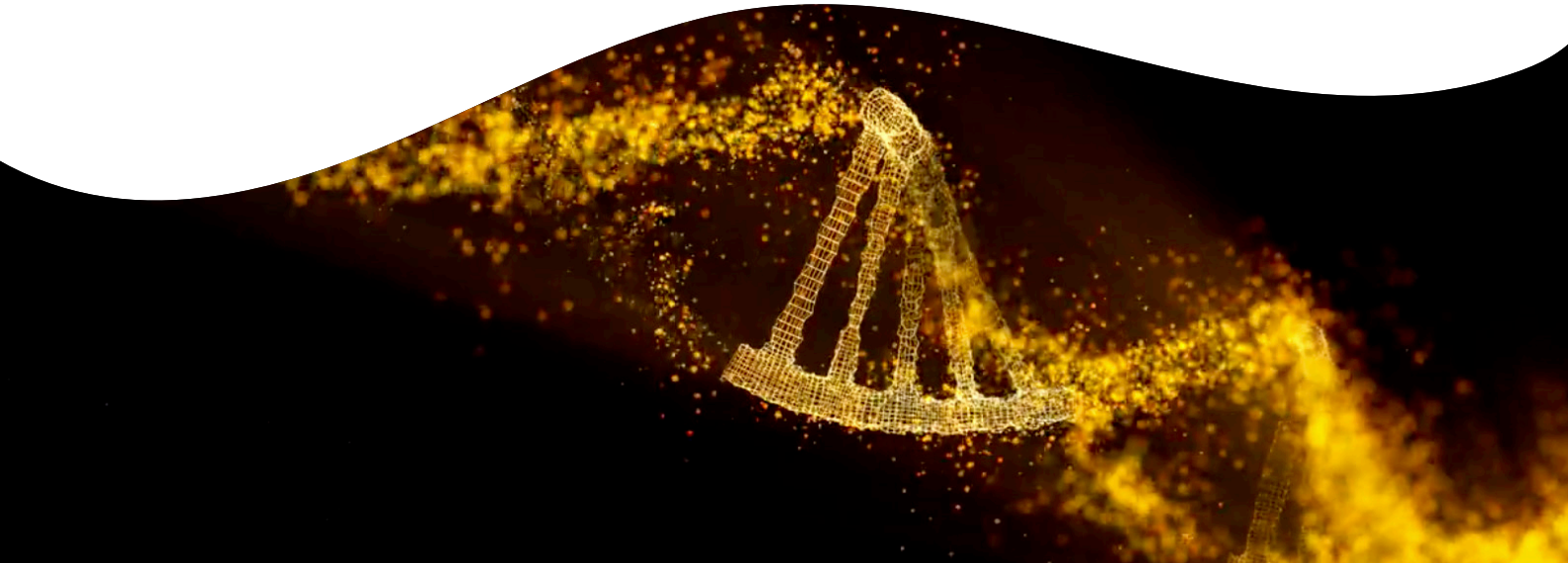
APPLICATION NOTE

Revvity's Pin-point™ base editing system: a novel nuclease-agnostic modular platform for increasing the targeting scope and precision of next-generation gene editing technology

Introduction

Base editing is a next generation gene editing technology that can introduce precise changes into the genome. Originally developed by David Liu and colleagues at Harvard University in 2016[1], base editing has made rapid progress towards the clinic. While base editing systems can knock out genes of interest by introducing premature stop codons[2] or splice site disruptions[3] and can sometimes introduce specific point mutations for gene correction[4], the technology remains limited in its ability to access all potential therapeutic targets[5,6].

One limitation of CRISPR-Cas based gene editing systems is the reliance on the protospacer adjacent motif (PAM) requirement for target recognition. The PAM sequence dictates where the Cas enzyme will bind to the target DNA sequence, and therefore determines the area of the DNA that the base editor can effect (known as the base editing window). Wildtype Cas9 is reliant on an NGG PAM, where N is any base. Whilst G-rich PAMs are often used by Cas9 variants, other Cas variants have a variety of alternate PAM sequence requirements, including Class II Type V enzymes that utilize a T-rich PAM. Some PAM-less, or near PAM-less enzymes have been developed that have relaxed PAM binding requirements and therefore have the potential to further expand the base editing window opportunities in the genome[7]. In 2020, a bioinformatic study of CRISPR-Cas base editing systems identified that of the human SNVs related to monogenic diseases that could be corrected by cytosine base editors, only about 25% could be addressed with the editing windows of available systems[6].



