CRISPR-Cas9 donor DNA template optimization and 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.

Whether you are a new or experienced investigator, this guide provides you valuable strategies for achieving high-fidelity HDR of CRISPR-Cas9–based editing. Part I outlines important parameters and design considerations for optimizing single-stranded oligodeoxynucleotide (ssODN) donor templates. Part II examines technical considerations for using Cas9 nickases to generate staggered double-strand breaks (DSBs).

Part I. Optimizing donor DNA templates for CRISPR-Cas9 homology-directed repair

Here, we provide technical recommendations for optimizing donor DNA templates to insert specific sequences via HDR. Based on our data as well as those of other groups, we recommend using ssODN donor templates rather than double-stranded DNA (dsDNA) donor templates. We discuss design issues, experimental considerations, and make recommendations for optimizing donor template homology arms and sequence structure relative to the target site. Custom Ultramer or Megamer oligonucleotides can be used as donor templates, depending on the size of the intended insertion sequence.

Part II. 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 specificity. 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 performance. These enzymes can be directly substituted for prior Alt-R Cas9 enzymes in the following protocols.

Part I. Optimizing donor DNA templates for CRISPR-Cas9 homology-directed repair

Using Ultramer Oligonucleotides

Introduction

CRISPR-Cas9 genome editing is changing the landscape of genomics due to its ease of use and ability to create double-stranded breaks (DSB) at almost any locus of interest [1]. Genome stability in eukaryotic cells requires a mechanism for the efficient repair of DNA lesions. Cells rely on 2 canonical pathways to repair double-stranded DNA breaks: the non-homologous end-joining (NHEJ) pathway, which is error-prone, and the cellular homology-directed repair (HDR) pathway, which faithfully copies the genetic information from a related sequence and seals the DSB in an error-free manner.

Researchers take advantage of the different properties of the two pathways to understand gene function or model disease-causing mutations. They disrupt gene function by introducing frameshifts or other changes to the open reading frame of their target gene through NHEJ for loss-of-function studies, a process called gene "knockout", which is akin to tearing a page out of a book. Alternatively, they seamlessly insert (or "knock-in") exogenous sequences such as selectable markers, fluorescent tags, or other functional units through the endogenous HDR pathway, which some call "true" genome editing. While NHEJ based gene disruption is fairly well understood and relatively efficient as a research tool, knock-in of foreign DNA by HDR remains a challenge, particularly in mammalian cells [2–4].

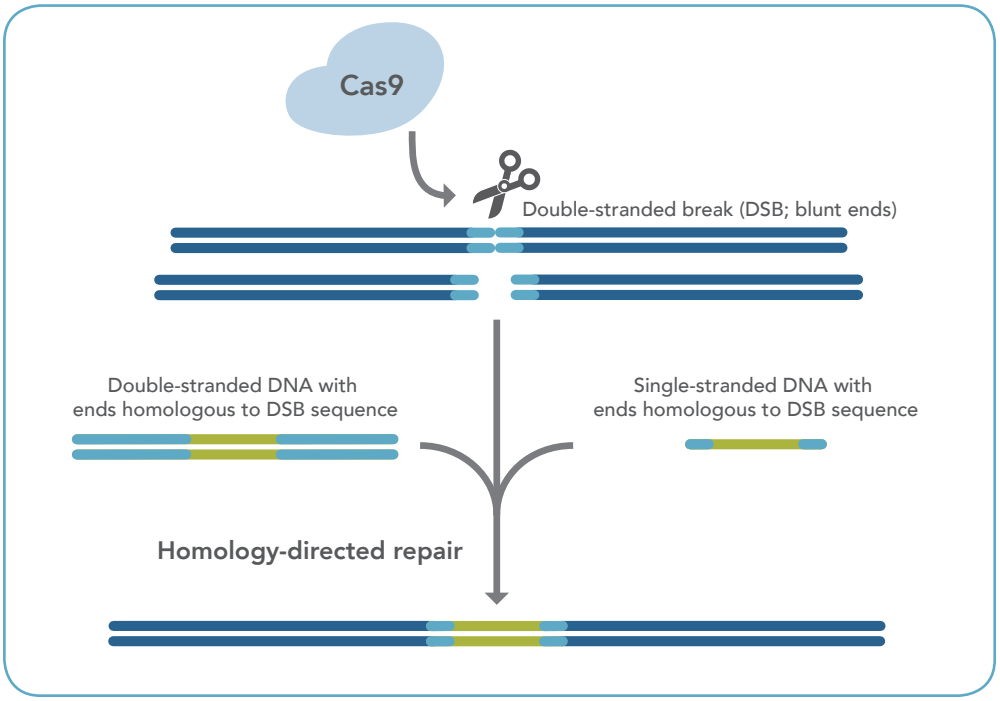


Figure 1. HDR insertion of an exogenous donor sequence at a specific genomic location. Cas9 is directed to cut at a specific target by a guide and transactivating RNA complex. The Cas9:RNA complex is delivered along with either double-stranded or single-stranded donor templates with the desired insert (green) and flanking homology arms (light blue; see sidebar for definitions), which mediate the exchange of DNA sequence information via HDR repair pathway.

To enable a homology-based recombination, the HDR repair mechanism requires that the donor DNA features stretches of homologous sequence to the region immediately located around the double-stranded breaks. This donor DNA harboring overlaps of sufficient lengths must be delivered simultaneously with the Cas9 ribonuclease (RNP) complex (formed by the Cas9 endonuclease and the triggering RNA system) as illustrated in Figure 1 [5].

In this application note, we summarize our recent findings using CRISPR-Cas9 in HDR applications, and provide stepwise guidance for maximizing HDR rate in your own genome editing experiments (Figure 2). These recommendations apply to HDR-mediated insertion of short fragments, such as epitope tags, or sequence replacement and correction of point mutations.

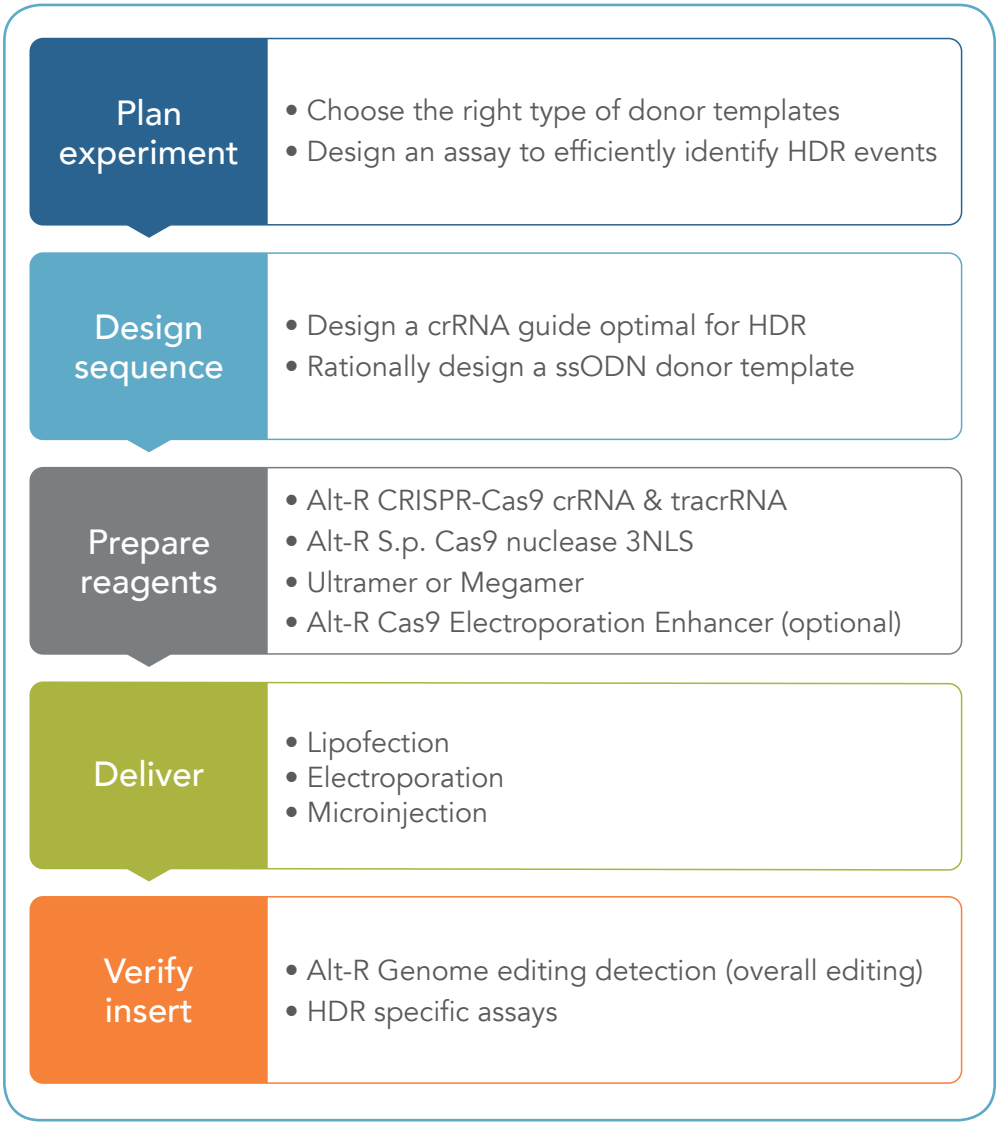


Figure 2: Experimental workflow for HDR using co-delivery of synthetic crRNA, tracrRNA, Cas9 endonuclease, and HDR donor template.

Definitions

crRNA (CRISPR RNA) and tracrRNA (transactivating CRISPR RNA): The crRNA contains both the 20-base protospacer element and additional nucleotides that are complementary to the tracrRNA. The tracrRNA hybridizes to the complementary region of the crRNA and the combined crRNA and tracrRNA interact with the Cas9 endonuclease, activating the editing complex to create double-stranded breaks at specific sites within target genomes. These 2 native RNA molecules can be synthetically generated for use in genome editing experiments. IDT scientists have modified these RNAs in length and composition to optimize genome editing efficacy, especially when pre-complexed with a CRISPR nuclease and delivered to cells as an RNP.

HDR (homology directed repair): A cellular mechanism for repair of double-stranded breaks in genomic DNA involving homologous recombination of a donor DNA sequence into the genome. Scientists have taken advantage of this cellular mechanism to insert desired sequences into specific genomic locations after a DSB is generated by CRISPR-Cas9 cleavage.

