Using CRISPR-Cas9 HDR positive controls

Alt-R™ CRISPR-Cas9 HDR positive controls are experimentally confirmed controls that consist of both a guide RNA (available as the 2-part crRNA:tracrRNA, or the single guide sgRNA), and a single-stranded HDR donor template.

The provided guides target either the *AAVS1* (human), or *Rosa26* (mouse), safe-harbor locus to create a CRISPR-Cas9–mediated double-stranded break. The provided HDR donor templates will introduce a six-base insertion (EcoRI restriction enzyme recognition sequence) at the CRISPR-Cas9 cleavage site in cells that have undergone HDR. The guide and HDR donor template sequences for both human- and mouse-specific designs are shown in **Table 1** with the EcoRI insertion sequence indicated in red.

Table 1. Positive control guide RNA and HDR donor templates.*

Species	Guide (spacer sequence)	HDR donor†	Edit location
Human (*Homo sapiens*)	CCTCTAAGGTTTGCTTACGA	AGCCATCTCTCTCCTTGCCAGAACCTCT AAGGTTTGCTTAGAATTCCGATGGAGC CAGAGAGGATCCTGGGAGGGAGAGCT TGGCA	chr19:55115594
Mouse (*Mus musculus*)	TAACAACTCAGAGCGACTTT	CTCCACTGCAGCTCCCTTACTGATAACA ACTCAGAGCGACGAATTCTTTGGGAGA GCAAGTGCTTCCTGCCTCCAAAACAGC CCAA	chr6:113076232

* Positive controls (guide RNA and HDR donor template) are available at **www.idtdna.com/HDRDonorOligos**.

† GAATTC = EcoRI recognition site

Confirmation of insertion

CRISPR genome editing takes place within 48–72 hr in the cell lines we tested. Typically, we collect genomic DNA 48 hr after the delivery of CRISPR-Cas9 reagents, then assess the overall editing efficiency of both NHEJ and HDR pathways.

For the quantification of HDR events, one of the following three methods can be employed:

1. **Restriction fragment length polymorphism (RFLP) assay**

   Insertion of a restriction enzyme recognition sequence facilitates the subsequent confirmation of successful HDR using RFLP analysis. Primers should be carefully designed to prevent unwanted amplification of donor DNA, which has the potential to lead to false positive results. After PCR amplification, the restriction enzyme EcoRI can be used to enzymatically digest the PCR product, and the frequency of insertion can then be estimated by measuring the resulting fragments.

Table 2. Primers used to amplify the *AAVS1* or *Rosa26* region by PCR during an RFLP assay.

Target	Primer	Sequence*	Full-length (bp)	Fragment 1 (bp)	Fragment 2 (bp)
Human *AAVS1*	Forward	GCCAAGGACTCAAACCCAGA	1037	561	476
	Reverse	CCCCGTTCTCCTGTGGATTC			
Mouse *Rosa26*	Forward	CGAGGCGGATCACAAGCAAT	571	431	140
	Reverse	GTGCAAGCACGTTTCCGACT			

* Order these primers as custom oligos (access ordering from the **Oligo Entry** page at **www.idtdna.com/DNA**.)

2. **Sanger sequencing**

   To check the DNA sequence of your edited locus at the single-nucleotide level, use Sanger sequencing. This is a straightforward and cost-effective method to check on-target editing at a single locus. Various software programs are available online to analyze complex Sanger sequence traces representing samples of mixed sequences after CRISPR-Cas9–mediated editing.

3. **Next generation sequencing**

   Next generation sequencing (NGS) 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. NGS provides an efficient, quantifiable, and comprehensive approach for **measuring the levels of on- and off-target editing**.

Performance of HDR positive controls

HDR rates will vary with cell type and delivery conditions; HDR rates in several common cell lines are shown in **Figure 1.**

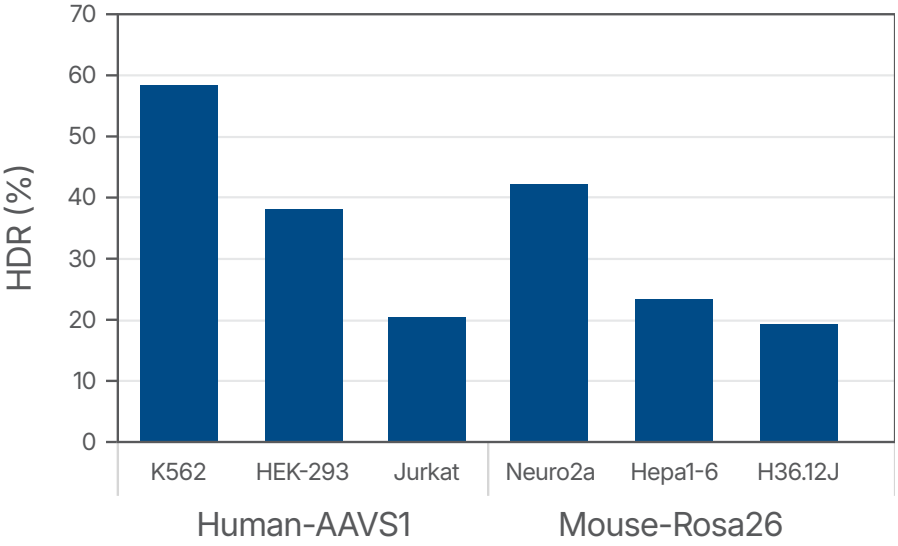

Figure 1. Representative positive control HDR rates in commonly used cell lines. RNP complexes (2 µM) consisting of Alt-R *S.p.* Cas9 Nuclease V3 complexed with Alt-R crRNA and tracrRNA targeting either the *AAVS1* or *Rosa26* safe-harbor locus were delivered by the 4D-Nucleofector™ System (Lonza). Alt-R 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 (Neuro2a, Hepa1-6, and H36.12J). N=1.

For more information, go to: **idtdna.com/ContactUs**

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