# Improved methods for CRISPR-Cas9 homology-directed repair (HDR) for efficient, high-fidelity genome editing

Whether you are a new or experienced investigator, this application note describes strategies for achieving high-fidelity HDR of CRISPR-Cas9–based genome editing. As outlined below, Part I introduces important concepts for CRISPR HDR, Part II describes considerations for optimizing single-stranded oligodeoxynucleotide (ssODN) repair templates for small edits, Part III provides recommendations for designing double-stranded DNA (dsDNA) repair templates for large insertions, and Part IV discusses delivery of HDR reagents and options for the analysis of HDR editing.

## Part I. General considerations for designing HDR experiments

In this section, we provide an introduction to CRISPR HDR and discuss important considerations for any HDR experiment, including guide and template selection.

## Part II. Optimizing ssODN templates for small CRISPR-Cas9 HDR edits

This section provides technical recommendations for optimizing donor DNA templates to generate specific small edits (single base conversions or short insertions) via HDR. We recommend using ssODN repair templates rather than double-stranded DNA (dsDNA) for insertions < 120 bp. Custom Alt-R™ HDR Donor Oligos can be used as repair templates for sequences ≤ 200 nt. We discuss design and experimental considerations such as optimizing repair template homology arm length, selection of the ssODN strand, and use of blocking mutations to prevent Cas9 re-cutting.

## Part III. Optimizing dsDNA templates for large HDR insertions using CRISPR-Cas9

For large insertions (>120 bp) we recommend the use of long dsDNA repair templates. Alt-R HDR Donor Blocks can be used as repair templates for sequences up to 3 kb. Part III discusses the benefits of using modified dsDNA templates and important design considerations such as optimal homology arm lengths and use of blocking mutations to prevent Cas9 recutting.

## Part IV. Considerations for delivery and evaluation of HDR editing

We conclude this application note by describing experimental factors relating to the delivery of HDR and CRISPR reagents and describe various methods for the downstream analysis of HDR editing.

# Part I. General considerations for designing HDR experiments

## Introduction

CRISPR-Cas9 genome editing is changing the landscape of genomics due to its ease of use and ability to create double-strand 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 two main 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 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. Gene function is disrupted by utilizing the NHEJ pathway to introduce frameshifts or other changes to the open reading frame of a target gene for loss-of-function studies, a process called gene "knockout." Alternatively, researchers seamlessly insert (or "knock in") exogenous sequences such as selectable markers, fluorescent tags, or other functional units through the endogenous HDR pathway. While NHEJ-based gene disruption is fairly well understood and relatively efficient as a research tool, knock-in of foreign DNA by HDR can be a challenge [2–4].

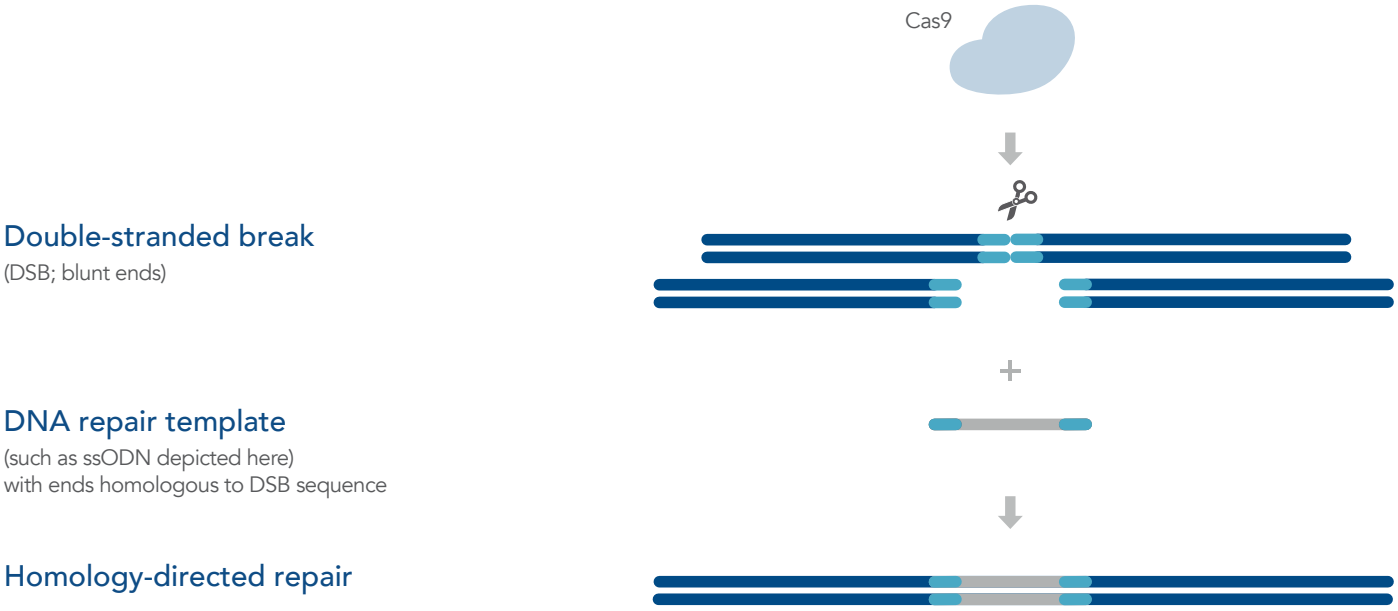



**Figure 1. HDR insertion of an exogenous donor sequence at a specific genomic location.** Cas9 is directed to cut at a specific target by sgRNA or a crRNA:tracrRNA complex. The Cas9:RNA complex is delivered along with a repair template with the desired insert (gray) and flanking homology arms (light blue), which mediate the exchange of DNA sequence information via the HDR repair pathway.

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

In this application note, we summarize our recent findings using CRISPR-Cas9 in HDR research applications and provide stepwise guidance for maximizing HDR rates in your own genome editing experiments (Figure 2). These recommendations apply to HDR-mediated insertion of small edits, such as epitope tags or the correction of point mutations (Part II), and to the insertion of large fragments, such as fluorescent tags or functional genes (Part III).


**Figure 2. Experimental workflow for HDR using co-delivery of synthetic guide RNA (gRNA), Cas9 endonuclease, and HDR repair template.**

## Definitions

**gRNA (guide RNA)**: The CRISPR RNA (crRNA) used by Cas9 contains both the 20-base protospacer element and additional nucleotides that are complementary to the tracrRNA. The transactivating CRISPR RNA (tracrRNA) hybridizes to the complementary region of the crRNA. The combined crRNA and tracrRNA interact with the Cas9 endonuclease, activating the editing complex to create double-strand 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 efficiency, especially when pre-complexed with a CRISPR nuclease and delivered to cells as an RNP. Alternatively, single guide RNA (sgRNA) can be used instead of the combination of crRNA and tracrRNA. sgRNA contains the crRNA and tracrRNA sequences connected by a hairpin-like loop sequence. Guide RNA (gRNA) can be either the crRNA:tracrRNA complex, or just the sgRNA.

**HDR (homology-directed repair)**: A cellular mechanism for repair of double-strand 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 repair template**: A 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). May also be referred to as an **HDR donor template**. A repair 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)**: A cellular pathway that repairs double-strand breaks in genomic DNA. It is the most frequent 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 useful when precise introduction of genetic material is desired.

**Non-homologous integration / Blunt insertion**: An unintended repair event resulting from the misincorporation of a linear dsDNA HDR repair template, where the free ends of the dsDNA template are recognized by NHEJ repair factors and directly ligated into a DSB. This misincorporation can occur at the CRISPR-Cas9 on-target DSB (resulting in the duplication of one or both homology arms, as the resolution of the two junctions occurs independently), at Cas9 off-target sites, or at naturally occurring endogenous DSBs. The frequency of non-homologous integration can be reduced through modification of the dsDNA HDR template [6-8].

**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, or alternatively, it is composed of sgRNA and Cas9. Delivery of these CRISPR components in the form of RNP provides optimal genome editing efficiency and reduces unwanted off-target cutting [9].

**PAM (protospacer-adjacent motif)**: The sequence feature recognized by a specific CRISPR endonuclease hybridized with a gRNA. For Cas9 endonuclease, the PAM sequence is NGG.

**Targeting strand**: The genomic DNA strand complementary to the gRNA. The targeting strand is also known as the protospacer element.

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

**Homology arm**: The sequence within the HDR repair template that matches either side of the genomic cut site. The homology arms required for long donors are significantly longer than for short donors.

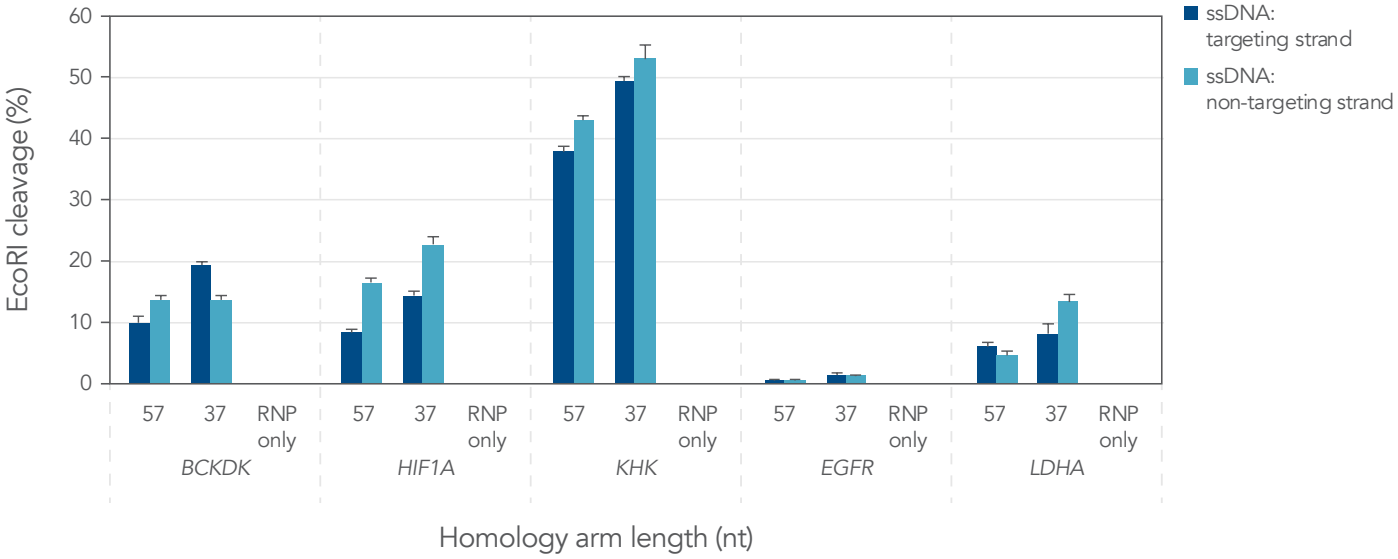
**ssODN**: single-stranded oligodeoxynucleotide.

# gRNA selection for HDR optimization

The efficiency of HDR insertion varies with the PAM site location, independent of the efficiency of Cas9 cleavage [9]. However, high levels of editing are indeed needed to achieve good HDR. For initial screening, we recommend testing at least 2–4 CRISPR-Cas9 guides close to where the sequence modification is desired. Figure 3a shows the HDR integration rate at 5 different genomic locations of a short fragment (6 bases) containing a recognition site for the 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 restriction endonuclease digestion. The HDR efficiency, as measured by the percentage of EcoRI digestion, is site-specific even though all of the sites tested here generated DSBs efficiently.