HDR donor template: A single- or double-stranded DNA sequence containing a desired insert sequence flanked by homology arms that are complementary to the adjacent sequence of a planned break in genomic DNA (Figure1). A donor template is included in CRISPR-Cas9 HDR experiments, allowing scientists to create desired point mutations, incorporate tags, or add other functional units into a specific genomic location.

NHEJ (non-homologous end joining): An evolutionarily conserved cellular pathway that repairs double-stranded breaks in genomic DNA. It is the most likely mechanism of repair for CRISPR-Cas9 mediated genome editing. As opposed to HDR, NHEJ occurs without the need for an additional homologous sequence to guide repair, and relies on microhomologies present in single-stranded overhangs for the re-ligation of two ends. NHEJ is a homology-independent, error prone repair pathway and thus is not favored when precise introduction of genetic material is desired.

RNP (ribonucleoprotein): A molecular complex comprising both RNA and protein elements. In CRISPR-Cas9 genome editing, the RNP is composed of hybridized crRNA, tracrRNA, and the Cas9 endonuclease. Delivery of these CRISPR components in the form of RNP provides an optimal genome editing efficiency and reduces unwanted off-target cutting [6].

PAM (protospacer adjacent motif): The sequence feature recognized by a specific CRISPR endonuclease, once hybridized with a crRNA. For Cas9 endonuclease, PAM=NGG.

Targeting strand: The genomic DNA strand complementary to the crRNA, protospacer element.

Non-targeting strand: The genomic DNA strand containing the PAM site, which is complementary to the targeting strand.

Homology arm: The sequence within the donor template that matches either side of the genomic cut site. The homology arms required on dsDNA donors are significantly longer (500–1000 bp) than the ones on ssDNA donors (30–60 nt).

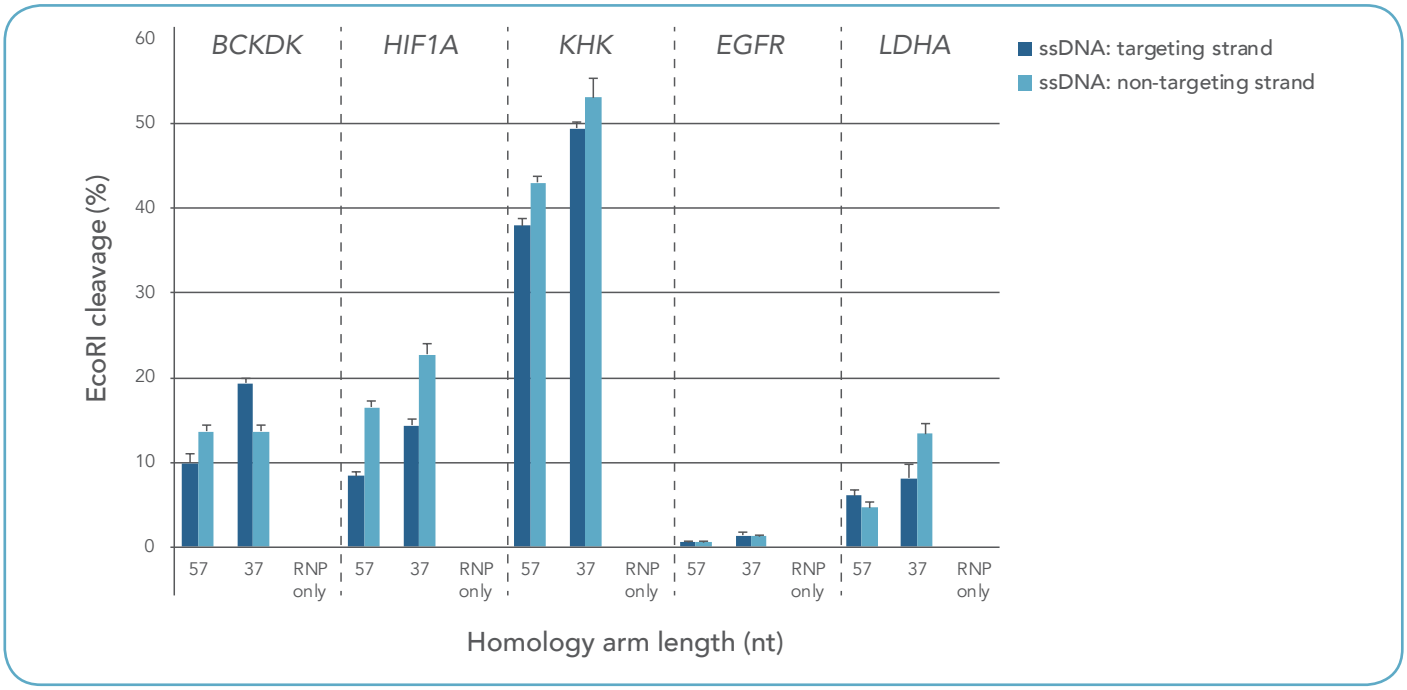
ssODN: single-stranded oligo deoxynucleotides.

crRNA guide selection for HDR optimization

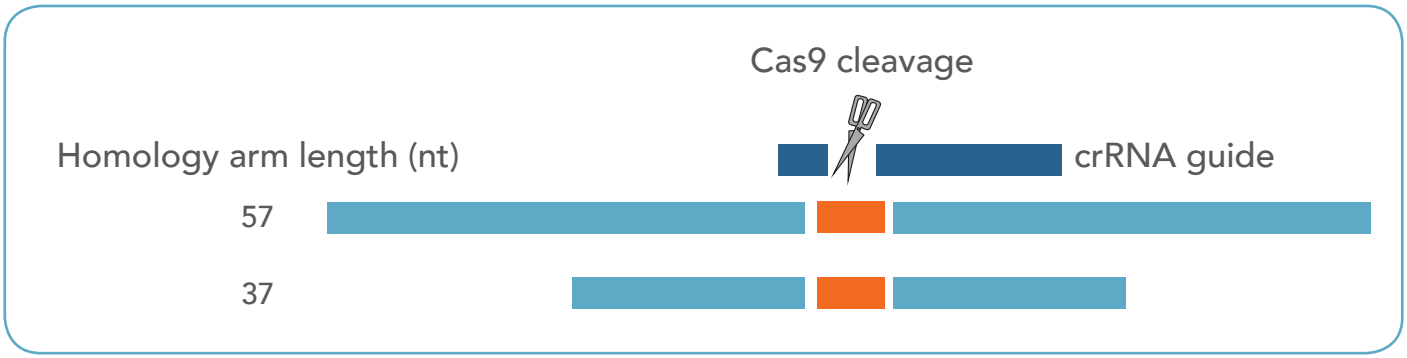
The efficiency of HDR insertion varies with the PAM site location, independent of the efficiency of Cas9 cleavage [6]. For initial screening, we recommend testing at least 2–4 CRISPR-Cas9 PAM sites close to where the sequence modification is desired. Figure 3 shows the HDR integration rate at 5 different genomic locations of a short fragment containing recognition site for EcoRI restriction enzyme. Introduction of a novel restriction site during HDR creates a restriction fragment length polymorphism (RFLP), which allows for the subsequent interrogation of the HDR event by enzymatic cleavage of that restriction endonuclease. The HDR efficiency, as measured by the percentage of EcoRI digestion, is clearly site-specific even though all of the sites tested here generated DSB efficiently. While the crRNA efficacy at available PAM sites might not always be optimal, the ultimate HDR efficiency can potentially be improved by a rational design of the HDR template, which we will discuss in detail in the following sections.

It is also important to consider the position of each guide sequence relative to the desired change. Ideally, the cleavage site generated by the Cas9 enzyme should be in close proximity to where mutations are to be introduced [7]. The HDR rate decreases dramatically when the template insertion is just 5–10 bases away from the cut site (Figure 4). However, not every insertion site will have PAM sequences available in the immediate vicinity. In these situations, choose the most active crRNA that is as close as possible to the intended insertion site for the best chance of efficient HDR.

The HDR rate can also differ greatly among cell types. In our internal testing, we have noticed lower HDR efficiencies in transformed cells compared to immortalized cell lines derived from the same species (data not shown). In addition, while HDR in general tends to be less efficient in cancer cells lines, HDR potential in primary cells may be highly variable. All the data described herein is derived from studies on HEK-293, an immortal cell line that is competent at CRISPR/Cas9-mediated HDR.



A. Variation of HDR efficiency with different donor template designs.



B. HDR template design and Cas9 cleavage site.

Figure 3: HDR efficiency varies with the choice of PAM sites. (**A**) HEK-293 cells were transfected with 10 nM RNP (Alt-R S.p. Cas9 Nuclease 3NLS complexed with Alt-R CRISPR-Cas9 crRNA and tracrRNA) and 3 nM HDR template (standard desalted Ultramer oligonucleotide) using 1.2 µL Lipofectamine® RNAiMAX Transfection Reagent (Thermo Fisher). Genomic DNA was isolated 48 hr after lipofection followed by PCR amplification of the target regions. PCR products were digested with EcoRI and analyzed using a Fragment Analyzer™ (Advanced Analytical) to determine rate of EcoRI site insertion as a proxy for HDR efficiency. (**B**) 3′ and 5′ homology arms of either 37 nt or 57 nt were placed flanking the EcoRI site for interrogating the effect of homology arm length on HDR. The horizontal orange bar represents the EcoRI cleavage site on the HDR template.

