISPR-Cas9 and novel cas fusion proteins leveraging specific DNA targeting ability combined with deaminases or reverse transcriptases have revolutionized genome editing. However, their efficacy heavily relies upon protein variants, targeting single guide RNAs, and surrounding DNA sequence context within the targeted loci. This necessitates the need for efficient and rapid screening methods to evaluate these editing reagents and designs. Existing plasmid-based reporters lack flexibility, being fixed to specific DNA sequences, hindering direct comparisons between various editing approaches. To address this, we developed the versatile genome editing application reporter (V-GEAR) system. V-GEAR comprises genes detectable after desired editing via base editing, prime editing, or homology-directed repair within relevant genomic contexts. It employs a detectable synthetic cell surface protein (RQR8) followed by a customizable target sequence resembling genomic regions of interest. These genes allow for reliable identification of corrective editing and cell enrichment. We validated the V-GEAR system with base editors, prime editors, and Cas9-mediated homology-directed repair. Furthermore, the V-GEAR system offers versatility by allowing transient screening or stable integration at the AAVS1 safe harbor loci, rapidly achieved through immunomagnetic isolation. This innovative system enables direct comparisons among editing technologies, accelerating the development and testing of genome editing approaches.

INTRODUCTION

Precise genome engineering is a powerful tool to induce nucleotide alterations within targeted loci with the capacity to correct monogenic disease mutations or alter relevant cellular mechanisms for therapeutic benefits.[1,2] Many of these potentially correctable diseases currently have no effective standard treatment or pharmaceuticals. The development of new genome editing techniques, such as CRISPR-Cas9, has positioned targeted gene therapies to treat these diseases as a valid primary treatment option for future patients. Using CRISPR-Cas9 to create double-stranded breaks (DSBs) has allowed researchers to create targeted insertions and deletions (indels) through non-homologous end-joining at incredible efficiencies.[3–6] Simultaneously delivering donor DNA with homology to the DSB region allows for homology-directed repair (HDR), incorporating small or large DNA sequences into the chromosome.[7,8] This technology has revolutionized the field of gene therapy and provides the potential for corrective editing for many monogenic diseases.

Nucleotide deaminases enzymatically convert single nucleotide bases, which further expands the capability to correct monogenic diseases. These base editor (BE) proteins have been fused to a partially enzymatically disabled Cas9 protein (Cas9n, or "nickase"), exploiting the programmable targeting ability of Cas9 to enable nucleotide-specific amendments to genomic DNA.[9] The fusion of these deaminase proteins with Cas9n results in formation of adenine base editors or cytosine BEs, which create genomic A:T to G:C or C:G to T:A changes, respectively.[10–17] More recently, prime editors (PE) were developed that utilize a reverse transcriptase (RT) fused to Cas9n. Prime editor guide RNAs (pegRNAs) contain the Cas9 single guide RNA (sgRNA) to target specific DNA sequences within the genome, a primer binding sequence, and an RNA reverse transcription template, also encoded in the same guide RNA molecule, enabling genomic nucleotide sequences to be inserted, replaced, or deleted.[18,19] Cas9n generates single-stranded DNA (ssDNA) that can hybridize with the pegRNA allowing it to serve as the RT template. Cas9n has also been shown to prevent the formation of unwanted indels compared with Cas9.[20,21] Unlike Cas9 HDR, BEs and PEs avoid DSBs and the need for DNA donor delivery, which are associated with poor engineering efficiency, stochastic editing outcomes, and decreased cell viability of engineered cell populations.[22–26]

Genome editing technologies have been in a state of rapid evolution over the past decade,[27] in part due to the deployment of the CRISPR-Cas9 system. Computational models for Cas9 nuclease and BE sgRNAs have been able to narrow down the pool of potential sgRNAs.[28] However, predicting the efficacy of these sgRNA designs and identification of optimal BE sgRNAs and PE pegRNAs remain an issue.[29–31] Identification of optimal sgRNAs thus rquires screening a variety of sgRNAs or pegRNAs.[32] Moreover, the efficacy of these

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Correspondence: Branden S. Moriarity, Department of Pediatrics, University of Minnesota, Minneapolis, MN 55455, USA.
E-mail: mori0164@umn.edu

genome engineering tools depends on a multitude of factors including sequence context of the target site, sgRNA design and formulation, level of enzyme expression, and cellular DNA repair context. While deep learning strategies and predictive sgRNA design software have been developed and utilized to improve editing efficiencies, refinements for precise editing prediction are still desired.[32–34] A screening tool to directly test multiple sgRNAs, protein variants, and editing methods such as HDR, BE, and PE would reduce the time to identify optimal engineering approaches.

Several BE reporters, and recently a PE reporter, have provided ways to enrich engineered cell populations.[35–39] These methods also show engineering a transient reporter system correlates with the genomic editing of a cell population. However, these approaches often require the use of multiple sgRNAs, one targeting the transient reporter system as well as an sgRNA targeting the genomic loci of interest. More recently, a fluorescent PE and enrichment reporter (fluoPEER) system allowed researchers to utilize the same pegRNA for the transient plasmid reporter and the genomic loci.[40] Inspired by multiple previous reporters[35–39,41] and to provide rapid functional testing of current prediction methods, we designed a versatile genome editing application reporter (V-GEAR). Similar to past systems, this reporter cassette activates reporter genes upon corrective editing of transiently transfected or stably integrated plasmid constructs. Notably, each V-GEAR reporter enables screening of HDR, BE, and PE in a single construct for direct comparison of efficiencies. Our system incorporates a genomic locus sequence of interest (30 base pairs [bp]–400+ bp in size tested), thereby eliminating the need for patient-specific cells or special cell lines harboring the mutation of interest. Upon corrective editing, downstream reporter genes are transcribed and productively translated. This V-GEAR system enables rapid screening of sgRNAs, and editor protein variants, such as Cas enzymes, BEs, or PEs. Our experiments demonstrate that V-GEAR editing compares reliably to on-target genomic editing, including the level of bystander editing activity. This reporting system is also useful for the enrichment of edited cells and can be rapidly integrated into the "safe harbor loci," *AAVS1* for high-throughput screening assays. Thus, the V-GEAR system is an efficient, reliable, and multifaceted tool that will provide rapid data on target-specific engineering.

RESULTS

Design and development of a V-GEAR system

In order to rapidly assess genome editing events using a broad spectrum of editing approaches, we designed a novel plasmid reporter that can be delivered alongside editing reagents that will express reporter proteins upon successful genome editing. We have termed this a versatile genome editing application reporter (V-GEAR). This reporter is transfected alongside DNA editing reagents, such as Cas9 nuclease, BEs or PEs in order to assess editing efficiencies and potential bystander edits. To accurately measure genomic editing within the reporter and test multiple editing approaches, we sought to develop a modular reporter that can accommodate large (30–400+ bp) sequences of DNA that mimic endogenous DNA target sequences. The utilization of larger target DNA sequences uniquely enables testing of HDR, BE, and prime/twin PE while simultaneously accounting for editing efficacy in a sequence context-dependent manner.

