Improved specificity and efficacy of base-editing therapies with hybrid guide RNAs

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Abstract

Phenylketonuria (PKU), hereditary tyrosinemia type 1 (HT1), and mucopolysaccharidosis type 1 (MPSI) are autosomal recessive disorders linked to the phenylalanine hydroxylase (*PAH*) gene, fumarylacetoacetate hydrolase (*FAH*) gene, and alpha-L-iduronidase (*IDUA*) gene, respectively. Potential therapeutic strategies to ameliorate disease include corrective editing of pathogenic variants in the *PAH* and *IDUA* genes and, as a variant-agnostic approach, inactivation of the 4-hydroxyphenylpyruvate dioxygenase (*HPD*) gene, a modifier of HT1, via adenine base editing. Here we evaluated the off-target editing profiles of therapeutic lead guide RNAs (gRNAs) that, when combined with adenine base editors correct the recurrent *PAH* P281L variant, *PAH* R408W variant, or *IDUA* W402X variant or disrupt the *HPD* gene in human hepatocytes. To mitigate off-target mutagenesis, we systematically screened hybrid gRNAs with DNA nucleotide substitutions. Comprehensive and variant-aware specificity profiling of these hybrid gRNAs reveal dramatically reduced off-target editing and reduced bystander editing. Lastly, in a humanized *PAH* P281L mouse model, we showed that when formulated in lipid nanoparticles (LNPs) with adenine base editor mRNA, selected hybrid gRNAs revert the PKU phenotype, substantially enhance on-target editing, and reduce bystander editing *in vivo*. These studies highlight the utility of hybrid gRNAs to improve the safety and efficacy of base-editing therapies.

Introduction

In vivo CRISPR base editing is an emerging new therapeutic approach to efficiently and precisely make genomic alterations, including the direct correction of pathogenic variants, in a patient's body[1,2]. Early results from a clinical trial of base editing to inactivate the *PCSK9* gene

in patients with familial hypercholesterolemia have provided a strong proof of concept of the efficacy of adenine base editing in the human liver *in vivo*[3]. These results motivate the development of liver-centered base-editing therapies to address a wide variety of genetic disorders, such as inborn errors of metabolism. In previous studies, we have demonstrated the ability of *in vivo* base editing to definitively address three such disorders in postnatal or prenatal mouse models: (1) correction of the recurrent *PAH* P281L variant or *PAH* R408W variant in humanized mouse models of PKU[4,5]; (2) correction of the mouse ortholog of the recurrent *IDUA* W402X variant in a mouse model of MPSI[6]; and (3) inactivation of the mouse ortholog of the *HPD* gene in a mouse of model of HT1[7].

The eighth-generation adenine base editor, ABE8.8-m (ABE8.8), has now been validated in preclinical non-human primate studies and in the aforementioned clinical trial.[3,8,9] As with other adenine base editors, it can efficiently catalyze A→G edits on the sense or antisense strands of genes and correct a substantial proportion of human pathogenic variants. ABE8.8 also has among the narrowest editing windows of the eighth-generation adenine base editors, in contrast to adenine base editor 8.20-m (ABE8.20), which has one of the widest editing windows[10]. If the position of the target adenine base lies within the window of ABE8.8, a narrow window limits the potential for two undesirable consequences of base editing, bystander editing and off-target editing. Bystander editing of one or more adenine bases near the target adenine base can be counterproductive if it introduces new pathogenic variants alongside the desired corrective editing of the original pathogenic variant. Off-target editing, a general concern with any type of CRISPR editing, entails mutagenesis at genomic sites other than the desired on-target site. Although base editors largely avoid the liabilities of nuclease editing, such as large genomic deletions and chromosomal rearrangements induced by double-strand breaks, off-target base

edits in or near tumor suppressor genes and oncogenes could increase the risk of carcinogenesis. Thus, strategies besides the use of a narrow-window base editor that can simultaneously reduce off-target editing and bystander editing would be of substantial value in improving the safety of base-editing therapies.