When selecting guides in your area of interest, it may be helpful to first assess HDR potential using a short insertion (as described here) prior to attempting larger insertions. Selection of guides with high overall editing efficiency is recommended, as this maximizes the potential for HDR. When possible, avoid use of low-activity guides, as they will limit HDR rates. While the gRNA 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.

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




B. HDR template design and Cas9 cleavage site.

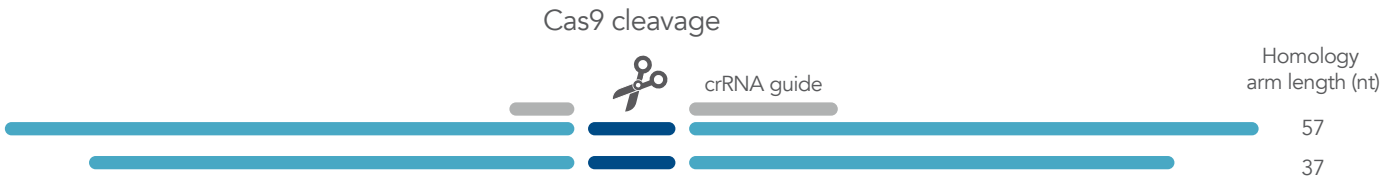



**Figure 3. HDR efficiency varies with the choice of PAM sites.** (**A**) HEK-293 cells were transfected with 10 nM RNP (Alt-R *S.p.* Cas9 Nuclease v3 complexed with Alt-R CRISPR-Cas9 crRNA and tracrRNA) and 3 nM HDR template (standard desalted Ultramer™ DNA Oligonucleotide) using 1.2 µL Lipofectamine® 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™ (Agilent) to determine rate of EcoRI site insertion as a proxy for HDR efficiency (n=3). (**B**) 3′ and 5′ homology arms of either 37 or 57 nt were placed flanking the EcoRI site for interrogating the effect of homology arm length on HDR. The dark blue bar represents the EcoRI cleavage site on the HDR template.

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 [10]. The HDR rate decreases dramatically when the template insertion is just 5–10 bases away from the cut site (Figure 4A). However, not every insertion site will have PAM sequences available in the immediate vicinity. In these situations, choose the most active gRNA that is as close as possible to the intended insertion site for the best chance of efficient HDR (Figure 4C). Incorporation of silent mutations within the HDR repair template can improve HDR efficiency in these cases, as will be presented in Parts II and III.

The HDR rate can also differ greatly among cell types. In particular, HDR potential in primary cells may be highly variable. We have data from a variety of cell lines, and have determined optimal conditions in many of them for CRISPR/Cas9-mediated HDR. HDR positive control sequences mediating short insertions are available for human and mouse species (Figure 5) and are recommended when first attempting HDR in these systems.

To enable design of optimal gRNA and HDR donors, IDT has developed the Alt-R HDR Design Tool. This tool provides optimized HDR repair template design and Cas9 guide RNA selection. The higher HDR rates result from clear design rules based on extensive wet bench testing. Simply provide basic information about your desired target site, then use the HDR tool to design and visualize your desired edit within the sequence. The HDR design tool will provide the recommended gRNAs and HDR repair template for your desired specifications.

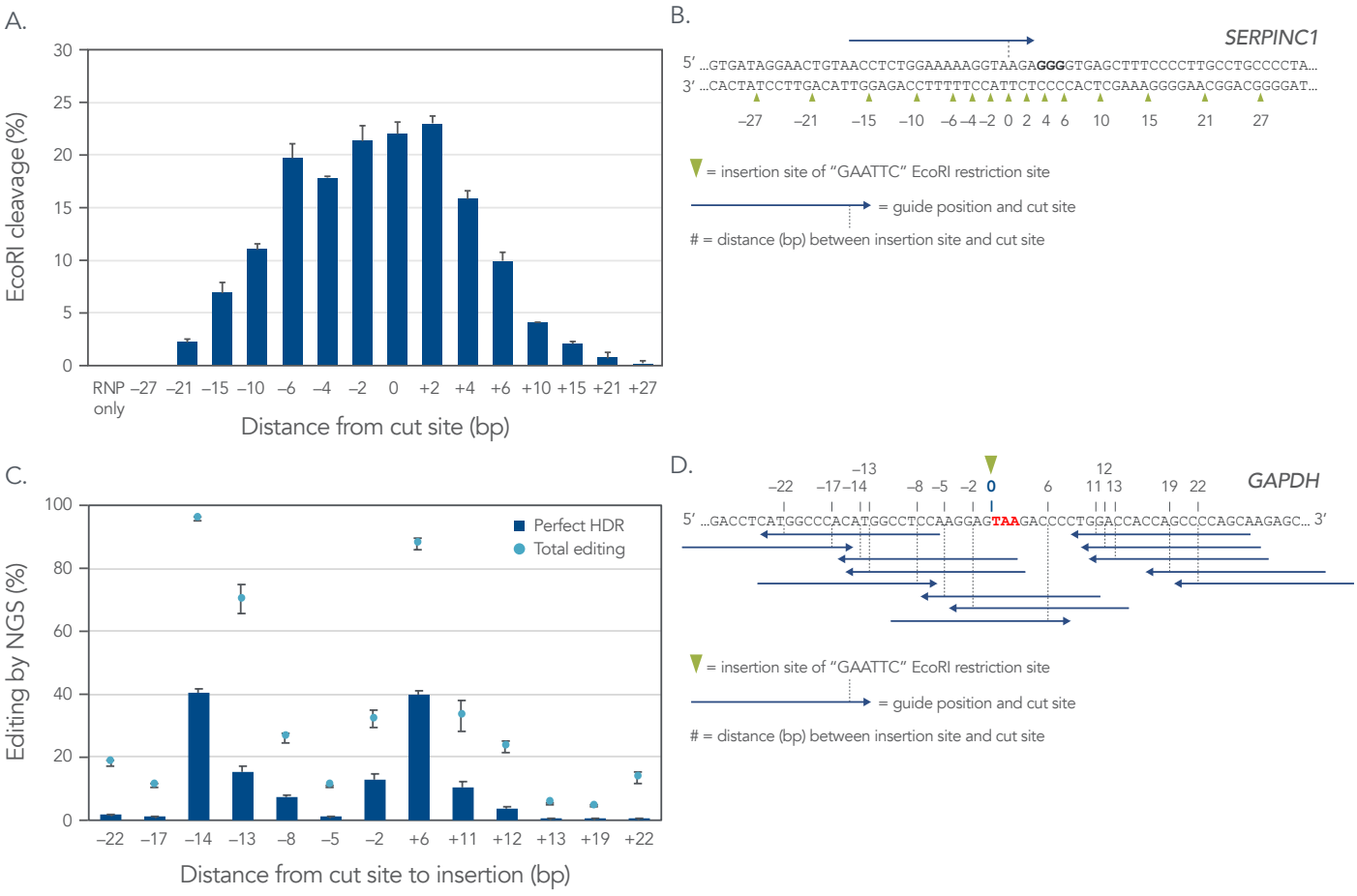



**Figure 4. HDR efficiency depends on HDR edit location relative to CRISPR-Cas9 cut site and on overall guide activity.** (**A**) HDR rates decrease significantly at positions >10 bases away from the Cas9 cut site. Alt-R CRISPR-Cas9 RNP targeting the human gene SERPINC1 was delivered into HEK-293 cells at a concentration of 4 µM via electroporation, together with a series of ssODN repair templates that gradually increase the distance in both directions between the cut site and the EcoRI insertion site as depicted in (**B**). All ssODN templates were designed using the sequence of the non-targeting strand, with inserts flanked by 40 nt homology arms. Additionally, 2 µM Alt-R Cas9 Electroporation Enhancer was included in the reaction to increase editing efficiency. Genomic DNA was isolated 48 hr after electroporation, and the rate of HDR insertion was assessed by EcoRI cleavage (n=3). (**C**) HDR rates are increased by the selection of high activity guides. Alt-R CRISPR-Cas9 RNPs were generated with guides targeting the human gene GAPDH with varying distances between the Cas9 cut site and the intended HDR edit as depicted in (**D**). ssODN repair templates were designed to introduce an EcoRI recognition site inserted before the GAPDH stop codon and incorporated 40 nt homology arms from the Cas9 cut and the insertion site. 2 µM Cas9 RNP, 2 µM ssODN template, and 2 µM Cas9 Electroporation Enhancer were delivered into K562 cells via electroporation. Genomic DNA was isolated 48 hr after electroporation, and editing levels were assessed by NGS using the rhAmpSeq CRISPR Analysis System (n=3). Both the non-targeting or targeting strand were tested; the data presented reflect the preferred strand (as further discussed in Part II).

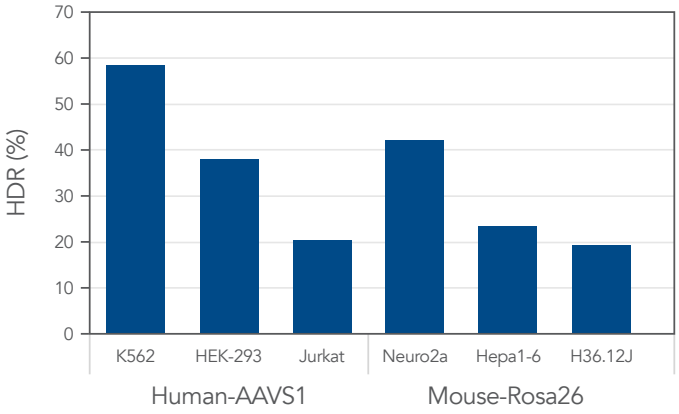



**Figure 5. Representative postive control HDR rates in commonly used cell lines.** RNP complexes (2 µM) consisting of Alt-R *S.p.* Cas9 Nuclease V3 complexed with Alt-R crRNA and tracrRNA targeting either the AAVS1 or Rosa26 safe-harbor locus were delivered by the 4D-Nucleofector™ System (Lonza). Alt-R HDR donor templates were included at 0.5 µM (K562, HEK-293, Jurkat human cells) or 3 µM (Neuro2a, Hepa1-6, H36.12J murine cells) along with 2 µM of Alt-R Cas9 Electroporation Enhancer. HDR efficiency was measured by amplicon sequencing on an Illumina™ MiSeq™ system (K562, HEK-293, Jurkat) or by EcoRI restriction fragment length polymorphism (RFLP) assay, n=1 (Neuro2a, Hepa1-6, H36.12J).

# Selection of DNA templates

Single-stranded oligodeoxynucleotides harboring the desired exogenous sequences have become common in HDR applications, due in combination to their high HDR efficiency, low toxicity, and the ease of chemically synthesizing diverse sequences [11,12]. 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), IDT recommends using ssODN templates such as Alt-R HDR Donor Oligos.

For longer insertions, Alt-R HDR Donor Blocks are available up to 3000 bp in length. Alt-R HDR Donor Blocks are modified linear dsDNA fragments generated from clonally purified DNA, which offers the greatest purity available. Utilizing the same high-fidelity synthesis process as IDT gBlocks™ HiFi Gene Fragments, Alt-R HDR Donor Blocks incorporate advanced chemical modifications placed within terminal universal sequences. These modifications boost HDR rates and inhibit the occurrence of non-homologous (blunt) integrations. Additionally, the use of terminal universal sequences allows for increased consistency and speed of production.

