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APPLICATION NOTE

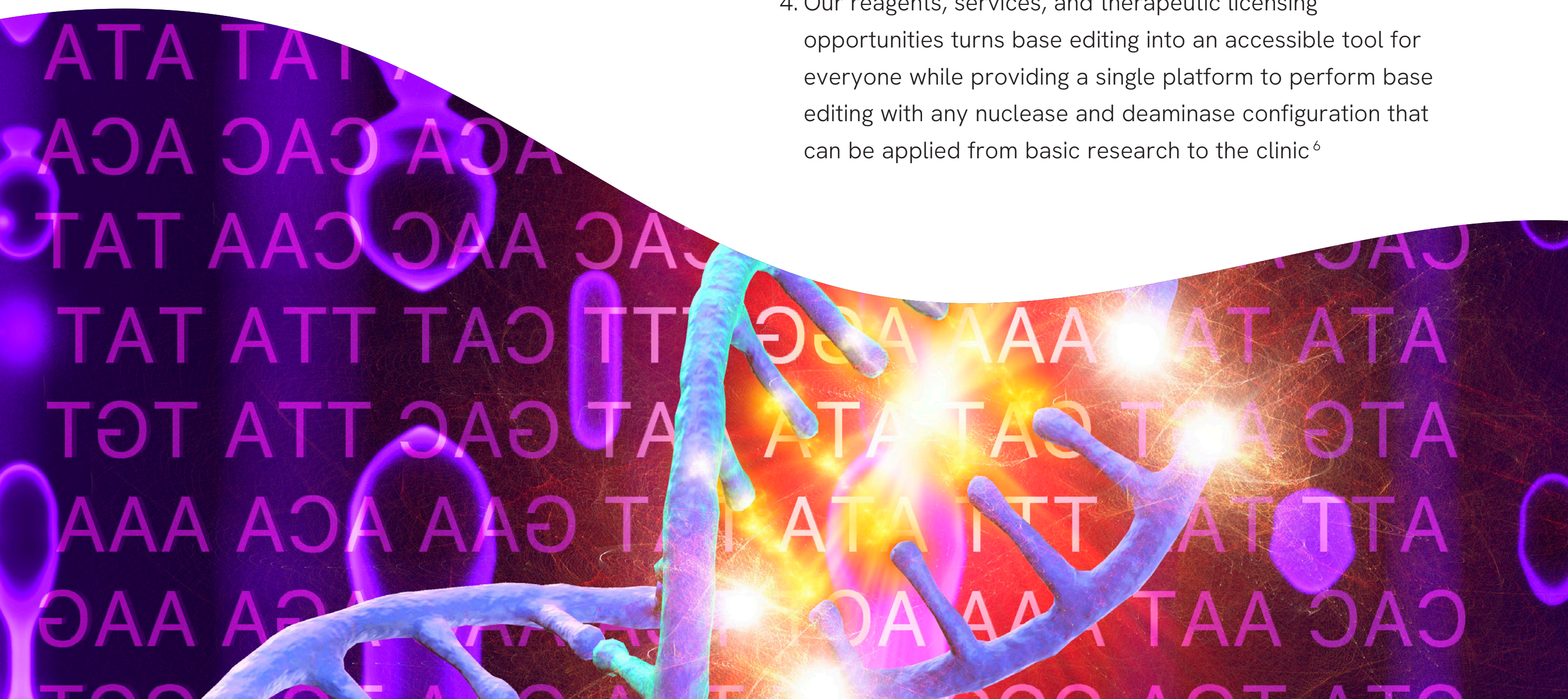
Designing and evaluating single guide RNAs for introducing protein knockout with the Pin-point base editing platform.

Designing reagents for gene editing experiments can be tricky. Factor in the complexities of base editing – such as trying to change a single base within the base editing window – all while making sure the edit is on the right strand, and it can quickly feel like a daunting task. In this application note we'll walk you through designing a guide RNA spacer sequence for a base editing experiment and demonstrate our approach to evaluating several candidate guide RNAs to identify the best one for generating a functional knockout.