This V-GEAR cassette has three specific markers (Figure 1A), one to indicate successful delivery of the engineering reporter plasmid and two that become activated as the intended genome edit takes place. The V-GEAR cassette constitutively expresses RQR8, a synthetic cell surface receptor with extracellular epitopes of CD34 and CD20 to indicate successful delivery of the reporter system within target cells.[42] RQR8 is followed by an interchangeable target region located in the 5′ region of eGFP. As eGFP fusions have been made with thousands of genes and peptide sequences without quenching its fluorescence,[43,44] we posited that insertion of the target DNA into the 5′ end of eGFP would provide a high probability of retaining reporter function (Figure 1A). The interchangeable target sequence incorporates a stop codon and/or a codon frameshift that prevents downstream reporter expression. The genomic codon frame and translation is not required to be mirrored in the V-GEAR interchangeable target sequence; only the nucleotide context is replicated. This flexibility enables us to position a nucleotide region of interest so that the desired target site results in an in-frame stop codon within the V-GEARs interchangeable target sequence. HDR and PE techniques may be used to correct any frameshift preventing reporter expression within the V-GEAR and are therefore not limited to the incorporation of a stop codon. However, to further enhance the adaptability of generating stop codons from genomic target regions of interest without altering the nucleotide sequence context, users can utilize any combination of frameshifts and/or the reverse complement of genomic sequences for placement within the interchangeable target sequence. This approach also broadens the capabilities of V-GEAR for BE testing (Figures 1B and S1).

A replaceable secondary editing reporter can be used depending upon the experimental need. For example, a luciferase secondary reporter allows for a spectrophotometer readout and a sodium/iodide symporter (NIS) enables *in vivo* imaging in larger animals. Additionally, a drug resistance gene may allow for cell selection. Enriching cells on these conditionally expressed proteins (i.e., eGFP) from the V-GEAR plasmids by fluorescence-activated cell sorting (FACS) or drug selectable marker could therefore enrich genetically edited cell populations, as previously reported.[36,39,41,45] Each V-GEAR construct also contains *AAVS1* "safe harbor" homology arms for integration into the cellular genome for increased throughput assays. Notably, we leveraged a recently described homology-mediated end-joining (HMEJ) approach that uses 48 bp of homology to *AAVS1* and allows for highly efficient site-specific integration, particularly when using large cargo.[46,47] Expression of the RQR8 receptor in this context allows for rapid, stable cell line generation using simple immunomagnetic or FACS enrichment (Figure 1C). In sum, the V-GEAR system is truly a versatile reporter designed for testing many editing reagents, and within a transiently transfected or stable cell line context.

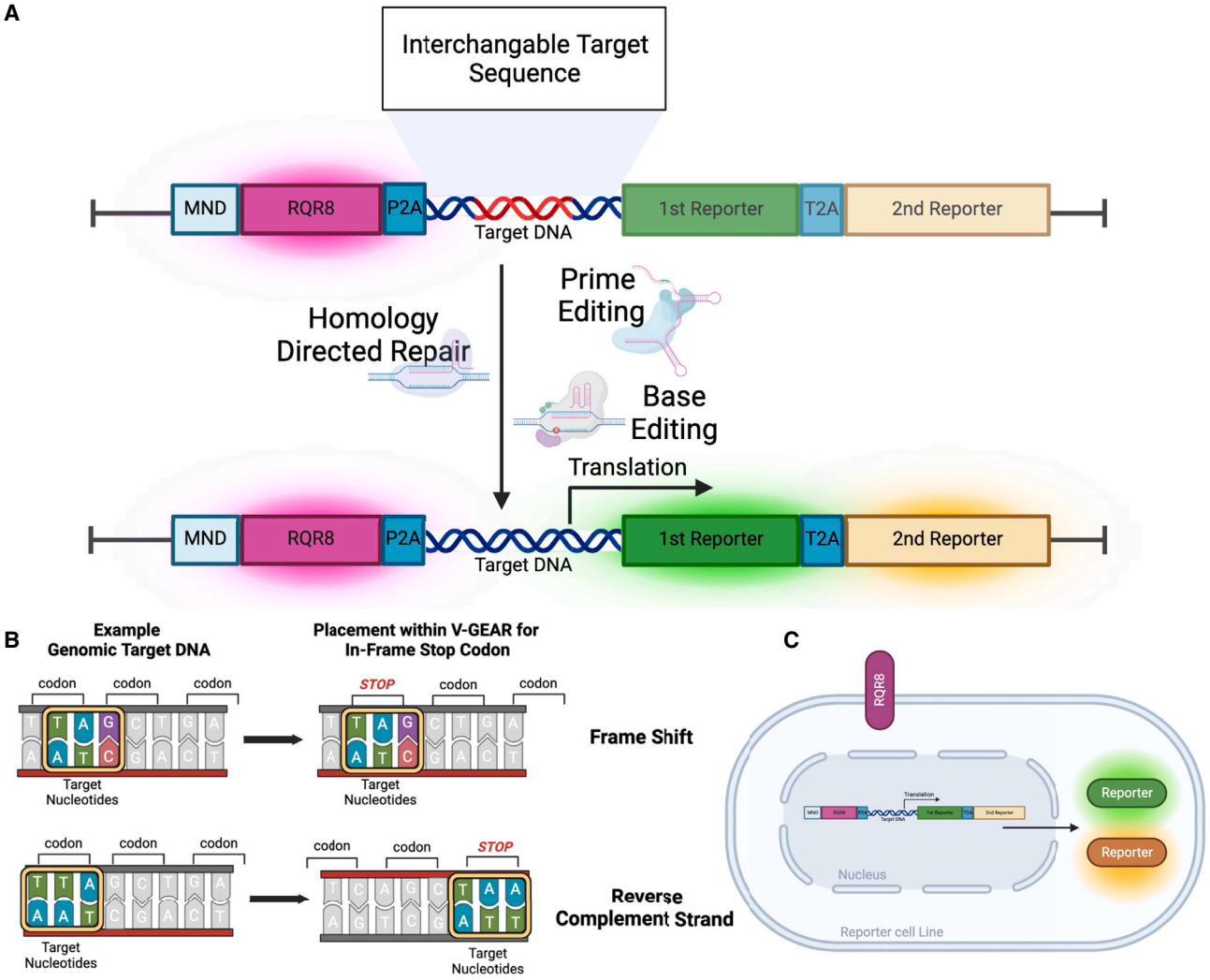

Figure 1. Design of a versatile genome editing application reporter (V-GEAR) system

(A) Construct design of the V-GEAR system configuration comprising the MND promoter, RQR8, target sequence of interest, eGFP, and a secondary reporter gene. Constitutive expression of RQR8 indicates V-GEAR cassette presence. Conditional expression of the eGFP and secondary reporter genes indicates successful gene editing of the interchangeable target sequence. (B) Illustrative examples of genomic target nucleotides being mirrored within the interchangeable target sequences that produce in-frame stop codons. This is accomplished by any combinations of using codon frame shifts and/or reverse complement DNA sequences. (C) Integration of the V-GEAR cassette into a stable cell line depicting membrane-bound RQR8 and conditionally expresses engineering reporters.