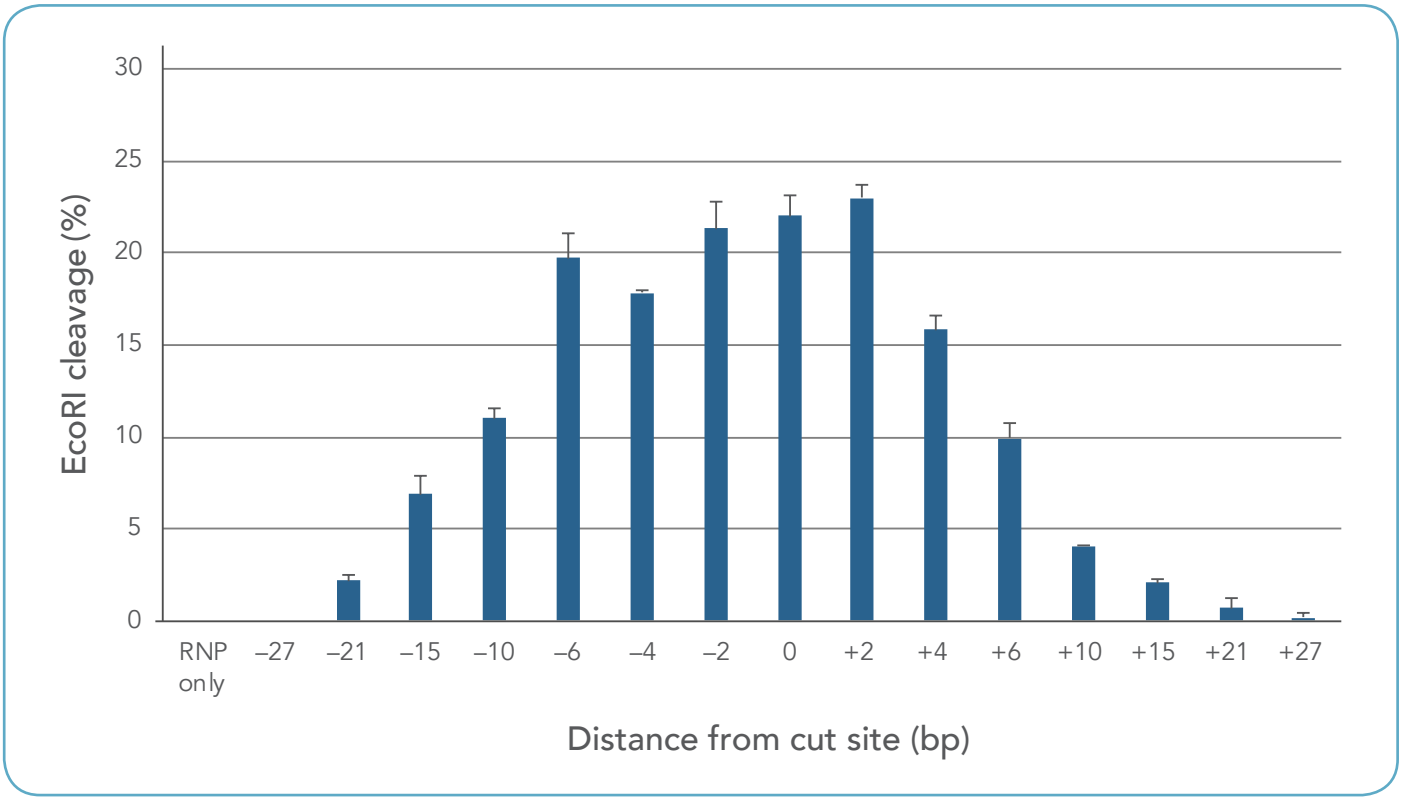


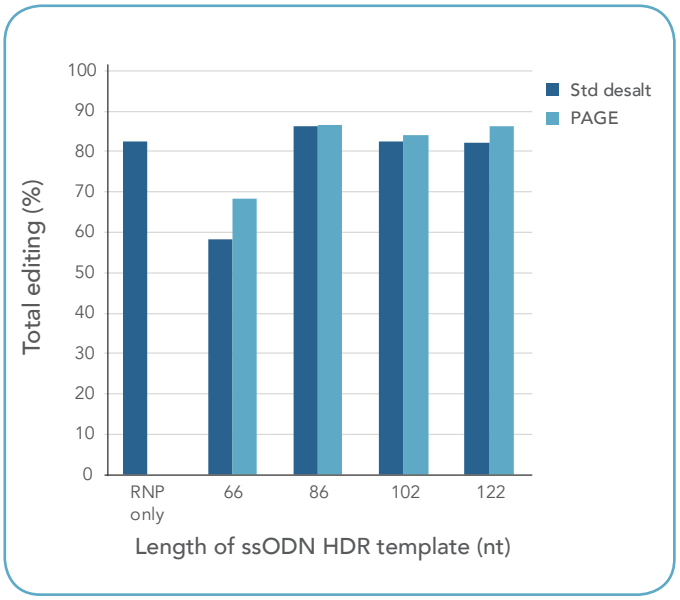
Figure 4: HDR rate decreases significantly at positions >10 bases away from CRISPR-Cas9 cut site. Alt-R CRISPR-Cas9 RNP targeting the human gene *SERPINC1* was delivered into HEK-293 cells at a concentration of 4 µM via electroporation, together with a series of ssODN donor templates that gradually increase the distance in both directions between the cut site and the EcoRI insertion site. All ssODN donor inserts tested were designed using the sequence of the non-targeting strand, flanked by 40 nt homology arms. 2 µM Alt-R Cas9 Electroporation Enhancer was included in the reaction for higher editing efficiency. Genomic DNA was isolated 48 hr after electroporation and the rate of HDR insertion was assessed by EcoRI cleavage.

DNA templates: dsDNA vs. ssDNA template

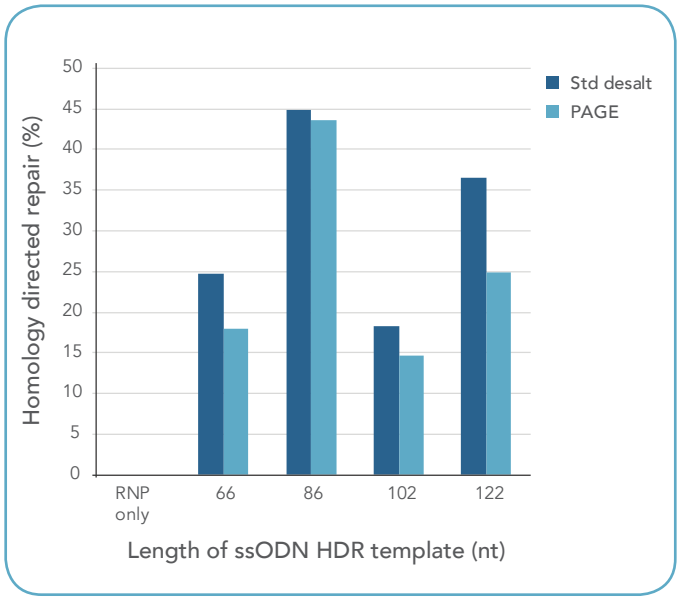
In the past, dsDNA templates have been used extensively for HDR donor sequences. However, dsDNA donors are more readily incorporated by the dominant NHEJ process. This blunt end incorporation of the donor sequence can result in duplication of the homology arms or partial incorporation of the dsDNA template. Furthermore, dsDNA templates can be deleterious to cultured cells. Delivery of linear or plasmid dsDNA often results in poor lipofection efficiency, and/or cytotoxicity.

Single-stranded oligo deoxynucleotides (ssODN) harboring the desired exogenous sequences have recently become more common in HDR applications, in part because the homology arms do not need to be as long as with dsDNA donor templates, yet they can show higher insertion efficiency [8, 9]. As a rule of thumb, for sequence replacements or small insertions shorter than 120 bp (e.g., addition of stop codons, protein functional sites, antibody reactive epitopes, and detectable tags), we recommend using ssODN templates such as Ultramer Oligonucleotides. For longer insertions, Megamer Single-Stranded DNA fragments, which are up to 2000 bases in length, are available.

Custom Ultramer Oligonucleotides provide high-fidelity templates up to 200 nt that are well suited for HDR. Standard desalted Ultramer Oligonucleotides can be used for introduction of point mutations or insertions up to 120 nt without the need for further purification. As shown in Figure 5, a series of ssODN donor templates with various lengths were designed to insert a novel restriction site and purified by either standard desalt or PAGE (polyacrylamide gel purification). Compared to standard desalted ssODN templates, PAGE purification did not improve the total editing efficiency (Fig 5A) or the HDR rate (Fig 5B), as determined by amplicon sequencing.



A. Totaling editing efficiency—standard desalt- vs. PAGE-purified ssODN template.



B. HDR efficiency— standard desalt- vs PAGE-purified ssODN template.

Figure 5: Standard desalted Ultramer Oligonucleotides are ideal ssODN templates for introducing point mutations or short insertions. 10 nM RNP (Alt-R S.p. Cas9 Nuclease 3NLS complexed with Alt-R CRISPR-Cas9 crRNA and tracrRNA) were transfected into HEK-293 cells using 1.2 µL CRISPRMAX™ (Thermo Fisher Scientific), together with 3 nM Ultramer Oligonucleotide templates purified by either standard desalt or PAGE methods. Note that 66-/102-nt ssODN donors contain symmetrical homology arms of 30 nt long while the homology arms on 86-/122-nt donors are 40 nt at length. To assess editing efficiency, genomic DNA samples were harvested 48 hr after transfection, followed by a PCR amplification of the target locus. The PCR products were subsequently analyzed using a targeted NGS approach. Amplicons were run on a MiSeq® System (Illumina) and sequencing data were analyzed using a proprietary processing program developed in-house to allow a concurrent quantification of (**A**) total editing events and (**B**) the percentage of perfect sequence incorporation.

IDT also offers Megamer Single-Stranded DNA Fragments, which are single-stranded DNA from 201 to 2000 bases long. Megamer DNA Fragments are synthesized using clonally purified DNA, which offers the greatest fidelity available, and they are sequence-verified via NGS prior to shipment. Megamer DNA Fragments have demonstrated superior performance in animal genome engineering [6, 10]. The availability of these custom, synthetic, long ssDNAs greatly increases the versatility of long fragment incorporation.

Rational design of a ssODN donor template

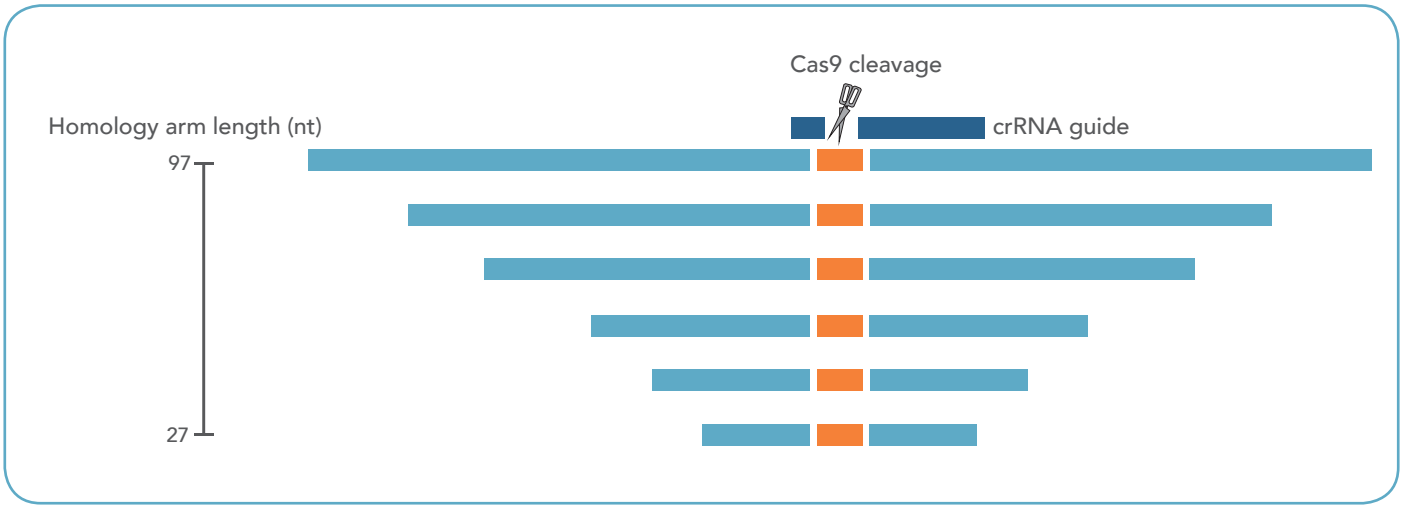
Unlike dsDNA donors, template sequences for ssDNA HDR donors can be designed to bind to either the targeting or non-targeting strand. As shown in Figure 3, HDR at certain sites appears to be more efficient when one strand is used over the other. Although efficiency varies between sites and is not always predictable, our research suggests that use of an ssDNA donor sequence with homology arms identical to the non-targeting strand (i.e., the strand containing the PAM sequence) gives higher HDR efficiency in many cases. For a comprehensive study, we still recommend targeting both strands when possible. However, the success rate may be improved by designing the ssODN donor oligo identical to the non-targeting strand when the number of templates tested for each PAM site is limited. Note that most of our testing involved small insertions. Large insertions are currently being studied at IDT and may behave differently.

