CRISPR-Cas9 nickase mutants promote homology-directed repair for efficient, high-fidelity genome editing

Strategies that promote high-fidelity homology-directed repair (HDR) over error-prone non-homologous end joining (NHEJ) are essential for CRISPR-Cas9 applications where minimal off-target activity is critical. This guide provides valuable strategies for achieving high-fidelity HDR of CRISPR-Cas9–based editing using Cas9 nickases to generate staggered double-strand breaks (DSBs).

Generating staggered DSBs with Cas9 nickases minimizes off-target effects

Unlike wild-type (WT) Cas9, which creates blunt-ended DSBs, Cas9 nickases can only create single-strand nicks. Paired nickase strategies leverage that feature to favor HDR and increase targeting selectivity. Cooperative, offset nicking by a pair of Cas9 nickase mutants generates staggered DSBs with 5′ or 3′ overhangs that preferentially recruit HDR machinery. Any off-target cleavage events that may occur will generate single-strand nicks that are repaired through the high-fidelity base excision repair (BER) pathway.

In addition, the requirements for creating a DSB via Cas9 nickases are considerably more stringent than with WT Cas9: simultaneous nicking directed by two non-identical gRNAs substantially increases the number of specifically recognized bases in the target site.

In accordance with previous studies, our data confirm that cooperative, offset nicking is both effective and efficient with careful experimental design and optimization. The latest versions (V3) of Alt-R™ Cas9 enzymes are optimized to deliver the highest editing. These enzymes can be directly substituted for prior Alt-R Cas9 enzymes in the following protocols.

Generating staggered DSBs with Cas9 nickases minimizes off-target effects

Abstract

Two amino acid mutations (D10A and H840A) in *S. pyogenes* Cas9 catalytic domains can be independently introduced into wild-type (WT) Cas9 protein to produce proteins capable of inducing single-stranded nicks rather than double-stranded breaks (DSBs). To create DSBs with Cas9 nickases, specific binding of two guide RNAs (gRNAs), located on opposite strands and in close proximity to each other, is required. In this study, we tested multiple guide pairs with distinct orientations and spacing to identify guide designs that result in optimal genome editing with Cas9 nickases. In addition, by testing and comparing a variety of donor template designs, we investigated the ability of Cas9 nickases to mediate homology-directed repair (HDR) events in human cells.

Introduction

The CRISPR-Cas9 (clustered, regularly interspaced, short palindromic repeats–CRISPR-associated protein 9) system has been widely used to perform site-specific genome editing in eukaryotic cells. WT Cas9 protein derived from *S. pyogenes* contains two endonuclease domains (RuvC and HNH) that function together to generate blunt-ended DSBs by cleaving opposite strands of double-stranded DNA (dsDNA). Inactivating one of the two endonuclease domains results in the formation of Cas9 nickase mutants that introduce a single nick in one strand of the target dsDNA. The RuvC mutant (Cas9 D10A) generates a nick on the targeting strand (gRNA complementary), while the HNH mutant (Cas9 H840A) generates a nick on the non-targeting strand (gRNA non-complementary) (**Figure 1**).

DSBs are known to be essential for efficient genome editing. To generate DSBs with a single nickase, a pair of gRNAs targeting opposite DNA strands is required. The NGG protospacer adjacent motif (PAM) of the two guides can either face outwards (PAM-out) or towards each other (PAM-in). This application note summarizes our recent discoveries in developing the utility of Cas9 nickases for genome engineering.

We show that both Cas9 D10A and Cas9 H840A demonstrate highest editing efficiency with guide pairs in a PAM-out configuration. In addition, we find that Cas9 D10A is generally more potent at mediating HDR events compared to its Cas9 H840A counterpart.

This article will show the following:

- Combined use of a Cas9 nickase with a pair of gRNAs creates DSBs with overhangs
- Cas9 nickases demonstrate higher editing efficiency with gRNA pairs in PAM-out configuration compared to a PAM-in configuration
- Single-stranded oligo deoxynucleotides (ssODNs) are the preferred donor template for nickase-mediated HDR
- Cas9 D10A is more potent in mediating HDR than Cas9 H840A
- Case study: Using Cas9 D10A nickase to introduce a new restriction recognition site at intended genomic loci in human cells
- Key points before starting your own Cas9 nickase experiment

Results and discussion

Combined use of a Cas9 nickase with a pair of gRNAs creates DSBs with overhangs

The cleavage activity of the *S. pyogenes* Cas9 endonuclease is mediated through the coordinated functions of two catalytic domains, RuvC and HNH [1,2]. The RuvC domain cleaves the non-targeting strand, which contains the PAM sequence, while the HNH domain cleaves the targeting strand, which is complementary to the guide RNA. A nickase variant can be generated by alanine substitution at key catalytic residues within these domains: Cas9 D10A has an inactivated RuvC and Cas9 H840A has an inactivated HNH. While single nicks are predominantly repaired by the high-fidelity base excision repair pathway [3], nicking of both strands by paired gRNAs that are appropriately spaced and oriented leads to the formation of a site-specific DSB. As opposed to the blunt-ended DSBs created by WT Cas9, the use of two nicking enzymes generates 5′ (Cas9 D10A) or 3′ (Cas9 H840A) overhangs along the target (**Figure 1**). Since simultaneous nicking via a pair of gRNAs substantially extends the number of specifically recognized bases in the target site, this approach can be leveraged to reduce off-target effects [4].