Introduction

Base editing is a next-generation gene editing method used to change a single base in the genome using an RNA-guided nuclease and a deaminase [1,2]. Revvity's Pin-point™ base editing platform is a technology that brings any RNA-guided nuclease and deaminase together through interaction with an RNA aptamer on the single guide RNA (sgRNA) scaffold (Figure 1). This aptamer-recruitment approach has several advantages:

1. The modularity of the platform allows for nuclease and deaminase flexibility so our customers can easily optimize the targeting specificity and efficiency for their application [3]
2. The nature of the aptamer recruitment means that the Pin-point platform can use a single nicking enzyme to do simultaneous knockout and site-specific transgene knock-in [4]
3. Multiple deaminases can be recruited to different areas of the genome simultaneously to make highly complex disease models or corrections [5]
4. Our reagents, services, and therapeutic licensing opportunities turns base editing into an accessible tool for everyone while providing a single platform to perform base editing with any nuclease and deaminase configuration that can be applied from basic research to the clinic [6]



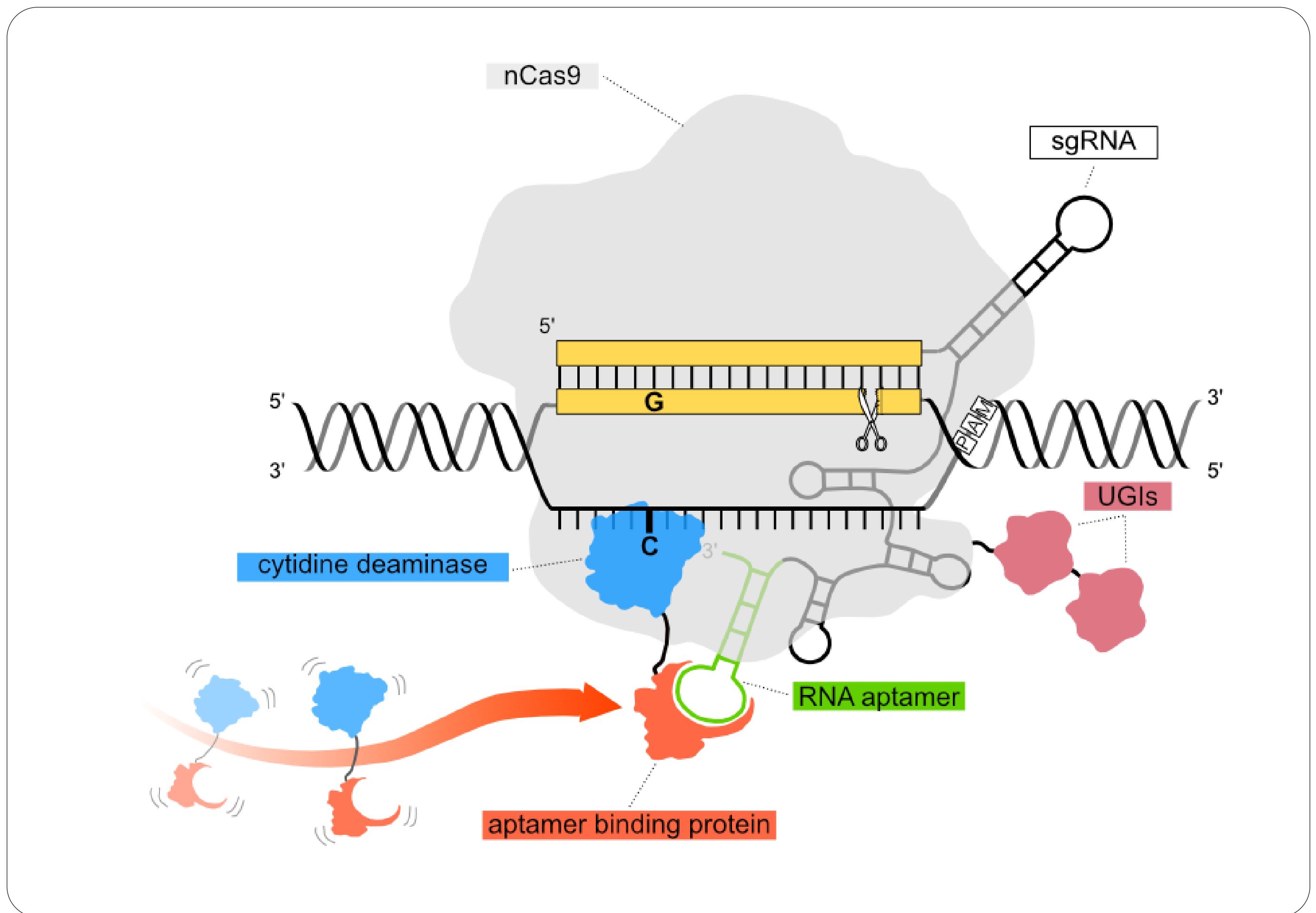


Figure 1: The Pin-point platform depicted in an nCas9 and cytidine deaminase configuration. The nCas9 is recruited to the target via the sgRNA targeting sequence (yellow), while the RNA aptamer (green) recruits the deaminase (blue) via the aptamer binding protein (orange). Cytidine residues on the same strand as the PAM, opposite of the sgRNA binding strand, will be modified by base editing.