Homology arms are the donor template sequence elements that match either side of a cut site, which must be included for HDR. Homology arm length can be site-specific, but we tested a variety of arm lengths to identify an optimal size range. As shown in Figure 6, we designed 6 HDR templates with the same 6-base EcoRI restriction site centered on the Cas9 cleavage site, with symmetrical homology arms varying from 27 to 92 bases. In agreement with previous studies [11], robust HDR was consistently observed when the homology arms were 30–60 nt long. While the rate of dsDNA insertion appeared to be high at most of the tested sites, closer scrutiny of the fragment sizes indicated that dsDNA templates were integrated by both NHEJ and HDR, with the former occurring by blunt end incorporation. The involvement of NHEJ pathway often yields duplication of the homology arms, which was not observed when ssODN was used as the donor template (data not shown). We did not observe a consistent improvement of HDR efficiency with the use of ssODN template designed asymmetrically versus symmetrically, regardless of whether the additional length of homology was introduced on the PAM-distal or -proximal side (data not shown).

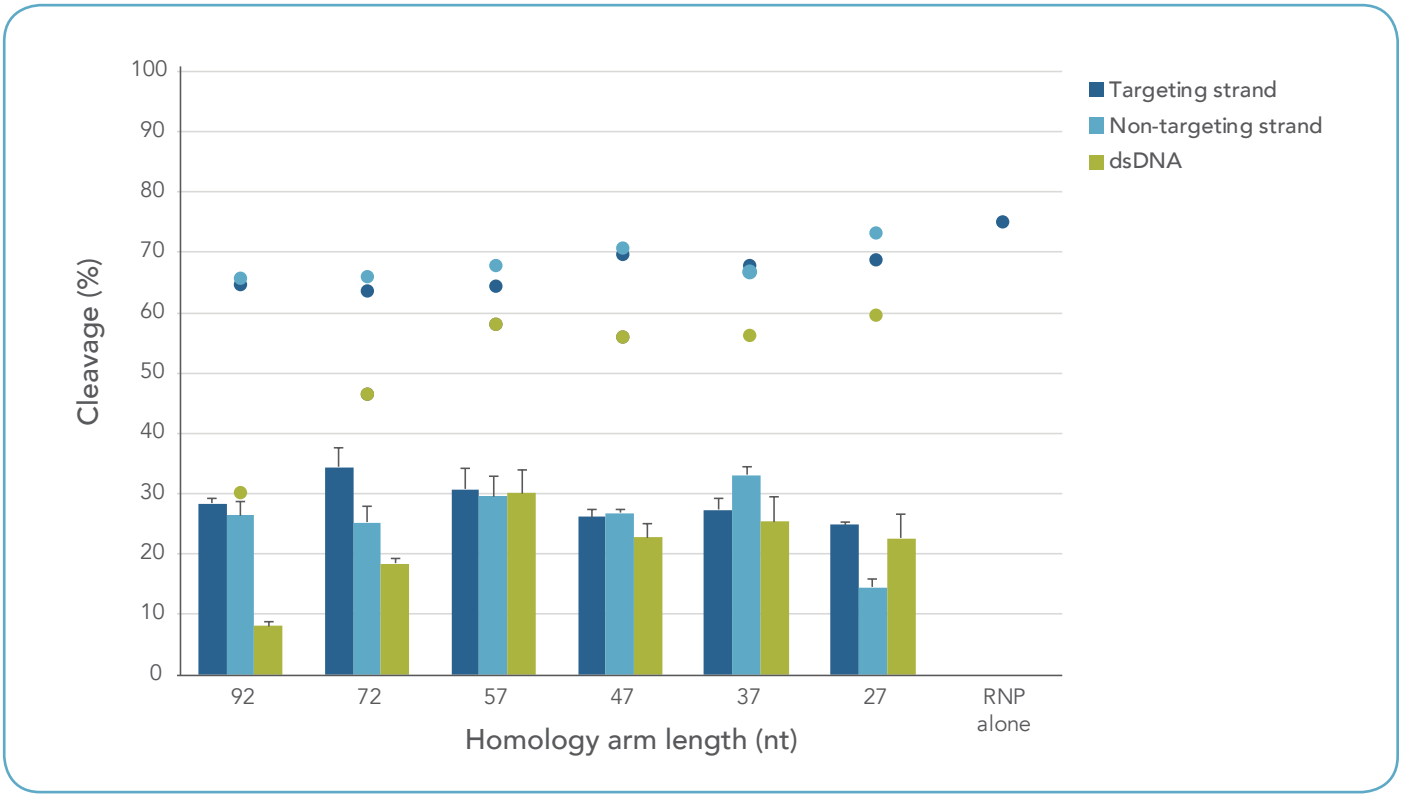
It has also been reported that the addition of phosphorothioate bonds (PS) on each end of a ssODN template increases HDR efficiency over their unmodified counterparts [12]. These modifications may be beneficial in some circumstances. However, at least in HEK-293 cells, we have typically found no advantage to add PS linkages at the termini of a donor template to protect against nuclease digestion.

The introduction of silent mutations into the protospacer sequence or mutating the PAM site itself when an intact CRISPR recognition site is present on the HDR template will help prevent the HDR template from reconstituting the crRNA recognition site near, and avoid unwanted re-cutting at the same locus after the repair.

A double-cut strategy can also be used, which is based on a hypothesized synergistic effect of two Cas9 cutting events [13]. The occurrence of a second Cas9-mediated cleavage directed to a proximal location may result in an open chromatin conformation favored for genome editing and thereby improve the enzyme accessibility of certain PAM sites. This could be potentially beneficial in some biological systems, or for genomic regions that are difficult to access, but it also adds another layer of experiment complexity. Currently, we have limited experience in applying this strategy in HDR experiments.



A. HDR template designs with various homology arm lengths.

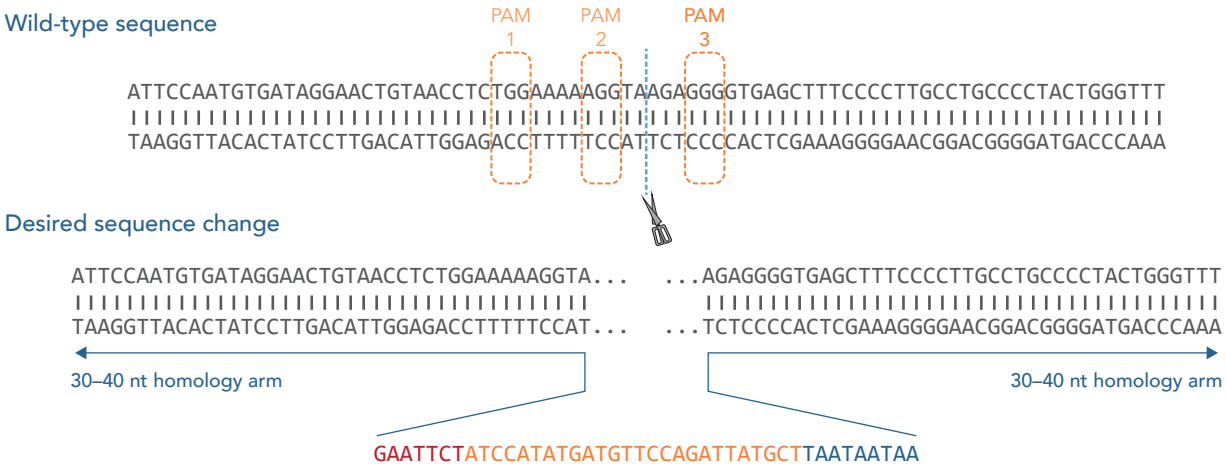


B. Assessment of HDR mediated by various HDR template designs.

Figure 6: The efficiency of HDR mediated by ssODN template is optimal for homology arms 30–60 nt long. (**A**) 6 HDR template designs with the same 6-base, EcoRI restriction site insert, and including symmetrical homology arms identical to either strand of the genome ranging from 27 to 92 bases long, were synthesized as dsDNA or ssDNA. HEK-293 cells were transfected via lipofection with 10 nM Alt-R CRISPR-Cas9 RNP targeting human *EMX1* gene and 3 nM single- or double-stranded HDR template. Genomic DNA was isolated 48 hr post lipofection, and PCR amplified with primers designed outside of the flanking arms of the HDR oligo sequence. The horizontal orange bars represent the EcoRI recognition site along the template sequence. (**B**) PCR products were digested with T7EI to determine total editing (dots), or subject to EcoRI digestion to determine the rate of HDR insertion (bars).