The base editing window can also be amended through a combination of deaminase usage and base editor configuration. Base editing can use cytosine deaminases (introducing a C:G to T:A base change) or adenine deaminases (introducing a A:T to G:C change). Looking at cytosine deaminases, for example, there are variations in editing window and efficiency between classes, types, and species[8,9]. Additionally, use of different linkers and configurations of the entire base editing module can also influence on-target editing efficiency and precision[10,11].

Here, we demonstrate the unique modularity of the Pin-point™ base editing platform, first described by Collantes *et al*[12], and its advantages towards addressing difficult-to-reach genomic targets. The novel screening approach described allows for simple and fast evaluation of RNA-guided nucleases, deaminases, various guide RNA designs, and aptamer combinations to enable and optimize a variety of therapeutic base editing applications.

Results

The Pin-point platform is comprised of three different components (Figure 1). An RNA-guided nuclease is directed to the genomic locus of interest by an aptamer-containing gRNA where the deaminase is recruited through an aptamer binding protein. The RNA-guided nuclease part of the complex unwinds an R-loop in the DNA and gives single strand access to the deaminase for base conversion. When a specific nickase Type II nuclease is used, a nick is made in the targeted strand, initiating a DNA repair mechanism that enhances the desired base change. When a deactivated Type II or Type V nuclease is used, the converted base is incorporated into the DNA through the next round of DNA replication. All three components of this base editing system can be configured and optimized for specific applications and targets.

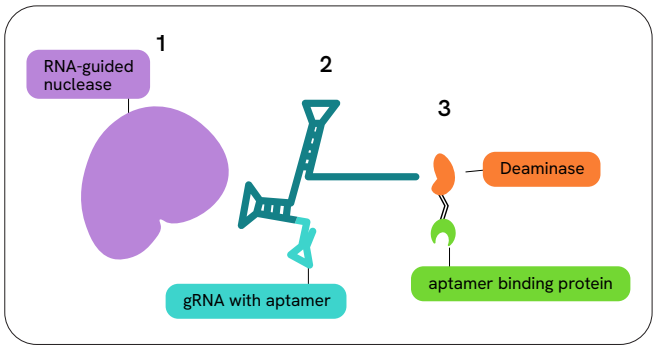


Figure 1. The modular components of the Pin-point base editing system. 1) An RNA-guided nuclease potentially in nickase or deactivated form. 2) A single guide RNA, crRNA, or a crRNA:tracrRNA with an aptamer, specifically designed to work with the RNA-guided nuclease. 3) A deaminase recruited by an aptamer binding protein.

Differences between nuclease types and variants range in targeting specificity, binding efficiency, enzyme size, and PAM requirements, which can enable greater access to the genome. The Pin-point system is highly versatile and can be adapted to work with RNA-guided nucleases that utilize either crRNA:tracrRNA, sgRNA, or crRNA only. As a proof-of-concept demonstration, we have evaluated the Pin-point system with several Type V and Type II enzyme variations (Table 1). Here we describe the outcomes from a subset of those preliminary evaluations.

Table 1. The Pin-point base editing system has been preliminarily evaluated with a variety of Type II and Type V nucleases. Table is up to date at time of publication, July 2023.

	Type II			Type V						
	A	**B**	**C**	**D**	**E**	**F**	**G**	**H**	**I**	**J**
Enzyme activity	nickase	nickase	nickase	deactivated	deactivated	deactivated	deactivated	deactivated	deactivated	deactivated
Demonstrated nuclease activity in mammalian cells	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
Demonstrated with the Pin-point system	✓	✓	In progress	✓	In progress	✓	In progress	✓	✓	In progress
sgRNA optimized	✓	In progress		In progress		✓		✓	✓	
Enzyme optimized	✓					✓				
Confirmed at multiple targets (2+)	✓	In progress				✓		✓	✓	
Demonstrated in multiple cell types (2+)	✓	In progress				✓		✓		
Demonstrated with multiple deaminases (2+)	✓							✓	✓	