Transiently transfected V-GEARs reliably assess HDR and BE-mediated editing

To first test the V-GEAR system, we designed two unique V-GEAR cassettes. The first contains the splice donor of the *PDCD1* gene positioned as in-frame stop codon (TAG), flanked bilaterally by 30 nucleotides of the endogenous *PDCD1* sequence. Successful engineering of this reporter gene will convert the stop codon to an amino acid codon and allow eGFP and Red Luciferase expression while simultaneously editing and halting genomic expression of PD1 protein. The second V-GEAR cassette contains a section of the *Artemis* gene with the severe combined immunodeficiency A (SCID-A) mutation, which results in an in-frame stop codon (TAA) preventing proper translation of Artemis protein.[48] This stop codon and surrounding 27-nucleotide sequences were inserted into the V-GEAR cassette with NIS as a secondary reporter. Corrective gene editing of the TAA stop codon to an amino acid codon allows for translation of eGFP and NIS.

We electroporated the *PDCD1* V-GEAR into K562 cells along with sgRNA and an adenine BE, ABE8e-NG.[16] Cells were analyzed via flow cytometry for RQR8 to assess the presence of the reporter, PD1 to assess expression of the genomically encoded *PDCD1* gene, and eGFP signal to indicate successful conversion of the V_GEAR stop codon (Figure 2A). Control cells that were transfected with the

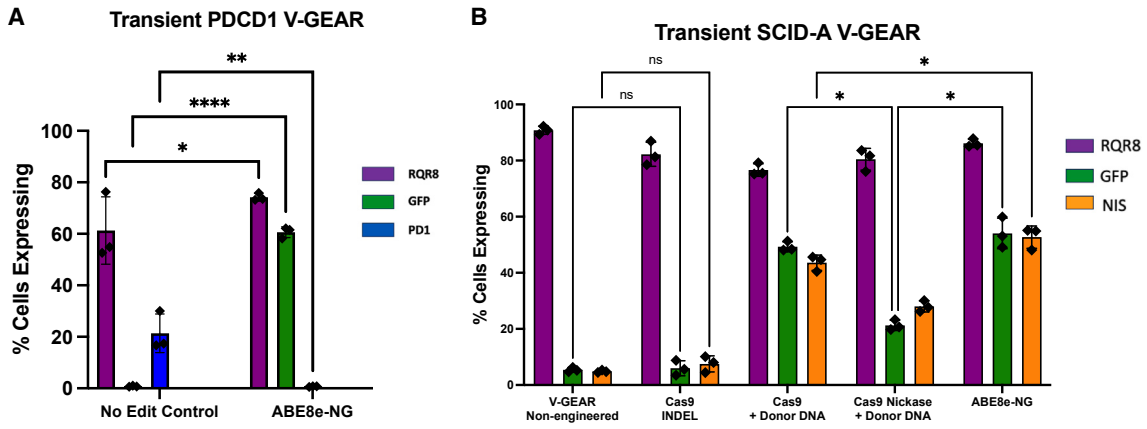

Figure 2. Assessment of base editing and homology-directed repair using transiently transfected V-GEARs

(A) Flow cytometry results of transiently transfected *PDCD1* V-GEAR with conditionally expressed reporter eGFP, in K562 cells treated with ABE8e-NG and sgRNA targeting the splice donor of *PDCD1* exon 1. (B) Flow cytometry results of transiently transfected SCID-A V-GEAR with conditionally expressed reporter eGFP and NIS, in K52 cells treated with ABE8e and sgRNA or homology-directed repair targeting the SCID-A mutation of *Artemis*. *N* = 3 technical replicates. Statistical analysis: two-way ANOVA $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

PDCD1 V-GEAR without gene editing reagents had approximately 61% RQR8 expression and eGFP signal <1%. V-GEAR transfection along with adenine BE and sgRNA targeting the V-GEAR and the endogenous splice donor of *PDCD1* induced eGFP expression in 60% of RQR8+ cells and PD1 expression was reduced to less than 1% (Figures S2 and S3).

Next, we compared the efficacy of several editing proteins within the SCID-A V-GEAR (Figure 2B). Using adenine BE, Cas9, and Cas9 nickase for HDR, we compared editing efficiencies by measuring eGFP and NIS signals. Transiently expressed SCID-A V-GEAR had approximately 5.4% eGFP and 4.84% NIS signal of RQR8+ cells at baseline, indicating a low level of background expression. Testing several editing methods using sgRNAs, adenine BE, or Cas9 variants with single-stranded donor DNA (ssDNA) for HDR increased reporter gene signal indicating a conversion of the TAA stop codon to TGG within the V-GEAR (Figures 2B and S4). As expected, the adenine BE provided the most robust editing of the target TAA sequence coinciding with recent reports of improved editing using BE proteins.[16] Notably, Cas9 induced double-strand breaks that result in indel formation did not significantly increase the reporter eGFP and NIS expression. Sanger sequencing analysis of the transient SCID-A V-GEAR indicated indel rates >60% (Figure S5). These data demonstrate the utility of V-GEARs for assessing both HDR and BE editing frequencies with little background activation due to indel formation.

V-GEARs can assess pegRNA design that reliably correlates with chromosomal genome editing

Prime editing and subtle variants, such as twin prime, have enabled researchers to insert and delete large (>400 bp) sequences of DNA.[18,22–24] However, the ability to screen multiple pegRNAs as well as editor proteins to achieve the desired efficiency has been a challenge for researchers. To investigate nucleotide insertions and deletions, a *CCR5* V-GEAR was created by inserting a 409-bp region of the *CCR5* gene into the V-GEAR plasmid followed by out-of-frame eGFP and Red Luciferase reporter genes (Figure 3A). Successful insertion of a 38-bp attB sequence through PE brings the eGFP and Red Luciferase in-frame. This reporter cassette allowed us to test several pegRNA configurations previously reported that will simultaneously insert a 38-bp attB sequence and delete endogenous nucleotides of the human *CCR5* gene.[49]