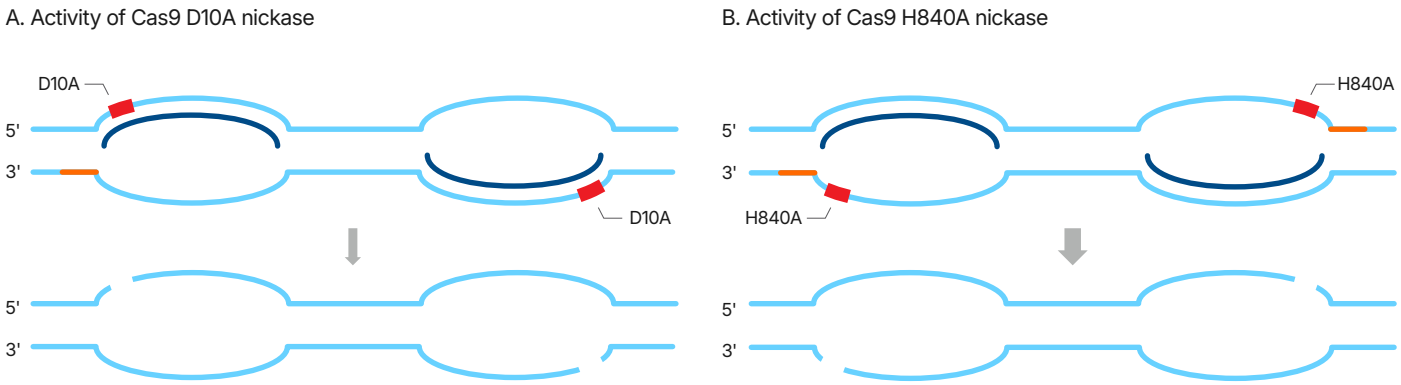
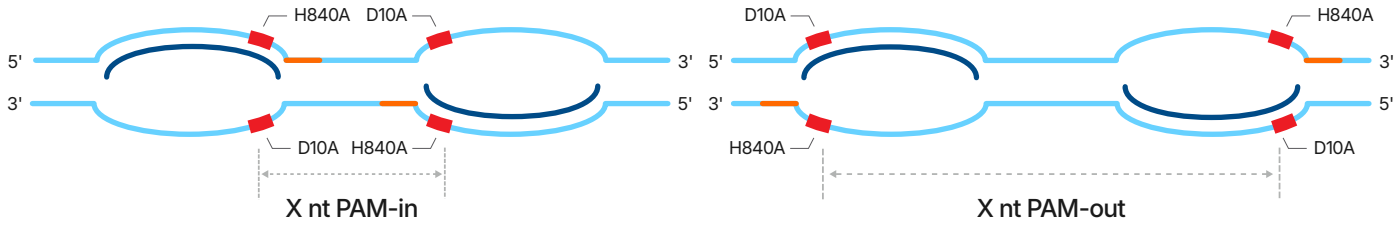


Figure 1. Schematic of DNA lesions generated by two nickases: Cas9 D10A and Cas9 H840A. PAM sites are shown as orange lines, while the corresponding cut sites (red boxes) are indicated by gray arrows. After nicking with a guide pair that targets opposite strands, Cas9 D10A yields a 5′ overhang (**A**), while Cas9 H840A results in a 3′ overhang (**B**).

Cas9 nickases demonstrate higher editing efficiency with gRNA pairs in PAM-out configuration compared to a PAM-in configuration

Because of the high-fidelity, single-nick repair pathway in cells, neither a single guide nor dual guides targeting the same strand leads to efficient genome editing in nickase ribonucleoprotein (RNP) transfection experiments (data not shown). In contrast, nicking both strands with paired guides is effective at introducing gene disruptions [**5**]; however, several factors may impact the efficiency of the cooperative nicking, such as overhang type and hindrance effect between two adjacent Cas9 RNP complexes. Some of these factors can be characterized by testing multiple gRNA pairs with distinct orientations and spacing. To systematically assess how gRNA designs affect indel formation, we designed sets of paired crRNAs against the human *HPRT1* gene with target cut sites that were separated by 40–130 base pairs (bp) and PAM sites facing either outwards (PAM-out) or inwards (PAM-in), as shown in **Figure 2A**. In this experiment, the two Cas9 nickase variants were assessed for their efficiencies in generating indels in human HEK-293 cells. In agreement with a previous study using Cas9 nickases [**6**], increased editing was observed when PAM sites faced towards the outside of the target region (PAM-out), whereas WT Cas9 generated high level of indels regardless of PAM orientation (data not shown). Furthermore, Cas9 D10A-mediated genome editing was increased even more when the two cleavage sites were 40–70 bp apart, while Cas9 H840A favored a distance of 50–70 bp (**Figure 2B**). During these experiments, we also discovered that higher editing efficiency is observed when using paired RNPs that were formed in separate reactions, as opposed to using RNPs that were formed by mixing the paired crRNAs, tracrRNA, and nickases in a single tube (data not shown).