We recommend 30–60 nt lengths for homology arms for short ssODN donors (e.g. Alt-R HDR Donor Oligos; <200 nt total length). For longer dsDNA HDR donors (e.g., Alt-R HDR Donor Blocks; 200–3000 bp total length), we recommend 200–300 bp lengths for homology arms. The Alt-R HDR Design Tool can design both short and long donors, and automatically selects either Alt-R HDR Donor Oligos or Alt-R HDR Donor Blocks in the output, depending on the final donor length. The Alt-R HDR Design Tool can be found at www.idtdna.com/pages/tools/alt-r-crispr-hdr-design-tool.

# Alt-R HDR Enhancer V2 for superior HDR efficiency

For HDR experiments, we recommend using the Alt-R HDR Enhancer V2, which is a small molecule compound with an improved ability to increase the rate of HDR. To increase HDR efficiency, our scientists conducted extensive research on the modulation of repair pathways within the context of CRISPR-Cas9 mediated genome editing. Those efforts led to the development of a small molecule reagent that effectively diverts repair pathways towards HDR, successfully enhancing overall HDR efficiency. The Alt-R HDR Enhancer V2 has increased potency and improved cell viability over previously tested compounds. Examples of the benefit to using Alt-R HDR Enhancer V2 will be shown for both short and large HDR insertions in the following sections.

# Part II. Optimizing ssODN templates for small CRISPR-Cas9 HDR edits

## Introduction

The use of chemically modified ssODN templates has been shown to increase HDR efficiency over unmodified donors when generating small insertions [13]. Research at IDT has further improved HDR efficiency by developing Alt-R HDR Donor Oligos. In addition to the published phosphorothioate bonds (PS) on each end, these templates contain an IDT proprietary end-blocking modification to provide increased stability and improved HDR rates (Figure 6A). Based on the high quality of IDT Ultramer DNA Oligos, these repair templates provide high HDR rates for introducing insertions up to 120 bp. HDR rates with these ssODN templates can be further improved with the use of Alt-R HDR Enhancer V2 (Figure 6B). The following section will provide guidance on strand preference, optimal lengths for homology arms, and the use of silent mutations to disrupt Cas9 re-cutting of the HDR edit.

## Strand preference and optimal homology arm lengths with Alt-R HDR Donor Oligos

Template sequences for ssDNA HDR donors can be designed to correspond 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 instead of the other. Although efficiency varies between sites and is not always predictable, our research suggests that when an insertion is placed at the cut site, there is little or no universal strand preference. We recommend testing HDR donors matching both strands to determine which yields higher HDR in these cases. However, HDR edits placed outside the optimal 5–10 bp distance from the cut site do show specific strand preferences (Figure 6C). The targeting strand is preferred for PAM-proximal edits (placed on the PAM-containing side of the Cas9 cut site). The non-targeting strand is preferred for PAM-distal edits (placed on the opposite side of the Cas9 cut site relative to the PAM). Additional mutations to prevent Cas9 recutting are often required in these cases, as will be discussed in the following section.

Homology arms are the repair template sequence elements that match either side of a cut site, which must be included for HDR. Optimal homology arm length can be site- or system-specific, but we tested a variety of arm lengths to identify an optimal size range. As shown in Figure 6D-E, we designed six 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 [14], reliable HDR was consistently observed when the homology arms were 30–60 nt long. We did not observe a consistent improvement of HDR efficiency with the use of ssODN templates designed asymmetrically versus symmetrically, regardless of whether the additional length of homology was introduced on the PAM-distal or -proximal side (data not shown).

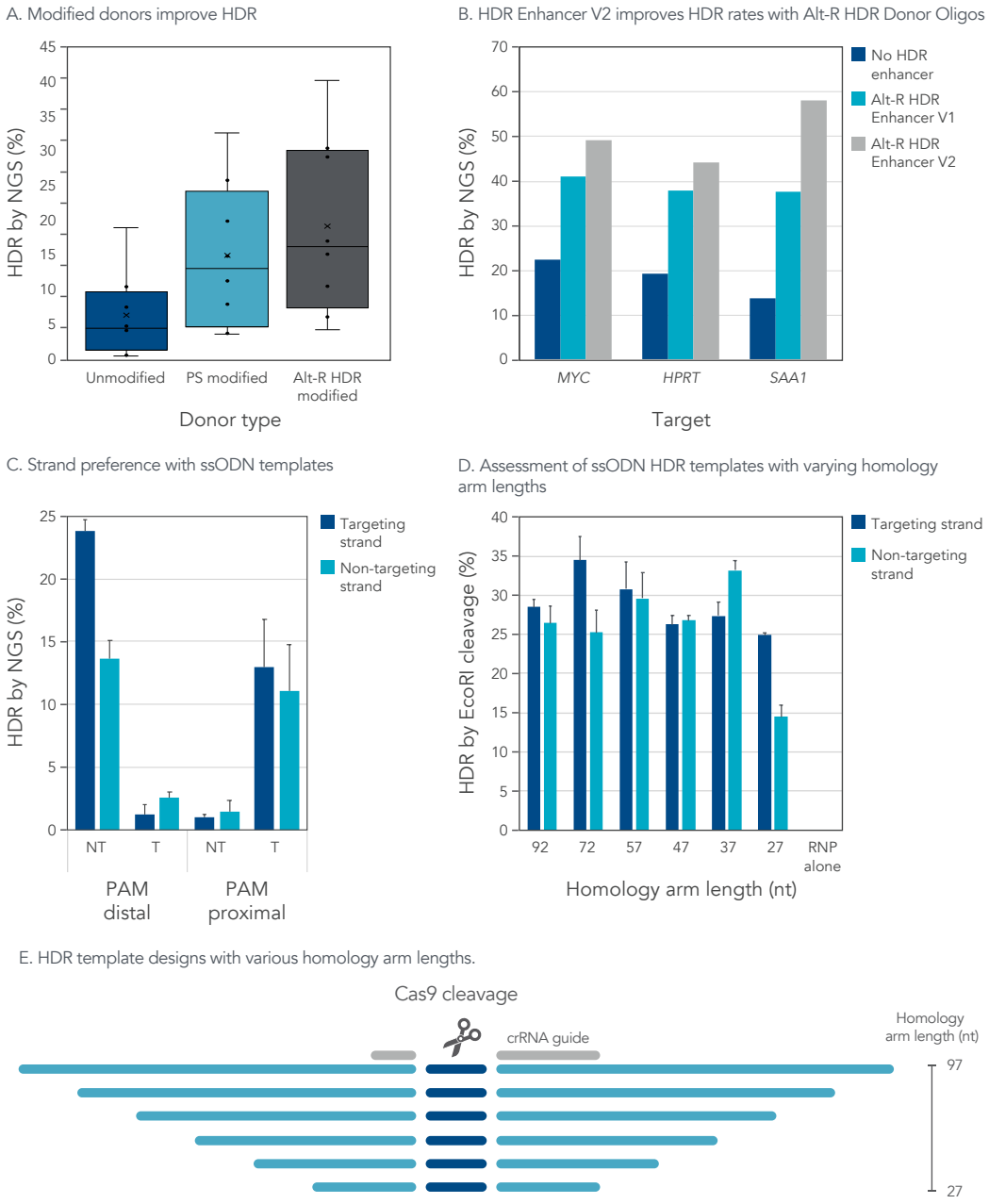



**Figure 6. HDR efficiency with ssODN templates can be improved by donor modification, small molecule enhancers, appropriate strand selection, and optimal homology arm lengths (30–60 nt).** (**A**) HeLa and Jurkat cells were electroporated with 2 µM Alt-R CRISPR-Cas9 RNP complexes targeting four genomic loci along with 2 µM Alt-R Cas9 Electroporation Enhancer and 0.5 µM single-stranded HDR repair template using the Nucleofector™ System (Lonza). Donor templates contained no modification (Unmodified), 4 phosphorothioate linkages (2 at each end of the template; PS-modified), or the Alt-R HDR modification (Alt-R HDR modified). Genomic DNA was isolated at 48 hours (HeLa) or 72 hours (Jurkat) after electroporation, and HDR efficiency was measured by amplicon sequencing on the Illumina MiSeq® system (V2 chemistry, 150 bp paired-end reads), n=8. These results show that Alt-R HDR Donor Oligos improve HDR efficiency over other ssODN templates. (**B**) We delivered 2 µM RNP complexes (targeting 3 genomic loci), along with 3 µM Alt-R Cas9 Electroporation Enhancer and 0.5 µM Alt-R HDR Donor Oligo to HeLa cells by electroporation using the 4D-Nucleofector™ System (Lonza). Cells were grown in media with either no HDR enhancer, 30 µM Alt-R HDR Enhancer, or 1 µM Alt-R HDR Enhancer V2 for 24 hours post-transfection, followed by a media change. Genomic DNA was isolated 48 hours after electroporation, and HDR efficiency was measured by NGS (n=1) as previously described in Figure 5. These results show that Alt-R HDR Enhancer V2 improves HDR rates when used with Alt-R HDR Donor Oligos. (**C**) A 6-base insertion was placed 25 bases from the cut site of a guide targeting the *SERPINC1* or *HPRT1* locus in either direction. ssODN templates corresponding to either strand were designed with mutations to disrupt Cas9 recutting (as discussed in Figure 7). We delivered 4 µM Cas9 RNP, 4 µM Cas9 Electroporation Enhancer, and 0.5 µM Alt-R HDR Donor Oligo to HeLa cells by electroporation using the Nucleofector™ System (Lonza). Genomic DNA was isolated 48 hours after electroporation, and HDR efficiency was measured by NGS (n=3). These results show that strand preference with ssODN HDR templates is dependent on the HDR edit location relative to the Cas9 cut site. (**D**) ssODN HDR templates mediating the insertion of a 6-base EcoRI restriction site into the human *EMX1* gene were designed with homology arms ranging from 27 to 92 bases long (as depicted in (**E**). HEK-293 cells were transfected via lipofection with 10 nM Alt-R CRISPR-Cas9 RNP and 3 nM ssODN templates corresponding to the targeting or non-targeting strand. Genomic DNA was isolated 48 hr post lipofection and PCR-amplified with primers designed outside the flanking arms of the ssODN template. PCR products were digested with EcoRI to determine the rate of HDR insertion by RFLP analysis (n=3).

# Rational design of Alt-R HDR Donor Oligos

The introduction of silent mutations into the protospacer or PAM sequence when an intact CRISPR recognition site is present on the HDR template will help prevent the HDR event from reconstituting the crRNA recognition site, and avoid unwanted recutting at the same locus after the repair. The Alt-R HDR Design Tool allows for silent mutation incorporation using empirically defined rules to provide the highest HDR rates, as described in the following case study [15].

While not yet incorporated into the rules governing the Alt-R HDR Design Tool, IDT researchers have made further progress on understanding optimal designs for HDR edits placed outside the recommended 5–10 bp distance from the Cas9 cut site [15]. As previously shown (Figure 6C), a strong strand preference can be observed when an HDR edit is oriented in either a PAM-proximal or PAM-distal orientation relative to the Cas9 cut site. Furthermore, these HDR edits display a preference for silent mutations that are placed on the same side of the DSB as the desired HDR edit (Figure 7A–B). For PAM-distal HDR edits, the non-targeting strand containing silent mutations placed within the protospacer sequence ("Repair track") is preferred. In contrast, PAM-proximal HDR edits prefer the targeting strand containing silent mutations within the PAM to disrupt Cas9 recognition.