Basic design principles

Base editing can be used to make silent or missense amino acid changes, introduce premature stop codons or disrupt splice sites to generate functional protein knockouts [7,8]. In the case of a splice site disruption, it is recommended to change a conserved splice donor (SD) site sequence from GT to AT, or disrupt a conserved splice acceptor (SA) site sequence from AG to AA (Figure 2). For introducing a premature stop codon with a cytidine base editor (CBE), it is recommended to target glutamine, arginine or tryptophan residues (Table 1).

In general, when designing sgRNAs to generate functional knockouts, avoid targeting the earlier or later exons, as targeting middle exons increases the probability to results in protein knockout [8]. Targeting exons coding for essential domains of the protein such as transmembrane domains for receptors is also a good strategy. Targeting non-symmetric exons should be prioritized because if a symmetric exon is targeted and skipped, the following exons will still be in-frame [9].

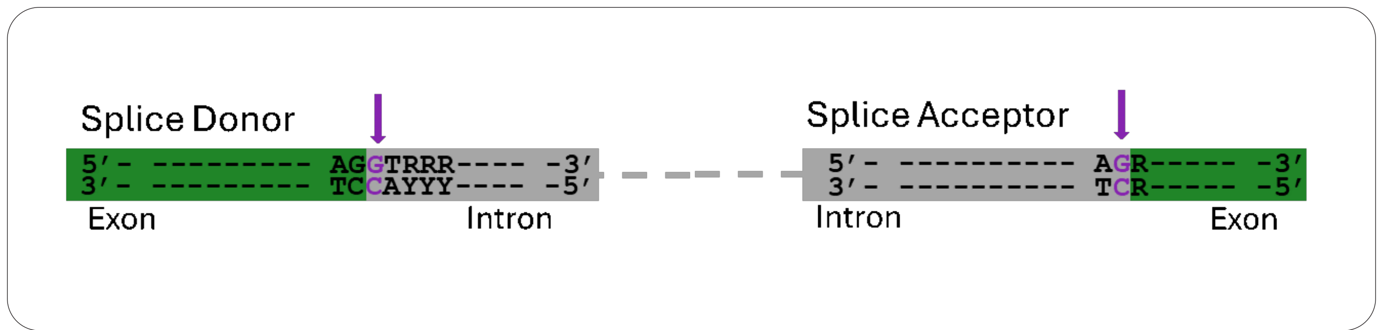


Figure 2: Target splice sites using a CBE. Diagram of splice donor and splice acceptor sites. Highlighted in purple is the base pair within the splice site that can be targeted by the CBE.