A. Schematic of PAM site orientation and nickase cleavage sites



B. Total editing efficiency of Cas9 nickases when varying PAM site orientation and spacing.

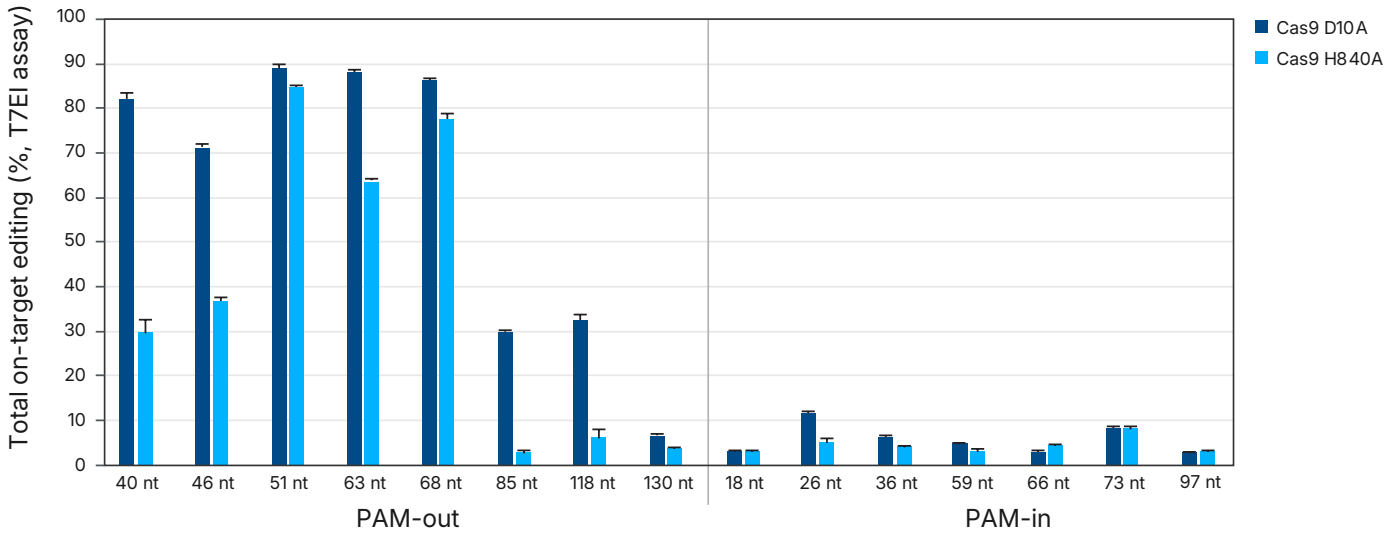


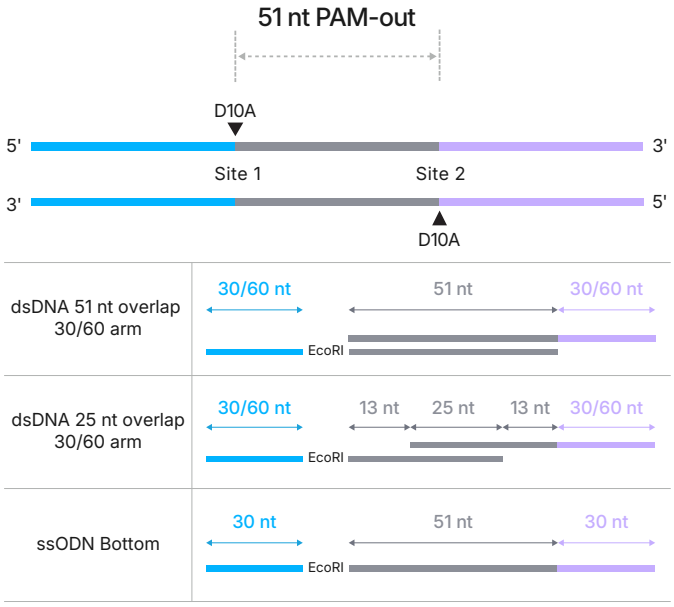
Figure 2. Optimization of guide RNA parameters governing Cas9 nickase-mediated genome editing. (A) Schematic illustration of guide designs in PAM-out/PAM-in configurations. PAM sequences are shown as orange lines, and protospacer sequences are shown in dark blue. The corresponding cut sites (red box) of the two Cas9 nickases are indicated by gray arrows. X represents the distance between the two cleavage sites. **(B)** In this study, 15 pairs of Cas9 guide RNAs were designed with distinct configurations to target the human *HPRT1* gene on chromosome X. Total editing efficiencies mediated by Cas9 variants (Cas9 D10A and H840A) were examined. The distances between cut sites within a pair and the orientation of the gRNA are indicated on the x-axis. HEK-293 cells were transfected with 5 nM of each ribonucleoprotein complex [RNP: Alt-R *S.p.* Cas9 D10A (dark blue) or H840A (light blue) Nickase 3NLS complexed with Alt-R CRISPR-Cas9 crRNA and Alt-R CRISPR-Cas9 tracrRNA] using 1.2 µL of Lipofectamine RNAiMAX transfection reagent (Thermo Fisher Scientific). Genomic DNA was isolated 48 hr after transfection. Total editing efficiency was determined using the Alt-R Genome Editing Detection Kit (T7 endonuclease I assay), n = 3.