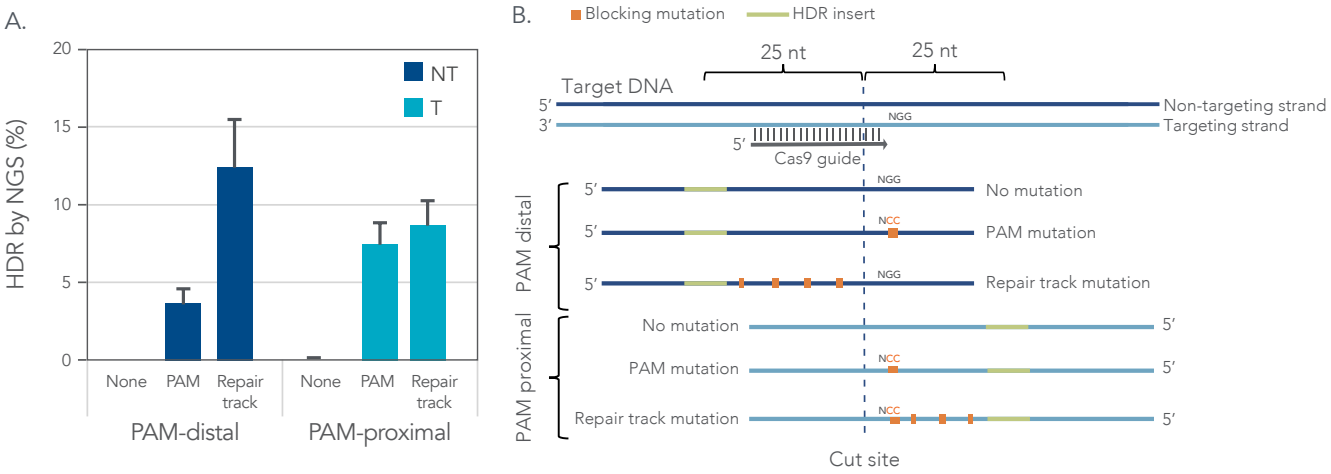



**Figure 7. Mutations to disrupt Cas9 recutting improve HDR rates when placed on the same side of the DSB as the desired HDR edit.** (**A**) Donor templates creating an EcoRI insertion 25 bases from the cut site at three genomic loci were delivered to Hela cells as the targeting (T) or non-targeting (NT) strand. Donor templates contained no further mutation (None), PAM mutation (PAM), or mutations in the repair track. RNP complexes (Alt-R S.p. Cas9 nuclease complexed with Alt-R CRISPR-Cas9 sgRNA) were delivered at 2 µM along with 2 µM Alt-R Cas9 Electroporation Enhancer and 0.5 µM donor template by nucleofection. Perfect HDR rates were determined by NGS (n=9). **B**) Schematic representation of donor templates used to generate PAM-proximal and PAM-distal insertions 25 bases from a Cas9 cut site with no further mutations (None), PAM mutations (PAM), or mutations in the repair track.

## Case study: Designing HDR repair templates to create a 3-base change in the human ACVR1C gene using the Alt-R HDR Design Tool

In this study, we evaluated the effect of silent mutations and donor strands on the HDR rate while making a 3-base change in the human *ACVR1* gene. We used the Alt-R HDR Design Tool to design HDR donor oligos for both the + and – strands and to select the corresponding guide RNA.

In step 1, we selected our species of interest (human–*Homo sapiens*), input the gene *ACVR1C*, selected the relevant NCBI transcript accession number (NM_145259.2) from the drop-down list, and input the desired edit location, chr2:157556230 (Figure 8).

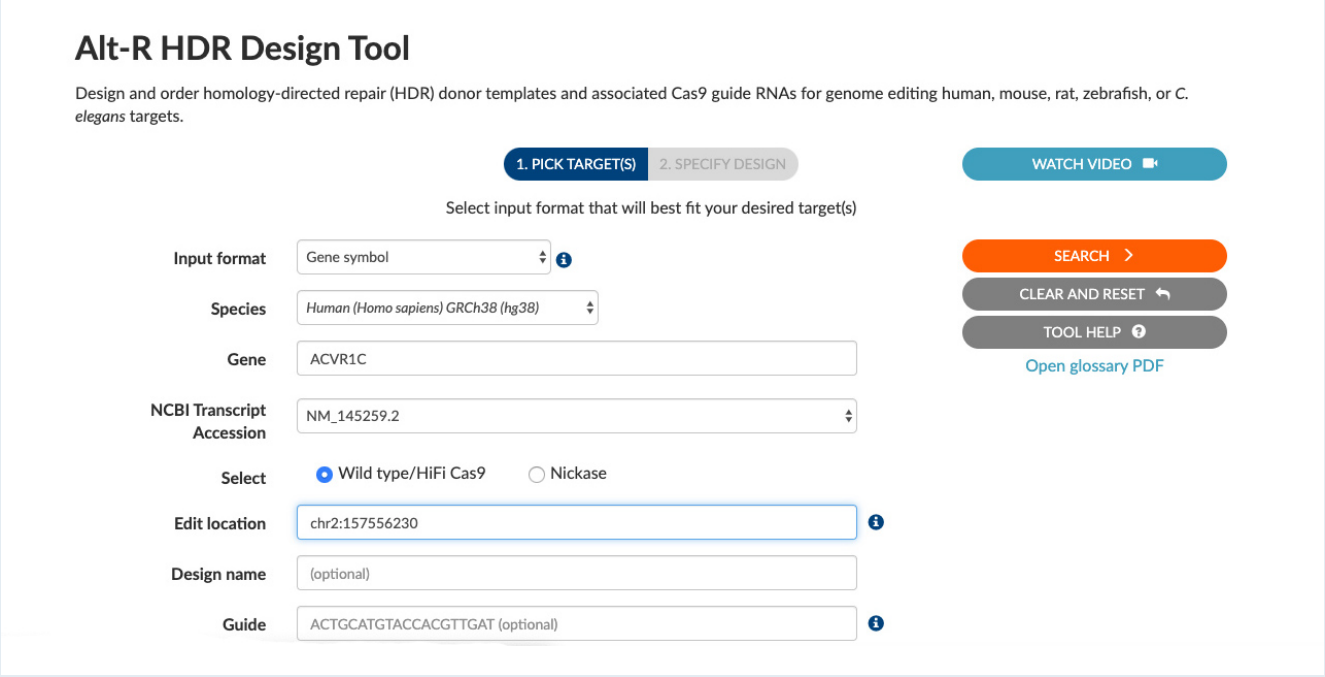



**Figure 8. The "Pick targets" (step 1) window in the Alt-R HDR Design Tool.** Here you will select species, gene, NCBI transcript accession number, and desired edit location.

You will specify the desired HDR mutation after clicking “Search” by. using the easy-to-navigate interface. In this case, we chose to introduce a CAT > ACA mutation at positions 157556230–157556232 of chromosome 2 (Figure 9).

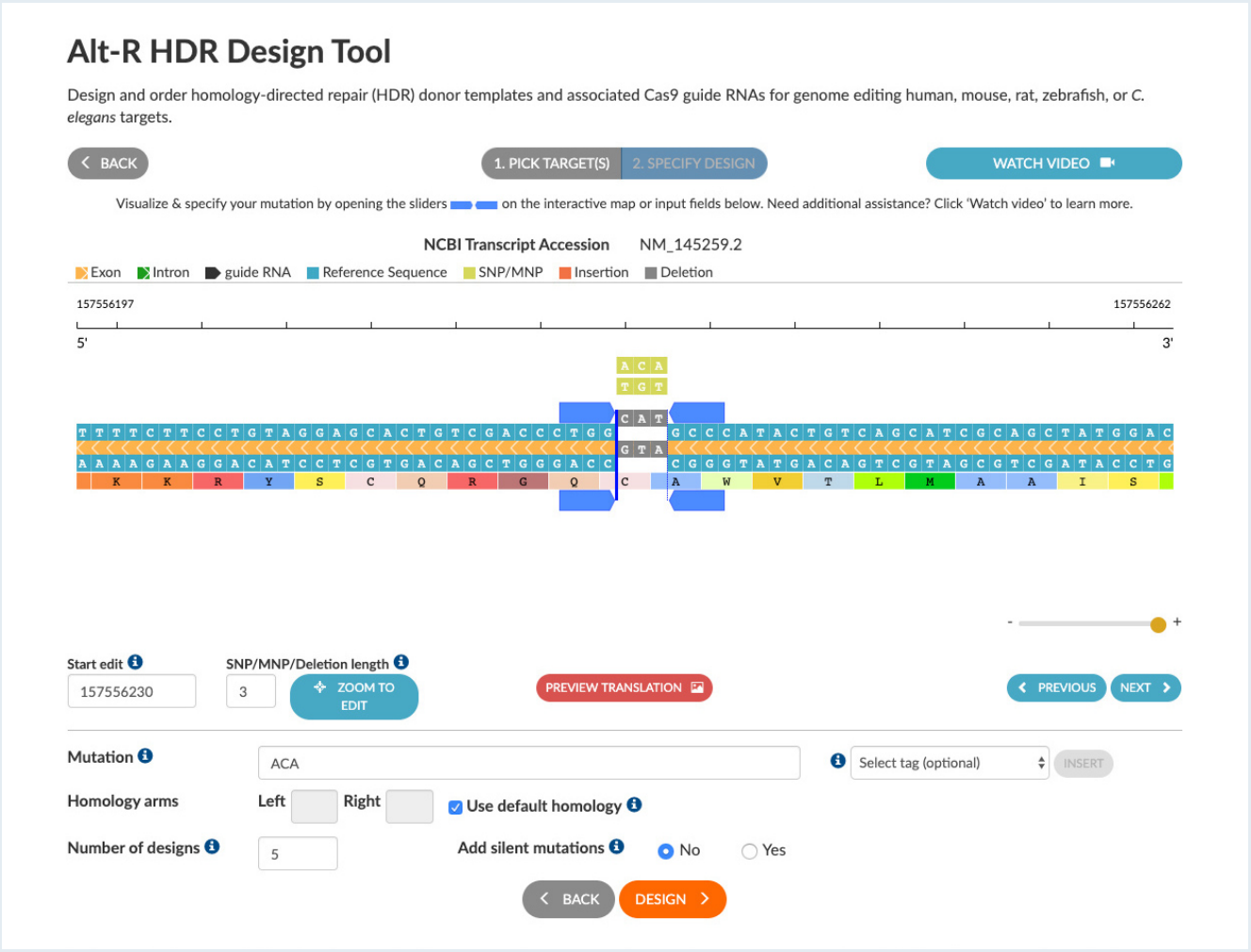



**Figure 9. The “Specify design” (step 2) window in the Alt-R HDR Design Tool.** Here you will specify the desired HDR mutation using the interactive map.

We wanted to examine the effect of adding silent mutations to prevent re-cleavage by Cas9 nuclease after the desired HDR event. Because there would be no need for silent mutations if the HDR mutation disrupts the gRNA on its own, we selected a gRNA from the design results that that would still direct Cas9 to the target site even after incorporation of the desired HDR mutation. We took advantage of the option in the "Specify design" window to test HDR donors both without silent mutations (silent mutations = no) and with silent mutations (silent mutations = yes). Both designs are shown in Figure 10. Default homology arm lengths (40 bases) were used. HDR templates were designed to match both the targeting and non-targeting strands.