In one such pilot evaluation, a Type V enzyme was paired with an aptameric guide RNA and cytosine deaminase to introduce C:G to T:A base edits in the genome. First, the enzyme sequence itself was optimized for efficient expression in mammalian cells, resulting in an observed 2-fold increase in activity (Figure 2A). Next, we screened a range of guide RNA designs with aptamers located at different positions within the gRNA scaffold and identified a design which further increased the activity an additional 50% for the target gene (Figure 2B). We next asked whether this updated configuration could address a second, more therapeutically relevant target and indeed observed editing at the second target without further optimization (Figure 2C). We then asked whether we could use the same updated configuration in clinically relevant T cells and saw that the system was effective both in the cancer cell line in which it was developed, as well as in primary T cells (Figure 2D).

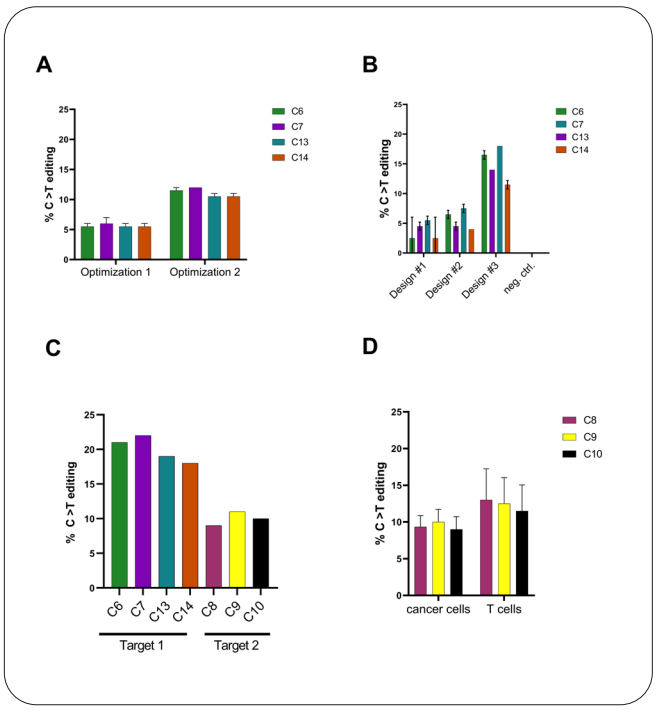


Figure 2. Preliminary evaluation of a deactivated Type V enzyme with the Pin-point base editing system. A) Optimization of a Type V nuclease resulted in a 2-fold increase in % C to T editing at the first target site. B) Optimization of the guide RNA design for the first target site resulted in an observed increase of editing efficiencies across the editing window with Design #3. C) This Pin-point system configuration was validated at two different target sites. D) The second target site was also validated in clinically relevant primary T cells.

In another example, we again demonstrated that the guide RNA design could be optimized for use with another deactivated Type V nuclease (Figure 3A). We then observed that when using guide RNA design 3, and pairing it with two different deaminases, we could further adjust the editing within the base editing window (Figure 3B).