ssODNs are the preferred donor template for nickase-mediated HDR

The ability to effectively and precisely introduce exogenous DNA fragments into a host genome is of enormous value for research that aims to understand gene function and to model disease-causing mutations. While WT Cas9-mediated gene knock-out proves to be very efficient in mammalian cells via the error-prone, non-homologous end joining (NHEJ) pathway, seamless insertion of genetic material via the cellular HDR pathway remains challenging. Similarly, Cas9 nickases have demonstrated efficacy in causing gene disruptions, but their utility in HDR-mediated sequence knock-in is less well defined.

ssODNs harboring the desired exogenous sequences are frequently used as the HDR repair template for small alterations of the target sequence. It was recently reported that a dsDNA donor with single-stranded overhangs facilitates HDR in cells transfected with WT Cas9 [**7**]. To test whether the same principle holds true in nickase experiments, we designed and generated a series of donor molecules with 3′ protrusions by annealing two ssODNs. Paired crRNAs that are specific for sites 1 and 2 in the human *HPRT1* gene were selected for this study, because this pair showed the highest editing efficiency in previous testing (51 nt PAM-out, **Figure 2B**). A novel restriction enzyme recognition site, EcoRI, was introduced into the template DNA, which allows for restriction fragment length polymorphism (RFLP) analysis to identify HDR events by a simple enzymatic cleavage assay (**Figure 3**).

A. Schematic of donor DNA templates for homology-directed repair experiments: single- vs. partially double-stranded templates



B. EcoRI digestion of PCR products to assess HDR insertion

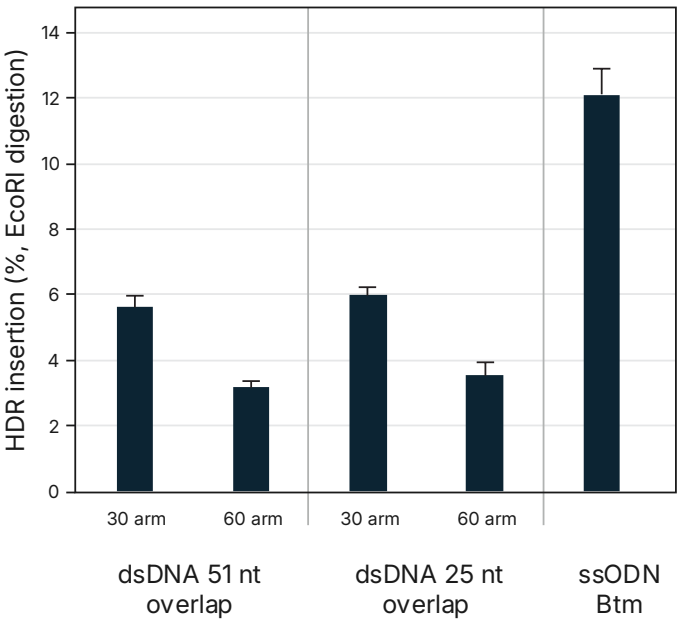


Figure 3. ssODN vs. partially overlapping dsDNA as donor for Cas9 D10A–mediated HDR. (**A**) Various donor DNA templates containing a 51 nt target region (gray) were designed and synthesized. Short dsDNA donors were prepared by annealing two synthetic ssODNs. Two different overlapping designs with either 30 or 60 nt homology arms (blue and purple) were tested in this experiment: either the 51 nt sequence between the two cleavage sites was fully complementary (dsDNA 51 nt overlap) or only the first 25 nt at the 5′ ends of the synthetic ssODNs overlapped (dsDNA 25 nt overlap). (**B**) Two Cas9 D10A RNPs (5 nM each) targeting *HPRT1* at cleavage sites 1 or 2 (51 nt apart) were co-transfected into HEK-293 cells with 3 nM of the various forms of DNA donors. Genomic DNA was isolated 48 hr after transfection. The targeted locus was amplified by PCR and subjected to restriction digestion with EcoRI to determine the percentage of HDR. Btm = Bottom strand, *n* = 3.

In this study, the length of single-stranded overhangs was either 30 or 60 nt (**Figure 3A**). A single-stranded donor (ssODN Btm) with 30 nt homology arms was included to allow a comparison between single- and double-stranded donor templates. As shown in **Figure 3B**, the use of partially overlapped dsDNA donors did not improve Cas9 D10A–mediated HDR efficiency under the conditions tested.