In this work, we identified or confirmed clinical lead gRNAs that, in combination with ABE8.8, efficiently achieve therapeutically desirable edits in *HPD*, *PAH*, or *IDUA* in human hepatocytes *in vitro*. We subjected these gRNAs to off-target screening analyses and verified several genomic sites with bona fide off-target mutagenesis in hepatocytes. To reduce the off-target editing at these sites, we explored a previously reported strategy, the use of hybrid gRNAs in which certain positions of the spacer sequence are substituted with DNA nucleotides[11–17]—a strategy compatible with the use of mRNA-LNPs for *in vivo* delivery. In systematically assessing the effects of series of hybrid gRNAs on the off-target profiles of clinical lead gRNAs, we unexpectedly observed that hybrid gRNAs have the potential to reduce bystander editing as well. Importantly, we found that hybrid gRNAs that eliminated off-target editing by the lead *PAH* P281L-correcting ABE8.8/gRNA combination *in vitro* did so without compromising *in vivo* efficacy in a humanized PKU mouse model; on the contrary, they substantially increased the desired corrective editing in the liver while simultaneously reducing unwanted bystander editing.

Results

Candidate therapeutic lead guide RNA sequences for three human diseases

In a previous study, we characterized a gRNA (herein termed "PAH1") targeting the human-specific protospacer sequence TCACAGTTCGGGGGTATACA, with the corresponding protospacer-adjacent motif (PAM) TGG, to correct the *PAH* P281L variant[4]. The variant adenine

base lies in position 5 of the protospacer sequence. A potential bystander adenine base lies nearby in position 3; bystander A→G editing would result in a splice site disruption that has been reported to be pathogenic for PKU[18] and thus would be undesirable. In combination with ABE8.8, the PAH1 gRNA results in efficient corrective editing of the P281L variant *in vitro* (in HuH-7 hepatocytes homozygous for the P281L variant, derived from the HuH-7 human hepatoma cell line) and *in vivo* (in mouse liver), with much lower but still sizeable levels of bystander editing[4]. Because of the ability of ABE8.8/PAH1 LNPs to normalize the elevated blood phenylalanine (Phe) levels that are the hallmark PKU phenotype observed in humanized P281L mice, PAH1 represents our therapeutic lead gRNA for a potential treatment for PKU patients with at least one copy of the *PAH* P281L variant.

In a parallel study, we characterized a gRNA (herein termed "PAH4") that corrects the *PAH* R408W variant *in vitro* and *in vivo*[5]. Because there are no NGG PAMs situated appropriately for adenine base editing of the *PAH* R408W variant, we screened PAM-altered and PAM-relaxed versions[19] of adenine base editors and identified an optimal combination of SpRY-ABE8.8 and the protospacer sequence GGCCAAGGTATTGTGGCAGC with an AAA PAM. The variant adenine base lies in position 5 of the protospacer sequence. Potential bystander adenine bases lie in positions 6 and 10; bystander A→G editing at position 6 would result in a benign synonymous variant, whereas editing at position 10 would result in a nonsynonymous variant that has been reported to be a pathogenic variant for PKU[20,21].

Lacking any previous data regarding adenine base editing to inactivate the *HPD* gene—our previous study assessed only cytosine base editing—we screened for precise disruption of a splice donor or acceptor site in the human *HPD* gene by adenine base editing. We sought to implement a high-throughput screening platform in which to introduce a base editor in

combination with a variety of gRNAs and rapidly evaluate the relative activities of the gRNAs in the target cell type of interest, primary human hepatocytes. We used lentiviral vectors harboring an expression cassette for an adenine base editor and a combination of an expression cassette for an individual gRNA and a genomic fragment that (1) spanned the potential target sequence of that gRNA, (2) included a unique barcode, and (3) was flanked by fixed sequences that allowed for PCR amplification with a common set of primers. For the screen, we chose ABE8.20, being a high-activity eighth-generation adenine base editor with a wide editing window (protospacer positions 3 to 9)[10] that permitted the greatest flexibility in gRNA selection for the disruption of splice donors (editing of the antisense A in the second position of the GT motif) or splice acceptors (editing of the sense A in the first position of the AG motif) in the *HPD* gene. We selected 16 gRNAs matched to protospacer sequences in the human *HPD* gene and with NGG PAMs, targeting splice sites ranged across the entire *HPD* gene (**Fig. 1a**). After production of the lentiviral library, we infected primary human hepatocytes with three doses (high:medium:low ratio of 25:5:1). After three days, we extracted genomic DNA, amplified the target sequences/barcodes from the genome-integrated lentiviral cassettes, and quantified editing by each gRNA with next-generation sequencing. We observed the highest levels of editing with gRNAs targeting either the splice acceptor of exon 7 or the splice donor of exon 8 (**Fig. 1a**). We prioritized the gRNAs designated “HPD3”, “HPD4”, “HPD5”, and “HPD20” (HPD3, HPD4, and HPD5 target consecutive protospacers shifted by one nucleotide) due to their efficient editing and because their protospacer sequences are a perfect match to the cynomolgus monkey ortholog of *HPD*, which would enable large animal studies using the same drug candidate intended for use in human patients.