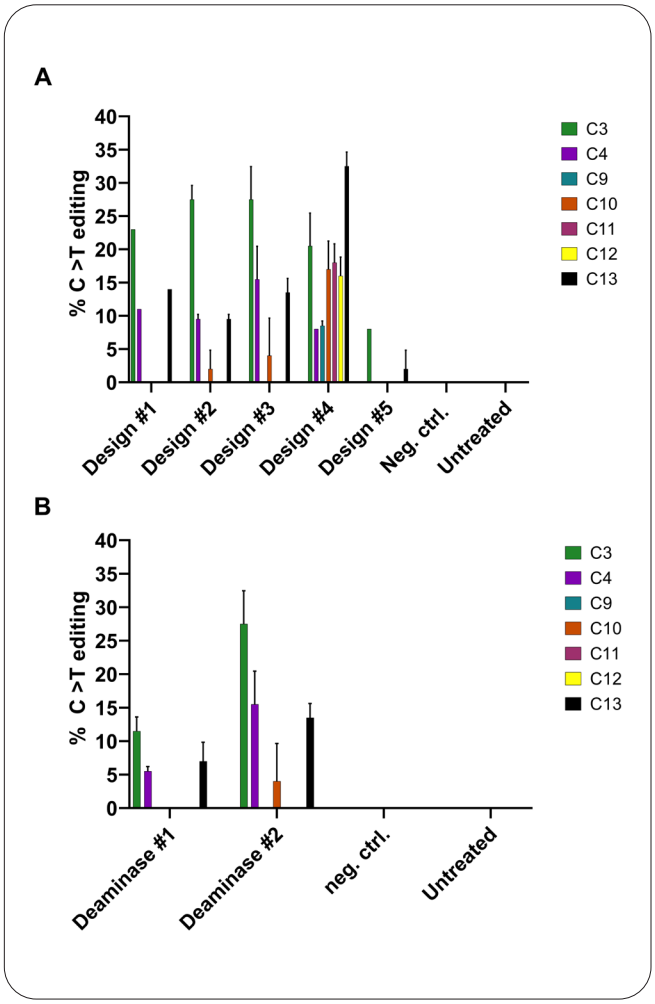


Figure 3. Preliminary evaluation of a second deactivated Type V enzyme with the Pin-point base editing system. A) Optimization of the guide RNA design resulted in different activity and editing results within the base editing window. B) Pairing the nuclease and optimized guide RNA design 3 with different deaminases resulted in different editing results within the base editing window.

To utilize a nickase Type II enzyme in the Pin-point base editing platform, we also first started with screening a range of aptamer-containing guide RNA designs specific to the nuclease and identified a design that worked well at multiple target sites (Figure 4A, manuscript submitted). We then screened specific target sequences with a variety of cytosine deaminases to identify the best configuration of the Pin-point platform to precisely edit the therapeutically relevant targets

(manuscript in progress). As an example of the data obtained with this screening approach, we identified that at the first target, deaminase 1 resulted in a clear single C to T base change in the target window (Figure 4B), whereas at a second target, it was deaminase 2 which resulted in a specific editing event at the C of interest (Figure 4C).