Cas9 D10A is more potent in mediating HDR than Cas9 H840A

Next, we optimized HDR efficiency by testing template design parameters, such as length of the homology arms, symmetry around the insertion site, strand preference, and choice of nickase variant. Using the best performing paired guides targeting *HPRT1* (51 nt PAM-out, **Figure 2B**), we compared HDR efficiencies of 20 ssODN template designs in human HEK-293 cells (**Figure 4A**). Again, an EcoRI restriction site, which functioned as a reporter for HDR efficiency, was introduced by various ssODN templates.

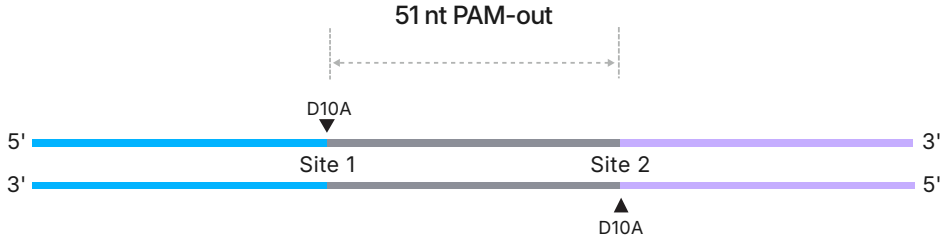
As shown in **Figure 4B**, dual nicking by Cas9 D10A engaged HDR more efficiently than Cas9 H840A activity. This is consistent with the observation described by Bothmer and colleagues, although they used a Cas9 HNH mutant (N863A mutation), which creates 3′ overhangs [**6**].

Homology arms are the donor template sequences that match the native sequence on either side of a cut site. For WT Cas9, higher HDR is often observed when the length of each homology arm of a ssODN template ranges from 30 to 60 nt. Here, we tested a series of HDR template designs whose homology arms were either 30 or 60 nt long (**Figure 4A**). Although the ideal length can be site specific, we did not see global HDR improvement with the use of longer arms, suggesting that 30 nt homology arms may be sufficient for HDR when using Cas9 nickases.

Unlike dsDNA donors, ssODN donor templates can be designed using the sequence of either strand of the template DNA (referred to as top or bottom strand in this study). Although HDR for certain designs appears to be favored when one strand is used instead of the other, we recommend testing both strands, because strand preference differs between designs and, thus, is not always predictable. We found no consistent enhancement of HDR efficiency when the ssODN template was designed asymmetrically versus symmetrically.

In addition to the distance between cut sites, the editing efficiency of individual guides in a pair also seems to affect the final HDR outcome. Therefore, we recommend that you verify the activity of each crRNA with WT Cas9 before starting an HDR experiment with a Cas9 nickase.

A. Schematic of ssODN designs for donor DNA templates for HDR experiments: varying homology arm length, target strand, and symmetry



ssODN name	Template structure	Intended edit	Length (nt)
30-E-0-30	30 nt 30 nt	EcoRI insertion + 51 nt deletion	66
60-E-0-60	60 nt 60 nt	EcoRI insertion + 51 nt deletion	126
30-E-51-30	30 nt 51 nt 30 nt	EcoRI insertion at left nick site (Site 1)	117
60-E-51-60	60 nt 51 nt 60 nt	EcoRI insertion at Site 1	177
30-51-E-30	30 nt 51 nt 30 nt	EcoRI insertion at right nick site (Site 2)	117
60-51-E-60	60 nt 51 nt 60 nt	EcoRI insertion at Site 2	177
30-E-30-0	30 nt 30 nt	EcoRI insertion at Site 1	66
60-E-51-9	60 nt 51 nt 9 nt	EcoRI insertion at Site 1	126
0-30-E-30	30 nt 30 nt	EcoRI insertion at Site 2	66
9-51-E-60	9 nt 51 nt 60 nt	EcoRI insertion at Site 2	126