Table 1: Stop codons that can be generated from cytidine deamination.

Target codons	Target amino acid	Introduced stop codon
CAA	Glutamine	TAA
CAG	Glutamine	TAG
CGA	Arginine	TGA
TGG	Tryptophan	TGA, TAG

Whether the purpose is to introduce a missense mutation or to generate a functional knockout, the targeted base will have to be in close proximity to a protospacer adjacent motif (PAM) relevant to the nuclease used in the Pin-point platform. For example, when using the Pin-point system with the Streptococcus pyogenes nickase Cas9 (nCas9) and a rat APOBEC deaminase, the sgRNA targeting sequence (sometimes referred to as the spacer, or protospacer) would be the 20 nucleotides 5′ to an NGG PAM motif.

The configuration of the Pin-point platform can also impact the base editing window, which is the target area accessible for deamination when the base editing machinery assembles at the target locus. With a Pin-point system configured with nCas9 and rat APOBEC, base editing is likely to happen at positions 4-7 (Figure 3).

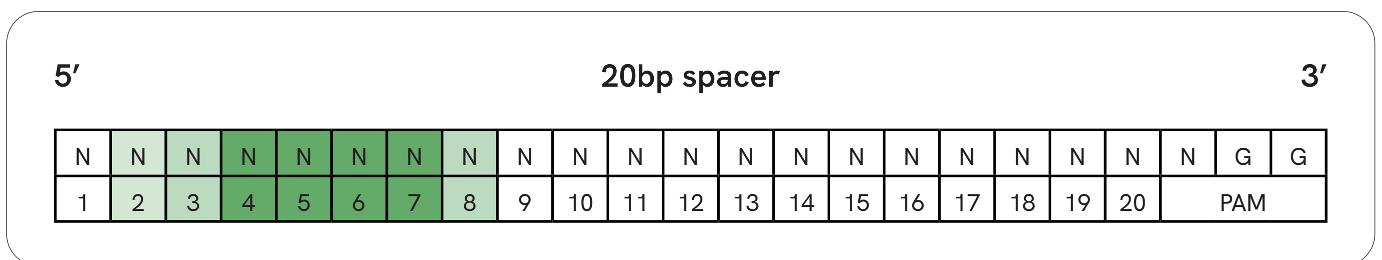


Figure 3: Base editing window. Dark green= potential for highest efficiency editing. Light green= potential for lower efficiency editing. White= potential for bystander editing.

Finally, a Pin-point cytidine base editor configuration based on rat APOBEC1 has specific dinucleotide context preferences (Figure 4). Prioritize sgRNA designs in which the sequence context is NTC and TCC and avoid ones where the sequence context is NGC and GCC (target C is underlined) [10]. Good sgRNAs should also be specific, meaning that they have limited complementarity to other areas of the genome with no fewer than three mismatches in total, or no fewer than 1 mismatch in the seed region (positions 11-20 of the protospacer) [11].

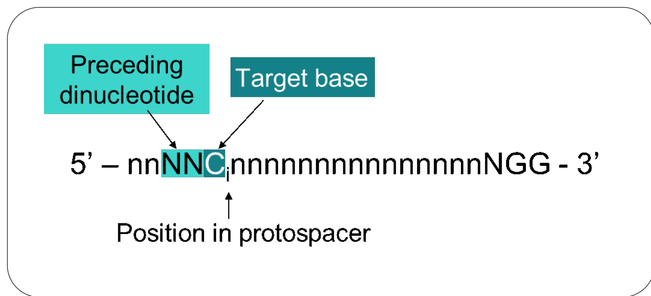


Figure 4: Dinucleotide context is defined by the identity of the target base, and the identity of the two bases immediately preceding the target.

In figures 5 A and B, two practical examples of base editing sgRNA design when the targeted C is on the forward or the reverse strand are reported.