Utilizing PE2[18] with six separate pegRNA combinations to insert the attB sequence, we observed that A260c and B291b twin prime combinations produce the highest luciferase signal and percent of eGFP-positive cells (Figures 3B, 3C, and S6). These same pegRNAs were then used with a new PE variant, PEmax.[49] PEmax had slightly improved editing efficiencies, although not significant, indicated by a higher level of luciferase signal and eGFP+ cells (Figures 3B and 3C). This result coincides with previously reported enhanced editing efficacy of PEmax compared with PE2.[19,50] As expected, all single pegRNAs had lower reporter gene expression for both PE proteins, which is consistent with the improved editing capabilities of twin PE.[49]

Next generation sequencing (NGS) was used to determine the percent reads that had the attB sequence installed within the V-GEAR as well as the genomic *CCR5* genomic locus. We found a clear correlation of reads with the inserted attB sequence within the transient V-GEAR and genomic *CCR5* (Figure 3D). Consistent with the V-GEAR reporter gene expression, the PEmax had increased engineering efficiency when compared with the PE2 variant using NGS analysis. Linear regression comparison of the transient V-GEAR and genomic PE correlate well ($R > 0.86$; $^{***}p < 0.0001$) (Figure 3E). In sum, these data demonstrate that V-GEARs are efficient tools that enable rapid

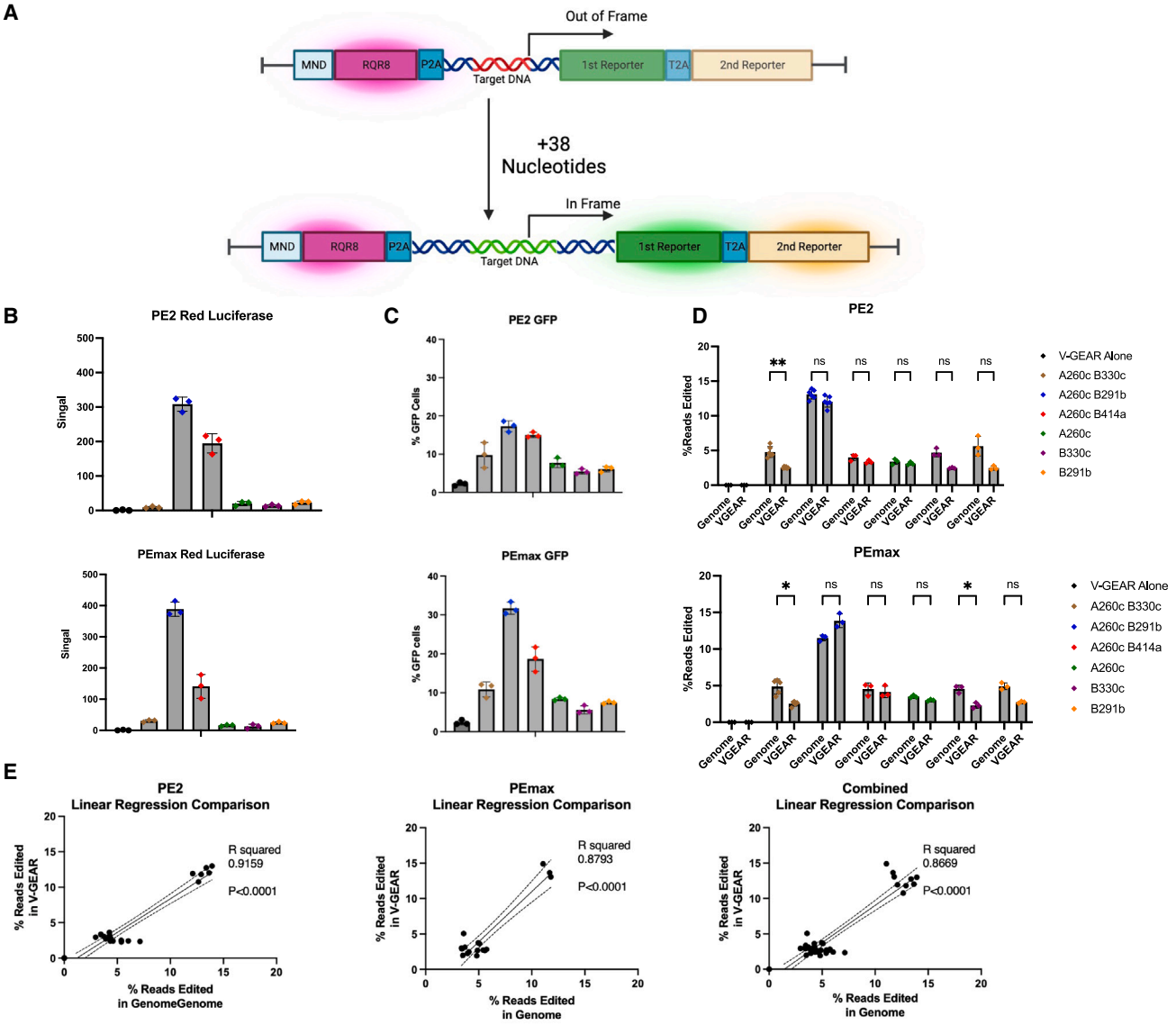

Figure 3. Assessment of prime and twin prime editing using transiently transfected V-GEARs

(A) Schematic of the *CCR5* V-GEAR containing the 409-nucleotide sequence of the *CCR5* gene. Nucleotide replacement and insertion of a 38-nucleotide attB sequence corrects codon frame for conditional eGFP and Red Luciferase expression. *CCR5* V-GEAR was transiently transfected in K562 cells with PE2 or PEmax and pegRNAs targeting *CCR5*. Bioluminescent and flow cytometry assays were performed 3 days post transfection. Results depict pegRNAs and PE variants for Red Luciferase (B) and eGFP (C) expression, respectively. (D) NGS results for V-GEAR and genomic editing with various pegRNAs and PE combinations for the insertion of the 38-nucleotide sequence encoding attB. (E) Linear regression analysis of the percent reads containing the attB sequence within the V-GEAR compared with the *CCR5* genomic loci. $N = 3$ technical replicates. Statistical analysis: two-way ANOVA $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

screening of multiple pegRNAs and PE protein variants that correlate well with time-consuming and costly NGS.