B. T7EI ssay for overall genome editing and EcoRI assay to assess HDR mediated by Cas9 D10A and H840A

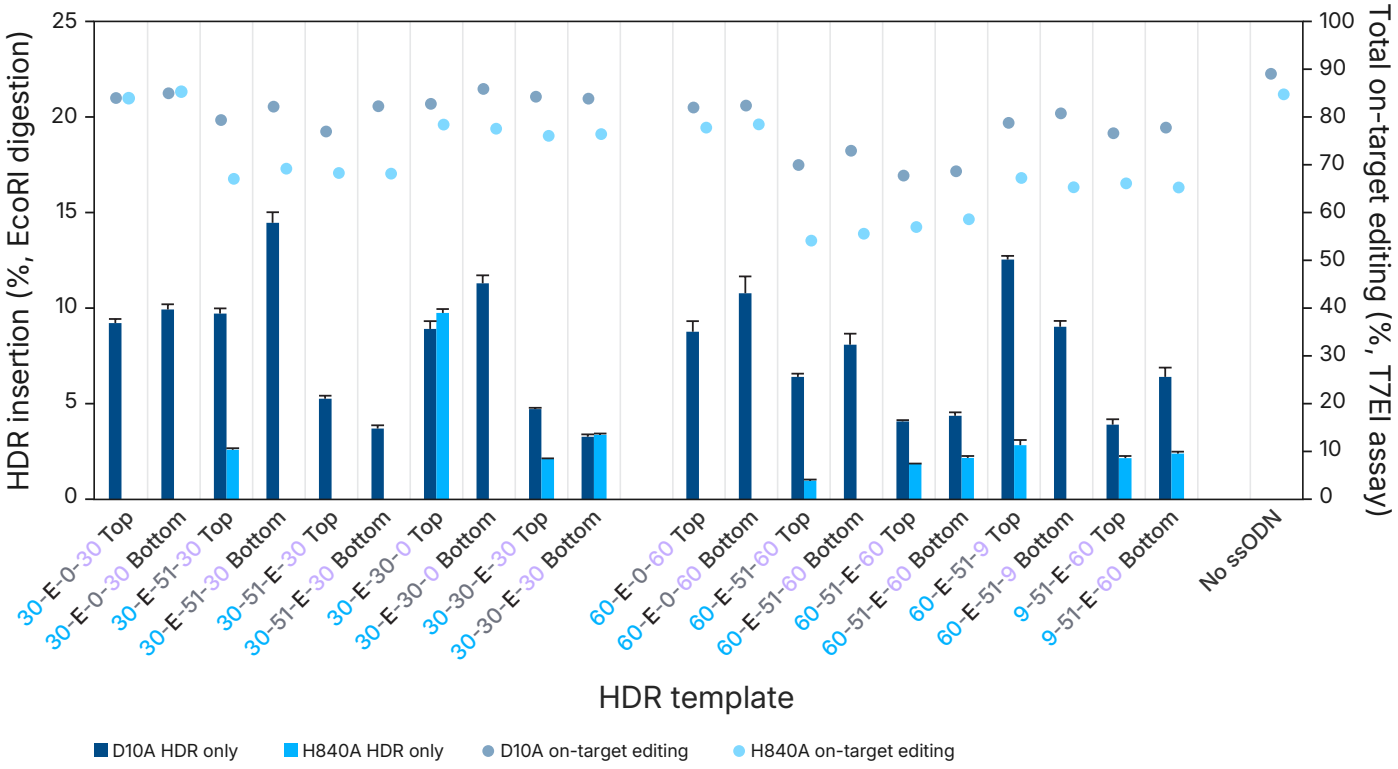


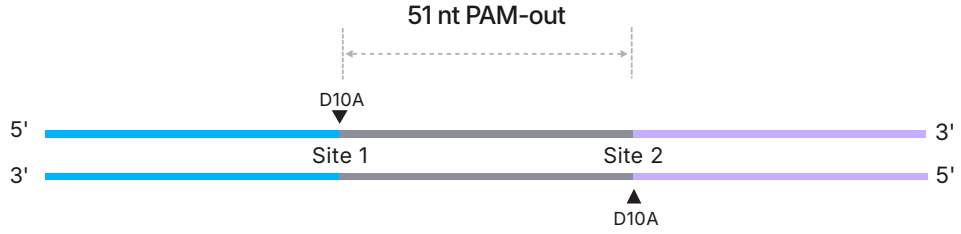
Figure 4. A systematic study of nickase-mediated HDR with a variety of ssODN template designs. (**A**) Schematic showing the structures of 20 HDR template designs (blue and purple = homology arms, gray = sequence between nick sites 1 and 2, black = EcoRI site). A 6-base EcoRI restriction enzyme recognition site was inserted using an ssODN repair template. The percentage of EcoRI digestion functioned as a proxy for HDR efficiency. For asymmetric donors (30/60-E-51-30/60 or 30/60-51-E-30/60), either 30 or 60 nt homology arms were placed on one side of the EcoRI site and 81 or 111 nt on the opposite side. Both top and bottom strands were tested. (**B**) Two Cas9 D10A RNPs (5 nM each, dark blue bars or circles) or two Cas9 H840A RNPs (5 nm each, light blue bars or circles) targeting *HPRT* at nick sites 1 and 2 were co-transfected into HEK-293 cells with 3 nM of the various forms of DNA donors (standard desalt **Ultramer™ DNA Oligonucleotides**) using 1.2 µL of Lipofectamine RNAiMAX reagent (Thermo Fisher Scientific). Genomic DNA was isolated 48 hr after transfection, and the target region was amplified by PCR. PCR products were digested with EcoRI (circles) or T7EI (bars) and analyzed via the Fragment Analyzer system (AATI) to determine the rate of EcoRI site insertion (HDR) as well as T7EI digestion (total editing), n = 3.