A

Activity window

1 20 PAM

Starting sequence

5′ – AGTTGAAAAATCCTTAGTTCAGGCCTGCGAATTAATGGCACGTCTCCACCT – 3′
3′ – TCAACTTTTTAGGAATCAAGTGCGGACGCTTAATTACCGTGCAGAGGTGGA – 5′

5'- AGUUCAGGCCUGCGAAUUAA - 3' Your gRNA spacer sequence

Edited sequence

1 20

5′ – AGTTGAAAAATCCTTAGTTTAGGCCTGCGAATTAATGGCACGTCTCCACCT – 3′
3′ – TCAACTTTTTAGGAATCAAATGCGGACGCTTAATTACCGTGCAGAGGTGGA – 5′

B

3'- UGAAGUCAGGUCGCUCAAUU - 5' Your gRNA spacer sequence

Starting sequence

5′ – AGTTGAAAAATGCCTAGTTCAGTCCTGCGAGTTAAACCCACGTCTCCACCT – 3′
3′ – TCAACTTTTTACGGATCAAGTCAGGACGCTCAATTTGGGTGCAGAGGTGGA – 5′

PAM 20 1

Activity window

Edited sequence

5′ – AGTTGAAAAATGCCTAGTTCAATCCTGCGAATTAAACCCACGTCTCCACCT – 3′
3′ – TCAACTTTTTACGGATCAAGTTAGGACGCTTAATTTGGGTGCAGAGGTGGA – 5′

20 1

Figure 5: Guide RNA design examples. A) Targeting a C on the forward strand aiming at modifying a C; B) editing a G, by targeting the C on the reverse strand. Identify the nucleotide to edit (in orange); in A) CAG is converted to a STOP codon (TAG); in B) a splice acceptor site (AG) is mutated. Consider that the NGG PAM is always located at the 3′ of the spacer on the targeted strand (targeted stand being the strand where the C substrate of the deaminase is located). Ensure that the targeted C is positioned in the activity window (darker green = potential for highest efficiency editing). Your spacer sequence will bind the non-targeted sequence and should be complementary to it, note that sequences are ordered from 5′ to 3′. In the example in A your spacer sequence is: 5′- AGUUCAGGCCUGCGAAUUAA - 3′. In the example in B your spacer sequence is: 5′-UUAACUCGCUGGACUGAAGU-3′.

Ultimately, the editing efficiencies and how the particular base edits translate into functional knockouts (if that is intended) must be experimentally determined. We recommend evaluating five to ten different spacer sequences, if possible. Below we report a sgRNA design and screening process to knockout the PCSK9 in human cells.

Results

Base editing sgRNA design

To design base editing sgRNAs that aim to knockout PCSK9, we first scanned the coding region and generated a list of 63 candidate sgRNAs that had a C in the base editing window (positions 3-8, PAM-distal, Figure 3) that, if edited, would introduce a premature stop codon or destroy a splice site. To reduce the number of sgRNAs for experimental validation, we followed a set of custom rules described below and refined that list to 21 final candidates.

When designing base editing sgRNAs for protein knockout, it is important to consider the gene and protein structure. In the case of PCSK9, the main protein coding isoform of the gene consists of 12 exons. Based on the published PCSK9 structure, sgRNAs targeting after exon 8 were excluded because they would likely not result in protein knockout [12]. sgRNAs that introduce a premature STOP codon in exon 1 were excluded to avoid the risk that transcription could start from alternative ATG codons located downstream of the introduced STOP codon. sgRNAs with the targeted C preceded by a non-favorable dinucleotide context (NG<u>C</u> and GC<u>C</u>) were excluded as well as editing activity in that context is expected to be lower than in the other dinucleotide configurations. We also excluded redundant sgRNAs, which are those that targeted the same C but with a slightly shifted editing window due to different/multiple PAM locations; instead, the ones where the C was best located in the editing window were prioritized.