Case study: Design HDR repair templates to add a HA tag plus 3 consecutive stop codons into human gene *SERPINC1* in HEK-293 cells

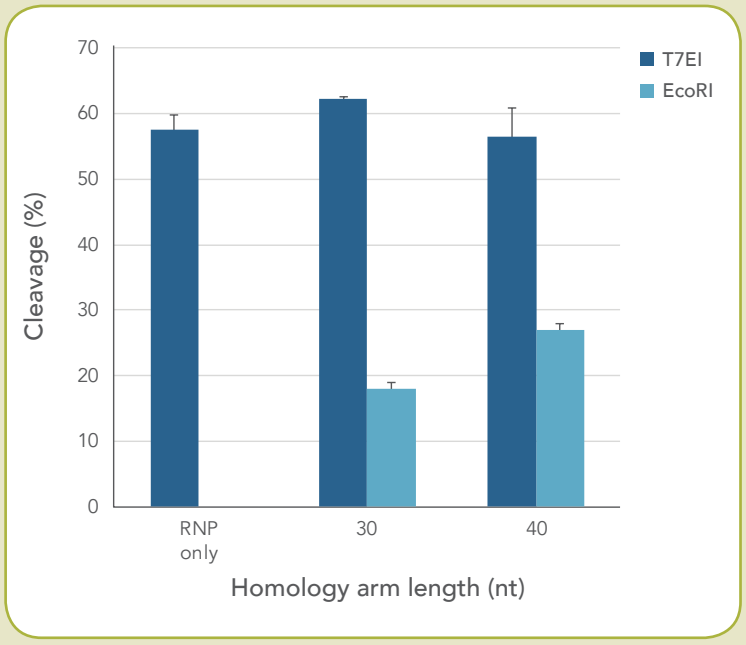
For this experiment, we planned to introduce a HA tag followed by 3 consecutive TAA stop codons at a specific location in the human *SERPINC1* gene. As shown in Figure 7A, 3 CRISPR-Cas9 PAM sites (PAM1–3, circled in red) juxtaposed with the insertion site are available and the PAM 3 site was selected in this study. The desired 42-nt insertion is positioned symmetrically in the center of the two ssODN HDR templates, with wild-type sequence extending 30 nt or 40 nt on each side of the cut site to create the homology arms (Figure 7B). Figure 7C shows the total editing efficiencies as well as the rates of HDR insertion when the two ssODNs were used. While Figure 7A shows the donor template sequences are identical to the non-targeting strand, HDR templates can also be designed to target both strands.



A. Target site in human *SERPINC1* and the corresponding donor template design.

Name	Nucleotide sequence (5' → 3')
20-nt protospacer	ACCTCTGGAAAAAGGTAAGA
ssODN donor template (30-nt arms)	GATAGGAACTGTAACCTCTGGAAAAAGGTAGAATTCTATCCATATGATGTTCCAGATTATGCTTAATAATAAAGAGGA GTGAGCTTTCCCCTTGCCTGCCCC
ssODN donor template (40-nt arms)	ATTCCAATGTGATAGGAACTGTAACCTCTGGAAAAAGGTAGAATTCTATCCATATGATGTTCCAGATTATGCTTAATAATAAAGAGGA GTGAGCTTTCCCCTTGCCTGCCCCTACTGGGTTT

B. Nucleotide sequences of the guide RNA and ssODN donors used in this study.



C. Effect of homology arm length.

Figure 7: HDR template design for introduction of a sequence tag. (**A**) Functional components of the insert are color-coded. The 6-base EcoRI recognition site (red), which was subsequently used for insertion detection, is shown, followed by the 27 nt HA tag (orange) and 3X TAA stop codons (blue). A silent G→A mutation (bold in green) was introduced to the PAM site to block further Cas9 targeting after the initial DSB is repaired. (**B**) 10 nM RNP (Alt-R S.p. Cas9 Nuclease 3NLS complexed with Alt-R CRISPR-Cas9 crRNA and tracrRNA) together with 3 nM Ultramer Oligonucleotide templates purified by standard desalt were transfected into HEK-293 cells using 1.2 µL CRISPRMAX Cas9 Transfection Reagent (Thermo Fisher). Genomic DNA samples were harvested 48 hr after transfection, followed by a PCR amplification of the target locus. PCR products were digested with T7EI to determine total editing (dark blue bar), or subject to EcoRI digestion to determine rates of HDR insertion (light blue bars).

Tips for HDR donor template design

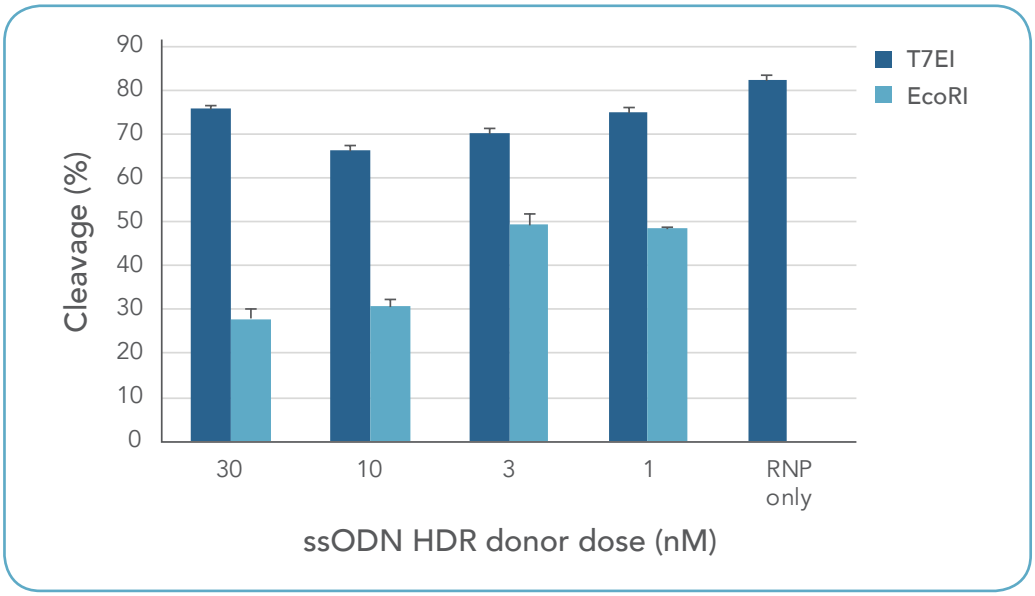
- Single-stranded HDR donor templates are better. When IDT Ultramer Oligonucleotides are used as single-stranded DNA donor templates, standard desalt is recommended. Incurring the extra cost for additional purification does not improve HDR experimental results.
- The cleavage site should be as close to the desired change as possible. HDR efficiency drops significantly once the cut site is more than 10 bases away from the desired mutation.
- Keep the desired mutations/insertions roughly centered in the donor template, flanked by 30–60 nt homology arms at both sides. This range delivers high levels of HDR for most small insertions.
- Design ssODN, HDR templates complementary to both strands where possible. The use of donor template identical to the non-targeting strand results in a higher HDR rate in some cases, but not always.
- It is important to assess whether the newly repaired sequence could still be recognized by the crRNA guide. To prevent the undesired re-cleavage of a repaired HDR locus, design the HDR donor template with one or more silent mutations within the protospacer sequence and/or PAM site to mitigate this risk.

Delivery

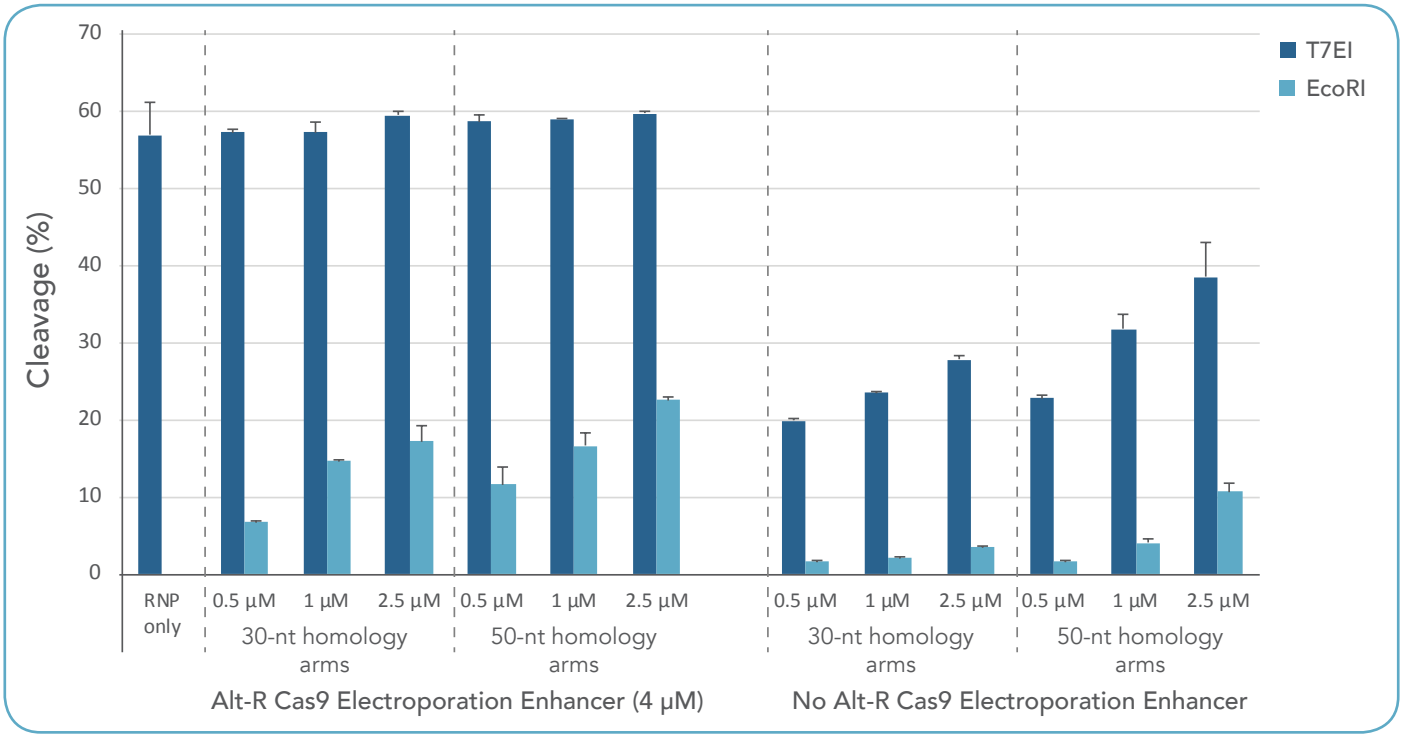
Extensive efforts have been devoted to optimizing delivery of CRISPR components to cultured cells. To accomplish the most efficient HDR, we recommend delivering an RNP consisting of Alt-R S.p. Cas9 Nuclease 3NLS complexed with Alt-R CRISPR-Cas9 crRNA and tracrRNA, concurrent with the ssODN donor template. This combination provides robust genome editing across most target sites and addresses issues that can arise with the use of other CRISPR-Cas9 editing methods, including a higher incidence of undesired off-target effects when Cas9 and the guide RNA are expressed from plasmid or viral templates. Figure 8A shows an example of successful HDR using the RNP format delivered by lipofection.