Noting that the best-performing *HPD*-targeting gRNAs in combination with ABE8.20 achieved editing of adenine bases towards the middle of the editing window (positions 4 through 7), for our subsequent studies we switched to ABE8.8 due to its narrow editing window. We retested the HPD3, HPD4, HPD5, and HPD20 gRNAs in combination with ABE8.8, now using plasmid transfection of ABE8.8 and gRNA in wild-type HuH-7 hepatocytes (as an orthogonal method to lentiviral infection of primary human hepatocytes) (**Fig. 1b**). All demonstrated efficient editing of their target splice site adenine, with HPD20 performing the best. Of note, all gRNAs demonstrated bystander editing, but such editing is expected to be of little consequence with respect to the inactivation of the *HPD* gene.

In a previous study, we identified a single mouse-specific gRNA targeting a protospacer with an NGG PAM that is appropriately positioned to correct the *Idua* W392X variant ("Idua1"), which is the mouse ortholog of the human *IDUA* W402X variant[6]. When delivered by adeno-associated viral (AAV) vectors, that gRNA in combination with ABE8.8 results in efficient corrective editing of the *Idua* W392X variant *in vivo* and *in utero* in mouse models of MPSI, improving survival and a variety of metabolic phenotypes. The corresponding human-specific gRNA for correction of the *IDUA* W402X variant (herein termed "IDUA1") targets the protospacer sequence GCTCTAGGCCGAAGTGTCGC with an AGG PAM. The variant adenine base lies in position 6 of the protospacer sequence, with no nearby adenine bases that are at risk for bystander editing. To ascertain the viability of IDUA1 as a clinical candidate, we used prime editing to generate a clonal HuH-7 hepatocyte cell line homozygous for the *IDUA* W402X variant. We transfected W402X HuH-7 cells with ABE8.8 and IDUA1 gRNA and observed efficient corrective editing of the W402X variant, with no bystander editing (**Fig. 1c**).

Accordingly, IDUA1 represents our therapeutic lead gRNA for a potential treatment for MPSI patients with at least one copy of the *IDUA* W402X variant.

Initial off-target analyses of candidate therapeutic lead guide RNA sequences

Most CRIPSR-based off-target assay techniques are designed to detect double-strand breaks in DNA, whether *in cellulo* or *in vitro*. However, base editors like ABE8.8 do not directly make double-strand breaks, and so conventional off-target assays such as GUIDE-seq do not accurately reflect off-target editing by base editors. We therefore utilized an ABE-tailored version of OligoNucleotide Enrichment and sequencing (ONE-seq) to nominate off-target sites[8,22], followed by genomic sequencing to verify these candidate sites. ONE-seq is a biochemical assay that uses a synthetic human genomic library selected by sequence similarity to the protospacer DNA sequence targeted by the gRNA—typically, up to 6 mismatches, or up to 4 mismatches plus up to 2 bulges, to sequences anywhere in the reference human genome. For any given gRNA, the library can comprise tens of thousands of oligonucleotides. The library is mixed *in vitro* with a ribonucleoprotein (RNP) comprising recombinant adenine base editor protein complexed with the synthetic gRNA. The RNP nicks certain oligonucleotide sequences on one strand and deaminates an adenine base on the other strand. EndoV is used to cleave the other strand adjacent to the deaminated base, resulting in the equivalent of a double-strand break. Next-generation sequencing (NGS) then quantifies the frequency with which each unique oligonucleotide sequence was edited *in vitro*, generating a rank-ordered list of candidate off-target sites, normalized to the on-target site—which receives a ONE-seq score of 1.0. Typically, the on-target site is at or near the top of the list. A site with a ONE-seq score of 0.01 has one-hundredth the *in vitro* editing activity of the on-target site.