Case study: Using Cas9 D10A nickase to introduce a new restriction recognition site at intended genomic loci in human cells

Ideally, to accomplish high HDR efficiency, the cleavage sites introduced by CRISPR-Cas9 enzymes should be in close proximity to where mutations are introduced. It is often recommended that the insertion site of desired modifications be no more than 20 bp away from the DSB, because a few bases further up- or downstream can have a dramatic effect upon the final HDR efficiency [**8**]. However, this poses a limitation to the current CRISPR-Cas9 system for efficient genome editing as PAM sites are not necessarily available in the immediate vicinity of the intended editing sites.

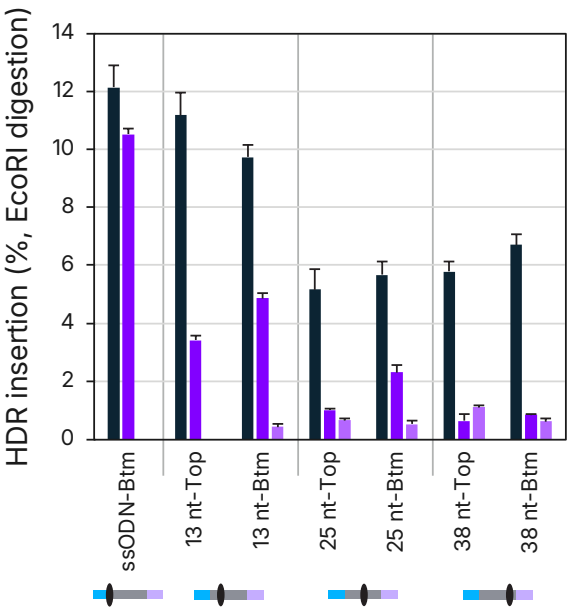
In this study, we examined how the incorporation rate of an exogenous element (EcoRI sequence) carried by the ssODN donor changes with distance to the DNA lesions (either double- or single-stranded breaks) and explored the possibility of using Cas9 D10A to insert intended sequence changes into a location distal to the flanked nicking sites. A series of ssODN donor templates were designed, in which the 6-base EcoRI sites were placed at different locations along the 51 nt sequence between cleavage sites 1 and 2 (**Figure 5A**). These templates mimic unfavorable scenarios where insertion sites are not immediately proximal to the dsDNA breaks. Overall HDR mediated by WT Cas9, as measured by the percentage of EcoRI digestion, decreased dramatically when the template insertion was ~10 bases away from where cleavage occurred. Increased HDR insertion was achieved by Cas9 D10A via either lipofection (**Figure 5B**) or electroporation (**Figure 5C**), even when the EcoRI site was intentionally positioned 13 or 25 nt from either of the cleavage sites. Notably, electroporation led to a more efficient HDR, possibly enabled by a higher transfection efficiency.

A. Schematic of ssODN designs for donor DNA templates for homology-directed repair experiments: varying position of EcoRI insertion site



Name	Structure	Sequence (5'→3')	Symbol
13 nt-Top	30 nt 13 nt 38 nt 30 nt	ACGTCAGTCTTCTCTTTTGTAATGCCCTGTAGTCTCTCTGTATGAATTCGTTATATGTCACA TTTTGTAATTAACAGCTTGCTGGTGAAAAGGACCCCACGAAGTGTTGGATATAAG	
13 nt-Btm	30 nt 13 nt 38 nt 30 nt	CTTATATCCAACACTTCGTGGGGTCCTTTTCACCAGCAAGCTGTTAATTACAAAATGTGACA TATAACGAATTCATACAGAGAGACTACAGGGCATTACAAAAGAGAAGACTGACGT	
25 nt-Top	30 nt 25 nt 25 nt 30 nt	ACGTCAGTCTTCTCTTTTGTAATGCCCTGTAGTCTCTCTGTATGTTATATGTCACGAATTC ATTTTGTAATTAACAGCTTGCTGGTGAAAAGGACCCCACGAAGTGTTGGATATAAG	
25 nt-Btm	30 nt 25 nt 25 nt 30 nt	CTTATATCCAACACTTCGTGGGGTCCTTTTCACCAGCAAGCTGTTAATTACAAAATGAATTC GTGACATATAACATACAGAGAGACTACAGGGCATTACAAAAGAGAAGACTGACGT	
38 nt-Top	30 nt 38 nt 13 nt 30 nt	ACGTCAGTCTTCTCTTTTGTAATGCCCTGTAGTCTCTCTGTATGTTATATGTCACATTTTGT AATTAAGAATTCCAGCTTGCTGGTGAAAAGGACCCCACGAAGTGTTGGATATAAG	
38 nt-Btm	30 nt 38 nt 13 nt 30 nt	CTTATATCCAACACTTCGTGGGGTCCTTTTCACCAGCAAGCTGGAATTCTTAATTACAAAAT GTGACATATAACATACAGAGAGACTACAGGGCATTACAAAAGAGAAGACTGACGT	
ssODN-Btm	30 nt 51 nt 30 nt	CTTATATCCAACACTTCGTGGGGTCCTTTTCACCAGCAAGCTGTTAATTACAAAATGTGACA TATAACATACAGAGAGACTGAATTCACAGGGCATTACAAAAGAGAAGACTGACGT	

