APPLICATION NOTE

A Pin-point™ base editing system configured with the compact Type V effector protein dCasMINI.

Introduction

Base editing is a revolutionary genome editing technique that enables precise and efficient modification of DNA sequences without causing a double-strand break. It was initially developed by David Liu and colleagues at Harvard University in 2016[1]. Their published method takes advantage of the precise targeting of the CRISPR-Cas9 system to bring a deaminase fused to a nuclease-deficient Cas9 to the site of interest, resulting in the conversion of a C-G base pair to a T-A base pair. Since then, several other base editing systems have been developed using various DNA-modifying enzymes, including adenine deaminases and glycosylases, to enable precise and efficient editing of specific nucleotides. Moreover, the use of the nickase $Cas9^{D10A}$ has increased the efficiency of the original base editor 2 to 6-fold by introducing a nick in the non-targeted strand, which promotes the use of the edited strand as a template to repair DNA[1].

Revvity's Pin-point™ base editing platform is a modular system composed of three structural components where one possible configuration includes (Fig.1): i) a RNA aptamer derived from the operator stem-loop of bacteriophage MS2 (MS2) fused to the gRNA, ii) an effector molecule, in this case composed of a cytidine deaminase fused to the MS2 coat protein (MCP), and iii) a nickase Cas9 fused to 2 molecules of the Uracil DNA Glycosylase (UNG) inhibitor, UGI[2]. Each of these components are delivered individually and then binds together in cells to form the complete base editing system. This modularity allows for the customization of each of the components individually to optimize the needs of the target site.

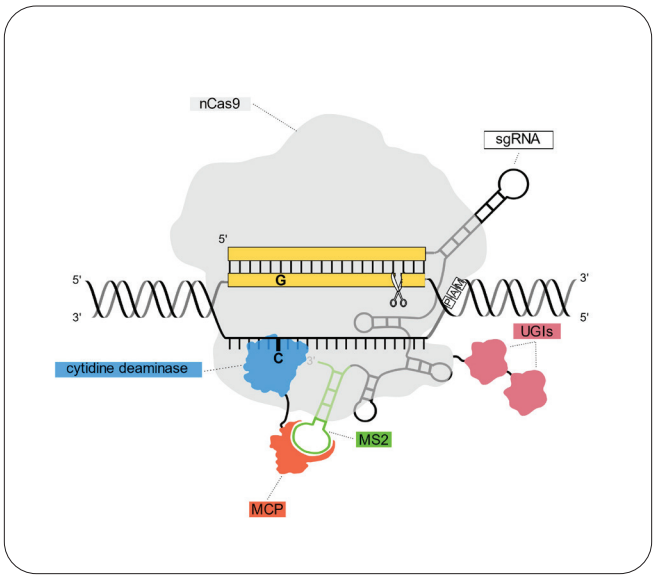

Figure 1: One configuration of the Pin-point base editing platform.

Schematic of one configuration of the Pin-point base editing technology. A SpCas9 nickase (nCas9) binds to the gRNA, the recruiting RNA aptamer (MS2) fused to the gRNA recruits the effector module. The effector molecule is composed by a deaminase fused to the aptamer binding protein (MCP). The recruitment of the deaminase to the target site forms an active complex capable of editing target cytosine residues on the unpaired DNA strand within the CRISPR R-loop.

A key factor that determines the accessibility of the target cytosine for editing is the protospacer adjacent motif (PAM) sequence, which is a short DNA sequence required for Cas enzymes to recognize the target site. The PAM sequence differs among different Cas enzymes, with Cas9 requiring a PAM sequence of NGG (where N is any nucleotide). The original Pin-point system was developed using nCas9, which restricts the scope of mutations that can be targeted to cytosines close to an NGG PAM. To address this, we explored the use of an alternative Class II Type V family of Cas effector proteins, which require a single effector for target recognition and cleavage, similar to Cas9. Type V proteins have a distinct PAM sequence, often more T-rich, which allows for the targeting of cytosines in previously inaccessible genomic regions. However, Type V proteins only have one nuclease domain which cuts both strands of DNA sequentially. Therefore, in order to achieve base editing, a dead version of any Type V Cas instead of a nickase must be used.

We demonstrate herein the flexibility of the Pin-point platform by exchanging nCas9 for the deactivated Type V protein dCasMINI, a modified version of Un1Cas12f1 optimized for maximum activity and with a low off-target profile[3,4]. We engineered a range of structures of the sgRNA molecule to look at the effect on editing efficiencies and profiles, highlighting the versatility of the Pin-point system for addressing different target sites. Moreover, we have generated data using two different deaminases with different editing efficiencies, an enhanced version of human Apobec3a[5] and Anolis carolinensis Apobec (AnoApobec), further demonstrating the flexibility of the Pin-point platform.

Results and discussion

In order to test the modularity and flexibility of the Pin-point platform, we substituted the nCas9 effector with dCasMINI, a promising Type V protein optimized to achieve high levels of activity and specificity[3,4], and ratApobec by hApobec3A and AnoApobec. We also engineered five different versions of the gRNA component. HEK293T cells were transfected with plasmids expressing the 3 components of the dCasMINI-based Pin-point platform and two different sites were analysed, VEGFA-1 and VEGFA-2. As depicted in Figure 2, editing levels between 0% and 32.5% were observed depending on the location of the cytosine inside the targeting window, locus, the deaminase and the gRNA design used, which clearly shows the flexibility of the system and the potential to optimize it.