For the ABE8.8/PAH1 combination, we had previously performed a preliminary ONE-seq analysis with a library of ≈25,000 oligonucleotides, followed by targeted amplicon sequencing of ≈50 top-ranked sites that verified one genomic site of low-level off-target mutagenesis in HuH-7 hepatocytes.[4] We now undertook a deeper analysis of the ONE-seq rank-ordered list, using hybrid capture sequencing to assess the entire set of 280 genomic sites with ONE-seq scores greater than 0.01 (**Supplementary Table 1**). We verified an additional six sites of off-target mutagenesis in ABE8.8/PAH1 mRNA/gRNA-transfected P281L HuH-7 hepatocytes (**Fig. 1d**). None of the seven sites raises a concern of oncogenic risk. The first site (PAH1_OT1) has 3 mismatches with the on-target protospacer, with an NGG PAM, and is in an intron of the *SPAG17* gene, which encodes a microtubule protein. PAH1_OT2 has 5 mismatches with the on-target protospacer, with an NGA PAM, and is in an intron of the *STYXL2* gene, which encodes a myosin-interacting protein. PAH1_OT3 has 2 mismatches and 2 consecutive bulges compared with the on-target protospacer, with an NGG PAM, and is in an intron of *LINC00882*, a long noncoding RNA. PAH1_OT4 has 6 mismatches with the on-target protospacer, with an NGG PAM, and is in an intron of DCLK1, which encodes a microtubule-associated protein. PAH1_OT5 and PAH1_OT6 each have 4 mismatches with the on-target protospacer, with an NGG PAM, and is intergenic and distant from any gene. PAH1_OT7 has 4 mismatches and 2 consecutive bulges compared with the on-target protospacer, with an NGG PAM, and is in an intron of *KCNJ10*, which encodes a potassium channel protein. None of the five implicated genes appear to be significantly expressed in the liver.

To evaluate the ABE8.8/HPD3, ABE8.8/HPD4, ABE8.8/HPD5, and ABE8.8/HPD20 combinations, we used two ONE-seq libraries: a combined library of ≈130,000 oligonucleotides for the cluster of closely spaced HPD3, HPD4, and HPD5 protospacers, and a library of ≈65,000

oligonucleotides for the HPD20 protospacer. Targeted amplicon sequencing of ≈50 top-ranked sites in ABE8.8/gRNA-treated wild-type HuH-7 hepatocytes verified one genomic site of off-target mutagenesis each for HPD3 and HPD4, and none for HPD5 and HPD20 (**Fig. 1b**, **Supplementary Tables 2–5**). Because of its more favorable on-target and off-target editing profiles, we prioritized HPD20 for further study. We performed targeted amplicon sequencing of an additional ≈50 top-ranked sites in ABE8.8/HPD20-treated HuH-7 cells to cover the full set of sites with ONE-seq scores greater than 0.01, with no off-target mutagenesis observed (**Fig. 1b**, **Supplementary Table 5**). These data support a choice of HPD20 as the therapeutic lead gRNA for a potential treatment for HT1 patients.

Finally, we evaluated the ABE8.8/IDUA1 combination with a ONE-seq library of ≈9,000 oligonucleotides. Remarkably, there were only two sites with ONE-seq scores greater than 0.01, indicating that IDUA1 is an unusually specific gRNA. In evaluating these sites with targeted amplicon sequencing in ABE8.8/IDUA1-treated W402X HuH-7 hepatocytes, we observed no off-target mutagenesis (**Fig. 1c**, **Supplementary Table 6**).

Screening of hybrid guide RNAs for reduction of off-target editing

Having verified seven genomic sites of off-target mutagenesis in ABE8.8/PAH1-treated P281L HuH-7 hepatocytes—with the PAH1_OT3 site displaying the most off-target editing, with 1.4% editing—we assessed whether hybrid gRNAs could reduce the off-target editing at these sites. We designed a series of 21 synthetic hybrid gRNAs ("PAH1_hyb1" through "PAH1_hyb21") with single, double, or triple DNA nucleotide substitutions ranging from positions 3 through 10 in the spacer sequence (**Fig. 2a**). We transfected ABE8.8 mRNA in combination with the standard PAH1 gRNA and each of the hybrid gRNAs into P281L HuH-7 hepatocytes and

assessed for on-target P281L corrective editing and PAH1_OT3 off-target editing in each of the samples (**Fig. 2b**). While most of the PAH1 hybrid gRNAs had comparable on-target editing to the standard PAH1 gRNA, several had modestly decreased editing, and a few had slightly increased editing.