The final list of sgRNAs is reported in Table 2. Of those, sgRNA 1 and 8 have two target C's because editing of either C would result in generation of a stop codon. We also performed primer design for each target region and PCR optimization to ensure that each target locus could be amplified and analyzed by Sanger sequencing. sgRNA 5 was excluded from testing as sequencing of the target region failed due to the complexity of the sequence.

Table 2: Protospacer sequences to target PCSK9 for knockout were designed. Target C residue(s) required to destroy splice sites or to introduce a stop codon (STOP) are in bold. SD= splice donor; SA= splice acceptor.

Name	Spacer + PAM	Targeted exon	Targeted C position	Target type
sgRNA 1	CAGGTT**CC**ACGGGATGCTCTGGG	3	7, 8	STOP
sgRNA 2	GATC**C**TGGCCCCATGCAAGGAGG	2	5	SA
sgRNA 3	GGTC**C**AGCCTGTGGGGCCACTGG	6	5	STOP
sgRNA 4	AAGAC**C**AGCCGGTGACCCTGGGG	7	6	STOP
sgRNA 5	CCTA**C**CTCGGGAGCTGAGGCTGG	6	5	SD
sgRNA 6	CTTA**C**CTGCCCCATGGGTGCTGG	8	5	SD
sgRNA 7	CAGGCC**C**AGGCTGCCCGCCGGGG	2	7	STOP
sgRNA 8	CCAGGTT**CC**ACGGGATGCTCTGG	3	8, 9	STOP
sgRNA 9	GCTTA**C**CTGTCTGTGGAAGCGGG	4	6	SD
sgRNA 10	ACGGATC**C**TGGCCCCATGCAAGG	2	8	SA
sgRNA 11	GATGAC**C**TGGAAAGGTGAGGAGG	7	7	SA
sgRNA 12	ATCA**C**AGGCTGCTGCCCACGTGG	7	5	STOP
sgRNA 13	GTCA**C**AGAGTGGGACATCACAGG	7	5	STOP
sgRNA 14	TCAGTA**C**CCGCTGGTCCTCAGGG	8	7	SD
sgRNA 15	CCCGCA**C**CTTGGCGCAGCGGTGG	1	7	SD
sgRNA 16	AAGGC**C**TGCAGAAGCCAGAGAGG	3	6	SA
sgRNA 17	AAGC**C**AGCTGGTCCAGCCTGTGG	6	5	STOP
sgRNA 18	ATGC**C**TGGTGCAGGGGTGAATGG	8	5	SA
sgRNA 19	TGGC**C**TGCTCGACGAACACAAGG	5	5	SA
sgRNA 20	CA**C**CTTGGCGCAGCGGTGGAAGG	1	3	SD
sgRNA 21	GAC**C**TGGAAAGGTGAGGAGGTGG	7	4	SA

Evaluation of editing efficiency and phenotypic validation

To test base editing efficiency and the ability of the sgRNAs to induce a functional knockout, we selected the human hepatocyte cell line HepG2 that expresses PCSK9. A sgRNA previously validated for high base editing efficiency in primary human T cells, was used as a positive control. HepG2 cells were electroporated with the Pin-point base editing configuration consisting of a nickase Cas9 mRNA, rat APOBEC mRNAs and the sgRNAs targeting the control or *PCSK9*. DNA was extracted 3 days after electroporation and C to T conversion was measured by Sanger sequencing (Figure 6A).

Of the 20 sgRNAs tested, sgRNA 1, 2 and 10 were the top performing with 49%, 45% and 61% C to T conversion at the target C, respectively. Guide RNAs that demonstrated editing efficiency around 20% or higher (sgRNA1, 2, 7, 8, 10, 11, 14, 16, and 18) were selected for a secondary screen and phenotypic validation (Figure 6B, 6C). The secondary screen validated initial findings with designs 1, 2, and 10 resulting in the highest percentage of C to T conversion at the target base(s) (Figure 6B).