V-GEAR editing corresponds with genomic editing and allows for enrichment of genome edited cells

Patient-specific cells harboring deleterious mutations can often be difficult to acquire or culture, making testing and characterization of genome engineering approaches challenging. A reporter system that recapitulates genomic editing in a similar fashion would thus be beneficial. To assess this in the context of V-GEARs, we simultaneously base edited the genomic *PDCD1* and the transient *PDCD1* V-GEAR with ABE8e-NG. Using Sanger sequencing and analysis through EditR software,[51] we observed no significant difference in editing efficiencies at the genomic loci vs. the transient V-GEAR (Figure 4A). Additionally, two bystander base edits flanking the *PDCD1* splice donor are edited in a nearly identical fashion in the V-GEAR

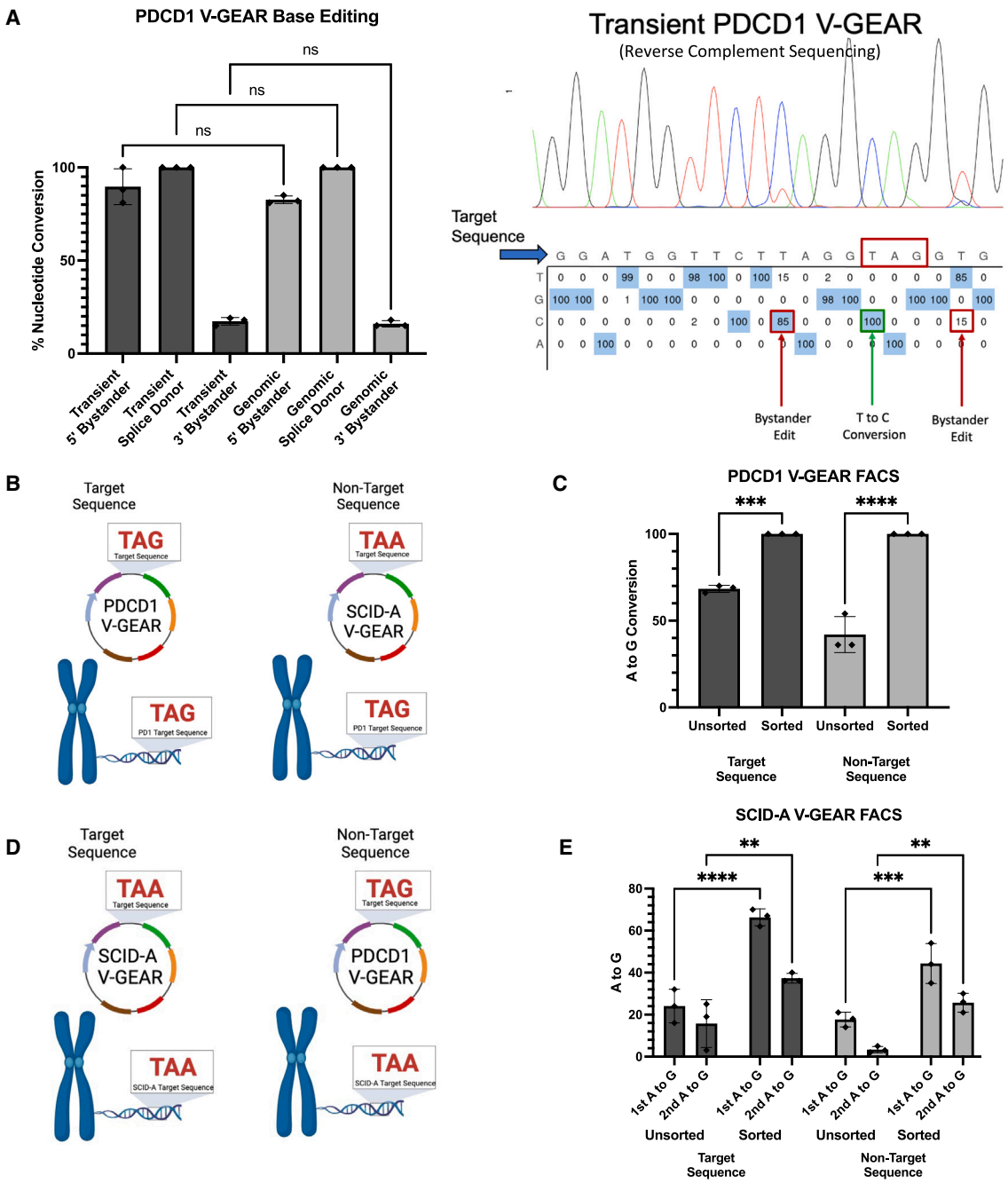

Figure 4. Editing outcomes in the V-GEAR correlate well with genomic DNA editing and the V-GEAR system enables enrichment of gene edited cells

(A) Sequencing results of the interchangeable target sequence transiently transfected *PDCD1* V-GEAR in K562 cells with ABE8e-NG and sgRNA targeting the spice donor of the *PDCD1* exon 1. Representative Sanger sequencing and EditR results of the *PDCD1* V-GEAR (right). (B) Diagram of the use of matched and unmatched V-GEARs when targeting *PDCD1* exon 1 splice donor with base editing. (C) Sequencing results of adenine base editor, ABE8e-NG, editing of the endogenous *PDCD1* loci pre and post FACS sorting of conditionally expressed eGFP+ cells transfected with *PDCD1* or SCID-A V-GEARs (i.e., matched vs. unmatched reporters). (D) Diagram of the use of matched and unmatched V-GEARs when targeting the *SCID-A* mutation with base editing. (E) Sequencing results of ABE8e-NG editing of the endogenous *Artemis* loci pre and post FACS sorting of conditionally expressed eGFP+ cells transfected with *PDCD1* or SCID-A V-GEARs (i.e., matched vs. unmatched reporters). $N = 3$ technical replicates. Statistics analysis: two-way ANOVA $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

reporter. As expected, K562 cells also lost PD1 protein expression as a result of targeted BE at the genomic locus (Figure S10). Thus, the rates of V-GEAR editing are comparable to genomic editing levels for both intended and bystander edits, which is ideal when assessing frequency and fidelity of editing several genome editing approaches.

Obtaining high-frequency editing in some cell populations can be challenging, particularly in primary cell types or cell lines with low transfection efficiency. Previous transient reporters have demonstrated enrichment of edited cell populations to overcome these challenges.[36,39,41] Thus, we sought to test if the V-GEAR system can be used in the same capacity to enrich genome edited cells. We first tested our *PDCD1* V-GEAR using ABE8e-NG by targeting the splice donor of the *PDCD1* loci coupled with FACS enrichment. K562 cells were thus transfected with the *PDCD1* V-GEAR containing the same splice donor region of *PDCD1* exon 1 and sgRNA (Figure 4B). FACS sorting of eGFP+ cells on day 3 post transfection demonstrated a significant enrichment in cells with genomic *PDCD1* loci editing as assessed by sequencing (Figure 4C). Next, we transiently transfected a non-target SCID-A V-GEAR with its own targeting sgRNA as well as a *PDCD1* targeting sgRNA to edit the genomic loci to test if using a non-target paired V-GEAR will also enable enrichment of edited cells (Figure 4B). eGFP enrichment was performed through FACS on day 3 and sequenced to determine genomic editing efficiency, which again demonstrated enrichment of gene edited cells (Figure 4C). To further confirm these observations, we transfected either an SCID-A V-GEAR into K562 cells containing the SCID-A mutation or a separate non-target *PDCD1* V-GEAR, requiring a distinct targeting sgRNA (Figure 4D). Using this approach, we again observed robust enrichment of genomic editing in the eGFP+ cell population of both the matched and mix-match non-target V-GEAR systems. This indicates that a non-target V-GEAR system that requires a separate targeting sgRNA can be used to enrich edited cell populations, which is in line with all previous gene editing reporters.[36,39,41] The ability to enrich edited cell populations further highlights the versatility of V-GEARs and enables researchers to quickly enrich gene edited cell populations.