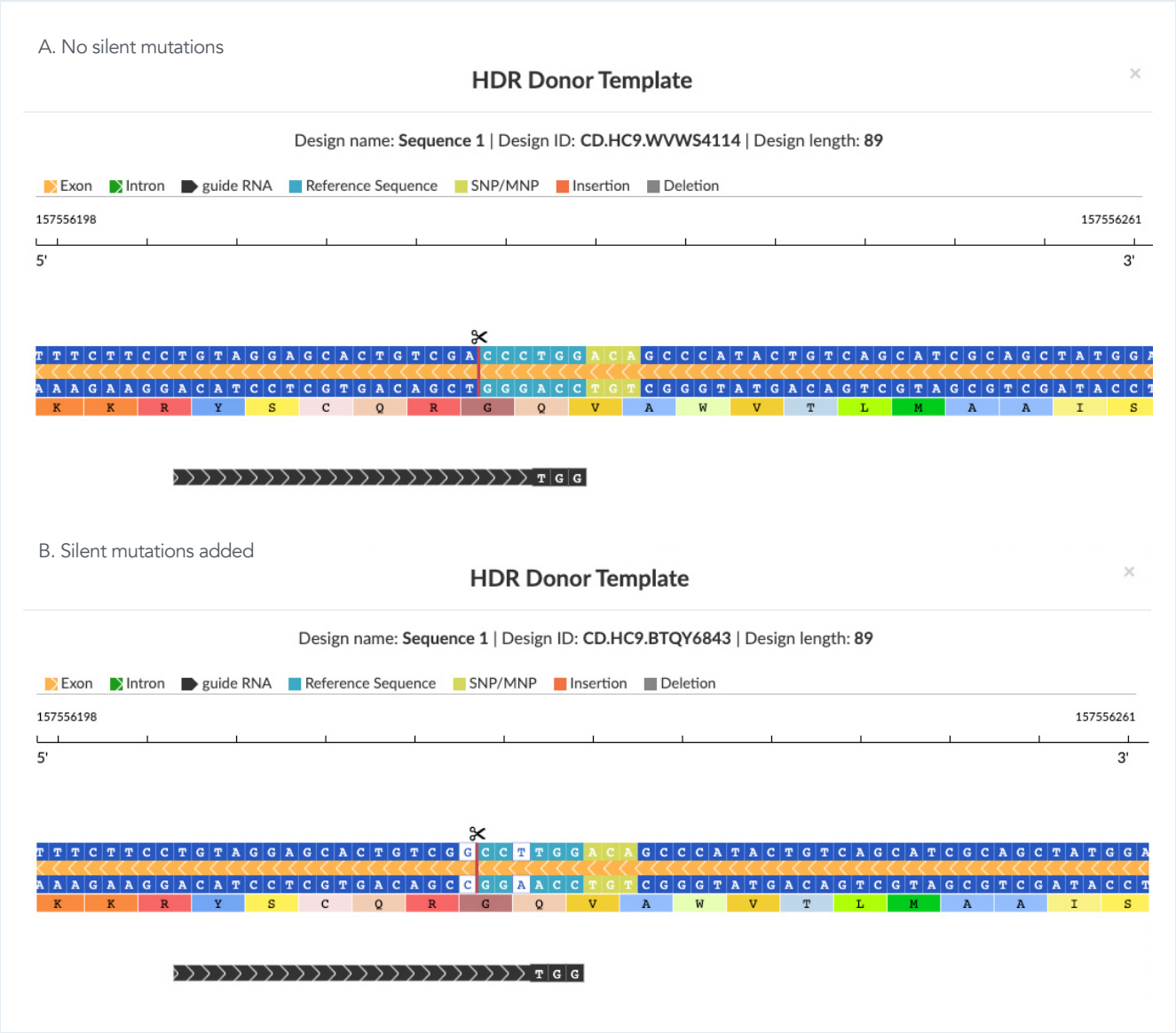



**Figure 10. Design results from the Alt-R HDR Design Tool.** We specified a desired HDR mutation (SNP/MNP; olive green boxes). (**A**) The results map without the addition of silent mutations. (**B**) The results map with silent mutations (white boxes) incorporated by the HDR Design Tool. SNP = single nucleotide polymorphism; MNP = multiple nucleotide polymorphism.

## Experimental results

HDR templates designed using the Alt-R HDR Design Tool were tested in HeLa cells. After completion of the HDR experiment, targeted sequencing was performed to determine rates of HDR. As expected, silent mutations added by the HDR Design Tool improved the rate of perfect HDR, independent of which donor strand was used (Figure 11). Therefore, an HDR template that introduces silent mutations into the target (protospacer) in the genomic DNA, in addition to introducing the desired mutations, increases the percentage of desired recombination events when the HDR mutation does not sufficiently prevent re-cleavage by Cas9, as this nuclease is still present in the cell after the initial cutting event.

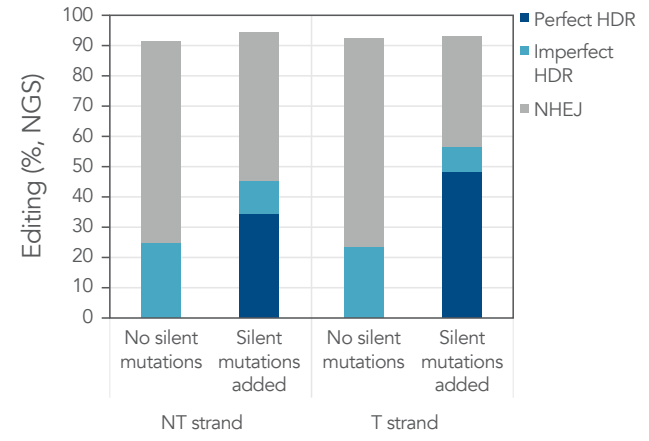



**Figure 11. Editing results after HDR.** Alt-R sgRNA and Alt-R S.p. Cas9 Nuclease V3 were combined to form an RNP complex. This was delivered to HeLa cells at 2 µM along with 2 µM Alt-R Cas9 Electroporation Enhancer, and 2 µM PS-modified Alt-R HDR donor oligos by the Lonza Nucleofection™ method. Data were analyzed by NGS using an internal analysis pipeline to determine the rates of perfect HDR, imperfect HDR, and NHEJ repair (n=1).

## Tips for Alt-R HDR Donor Oligo design

- Use of ssODNs such as Alt-R HDR Donor Oligos is recommended for small edits or insertions (<120 bp).
- High Cas9 editing efficiency maximizes the potential for HDR. When possible, avoid use of low-activity guides, as they will limit HDR rates. We recommend testing several guides within close proximity of your desired edit to screen out any low-efficiency guides prior to HDR experiments.
- The cleavage site for Cas9 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. (However, HDR outside this optimal spacing can be improved with optimized template designs.)
- Design ssODN templates with 30–60 nt homology arms extending from both the cut site and the desired HDR mutation. This delivers high levels of HDR for most small insertions.
- Test ssODN donors matching both strands of the genomic DNA when the HDR edit is at or near the cut site, as the preferred strand varies. When the HDR edit is >5–10 bases from the cut site, use the non-targeting strand for PAM-distal edits and the targeting strand for PAM-proximal edits.
- It is important to assess whether the gRNA target is disrupted by the desired HDR event. To prevent the undesired recleavage of a repaired HDR locus, design the HDR repair template with one or more silent mutations within the protospacer sequence and/or PAM site to mitigate this risk. When the HDR edit is >5–10 bases from the cut site, silent mutations placed on the same side of the DSB are preferred.
- Use the Alt-R HDR Design Tool to design HDR repair templates quickly and easily with recommended guides for your targets.

# Part III. Optimizing dsDNA templates for large HDR insertions using CRISPR-Cas9

## Introduction

While ssODNs such as Alt-R HDR Donor Oligos are an efficient solution for the generation of small precise edits, the introduction of longer knock-ins through CRISPR HDR can pose a challenge. Careful selection and design of the repair template can help improve HDR rates in such experiments. Here we present the benefits of using Alt-R HDR Donor Blocks, a novel modified linear dsDNA HDR template, and design considerations such as homology arm lengths and the disruption of Cas9 recutting for their use.

## Alt-R HDR Donor Blocks

Traditional templates for large HDR insertions >120 bp exist in a variety of formats and differ widely in the efficiency and accuracy of HDR, as well as in their cost, ease, and speed of synthesis. While plasmid donors offer the greatest flexibility in size and sequence complexity, their construction can be challenging and time consuming. Furthermore, their efficiency for HDR applications can be limited by high levels of toxicity [16]. Unmodified linear dsDNA templates, whether they be PCR amplicons or commercial solutions such as IDT gBlocks™ Gene Fragments, can be generated quickly but can lead to unintended edits through non-homologous (blunt) integration at any CRISPR-Cas9 or endogenous DSB [6, 7]. Finally, although viral template solutions are often high-efficiency and desirable for certain applications, their high cost and lengthy manufacturing times can pose challenges for researchers.

Due to these considerations, IDT researchers had previously recommended the use of long ssDNA templates, such as IDT Megamer™ Single-Stranded DNA Fragments, for longer knock-ins. While long ssDNA templates do avoid non-homologous integration into the genome, the enzymatic processes for producing these templates lead to long turnaround times, high costs, and limited yields for cell culture work. To better address the need for fast, cost-effective solutions for longer HDR knock-ins, IDT has developed Alt-R HDR Donor Blocks. These modified linear dsDNA HDR templates add universal terminal sequences to the ends of the donor that contain a proprietary modification pattern. These modifications are designed to increase HDR rates and reduce the occurrence of non-homologous integration at on- or off-target DSBs (Figure 12A-B), while the universal terminal sequences allow for consistent synthesis with a faster turnaround time and are not incorporated into HDR edits.

When compared to long ssDNA donors or unmodified dsDNA, Alt-R HDR Donor Blocks increase HDR rates while mitigating the risks of non-homologous integration associated with linear dsDNA (Figure 12A). Applying the Alt-R HDR Enhancer V2 to the cells can be used to drive the desired repair outcome further (Figure 12B and C). Additionally, use of long-read sequencing has improved interrogation of repair outcomes associated with large insertions [7]. While further improvements to this analysis method are ongoing, this approach has shown that long ssDNA templates are prone to partial integrations, where the 5′ junction relative to the donor molecule is incorporated at a lower rate (Figure 12D). In contrast, these partial integrations are not observed with the double-stranded Alt-R HDR Donor Blocks.