Lipofection is commonly used for established cell lines. For primary cells, non-dividing cells, and difficult-to-transfect cells, an alternative delivery approach, such as electroporation is often required. We have introduced optimized protocols for Amaxa nucleofection delivery of CRISPR reagents in HEK-293 cells, and Neon electroporation delivery of CRISPR reagents in Jurkat T cells. These protocols can also be utilized as the basis for optimization in your own HDR experiments. As shown in Figure 8B, robust HDR can also be achieved by optimized electroporation. Note that HDR efficiency increased significantly in the presence of Alt-R Cas9 Electroporation Enhancer, a purified carrier DNA that is specifically designed to improve delivery of Cas9 RNP in electroporation applications. In biological systems other than mammalian tissue culture, microinjection is a well-established approach to deliver genome-editing components into zygotes of major model organisms, such as mouse, *C. elegans*, and zebrafish.

Additional CRISPR-Cas9 RNP delivery protocols, developed at IDT or through external collaborations, are also available.



A. Lipofection experiment.



B. Electroporation experiment.

Figure 8: Robust HDR in tissue culture using lipofection or electroporation. (**A**) HEK-293 cells were transfected with 10 nM Alt-R CRISPR-Cas9 RNP using 1.2 µL Lipofectamine RNAiMAX Transfection Reagent (Thermo Fisher) along with varying doses of ssODN donor template, to target the human gene *EMX1*. The ssODN template contained a 6-base EcoRI restriction site flanked by 72-base, symmetrical homology arms. Genomic DNA was isolated 48 hr after lipofection followed by PCR amplification of the target locus. PCR products were digested with T7EI to determine total editing efficiency (dark blue bars) or subjected to EcoRI digestion to determine the rate of HDR insertion (light blue bars). (**B**) Alt-R CRISPR-Cas9 RNP, together with varying amounts of ssODN to target the human gene HPRT1, was delivered into HEK-293 cells at a concentration of 4 µM via electroporation using the Amaxa® Nucleofector® system (Lonza). Electroporation reactions contained either 4 µM Alt-R Cas9 Electroporation Enhancer, or no electroporation enhancer. ssODN templates with homology arms lengths of 30 or 50 nt were tested. Genomic DNA was isolated 48 hr after delivery. Total editing efficiency and HDR rate was assessed by T7EI and EcoRI digestion, respectively.

Guidelines for HDR template delivery

Lipofection

When performing lipofection, prepare the RNP and then add the single-stranded HDR template at a concentration of 1–3 nM before combining with the lipofection reagent for addition to cultured cells. Refer to the Alt-R CRISPR-Cas9 User Guide—ribonucleoprotein transfections for the recommended lipofection experimental set up and protocol.

Electroporation

Guidelines for electroporating CRISPR reagents depend on the electroporation instrument. IDT scientists have published optimized protocols for use with both the Amaxa Nucleofector System (Lonza) and the Neon® Transfection System (Thermo Fisher). The RNP complex should be formed before addition of the donor oligo.

For HDR mediated by the CRISPR-Cas9 system, add single-stranded HDR template to a final concentration of 2–4 µM along with the RNP and Alt-R Cas9 Electroporation Enhancer into the solution to be electroporated. Refer to the Alt-R CRISPR-Cas9 User Guide-ribonucleoprotein electroporation, Amaxa Nucleofector system or Alt-R CRISPR-Cas9 User Guide-ribonucleoprotein electroporation, Neon Transfection system for the recommended experimental set up and protocol.

Microinjection

Microinjection of murine zygotes with Alt-R CRISPR RNP complexes demonstrate higher HDR rates compared to other strategies, such as codelivery of sgRNA and Cas9 mRNA, possibly due to the immediate availability of functional RNP complexes following microinjection. IDT scientists have collaborated with researchers from multiple institutions to develop a mouse genome engineering strategy called Easi-CRISPR, which employs the use of long ssDNAs (Megamer Single-Stranded DNA Fragments) as repair templates, in combination with other Alt-R CRISPR components, for creating knock-in rodent models [10]. Successful HDR was achieved with the use of 5–20 ng/µL of the ssDNA template. Refer to the Mouse zygote microinjection protocol for the recommended experimental set up

Verification of insert

CRISPR genome editing takes place within 48–72 hr in the cell lines we have tested. Typically, we collect genomic DNA 48 hr after the delivery of CRISPR-Cas9 reagents and assess the overall editing efficiency of both NHEJ and HDR pathways using a mismatch detection analysis, such as the Alt-R Genome Editing Detection Kit. Total editing efficiency can be used as a preliminary assessment for overall levels of DSB generation. For the quantification of HDR events, one of the following methods can be employed:

1. **Design a PCR assay to target HDR template-specific sequence**
   Primers should be carefully designed to prevent unwanted amplification of donor DNA, which potentially leads to false positive results. For instance, one of the paired primers can be positioned outside of the flanking arm of donor DNA while the other primer specifically targets the repaired sequence.

2. **Incorporate a novel restriction enzyme recognition site**
   Insertion of a restriction enzyme recognition sequence facilitates the subsequent detection of successful HDR using a PCR-based approach, such as restriction fragment length polymorphism analysis. This is also the detection approach that we used repeatedly throughout this study.

3. **Add a tag sequence to enable downstream functional detection, such as flow cytometry**

4. **Design a PCR assay to amplify the edited region followed by sequencing analysis**

Next-generation sequencing offers rapid and cost-effective approaches for measuring desired changes to on-target loci, as well as identifying genome-wide, off-target cleavage events that result in mutagenic repair. Ideally, to verify the absence of off-target sites, cells that undergo genome engineering should be fully characterized by NGS methods.

Conclusion

CRISPR-mediated genome editing is arguably the most disruptive technology in the field of biology in the past decade. The technology, along with our understanding of associated cellular activities, has evolved at an unprecedented pace. This application note provides a summary of what we have learned for the optimization of HDR experiments. We, and the rest of the scientific community, still have a lot more to learn about HDR, particularly for the long fragment insertion or replacement. We will continue to update this application note by incorporating new knowledge as we make progress in our CRISPR research and development efforts.

Summary:

- Optimizing HDR experimental conditions is critical because of the generally lower baseline rate of HDR when compared to the error-prone NHEJ pathway.
- In comparison to conventional dsDNA templates which often show lower efficiency, blunt insertion, and toxicity, high fidelity ssODNs, such as Ultramer Oligonucleotides and Megamer Single-Stranded DNA Fragments, are preferred for HDR experiments.
- Because HDR efficiency differs from one site to the next, multiple PAM sites should be tested whenever possible. Select the most active crRNA design that cuts as close as possible to the intended insertion site.
- ssODN templates should be rationally designed to achieve an ideal HDR efficiency.
- Delivery may require extensive optimization in certain biological systems. Electroporation done in conjunction with the use of Alt-R Cas9 Electroporation Enhancer serves as an alternative delivery approach in tissue culture for cells that are not amenable to lipofection.
- Consider what strategy to use for insertion verification as early as possible, ideally this would mean starting from the experimental planning phase.

Contributors

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Part II. 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, 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).

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. Specifically, 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.

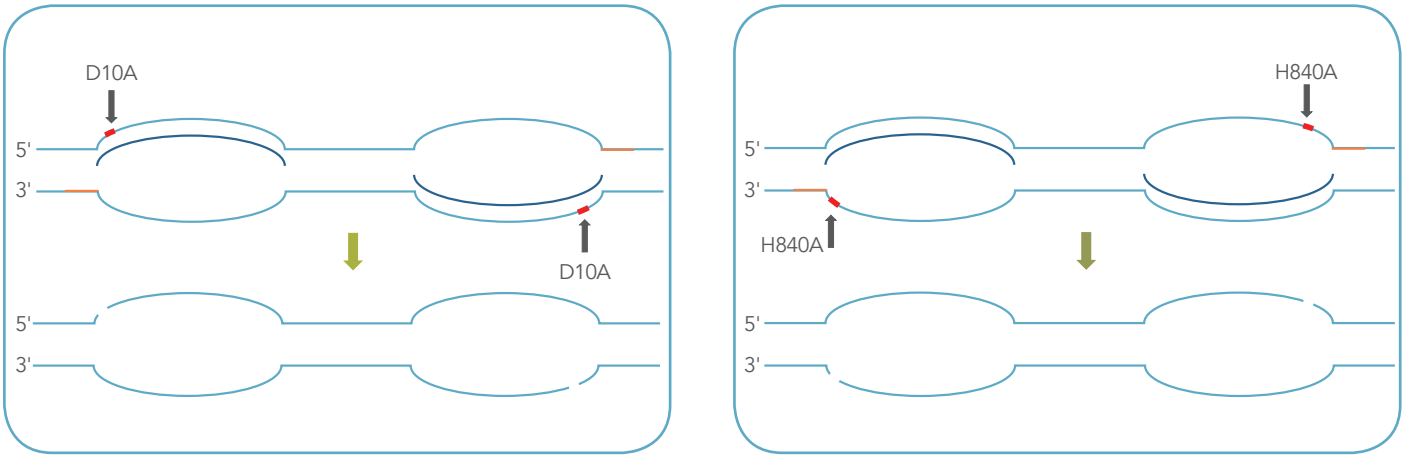
More specifically, this article will show the following:

- 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].



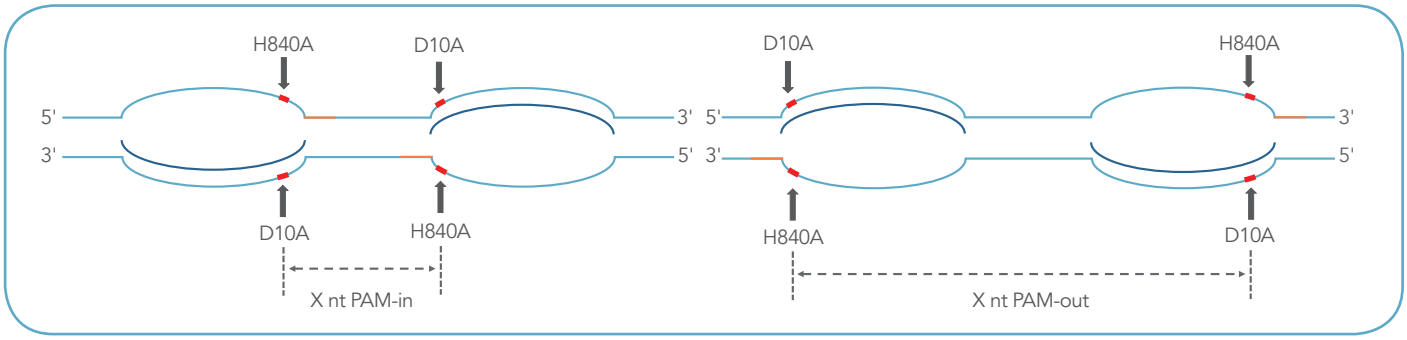
A. Activity of Cas9 D10A nickase. **B. Activity of Cas9 H840A nickase.**

Figure 1. Schematic of DNA lesions generated by two nickases: (A) Cas9 D10A and (B) 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**).