Rapid generation of stable cell lines harboring V-GEAR reporters for high-throughput assays

Providing a way to enable researchers to increase their throughput of screening editing methods, sgRNAs and protein editor variants would likely be valuable. The RQR8 protein constitutively expressed from V-GEARs allows for fast immunomagnetic isolation or FACS of stably integrated V-GEAR cells. Thus, we utilized the HMEJ homology arms of our V-GEAR plasmid to integrate the SCID-A and *PDCD1* V-GEAR systems into the *AAVS1* "safe harbor" locus in K562 cells. Enrichment of cells using either immunomagnetic beads or FACS significantly enriched for stably engineered cells (Figures 5A and S9). Notably, we did not observe expression of reporter genes eGFP and NIS in the enriched RQR8+ cells, indicating low to no background expression after stable integration of V-GEARs at *AAVS1*.

Upon corrective editing of the stable V-GEAR cell line through BE, the reporter genes were robustly expressed (Figures 5B, S7, and S8). Using a spectrophotometer plate reader to measure Red Luciferase activity, we also observed a significant increase in bioluminescent signal upon BE (Figure 5C). This bioluminescence reporter provides an additional approach to quantitatively measure editing activity without the requirement of a flow cytometer. Notably, sequencing analysis of the stably integrated SCID-A V-GEAR as well as a transiently transfected SCID-A V-GEAR indicate no significant difference in editing efficiencies (Figures 5D and 5E). These data demonstrate the V-GEARs can be integrated into a genomic locus to generate stable cell lines without impacting editing efficacy compared with its use in a transient transfection and without background expression of reporter genes.

DISCUSSION

Here, we describe a customizable V-GEAR system to address the challenges associated with validating and optimizing gene editing approaches in the context of user-defined target DNA sequences. The V-GEAR system offers a customizable solution that facilitates testing of sgRNAs, editing methods, protein variants, and the identification of potential bystander edits for almost any nucleotide sequence. Notably, V-GEARs can be utilized via transient transfection or rapidly stably integrated at the *AAVS1* safe harbor locus for high-throughput assays. Testing BE approaches is only constrained by the incorporation of an in-frame stop codon within the interchangeable target sequence, while nearly any HDR or PE editing event can be modeled. Much like past gene editing reporters, we also demonstrate that the V-GEAR system can be deployed in a transient manner for enrichment of gene edited cell populations.

Within possible interchangeable target sequence arrangements, care should be taken to ensure no upstream or downstream premature termination codons become in-frame, aside from the intended target nucleotides. This may limit the size allowable for the interchangeable target sequence that is mirrored from a genomic region of interest. Additionally, BEs are limited to targeting stop codon correction (ABE: TAA, TAG, TGA; CBE: TAG only). Thus, while reverse complement sequences can be used to expand upon possible placements of genomic DNA into the V-GEAR, not all desired sequences can be used. Prime editing efficiencies have been shown to be influenced by the chromatin state and packaging of DNA.[52,53] While the V-GEAR can incorporate the nucleotide sequence context of a genomic target, it will not be able to mirror the same chromatin packaging state, potentially resulting in an increased editing frequency within the V-GEAR and subsequent overestimation of editing. Furthermore, transient transfections of V-GEARs may introduce variability with flow cytometry readouts as this can result in a multitude of V-GEARs delivered to cell populations. Prime editing that has a higher propensity of 3′ indel formation relative to the target edit could further amplify an overestimation in editing efficiencies as it takes only one V-GEAR to express reporter proteins. Some variability may be controlled by stable integration into cell lines at the *AAVS1* loci, as indicated by the flow cytometry bimodal populations of the stable clones generated (Figures S6 and S7).