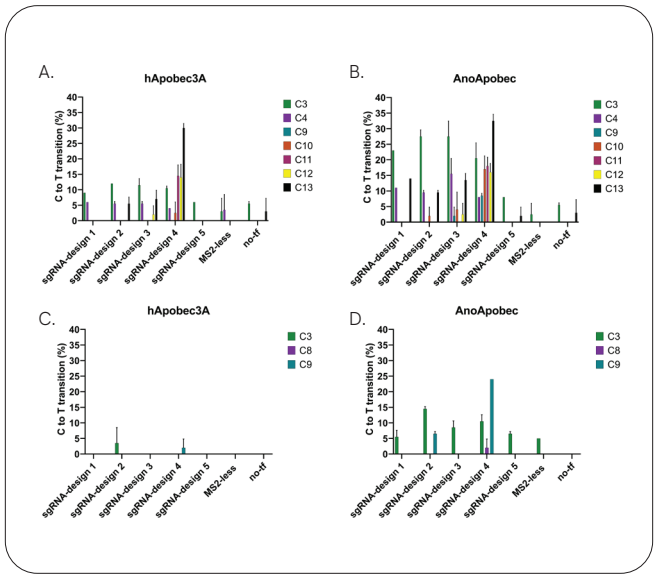

Figure 2. dCasMINI-based Pin-point platform is functional in HEK293T cells using two different deaminases and 5 sgRNA designs.

Sanger sequencing analysis shows that dCasMINI-based Pin-point platform introduces C to T transitions in different sites in HEK293T cell line. Two different regions targeted by two different gRNAs are shown: VEGFA-1 (A, B) and VEGFA-2 (C, D). Two different deaminases were used, hApobec3A or AnoApobec. X-axis represents different designs of the gRNA. Components were delivered by DNA plasmid transfection. No-tf: No transfection control

There are several layers of factors that seem to affect the editing efficiency. Firstly, there is a clear locus-dependent change in base editing efficiency: VEGFA-1 (Fig. 2A and 2B) is edited at higher efficiency than VEGFA-2 (Fig. 2C and 2D). Secondly, AnoApobec is a more efficient editor than hApobec3A for all the gRNA designs tested. Finally, there is an evident change both in the editing efficiency and specificity due to the gRNA design. This is relevant, as depending on the indication, there could be a bigger interest in achieving a very specific edit in a particular cytosine or a preference for using a broader editing window encompassing all possible cytosines accessible. For instance, the combination of dCasMINI, AnoApobec and gRNA design 2 offers more specific editing of the cytosine at position 3 in the targeting window of VEFGA-1, while we can target all cytosines present in the same site by using dCasMINI, AnoApobec and gRNA design 4. This difference is reproducible even if this position is challenging to sequence by Sanger Sequencing, as observed by background noise observed in the non-transfected (no-tf) condition. The same effect in the targeting window between gRNA design 2 and 4 is observed when using hApobec3A as a deaminase, but with a lower editing efficiency especially at the VEGFA-2 site (Fig 2A and 2B). Further experiments in a high-throughput manner are needed to confirm this effect and to select the right combination according to the project's goal.

By changing the three modules of the Pin-point platform, we demonstrate its highly flexible capabilities and potential to target any cytosine of interest, as we could, in principle, mix and match any Cas protein, deaminase, and gRNA design, adapting quickly to the needs of each application.

Material and methods

Cell culture and transfection

HEK293T cells were grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 100 U ml-1 penicillin/streptomycin. Twenty-four hours prior to transfection 10,000 cells were seeded into a single well of a 96-well plate to achieve ~ 70% confluency for transfection. After 24 hours the cells were lipid transfected with 200 ng of plasmid DNA (75 ng Cas vector, 75 ng Deaminase vector, and 50 ng sgRNA expression vector) using DharmaFECT Duo (Dharmacon™).

Cell lysis

Seventy-two hours after transfection, the medium was removed and the 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 lysates were then stored at -20˚C until PCR amplification.

PCR amplification of targeted regions

1 µl of cell lysate from each sample was used as template per PCR reaction. Regions of interest were PCR-amplified using GoTaq Hot Start polymerase (Promega). Reaction mixes were set up as follows:

Reagent	Volume
5x GoTaq buffer	5 µl
MgCl2 (25 mM)	2 µl
dNTPs (100 mM)	0.1 µl
Forward primer (10 uM)	1.25 µl
Reverse primer (10 uM)	1.25 µl
Nuclease-free water	15.4 µl
Total	**25 µl**

The PCR reaction was performed under the following thermocycling conditions:

Step	Temperature	Time
Initial denaturation	95°C	30 seconds
30 cycles	95°C 64°C 72°C	10 seconds 30 seconds 30 seconds
Final extension	72°C	2 minutes

Base editing analysis

The amplicons were sequenced using Sanger sequencing and base editing efficiencies were calculated 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 31 bp input guide sequence.

References

1. Komor, A. C., et al. Programmable editing of a target base in genomic DNA without double-stranded DNA cleavage. Nature **533**, 420–424 (2016)

2. Collantes, J. C. et al. Development and Characterization of a Modular CRISPR and RNA Aptamer Mediated Base Editing System. The CRISPR Journal **4**, 58–68 (2021)

3. Xu, X. et al. Engineered miniature CRISPR-Cas system for mammalian genome regulation and editing. Molecular Cell 81, 4333-4345 (2021)

4. Xin, C. et al. Comprehensive assessment of miniature CRISPR-Cas12f nucleases for gene disruption. Nature Communications 13: 5623 (2022)

5. Wang, X et al. Cas12a Base Editors Induce Efficient and Specific Editing with Low DNA Damage Response. Cell Reports 31, 9 (2020)

Revvity
940 Winter Street
Waltham, MA 02451 USA

(800) 762-4000
www.revvity.com