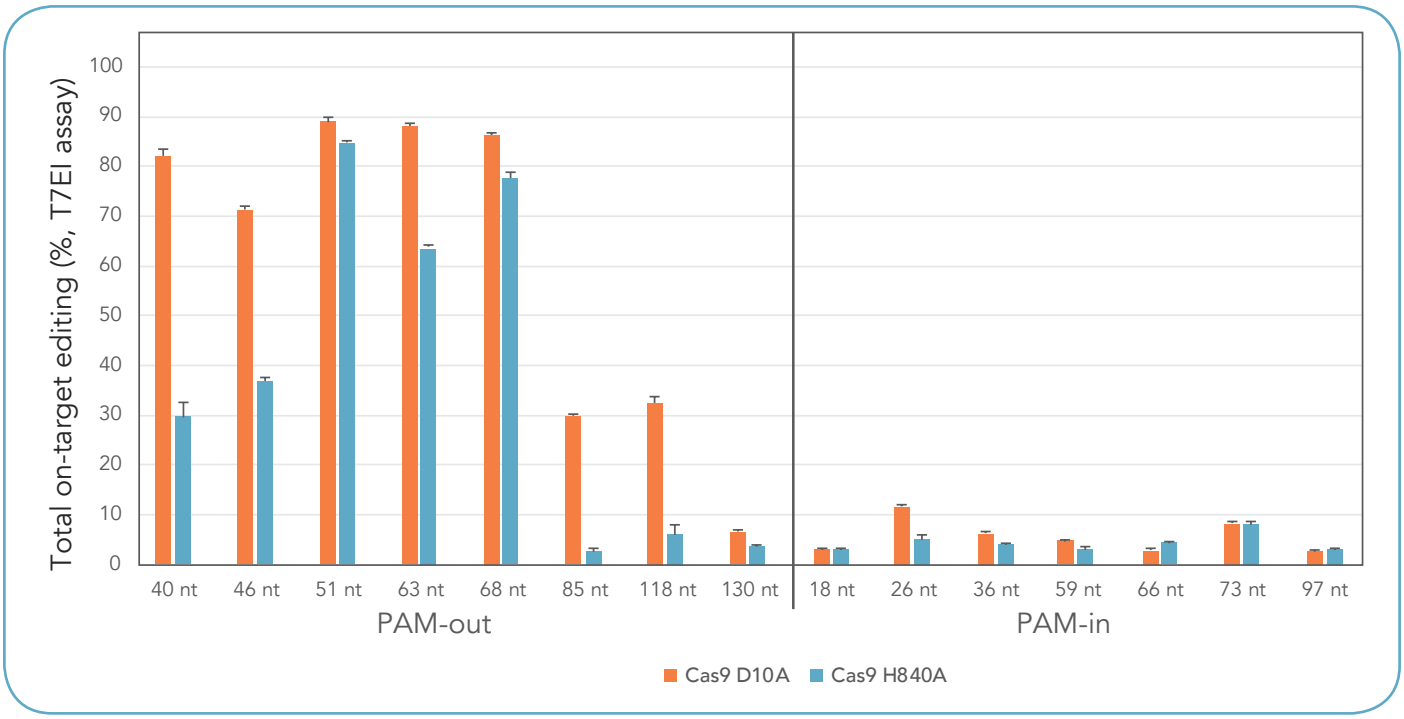
Cas9 nickases demonstrate higher editing efficiency with gRNA pairs in PAM-out configuration compared to a PAM-in configuration

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, a number of 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 *HPRT* 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], robust editing was only 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 more robust 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.



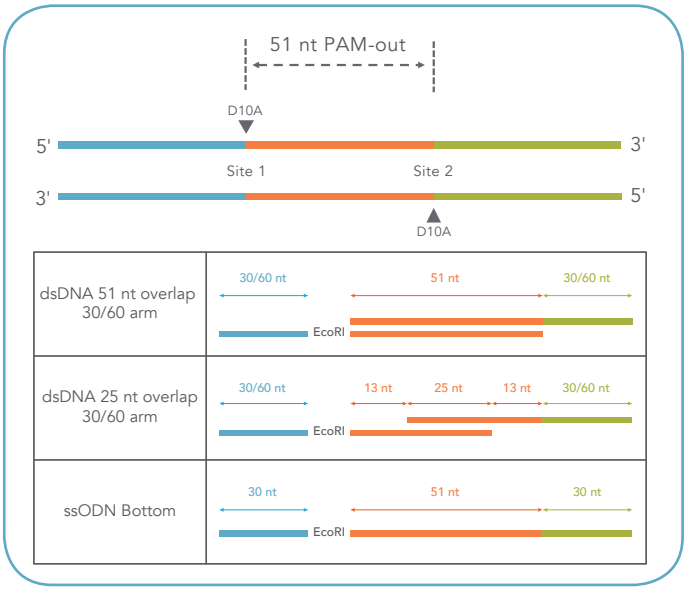
B. Total editing efficiency of Cas9 nickases when varying PAM site orientation and spacing.

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 *HPRT* 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 (orange) or H840A (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).

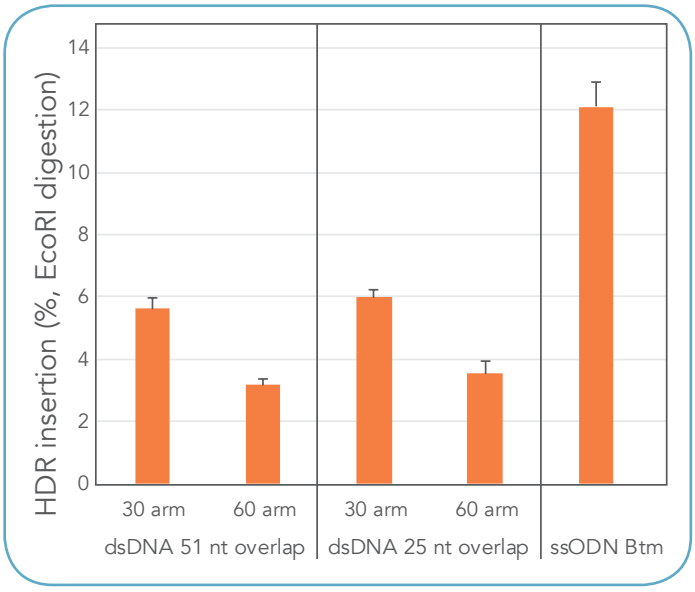
ssODNs are the preferred donor template for nickase-mediated HDR

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 *HPRT* 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 detect 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 (orange) 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 green) 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 *HPRT* 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.

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

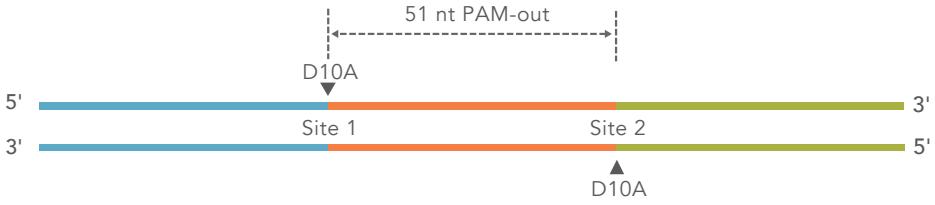
Next, we optimized HDR efficiency by testing template design parameters, such as length of the homology arms, symmetry around the insertion site, strand preference, and choice of nickase variant. Using the best performing paired guides targeting *HPRT* (51 nt PAM-out, Figure 2B), we compared HDR efficiencies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

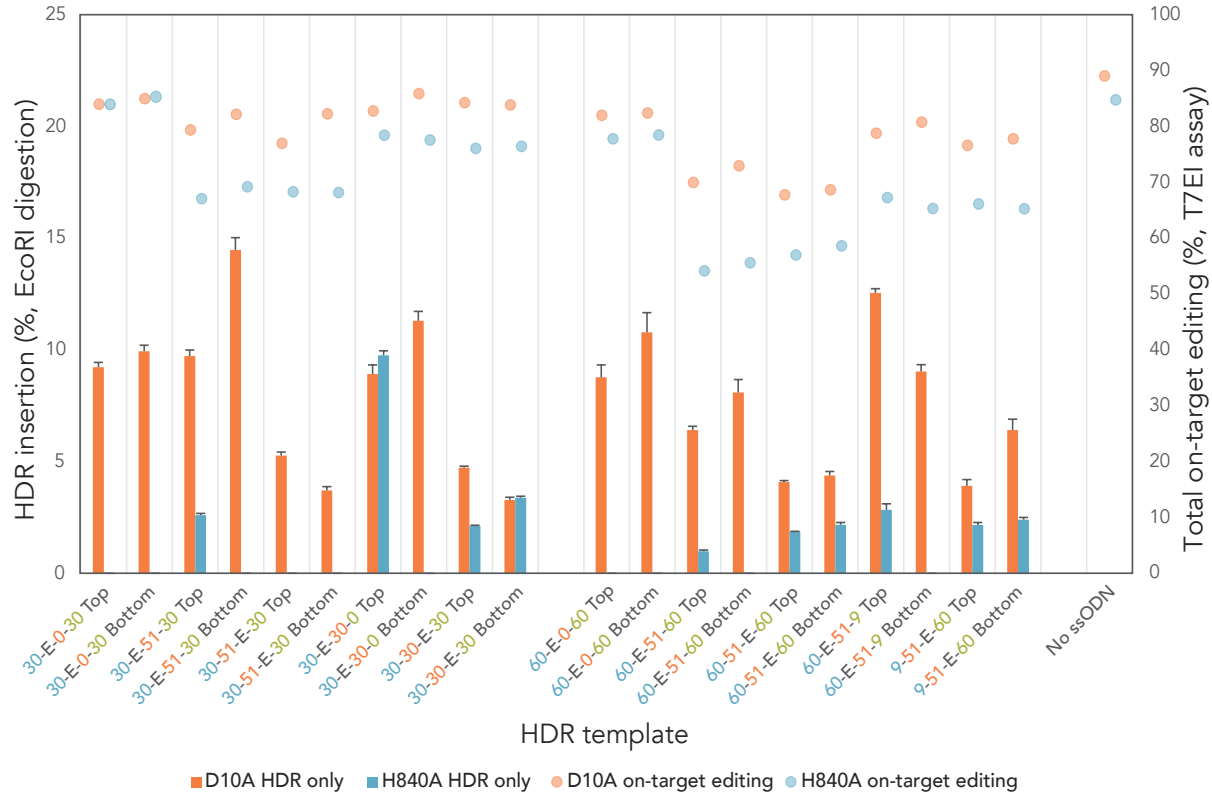
Unlike dsDNA donors, ssODN donor templates can be designed using the sequence of either strand of the template DNA (referred to as top or bottom strand in this study). Although HDR for certain designs appears to be favored when one strand is used instead of the other, we recommend testing both strands, 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.