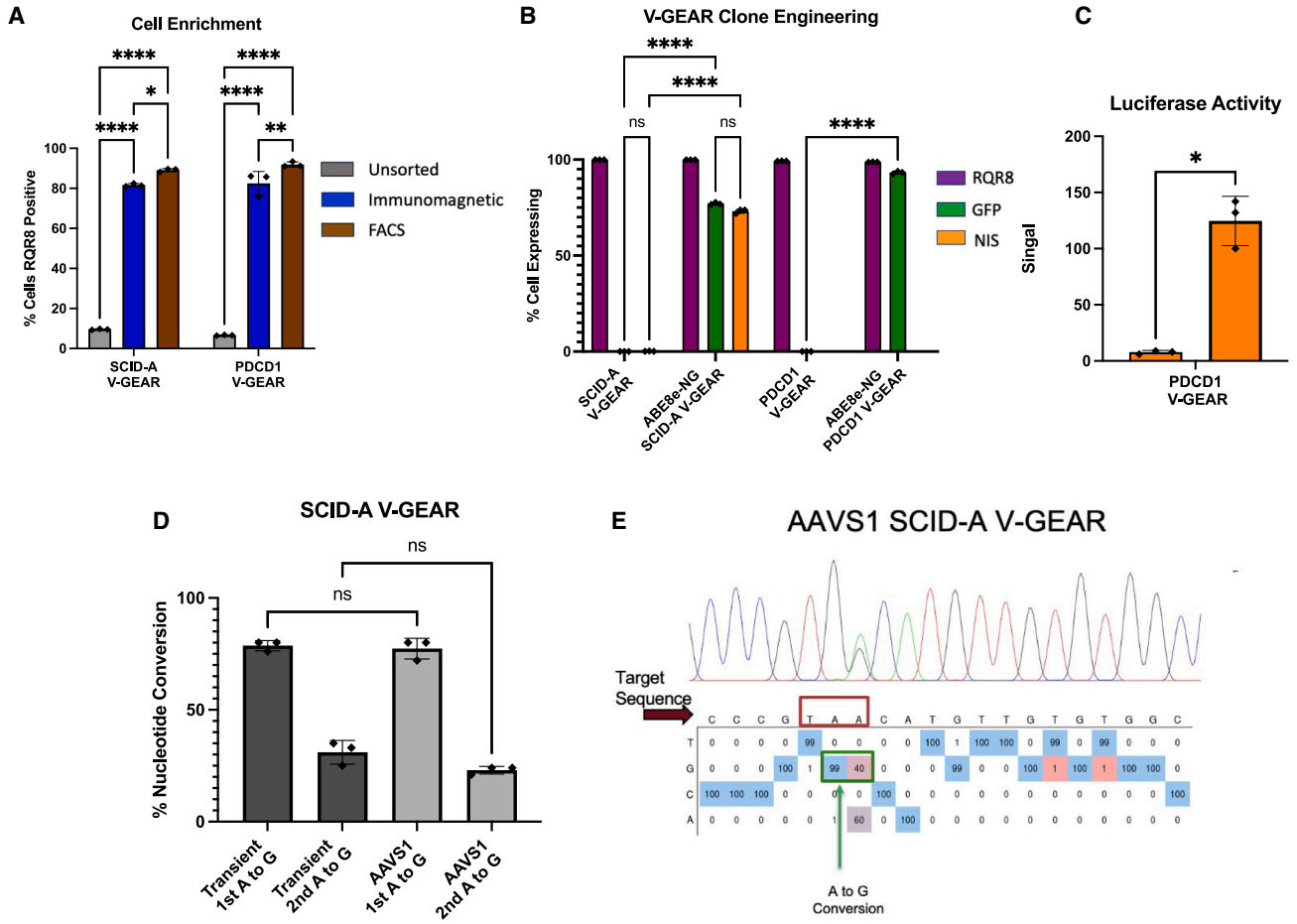

Figure 5. Rapid generation and testing of stable V-GEAR cell lines

(A) Flow cytometry results demonstrating enrichment of *AAVS1* knockin V-GEAR cells after immunomagnetic selection or fluorescence-activated cell sorting (FACS) on constitutively expressed RQR8 expressed by the V-GEAR cassette. (B) Flow cytometry results from a K562 clone with the SCID-A or *PDCD1* V-GEAR integrated into the *AAVS1* loci after base editor, ABE8e-NG treatment. Conditional expression of eGFP by PDCD1 and SCID-A V-GEAR, NIS expression by SCID-A V-GEAR alone. (C) Conditional Red Luciferase signal from a K562 clone with the *PDCD1* V-GEAR integrated into the *AAVS1* loci after base editor upon ABE8e-NG treatment. (D) Sequencing results of the interchangeable target sequence post ABE8e-NG base editing from a K562 clone with the SCID-A V-GEAR integrated into the *AAVS1* loci or transiently transfected (left). (E) Representative Sanger sequencing and EditR results (right). $N = 3$ technical replicates. Statistical analysis: One-way, two-way ANOVA $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

One of the key unique features of the V-GEAR system is the ability to substitute the interchangeable target DNA sequence. Several companies allow for rapid and cost-effective alterations of oligo nucleotides within the V-GEAR that can effectively mirror genomic regions of interest. In our experience, the process of incorporating a novel target sequence in the V-GEAR takes as few as 3 t 5 days to complete and often costs less than $100. Alternatively, oligonucleotides can be designed and cloned in-house. Because of this, it is now possible to rapidly customize beyond the singular target sequence of previous genome editing reporters.[35–39,41] The V-GEAR system also allows researchers to directly compare the outcomes of HDR, BE, and PE approaches using a single reporter construct, making the evaluation process more comprehensive and efficient. Moreover, it can allow for rapid testing of different sgRNAs, pegRNAs, and ssDNA DNA donor designs. Thus, the V-GEAR system is a complementary tool alongside existing *in silico* predictive algorithms and software for designing sgRNAs and pegRNAs. The effectiveness of these designs can vary depending on factors such as genic loci, chromatin structure and surrounding nucleotide context.[32–34] Although highly useful as a starting point for genome editing design, commonly used *in silico* prediction software cannot definitively determine editing efficacies and outcomes currently.[40] This highlights the need for functional testing within editing reporter assays, like V-GEAR, to accelerate the identification of optimal genome editing approaches.

Furthermore, the V-GEAR system can be utilized in a transient manner, much like past reporters, or readily integrated into the *AAVS1* safe harbor loci rapidly for improved throughput testing.[35–39,41] Stable reporter

integration eliminates the need for additional plasmids to be delivered along editing reagents, which can impact editing efficiencies.[54] Notably, the compact 48-bp homology arms for HMEJ integration within the V-GEAR system proves to be adequate for precise genome integration at *AAVS1*. Once integrated, the constitutively expressed RQR8 receptor assumes an additional role by facilitating enrichment of stably integrated V-GEAR cells through FACS or immunomagnetic isolation methods. This dual functionality further underscores the versatility and utility of the V-GEAR system. Utilization of a V-GEAR knockin cell line with arrayed sgRNA/pegRNA transfections will likely achieve a high-throughput and comprehensive assessment of optimal genome editing approaches. This approach should enable users to quantitatively measure the impact of various editing strategies and editing reagents. Furthermore, with simple Sanger sequencing of V-GEARs, one can obtain nucleotide-level resolution of the target sequence and surrounding region. This approach can ensure the utmost precision and reliability of an editing approach before deployment in precious patient samples. Incorporation of V-GEAR assays within the gene therapy workflow will thus provide valuable insights into the intricacies of the editing process.

The ability to rapidly integrate the V-GEAR system into the genome indicates that they may also be used as an *in vivo* reporter of gene editing activity. The V-GEAR system could be integrated into cells that are intended for engraftment in immunodeficient mice or directly used for animal transgenesis. In this context, RQR8 expression would serve as a reliable constitutive reporter, easily detectable in peripheral blood cells or tissue of transgenic or engrafted animal models. The murine *Rosa26* and *Polr2a* loci have been previously reported as safe harbor loci in mice and would therefore serve as ideal loci for V-GEAR integration.[55,56] Transgenic V-GEAR animal models could enable researchers to test *in vivo* genome editing delivery methods of editing tools in the intended human DNA sequence context. Secondary reporters, such as Luciferase or NIS, would allow for an *in vivo* imaging modality to identify editing efficacy and tissue tropisms of the delivery tools. Recently, a reporter animal model has been used to measure tissue-specific delivery, efficacy, and gene delivery agents by base editing a stop codon within a luciferase gene that has been integrated at the *Rosa26* loci of FVB.129S6(B6) mice.[57] While this *in vivo* reporter can screen tissue-specific targeting and cargo delivery methods, it is limited by base editing a luciferase gene. Use of the V-GEAR system *in vivo* will allow for testing editing within user-defined nucleotide sequences of interest and multiple editing modalities. To enable future development of murine models harboring V-GEAR reporters, we replaced the *AAVS1* HMEJ homology arms with homology arms targeting the murine *Rosa26* locus. We further validated stable integration of this V-GEAR reporter using a murine fibroblast cell line (Figure S11) and have deposited this plasmid on Addgene as a resource to the scientific community.

In summary, the V-GEAR system is a highly customizable, robust, cost-effective, and efficient tool that offers a comprehensive analysis of genome engineering approaches for nearly any target sequence of interest. Its versatility, fast readout, and ability to reduce the reliance on hard to obtain patient samples make it a valuable tool for researchers investigating challenging disease models and advancing genome engineering techniques.