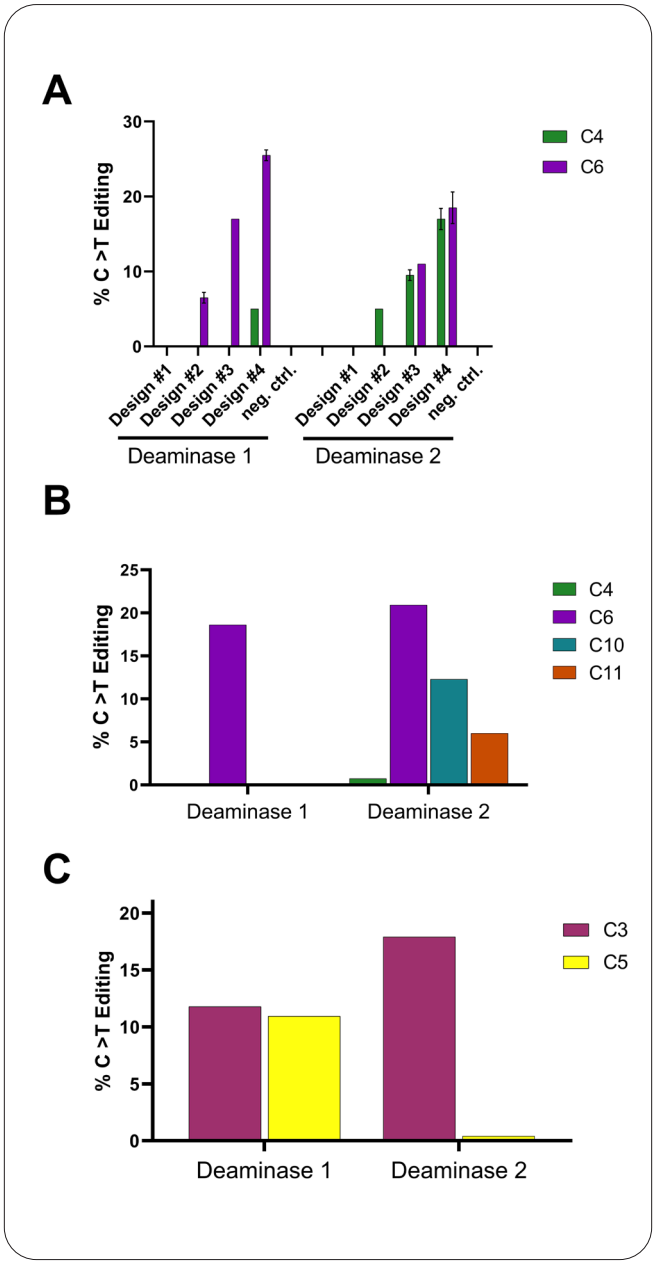


Figure 4. Preliminary evaluation of a nickase Type II enzyme with the Pin-point base editing system and subsequent impact on the base editing window. A) Optimization of the guide RNA design resulted in different activity and editing results within the base editing window. B,C) Pairing different deaminase and target guide RNAs resulted in different editing results within the base editing window.

Discussion

Here we demonstrate the benefits of the modular Pin-point base editing platform and its ability to be adapted for use with a sample of Type II and Type V Cas enzymes. Once the aptameric guide RNA scaffold is optimized, the system can be translated into different cell types and address different targets. Furthermore, different deaminases can be investigated for specific effects on the target window to precisely target a specific base change of interest.

Depending on the application, and whether bystander effects are tolerated (e.g. they may be tolerated when introducing a premature stop codon for gene knockout), the system can be further optimized to balance editing efficiencies, predicted off-targets, and editing precision. Due to the modular nature of the Pin-point system, elements can be swapped in and out for different applications and screened in high-throughput without time consuming cloning of each combination.

Material and methods

Cell culture and transfection

HEK293T and U2OS cells were seeded at 10,000 to 20,000 cells per well in a 96-well plate twenty-four hours prior to transfection. Cells were co-transfected using DharmaFECT™ Duo Transfection Reagent (Revvity, #T-2010) and different combinations of plasmid or synthetic gRNA and mRNA reagents for delivering the Pin-point components. For nickase Type II enzyme testing, HEK293T cells were generated to stably express a Cas9 nickase and synthetic gRNAs were co-delivered with deaminase mRNA. For deactivated Type V enzyme testing in primary T-cells, PBMCs were isolated from blood sources (e.g., CPD Blood bags, apheresis cones, leukopaks, etc.) by layering on Lymphoprep using SepMap columns (STEMCELL Technologies). Then total CD3+ T cells were isolated using negative selection with the EasySep Human T Cell Isolation Kit (STEMCELL Technologies). T Cells were checked by flow cytometry and then cultured in Immunocult XT media (STEMCELL Technologies) with 1x Penicillin/Streptomycin (Thermofisher) at 37C and 5% CO2. After 48-72 post-activation T cells were electroporated using the Neon Electroporator (Thermofisher) at 1600v/10ms/3 pulses with a 10 µl tip in 250k cells with deaminase mRNA, deactivated Type V enzyme mRNA, and synthetic gRNA.

Cell lysis and PCR

Seventy-two hours post transfection, cells were lysed in 100 µL of a buffer containing proteinase K (Thermo Scientific, #FEREO0492), RNase A (Thermo Scientific, #FEREN053 l), and Phusion HF buffer (Thermo Scientific, #F-518L) for 30 min at 56 °C, followed by a 5 min heat inactivation at 95 °C. Alternatively, cells were washed 1x with PBS and 50 µl of TrypLE express enzyme (Thermo Fisher Scientific) was added to each well. After the cells were dissociated, 100 µl of fresh DMEM was added and 20 µl of the resuspended cells were transferred to a 96 well plate and were incubated with 60 µl of DirectPCR lysis reagent (Viagen Biotech) under the following conditions: 55˚C for 45 minutes followed by 95˚C for 15 minutes. The cell lysate was then used to generate PCR amplicons spanning the region containing the base editing site(s). PCR amplicons between 400-1000 bp in length were generated and sequenced by Sanger sequencing.

Base editing analysis

Base editing efficiencies were calculated from Sanger sequencing reads and displayed as % C to T editing, using the Chimera™ analysis tool, an adaptation of the open-source tool BEAT. Chimera first determines the background noise to define the expected variability in a sample, using a geometric mean with outliers capped to the median value. Following this, Chimera subtracts the background noise to determine the editing efficiency of the base editor over the span of the input guide sequence.

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