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

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



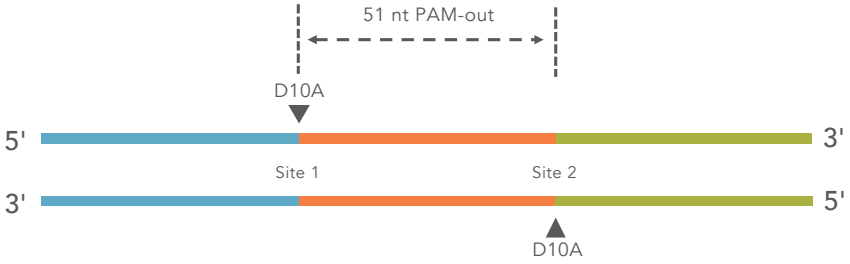
B. T7EI assay for overall genome editing and EcoRI assay to assess HDR mediated by Cas9 D10A and H840A.

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 green = homology arms, orange = sequence between nick sites 1 and 2, gray = 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, orange bars or circles) or two Cas9 H840A RNPs (5 nm each, 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 oligos) 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).

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

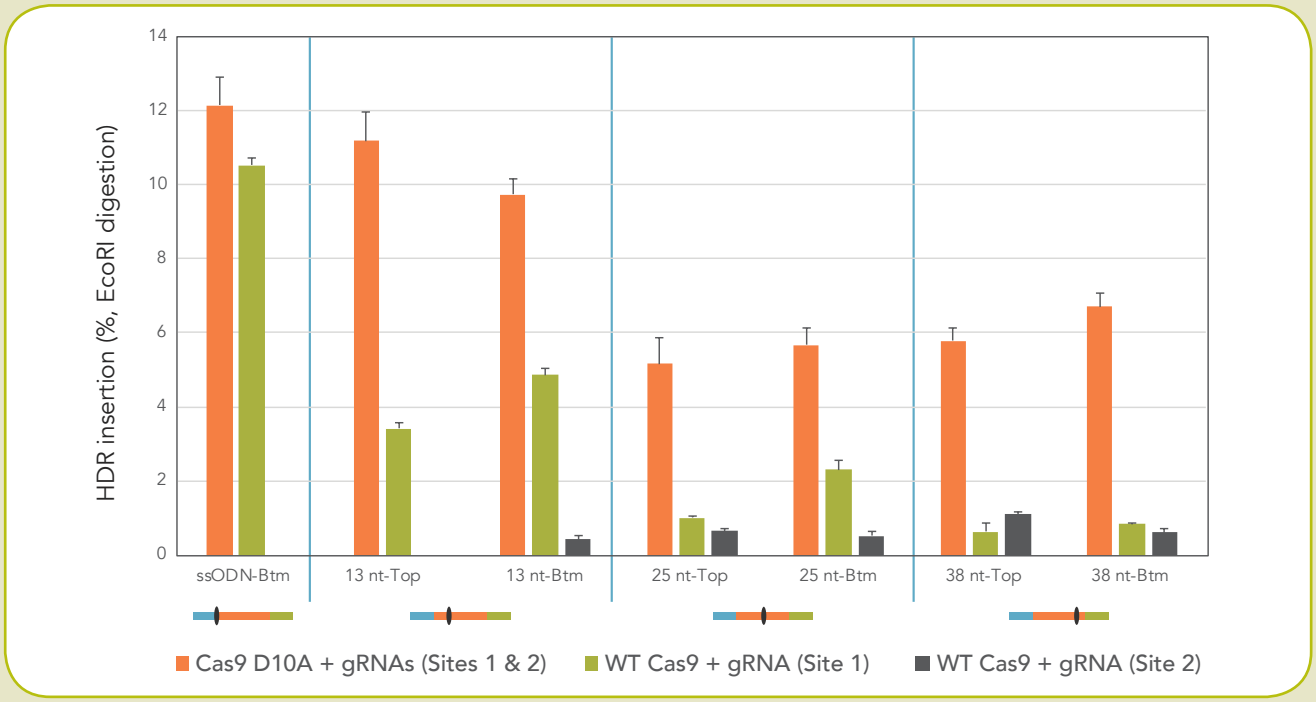
Ideally, to accomplish high HDR efficiency, the cleavage sites introduced by CRISPR-Cas9 enzymes should be in close proximity to where mutations are introduced. It is often recommended that the insertion site of desired modifications be no more than 20 bp away from the DSB, because a few bases further 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. More robust 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.

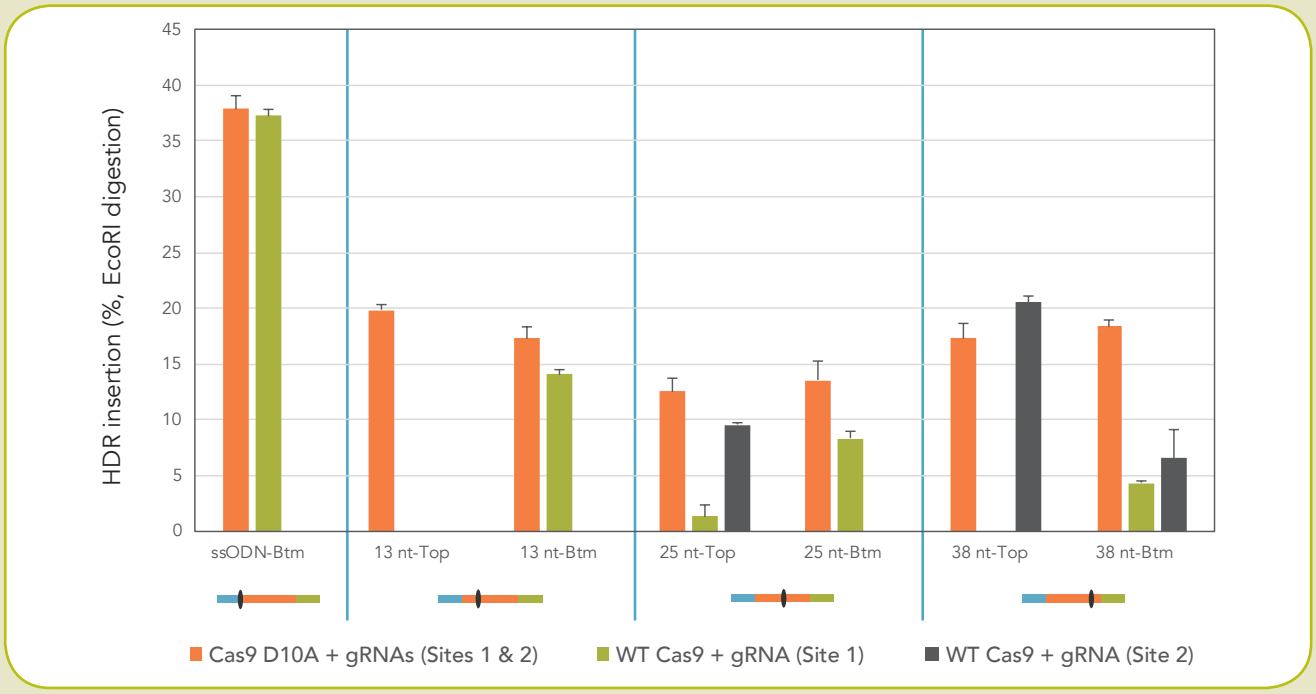


Name	Structure	Sequence (5' → 3')	Symbol
13 nt-Top	30 nt 13 nt 38 nt 30 nt	ACGTCAGTCTTCTCTTTTGTAATGCCCTGTAGTCTCTCTGTATGAATTCG TTATATGTCACATTTTGTAATTAACAGCTTGCTGGTGAAAAGGACCCCA CGAAGTGTTGGATATAAG	
13 nt-Btm	30 nt 13 nt 38 nt 30 nt	CTTATATCCAACACTTCGTGGGGTCCTTTTCACCAGCAAGCTGTTAATTA CAAAATGTGACATATAACGAATTCATACAGAGAGACTACAGGGCATTA CAAAAGAGAAGACTGACGT	
25 nt-Top	30 nt 25 nt 25 nt 30 nt	ACGTCAGTCTTCTCTTTTGTAATGCCCTGTAGTCTCTCTGTATGTTATAT GTCACGAATTCATTTTGTAATTAACAGCTTGCTGGTGAAAAGGACCCCA CGAAGTGTTGGATATAAG	
25 nt-Btm	30 nt 25 nt 25 nt 30 nt	CTTATATCCAACACTTCGTGGGGTCCTTTTCACCAGCAAGCTGTTAATTA CAAAATGAATTCGTGACATATAACATACAGAGAGACTACAGGGCATTA CAAAAGAGAAGACTGACGT	
38 nt-Top	30 nt 38 nt 13 nt 30 nt	ACGTCAGTCTTCTCTTTTGTAATGCCCTGTAGTCTCTCTGTATGTTATAT GTCACATTTTGTAATTAAGAATTCCAGCTTGCTGGTGAAAAGGACCCCA CGAAGTGTTGGATATAAG	
38 nt-Btm	30 nt 38 nt 13 nt 30 nt	CTTATATCCAACACTTCGTGGGGTCCTTTTCACCAGCAAGCTGGAATTC TTAATTACAAAATGTGACATATAACATACAGAGAGACTACAGGGCATTA CAAAAGAGAAGACTGACGT	
ssODN-Btm	30 nt 51 nt 30 nt	CTTATATCCAACACTTCGTGGGGTCCTTTTCACCAGCAAGCTGTTAATTA CAAAATGTGACATATAACATACAGAGAGACTGAATTCACAGGGCATTA CAAAAGAGAAGACTGACGT	

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



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 oligos) 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. (**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.

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, and we plan to update this application note with knowledge we gain through our extensive R&D efforts.

- 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 more robust 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.

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