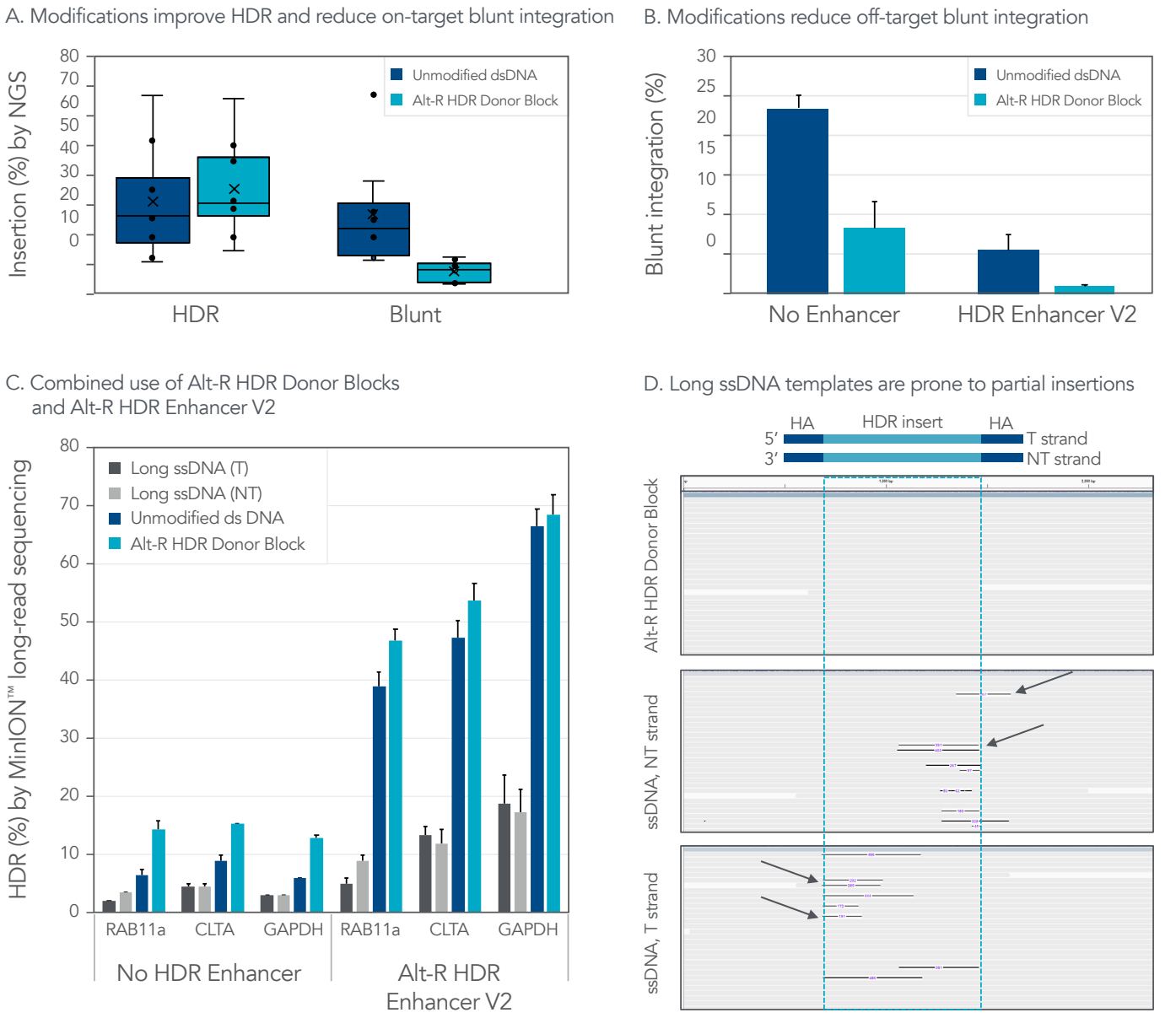



**Figure 12. Alt-R modified dsDNA templates result in higher HDR rates compared to unmodified dsDNA or long ssDNA with reduced rates of non-homologous integration.**(**A**) HDR templates introducing a 42 bp insert (40 bp HA) at 4 genomic loci were generated as either unmodified or Alt-R modified dsDNA. 2 µM Cas9 RNP and 250 nM dsDNA templates were electroporated into K562 and HEK293 cells using the Nucleofector™ System (Lonza). Genomic DNA was isolated from cells after 48 hrs and targeted amplification with rhAmpSeq was used to enrich the loci of interest. Following Illumina MiSeqTM 2x250 sequencing, data were analyzed via the CRISPAltRations™ data analysis pipeline (n=8). The results show that Alt-R modifications improve HDR rates and reduce on-target blunt integration. (**B**) A mock off-target DSB was generated by delivering 2 µM Cas9 RNP targeting the *SERPINC1* locus into HEK-293 cells as described above. dsDNA donor templates (50 nM) mediating GFP insertions at four alternative genomic loci were co-delivered with the mock off-target RNP. After electroporation, cells were plated in media with or without 1 µM Alt-R HDR Enhancer V2 with a media change after 24 hours. Genomic DNA was isolated 48 hours after electroporation. The SERPINC1 locus was PCR-amplified, and blunt insertion events were measured by size discrimination on a Fragment Analyzer (Agilent), n=4. The results show that combined use of Alt-R HDR Donor Blocks and Alt-R HDR Enhancer V2 reduces the rate of non-homologous (blunt) integration at off-target DSBs. (**C**) HEK-293 and K562 cells were electroporated with 2 µM Cas9 RNP complexes targeting three genomic loci along with 50 nM dsDNA or ssDNA donor templates using the Nucleofector™ System (Lonza). HDR templates were designed to mediate a GFP-tagging event (700 bp insert, 200 bp homology arms). dsDNA templates contained either no modifications (unmodified) or the Alt-R HDR Donor Block modification. Both the targeting (T) and non-targeting (NT) strands were tested for the long ssDNA templates. After electroporation, cells were plated in media with or without 1 µM Alt-R HDR Enhancer V2 with a media change after 24 hours. Genomic DNA was isolated 48 hours after electroporation. Editing was assessed by long-read amplicon sequencing on the MinION™ system (Oxford Nanopore Technologies) and processed using an internal analysis pipeline (n=4). The results show that Alt-R HDR Donor Blocks and Alt-R HDR Enhancer V2 provide the highest rates of HDR when compared to long ssDNA and unmodified dsDNA templates. (**D**) Repair events at the *CLTA* locus were assessed by PacBio SMRT™ long-read sequencing. HDR aligned reads were visualized using Integrative Genomics Viewer (IGV) to assess integration rates across the full HDR insert. While primarily complete HDR insertion events were observed for the dsDNA donors, use of long ssDNA donors resulted in partial HDR insertion events in which incomplete integration was observed at the 5′ junction of the ssDNA donor (arrows).

As with most CRISPR editing, blunt integration rates vary between sites and cell lines or systems. The data presented in Figure 12B represent an exaggerated case for off-target integration events, as the total editing at the mock off-target site was >90%. Blunt integration events at true Cas9 off-targets will be impacted by overall editing rates at these sites. Blunt integration at endogenous DSBs is expected to be a rare event. Any strategies that reduce the risk for Cas9 off-target editing (such as the use of Alt-R HiFi Cas9 Nuclease) will further mitigate the risk for off-target integration. Use of an NHEJ-inhibitory compound such as the Alt-R HDR Enhancer V2 will similarly reduce the risk for blunt integrations. Combined use of Alt-R HDR Donor Blocks, HiFi Cas9, and HDR Enhancer V2 will offer the lowest probability of unintended non-homologous integration while offering the highest rates of HDR.

# Optimal homology arm length

To investigate optimal homology arm lengths when using dsDNA HDR templates, we systematically varied the homology arm length for Alt-R HDR Donor Blocks for different size insertions at multiple genomic loci (Figure 13A). We first investigated the efficiency of incorporating a 700 bp or 2000 bp insert with homology arms ranging from 40 to 500 bp. Donors were delivered at either equal molar or equal mass amounts, as the delivery of dsDNA is often limited by mass due to toxicity. Due to this limitation, we focused on the results obtained with donors delivered at an equal mass. HDR rates plateaued at the 300 bp homology arm length for both large insertion sizes. We then assessed HDR efficiency for generating 120 or 500 bp inserts at three genomic loci with homology arm lengths varying from 40 to 300 bp. HDR rates plateaued at 100–200 bp homology arm lengths for the shorter insertions. Taking these findings together, we recommend 200–300 bp lengths as a default when designing Alt-R HDR Donor Blocks, but optimal lengths may vary with site, desired insertion, or system.

In addition to HDR rates, we assessed on-target blunt integration for these experiments (Figure 13B). We observed a decrease in blunt integration as the homology arm length increased and as the total donor length increased. Use of the Alt-R HDR Enhancer V2 reduced the on-target blunt integration rates to near background levels, regardless of homology arm length.

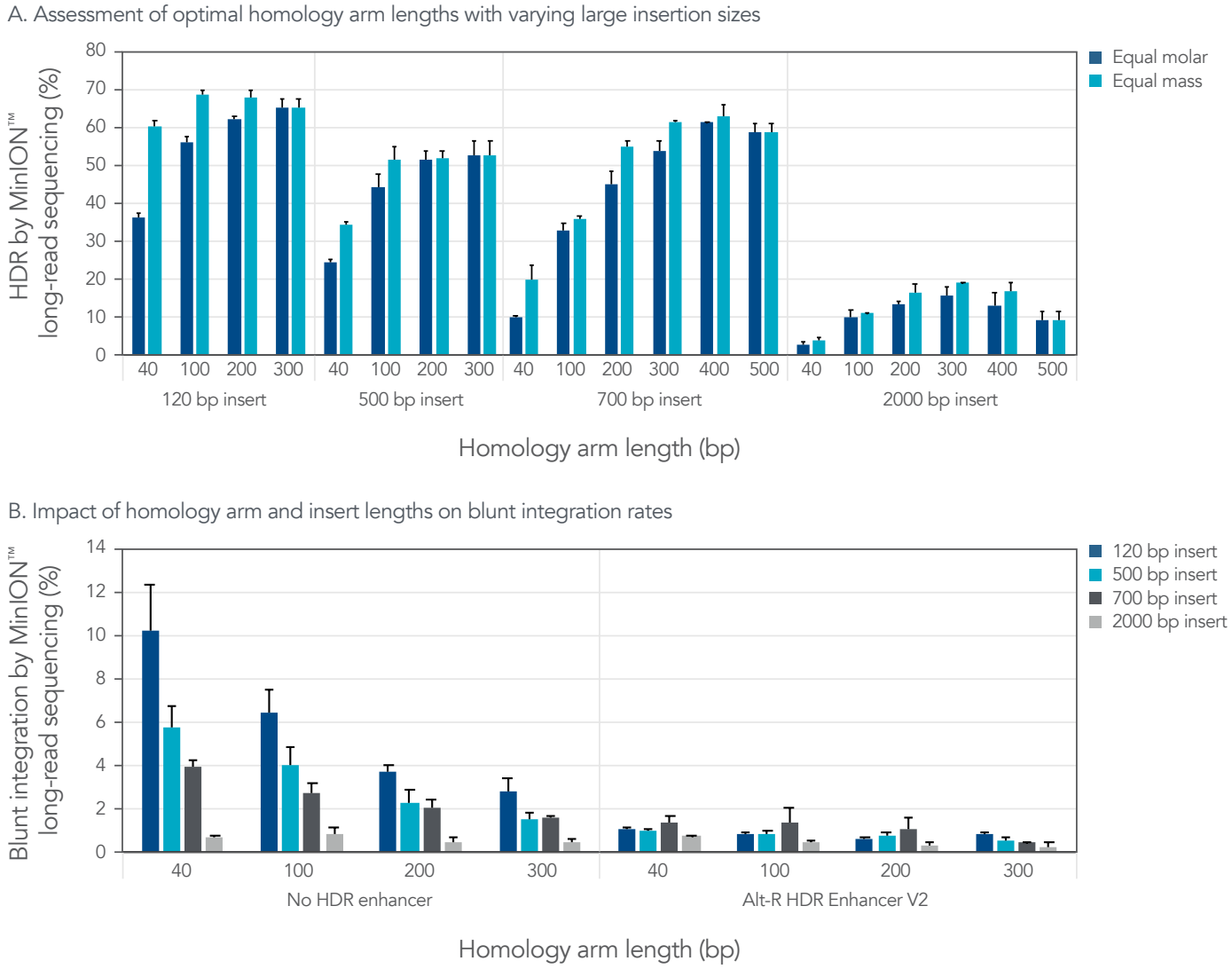



**Figure 13. Homology arm lengths of 200–300 bp result in the highest HDR efficiency with using Alt-R HDR Donor Blocks.** (**A**) K562 cells were electroporated with 2 µM Cas9 RNP complexes (targeting distinct genomic loci) and Alt-R HDR Donor Blocks mediating 120 or 500 bp (n = 3 genomic loci each) and 700 or 2000 bp insertions (n = 1 genomic locus each) using the Nucleofector™ System (Lonza). Donor templates were designed with homology arm lengths varying from 40 bp up to 500 bp and were delivered at equal molar or equal mass amounts (100 nM or 1.2 µg for 120 bp insert, 50 nM or 0.9 µg for 500 bp insert, 50 nM or 1.5 µg for 700 bp insert, and 25 nM or 1.3 µg insert for 2 kb insert). After electroporation, cells were plated in media with or without 1 µM Alt-R HDR Enhancer V2 with a media change after 24 hours. Genomic DNA was isolated 48 hours after electroporation. Editing was assessed by long-read amplicon sequencing on the MinION™ system (Oxford Nanopore Technologies) and processing using an internal data analysis pipeline. HDR rates shown represent cells with Alt-R HDR Enhancer V2 (n=2 per site). (**B**) On-target blunt insertion rates were assessed following long read sequencing (n=2 per site).