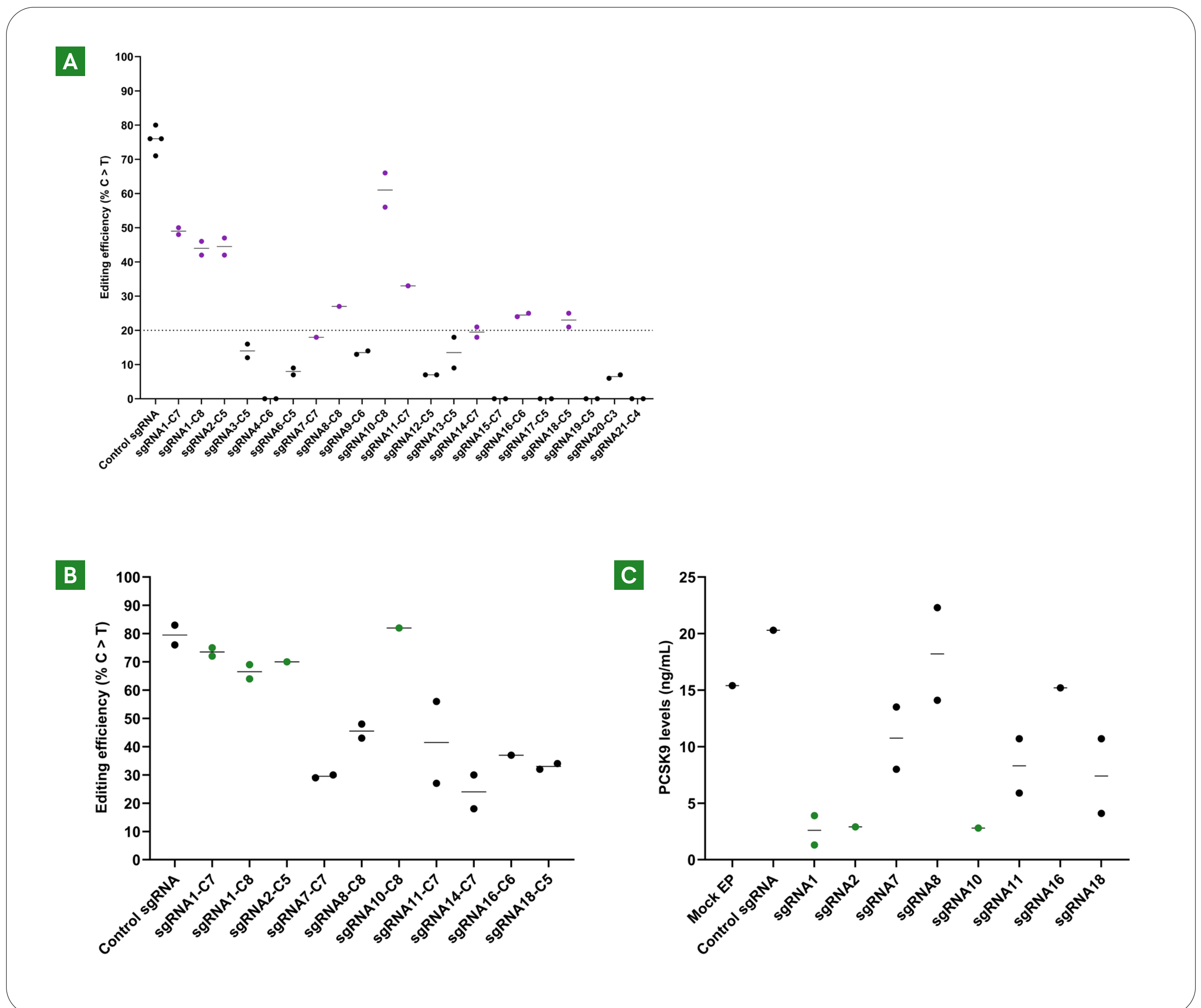


Figure 6: Data from the primary (A) and secondary (B, C) sgRNA screens. The percentage of C to T edits at the targeted C listed on the X axis was measured by Sanger sequencing (A, B). A) Comparison of base editing efficiencies. PCSK9 guides reaching 20% or higher editing efficiencies are shown in purple. B) The secondary screen confirmed the activity of the top performing sgRNAs (highlighted in green). C) PCSK9 levels in cell supernatant were measured by ELISA. Best performing sgRNAs are shown in green.