MATERIALS AND METHODS

Plasmid cloning

V-GEAR plasmid construct was synthesized into pAAV backbone (Addgene, plasmid #32395). Custom MND, RQR8, eGFP, Red Luciferase, and NIS were synthesized (Genscript) and cloned into the backbone replacing the CMV promoter sequence. Target sequences of *Artemis*, *PDCD1*, and *CCR5* were ordered and cloned into the target sequence region within the V-GEAR backbone (Genscript). For construction of the V-GEAR, *PDCD1* inserted 30-bp oligonucleotide regions surrounding the splice donor of exon one after the canonical "ATG" start codon of eGFP resulting in an in-frame stop codon. Construction of the V-GEAR SCID-A inserted a 48-bp oligonucleotide region surrounding the *Artemis* SCID-A "TAA" mutation after the "ATG" start codon of eGFP resulting in an in-frame stop codon. V-GEAR *CCR5* had a 409-bp oligonucleotide inserted after the "ATG" start codon of eGFP. Oligonucleotide synthesis and assembly was performed by Genscript. PEmax and PE2 were obtained from Addgene (#132775 and #174820). Plasmid preparations were cloned using DHB10 competent cells (Competent Cells) per manufacturer's protocols (ThermoFisher, #EC0113). DNA extraction kits (Invitrogen; Quick Plasmid Miniprep Kit #K210010 and Invitrogen; HiPure Plasmid Maxiprep Kit #K210006) were used per manufacturer's protocols. V-GEAR plasmids will be made available at Addgene (https://www.addgene.org/). PEmax and all plasmid cloning products were sequence confirmed via Sanger sequencing (Eurofins Genomics LLC; Louisville, Kentucky).

mRNA

ABE8e-NG and BE4 (Addgene #138491; Addgene #100802) were cloned (Genscipt) into pmRNA production vector and mRNA was produced commercially (Trilink Biotechnologies). Single guide RNAs (sgRNA) were designed using Synthego and SpliceR v1.2.0 online tool (https://moriaritylab.shinyapps.io/splicer/).[58]

Cell lines and clonal isolation

K562 cell lines were purchased from ATCC (ATCC CCL-243). Stocks were routinely tested for mycoplasma contamination (LookOut Mycoplasma PCR Detection Kit catalog MP0035). Cell lines were maintained in RPMI 1640 Medium (Thermo Fisher Scientific #11875093) with 10% FBS (Thermo Fisher Scientific #16000044) and 1% Pen/Strep (Corning #30-002-Cl) at 37°C with 5% CO_2. For clonal isolation, cells were diluted to single wells of a 96-well plate. Cells were expanded and RQR8 expression was determined through flow cytometry (ThermoFisher QBEND/10 PE #MA1-10205). Immunomagnetic enrichment was performed with EasySep PE Positive Selection Kit II (Stem Cell #17684).

Electroporation

For electroporations, 2×10^5 K562 cells were added to a combination of 1 μg of sgRNA (Integrated DNA Technologies) and 1.5 μg of either

Cas9, ABE8e-NG, BE4max mRNA (TriLink), or 1.5 μg PE2/PEmax plasmid. Cell reaction volumes were brought up to 10 μL of R Buffer (Thermo Fisher Scientific) for electroporation using the Neon Transfection System (Thermo Fisher Scientific). Cells were then loaded in 10-μL tips and electroporated in accordance with the manufacturer's instructions using settings of 1,450 V, 10 m width, and 2 pulses. Cells were immediately dispensed into 1 mL of culture media without Pen/Strep. Analysis was performed three days post electroporation unless stated otherwise.

PCR and sequencing

Genomic DNA was extracted from K562 cells 3 days or after electroporations with transient V-GEAR plasmid. DNA extraction and PCR were performed per Phire Tissue Direct PCR Master Mix manufacturer's protocols (#F170S). PCR products were purified using QIAquick PCR Purification Kit (Qiagen #28106). Sanger sequencing of purified PCR products was performed at Eurofins Genomics LLC, Louisville, Kentucky. Editing analysis was performed using the program MultiEditR (https://moriaritylab.shinyapps.io/multieditr/).[51] NGS and sequence analysis protocol is based on the 16S Metagenomic Sequencing Library Preparation from Illumina. Setup Indexing reaction utilized Nextera XT Index Set D (Illumina, Inc), 2× KAPA HiFi HotStart ReadyMix (Roche, Basel, Switzerland) and PCR-grade water. Samples were then purified by AMPure XP beads (Beckman Coulter, Indianapolis, IN) and quantified by Bioanalyzer (Agilent, Santa Clara, CA). A 300-cycle V2 sequencing kit (Illumina, Inc) was used to perform NGS on the Illumina MiSeq. The data were then analyzed using Crispresso2 software,[59] to qualitatively and quantitatively evaluate the outcomes of genome editing in which target loci were deep sequenced.

Flow cytometry and FACS

Three days after electroporation, 0.5–1 × 10^5 K562 cells were collected and stained with Viability Dye eFluor780 (ThermoFisher #65-0865-14 1:500 dilution) and fluorophore-conjugated antibodies for RQR8 (ThermoFisher QBEND/10 PE #MA1-10205), and NIS (R&D Systems Alexa Fluor 647 #FAB8367R). Cells were fixed after staining protocol with Fixation Buffer (Biolegend #420801). Samples were measured using the Cytoflex flow cytometer (Beckman Coulter) and analyzed using FlowJo v10 software. Fluorescent activated cell sorting was performed using a MACSQuant Tyto Cell Sorter (Miltenyi Biotec).

Luciferase assay

Luciferase assays were performed 3 days post electroporations; 0.5 × 10^5 K562 cells were removed and transferred to a black 96-well plate (ThermoFisher #237105). D-Luciferin, Potassium Salt (ThermoFisher #L2916) was added to each well to a final concentration of 150 μg/mL. Cells were shaken for 5 min prior to reading at 450 nm wavelength on a synergy 2 spectrophotometer (BioTek).

Graphical and statistical analysis

Graphs were generated using GraphPad Prism v10 and statistical significance was determined by either one-way ANOVA or two-way ANOVA. A p value <0.05 was considered significant. The data presented are shown as mean ± SD unless otherwise stated.

DATA AND CODE AVAILABILITY

The V-GEAR plasmids used within this study will be made available for non-commercial use through Addgene (https://www.addgene.org/). All reasonable requests can be made to the corresponding author at mori0164@umn.edu.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.omtm.2024.101253.

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AUTHOR CONTRIBUTIONS

E.W.K. designed experiments, performed assays, analyzed the data, and wrote the manuscript. K.L. and W.S.L. assisted with gene cassette designs. J.D.J. and J.J.P. assisted with experimental assays. J.B.B. performed next generation sequencing and related analysis. B.R.W. and B.S.M. supervised the research.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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