# Disruption of Cas9 recutting when guide selection is limited

In cases where guide selection is limited, disrupting Cas9 recognition sequences in the Alt-R HDR Donor Blocks increases the efficiency of HDR. Silent mutations placed within the protospacer sequence or PAM serve a dual purpose by preventing both the premature degradation of the dsDNA template and the recutting of the genomic DNA following correct HDR insertion. As with ssODN HDR templates, the preferred placement of silent mutations is dependent on the location of the desired insertion event relative to the Cas9 PAM site (Figure 14). When a 300 bp insert was introduced 20 bp from the cut site on the PAM-distal side, silent mutations placed within the protospacer sequence (i.e., repair track mutations) were more effective at promoting HDR. A single PAM mutation did boost HDR rates over an unaltered template but did not perform as well as protospacer mutations. When the same insert was placed on the PAM-proximal side with similar spacing, a PAM mutation was sufficient for disrupting Cas9 recognition and increasing HDR. In contrast, when the 300 bp insert was placed at the cut site and disrupted Cas9 recognition, no benefit to HDR was observed when a PAM mutation was included in the donor.

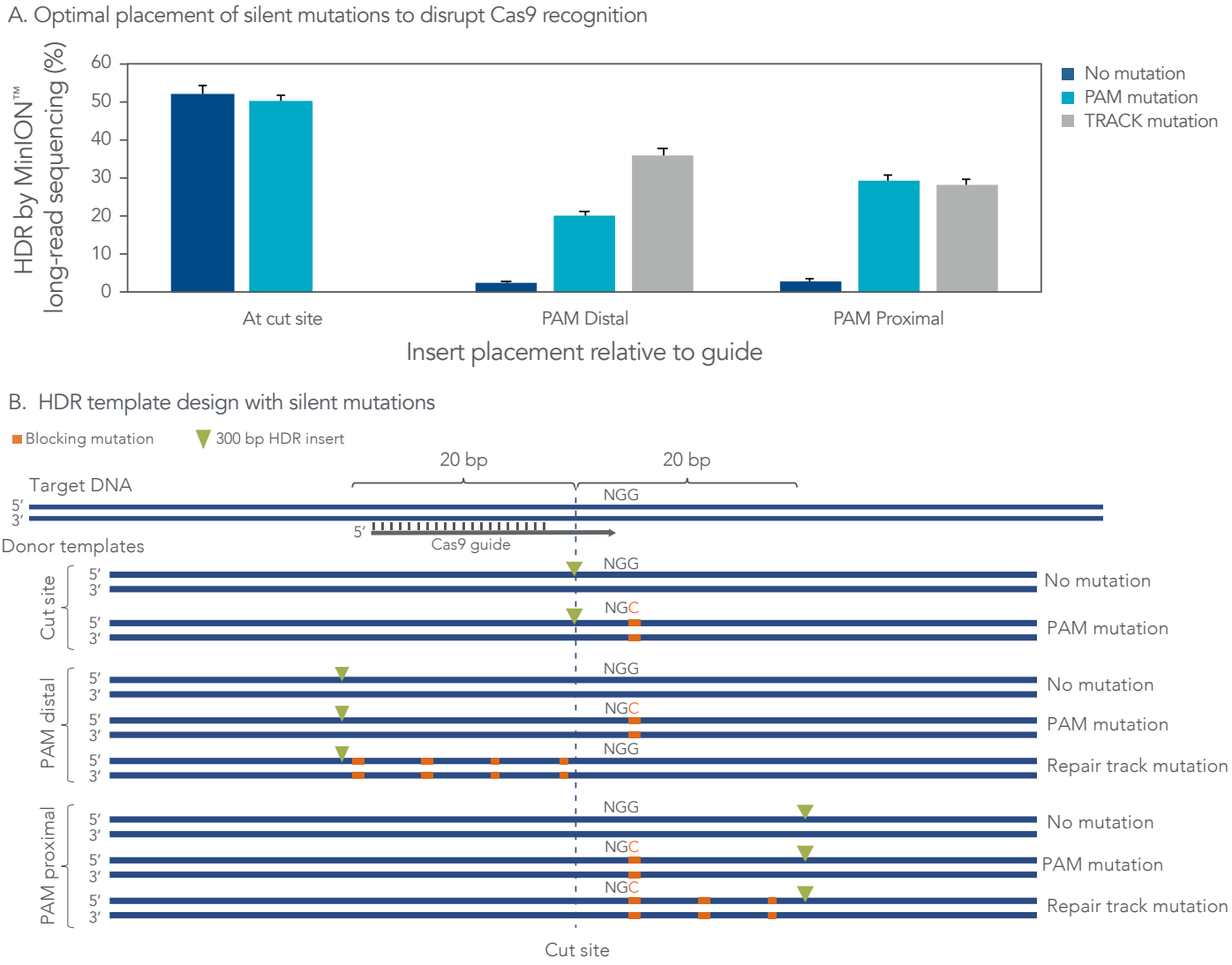



**Figure 14. Silent mutations placed on the same side of the DSB as the desired HDR edit result in highest HDR rates.** (**A**) K562 cells were electroporated with 2 µM Cas9 RNP complexes (targeting five genomic loci) and 75 nM Alt-R HDR Donor Blocks mediating 300 bp insertion either directly at the Cas9 cut site or 20 bp upstream or downstream from the cut site using the Nucleofector™ System (Lonza). Donor templates were designed with 200 bp homology arm lengths and included no further mutations, a PAM mutation (PAM), or mutations in the repair track (Track). Repair track mutations were placed at 6-base intervals. After electroporation, cells were plated in media with or without 1 µM Alt-R HDR Enhancer V2 with a media change after 24 hours. Genomic DNA was isolated 48 hours after electroporation. Editing was assessed by long-read amplicon sequencing on the MinION™ system (Oxford Nanopore Technologies) and processing using an internal data analysis pipeline. HDR rates shown represent cells with Alt-R HDR Enhancer V2 (n=10). (**B**) Schematic representation of donor templates used to generate PAM-proximal and PAM-distal insertions 20 bases from a Cas9 cut site with no silent mutations, PAM mutations, or mutations in the repair track.

# Considerations for midsize insertions

For midsize insertions around 120 bp, it is possible to synthesize repair templates as either Alt-R HDR Donor Oligos or as Alt-R HDR Donor Blocks. Insertions of this size still allow for the use of the recommended homology arm lengths with ssODNs (30–60 nt). However, the previously described investigations into optimal homology arm lengths for dsDNA templates indicated a potential benefit to using longer 100 bp homology arms for this insertion size (Figure 13). Therefore, we compared the use of Alt-R HDR Donor Oligos and Alt-R HDR Donor Blocks when generating a 120 bp insert at three genomic loci (Figure 15). Both reagents were designed according to the homology arm (HA) length recommendations for each donor type and delivered at an optimal concentration (40 nt HA length and 2 µM for ssODNs; 100 bp HA length and 300 nM for dsDNA). For 2 out of 3 sites, the use of Alt-R HDR Donors with longer homology arms resulted in higher HDR rates. For the third site, HDR rates were relatively similar between the reagents. When the imperfect HDR events were further investigated, the occurrence of partial integration events with the ssODN templates could be observed (data not shown).

Further studies into optimal reagents for mid-size insertions are ongoing. As optimal reagents and conditions may vary between cell lines or systems, we do not currently recommend one reagent over the other. In most cases, we expect the HDR Donor Oligos will offer an efficient HDR solution with a faster turnaround time and lower cost. However, in challenging systems with low HDR rates, it may be helpful to assess the efficiency of HDR with both reagents to determine which is preferred.

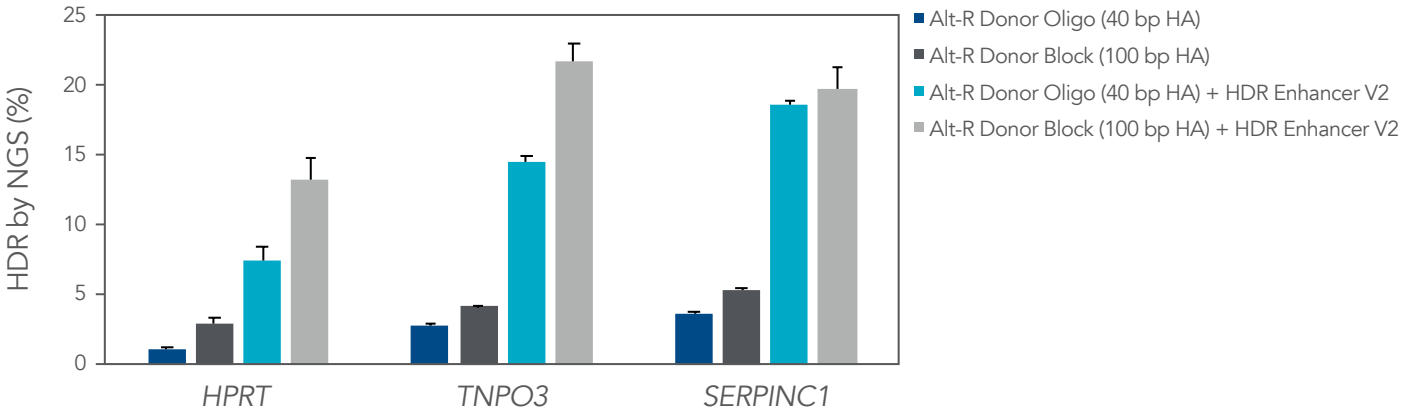



**Figure 15. Comparison between ssODN and dsDNA HDR templates for generating a 120 bp insertion.** HEK-293 cells were electroporated with 2 µM Cas9 RNP complexes (targeting four genomic loci) and either 2 µM Alt-R HDR Donor Oligos or 300 nM Alt-R HDR Donor Blocks mediating a 120 bp insertion using the Nucleofector™ System (Lonza). Donor oligos were designed as the non-targeting strand with 40 nt homology arms. Donor Blocks were designed with 100 bp homology arms. After electroporation, cells were plated in media with or without 1 µM Alt-R HDR Enhancer V2 with a media change after 24 hours. Genomic DNA was isolated 48 hours after electroporation. HDR efficiency was measured by amplicon sequencing on a MiSeq™ (Illumina) system (V2 chemistry, 250 bp paired-end reads), n=2.