As PCSK9 is a secreted protein, to validate the ability of the best performing sgRNAs to create a knockout of the gene, an ELISA assay to measure the protein levels was performed on cells supernatants three days post electroporation of the editing reagents (Figure 6C). High levels of base editing correlate with the measured PCSK9 levels. The top performing sgRNAs (sgRNA 1, sgRNA 2 and sgRNA 10) induced a significant decrease in PCSK9 protein levels in the cell pools, indicative of efficient PCSK9 knockout. These guides reduce PCSK9 levels to under 3 ng/mL, while 15.4 ng/mL and 20.3 ng/mL of PCSK9 protein were detected in the EP+ and control samples, respectively.

In this study, there was a significant correlation between the sgRNA designs with the highest editing efficiency (designs 1, 2, and 10 as shown in Figure 6B) and functional protein knockout (Figure 6C). However, not all designs that introduced C to T editing resulted in significant levels of PCSK9 protein reduction (e.g. sgRNA16). The data from these screens confirmed the functionality of sgRNA 1, a previously validated base editing sgRNA for PCSK9 [13,14], and it also enabled the identification of two new highly functional sgRNAs, sgRNA2 and sgRNA10.

Discussion

While editing efficiency and protein knockout correlated well in this study, this isn't always the case. The more that is known about the protein structure and function, including the location of previously validated guide RNAs for CRISPR knockout, the more likely it will be that designs targeting a similar area will be functional. Various base editing-independent factors specific to the gene structure and protein domains may impact the success of functional knock out. In cases where protein structure and domain functions are known, splicing out the active domain, or targeting amino acids essential to protein function will improve the chance of functional knockout. However, this is not always the case, and this makes predicting which base editing sgRNAs will result in a functional knockout rather complex.

In the future, the development of more comprehensive predictive models of functional knockouts will facilitate the design of more effective base editing sgRNAs. In this report we provide a road map for easy and efficient base editing. Furthermore, we outline the current guidelines for the design of sgRNAs that will efficiently and specifically edit the target base of interest.

For more information about designing and ordering sgRNAs, or to discuss utilizing Revvity's high throughput screening services for optimizing the Pin-point platform for your specific application, contact our team today.

Materials and methods

Cell culture and electroporation

HepG2 cells were cultured using MEM (Gibco) supplemented with 10% HI FBS (Gibco), 1% Glutamax (Gibco) and 1% Penicillin-Streptomycin (Gibco). 200,000 cells were electroporated with 2 µM sgRNA (Revvity),1.56 µg of Pin-point nCas9-UGI-UGI and 0.35 µg of Pin-point rAPOBEC1 (Revvity) using the Neon Electroporation System (ThermoFisher).

Base editing analysis and phenotypic validation

Three days after electroporation, HepG2 cells were lysed using DirectPCR lysis reagent (Viagen) and incubated at 55°C for 30 minutes, followed by 95°C for 30 minutes. Lysates were used to generate PCR amplicons spanning the region containing the base editing site(s). Amplicons between 300-800 bp in length were generated and sequenced by Sanger sequencing. 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. To measure PCSK9 protein concentration, cell supernatants were collected 9 days after electroporation and analysed using Legend Max Human PCSK9 ELISA pre-coated plates (BioLegend, now part of Revvity) according to manufacturer protocol.

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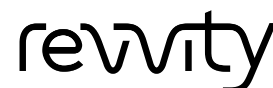

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