B. Lipofection experiment



C. Electroporation experiment.

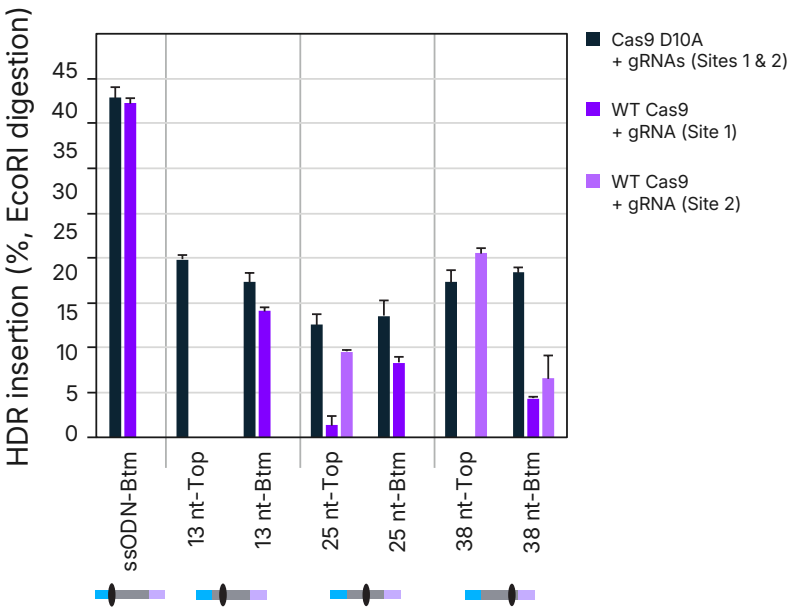


Figure 5. Cas9 D10A enables insertion of exogenous DNA fragment to sites distal from double-stranded breaks. (**A**) Single-stranded donor DNA templates, each containing an EcoRI site at a different location, were designed and synthesized. The position of the EcoRI site along the template sequence is highlighted in black. (**B**) gRNAs were used to target proximal cuts in the human *HPRT* gene at site 1 and/or site 2. WT Cas9 RNP (10 nM) or two Cas9 D10A RNPs (5 nM for each guide) were transfected into HEK-293 cells with 3 nM of a DNA donor (standard desalt Ultramer DNA Oligonucleotides) using 1.2 µL of Lipofectamine RNAiMAX reagent (Thermo Fisher Scientific). Genomic DNA was isolated 48 hr after transfection, and the target region was amplified by PCR. PCR products were subjected to digestion with EcoRI to determine the rate of EcoRI integration as a measure of HDR, *n* = 3. (**C**) WT Cas9 RNP (6 µM) or two Cas9 D10A RNPs (3 µM for each guide) were delivered into HEK-293 cells via electroporation along with 2 µM of a DNA donor and 3 µM of Alt-R Cas9 electroporation enhancer. HDR efficiency (EcoRI digestion) was examined 48 hr after delivery. gRNA = guide RNA (Alt-R crRNA annealed with Alt-R tracrRNA). RNP = ribonucleoprotein (gRNA annealed with Cas9 protein). Btm = bottom strand. Top = top strand, *n* = 3.

Conclusions

Key points before starting your own Cas9 nickase experiment

CRISPR-Cas9 genome editing technology, along with our understanding about the molecular mechanism underlying template repair, has been evolving at an unprecedented speed. This application note provides a summary of what we have learned about optimizing nickase experiments.

- Combinational use of Cas9 nickases and paired guides generates DSBs with either 5′ (Cas9 D10A) or 3′ (Cas9 H840A) overhangs.
- For both Cas9 D10A and Cas9 H840A, a higher level of genome editing is achieved when the guide pairs have a PAM-out orientation.
- Cas9 D10A is better at inducing genome editing when the two cleavage sites are 40–70 bp apart, while Cas9 H840A favors a distance of 50–70 nt.
- Cas9 D10A is more potent in mediating HDR than Cas9 H840A, despite generally comparable total editing efficiency.
- Cas9 D10A nickase may provide a noticeable advantage over WT Cas9 at mediating HDR further away from available cleavage sites.
- As best practice, the activity of each crRNA in a pair should be verified independently with WT Cas9 before being duplexed in experiments with Cas9 nickase RNPs.
- Activity is highest when RNP complexes are formed separately for each gRNA before co-delivery, rather than RNP formation as a single reaction containing both paired gRNAs.
- These design recommendations have been incorporated in the **HDR Design Tool** which greatly simplifies the guide RNA and donor template design process.

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Revision history

Version	Release date	Description of changes
4	September 2025	Updated to current branding
3	August 2023	Update to make this a stand alone application note for CRISPR-Cas9 nickase mutants
2	June 2019	Updates to full HDR application note
1	June 2018	Initital release

CRISPR-Cas9 nickase mutants promote homology-directed repair for efficient, high-fidelity genome editing

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