## Tips for Alt-R HDR Donor Block design

- Linear double-stranded DNAs are high-efficiency HDR repair templates for large edits. End modifications can increase HDR rates and mitigate the risk of non-homologous blunt integration of the donor. As such, Alt-R HDR Donor Blocks are recommended for introducing large insertions (>120 bp).
- High Cas9 editing efficiency maximizes the potential for HDR. When possible, avoid use of low-activity guides as these will limit HDR rates. We recommend testing several guides within close proximity of your desired edit to screen out any low efficiency guides prior to HDR experiments.
- The Cas9 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. (However, HDR outside this optimal spacing can be improved with optimized template designs.)
- Design dsDNA templates with 200–300 bp homology arms extending from both the cut site and the desired HDR mutation. This delivers high levels of HDR for most large insertions. For mid-sized insertions (120–500 bp), use of shorter homology arms (100–200 bp) may still result in optimal HDR.
- It is important to assess whether the gRNA target is disrupted by the desired HDR edit. To prevent the undesired degradation of the dsDNA template and the recleavage of any HDR events, design the HDR repair template with one or more silent mutations within the protospacer sequence and/or PAM site to mitigate recutting.
- Use the Alt-R HDR Design Tool to design HDR repair templates quickly and easily along with guides for your targets.

# Part IV. Considerations for delivery and evaluation of HDR editing

## Delivery of Alt-R CRISPR and HDR reagents

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 complexed with Alt-R CRISPR-Cas9 sgRNA or crRNA and tracrRNA, concurrent with the HDR repair template. This combination provides reliable genome editing at most target sites. Using RNP for delivery reduces off-target effects that can occur when Cas9 and the guide RNA are expressed from plasmid or viral templates. Figure 16A shows an example of successful HDR using an ssODN template with Cas9 in 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 used. We have developed protocols for Lonza Nucleofector™ delivery of CRISPR reagents in HEK-293 cells, and Neon™ (Thermo Fisher) electroporation delivery of CRISPR reagents in Jurkat T cells. These protocols can also be used as a starting point for optimization in your own HDR experiments. As shown in Figure 16B, high levels of HDR can be achieved by electroporation. Note that HDR efficiency with ssODN templates increased significantly in the presence of Alt-R Cas9 Electroporation Enhancer, a purified DNA that is thought to act as a carrier to improve delivery of Cas9 RNP in electroporation applications. Alt-R Cas9 Electroporation Enhancer is usually not needed when using Alt-R HDR Donor Blocks, since the dsDNA template is typically sufficient to act as a carrier for improved Cas9 delivery, and the addition of Electroporation Enhancer may increase cytotoxicity. However, this decision may require optimization for individual cell lines, as the use of Electroporation Enhancer may still be beneficial with some cells that are difficult to transfect.

Finally, Alt-R HDR Enhancer V2 can be used to substantially increase HDR efficiency. Alt-R HDR Enhancer V2 is an improved low-molecular-weight compound that inhibits NHEJ, tilting the balance of DNA repair pathways in favor of HDR. As shown in Figures 6B and 12C, use of this enhancer is highly beneficial for generating both small and large HDR edits and further reduces the potential for off-target integrations of linear dsDNA repair templates (Figure 12B). For use in cell culture applications, a concentration within the range of 1–2 µM in the final media with a change to fresh media without the enhancer after 12–24 hours gives high HDR rates (Figures 12B–C).

In biological systems other than mammalian tissue culture, microinjection is another 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 by external collaborations, are also available.

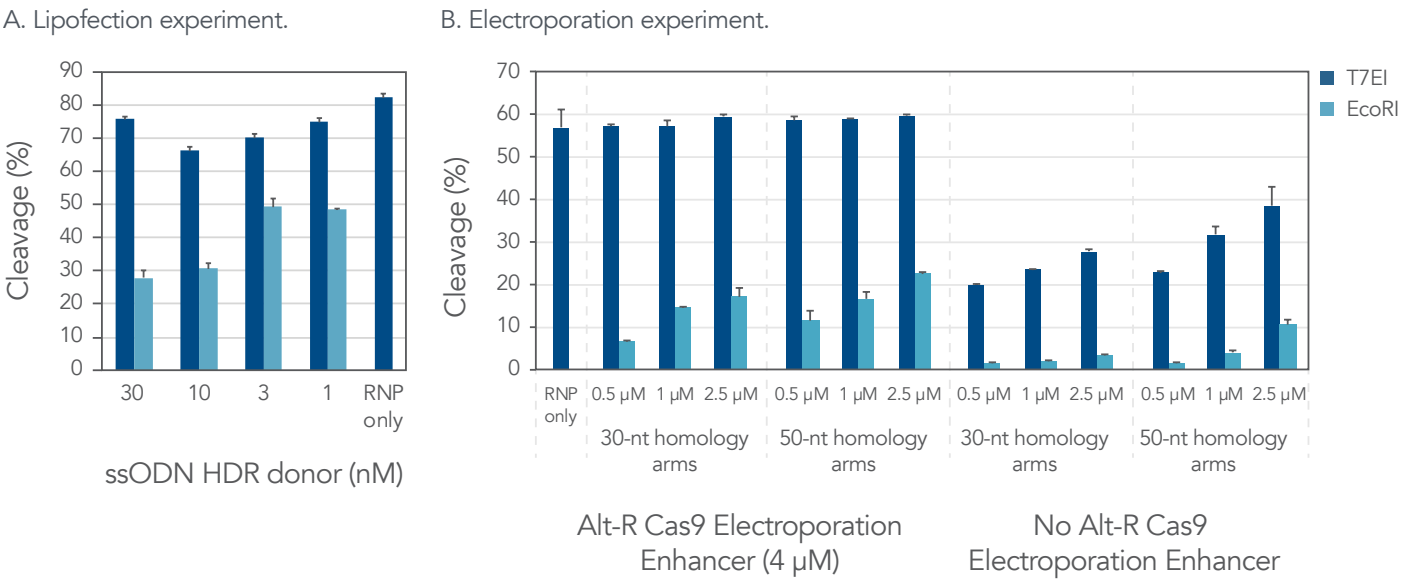



**Figure 16. Reliable HDR in tissue culture using lipofection or electroporation.** (**A**) Lipofection experiment. HEK-293 cells were transfected with 10 nM Alt-R CRISPR-Cas9 RNP using 1.2 µL Lipofectamine™ RNAiMAX Transfection Reagent (Thermo Fisher) along with varying concentrations of ssODN donor 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), n=3. (**B**) Electroporation experiment. Alt-R CRISPR-Cas9 RNPs, together with varying amounts of ssODN to target the human gene *HPRT1*, were delivered into HEK-293 cells at a concentration of 4 µM via electroporation using a Nucleofector system (Lonza). Electroporation reactions contained either 4 µM Alt-R Cas9 Electroporation Enhancer, or no electroporation enhancer. ssODN templates with homology arm lengths of 30 or 50 nt were tested. Genomic DNA was isolated 48 hr after delivery. Total editing efficiency and HDR rate were ssessed by T7EI and EcoRI digestion, respectively (n=3).

## Guidelines for HDR template delivery

**Lipofection:** When performing lipofection with ssODN templates, we recommend the use of RNAiMAX™ or CRISPRMAX™ (Thermo Fisher) with 10 nM RNP and 1–3 nM of Alt-R HDR Donor Oligos. Prepare the RNP and then add the ssODN HDR template before combining with the lipofection reagent for addition to cultured cells. Refer to the **Alt-R CRISPR-Cas9 User Guide—RNP transfections** for the recommended lipofection experimental setup and protocol.

**Note:** Lipofection is typically not used for long templates such as Alt-R HDR Donor Blocks due to high toxicity and poor delivery.

**Electroporation:** Guidelines for electroporating CRISPR reagents depend on the electroporation instrument. IDT scientists have published protocols for use with both the **Nucleofector™ System (Lonza)** and the **Neon® Transfection System (Thermo Fisher)**. The RNP complex should be formed before addition of the HDR repair template.

For HDR mediated by the CRISPR-Cas9 system, add Alt-R HDR Donor Oligos 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 **Homology-directed repair using the Alt-R™ CRISPR-Cas9 System and HDR Donor Oligos User Guide** for the recommended experimental setup and protocol.

For delivery of long templates such as Alt-R HDR Donor Blocks, optimal donor concentrations will vary between cell lines. Due to recognition of cytosolic dsDNA by innate immune response pathways, the final delivery amount is often limited by total mass; careful consideration of the final Alt-R HDR Donor Block concentration is recommended to avoid cytotoxicity. For initial optimization with Lonza Nucleofection, we recommend testing titrations within the range of 0.5–2 µg amounts. Refer to the **Homology-directed repair using the Alt-R™ CRISPR-Cas9 System and Alt-R HDR Donor Blocks User Guide** for experimental setup and protocol.

**Microinjection:** Microinjection of murine zygotes with Alt-R CRISPR RNP complexes shows higher HDR rates compared to other strategies, such as co-delivery of sgRNA and Cas9 mRNA, due to the immediate availability of functional RNP complexes following microinjection. Successful HDR was achieved with the use of 5–20 ng/µL of ssDNA templates [17]. IDT hosts several **demonstrated protcol** for CRISPR delivery in other systems that may be adapted for HDR.

# Evaluation of HDR editing

CRISPR genome editing takes place within 48–72 hr in the cell lines we have tested. Typically, we isolate genomic DNA 48 hr after the delivery of CRISPR-Cas9 reagents and assess the overall editing efficiency of both NHEJ and HDR pathways using an NGS-based approach or 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 a detection approach that we used repeatedly throughout this study. See Figures 3A, 4A, 4C, 7A, and 16A–B, for examples.

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 confirm the absence of off-target sites, cells that undergo genome engineering should be fully characterized by NGS methods. The new rhAmpSeq™ CRISPR Analysis System supports NGS analysis of CRISPR-Cas editing on Illumina® sequencing platforms. Our proprietary RNase H2 dependent PCR technology is used to generate amplicon libraries for targeted sequencing, while our advanced, cloud-based data analysis pipeline is used for quantification of NHEJ, perfect HDR, imperfect HDR, and microhomology-mediated end joining (MMEJ) editing [18]. This system provides a fully supported workflow for the analysis of short HDR edits.

   Long-read sequencing offers new and developing solutions for the analysis of large HDR knock-ins. This approach allows for the detection and characterization of varying repair events, including correct HDR, partial HDR insertions, blunt integration, and large deletions, and can be used to gather phased information regarding repair events from a pool of edited cells. Long-read sequencing is a method repeatedly utilized throughout this study using instruments from Oxford Nanopore Technology or PacBio SMRT™ long-read sequencing and an internal analysis pipeline.

# 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.

# Summary:

- Optimizing HDR experimental conditions is critical because of the generally lower baseline rate of HDR compared to the error-prone NHEJ pathway.
- Because HDR efficiency differs from one site to the next, multiple guides within your target region should be tested whenever possible. Select the most active gRNA design that cuts as close as possible to the intended insertion site.
- Select HDR templates according to the size of your desired insertion. Alt-R HDR Donor Oligos are recommended for small edits (SNPs or insertions <120 bp). Alt-R HDR Donor Blocks are recommended for large insertions (>120 bp).
- HDR templates should be rationally designed to improve HDR efficiency. The IDT HDR Design Tool will incorporate default homology arm lengths based on your donor type and can automatically select silent mutations to disrupt Cas9 recutting based on empirical data.
- Delivery may require extensive optimization in certain biological systems. Electroporation done in conjunction with the use of Alt-R Cas9 Electroporation Enhancer and Alt-R HDR Enhancer V2 can increase HDR rates in cultured cells.
- Consider what strategy to use for HDR evaluation as early as possible. Ideally, this would mean starting from the experimental planning phase.

## Contributors

Jessica Woodley, Mollie Schubert, Bernice Thommandru, Shuqi Yan, Michael Collingwood, Ashley Jacobi, Garrett Rettig, Maureen Young, Hans Packer, Ellen Prediger, Brian Wang

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Technical support: applicationsupport@idtdna.com