The PAH1 hybrid gRNAs had highly varied effects on OT3 off-target editing (**Fig. 2b**). Examining the single-substitution PAH1 hybrid gRNAs (PAH1_hyb1 through PAH1_hyb8), it was evident that certain positions in the spacer sequence have more influence than others. DNA nucleotide substitutions in positions 4 and 5 (PAH1_hyb2 and PAH1_hyb3) reduced the off-target editing the most, 0.3% compared to 1.4% with standard PAH1 (and to a baseline 0.05% in the negative controls) at the highest mRNA/gRNA dose. A substitution in position 8 actually increased the off-target editing to 1.7% at the highest dose. Some of the dual-substitution PAH1 hybrid gRNAs (PAH1_hyb9 through PAH1_hyb15) had even larger reductions of off-target editing, and some did not; inclusion of position 4 had the most benefit (PAH1_hyb9 and PAH1_hyb10, 0.1-0.2% at the highest dose). Triple-substitution PAH1 hybrid gRNAs (PAH1_hyb16 through PAH1_hyb21) had the most varied effects; substitutions in positions 4, 5, and 6 (PAH_hyb17) had the largest reduction of off-target editing (0.2% at the highest dose), and substitutions in positions 7, 8, and 9 (PAH_hyb20) had the largest increase in off-target editing (2.9% at the highest dose). Notably, none of the 21 PAH1 hybrid gRNA fully eliminated OT3 off-target editing.

As an orthogonal method of off-target profiling, we performed ONE-seq with ABE8.8 and each of the 21 PAH1 hybrid gRNAs (**Fig. 2c**). We used the number of genomic sites with ONE-seq scores greater than 0.01 as a metric of gRNA specificity—the fewer the sites, the less potential for off-target editing. Among single-substitution gRNAs, PAH1_hyb2 and PAH1_hyb3 had the

fewest sites, concordant with their superior effects on OT3 off-target editing. Among double-substitution gRNAs, PAH1_hyb9 and PAH1_hyb10 had the fewest sites (fewer than PAH1_hyb2 and PAH1_hyb3), again concordant with their superior effects on OT3 off-target editing. Among triple-substitution gRNAs, PAH1_hyb16 and PAH1_hyb17 had the fewest sites. Thus, there was good agreement between the results of the site-specific OT3 analysis and the more general ONE-seq off-target profiling for PAH1 hybrid gRNAs.

Because there was residual OT3 off-target editing with even the best of the 21 PAH1 hybrid gRNAs, we combined triple and double substitutions within a single hybrid gRNA to achieve an even better result. We chose the substitutions of PAH_hyb17 (positions 4, 5, and 6), which maximally reduced OT3 off-target editing while preserving on-target editing, and added the substitutions of PAH_hyb15 (positions 9 and 10), which substantially reduced OT3 off-target editing while slightly increasing on-target editing. We compared this new PAH1 hybrid gRNA (herein called "PAH1_hybA") directly against the standard PAH1 gRNA and negative controls via mRNA/gRNA transfection in P281L HuH-7 hepatocytes (**Fig. 2d**). At all seven verified PAH1 off-target sites, OT1 through OT7, the ABE8.8/PAH1_hybA combination reduced the detectable off-target editing to the background levels of the negative controls, while preserving on-target P281L corrective editing.

Next, we wished to assess whether the hybrid gRNA strategy could similarly modulate off-target editing activity with a therapeutic lead gRNA other than PAH1. We had not verified any genomic sites of off-target mutagenesis with the HPD20 gRNA, and so we were not able to perform any site-specific analyses as we did with PAH1. Instead, we used ONE-seq to generally assess the potential for off-target editing. We designed a full series of HPD20 hybrid gRNAs ("HPD20_hyb1" through "HPD20_hyb21"), with the same set of single/double/triple

substitutions in the same positions as the PAH1 hybrid gRNAs, ranging from positions 3 through 10 in the spacer sequence (**Fig. 2e**). Upon performing ONE-seq with ABE8.8 and each of the 21 HPD20 hybrid gRNAs, we observed patterns somewhat distinct from those of the PAH1 hybrid gRNAs (**Fig. 2f**). Among single-substitution gRNAs (HPD20_hyb1 through HPD20_hyb8), HPD20_hyb5 (position 7) and HPD20_hyb7 (position 9) had the fewest sites with ONE-seq scores greater than 0.01. Among double-substitution gRNAs (HPD20_hyb9 through HPD20_hyb15), HPD20_hyb9 (positions 3 and 4) had by far the fewest sites, followed by HPD20_hyb11 (positions 5 and 6). Among triple-substitution gRNAs (HPD20_hyb16 through HPD20_hyb21), HPD20_hyb17 (positions 4, 5, and 6) had the fewest sites of any of the HPD20 hybrid gRNAs. These results suggest that, in general, hybrid gRNAs can be used to reduce off-target editing at target loci, although empirical testing is needed to determine which specific hybrid gRNAs will perform best for each locus.

In vivo evaluation of editing by a hybrid guide RNA

Although our rational exploration of hybrid gRNAs, culminating in PAH1_hybA, was successful in rendering editing at the seven verified PAH1 off-target sites undetectable *in vitro* while preserving on-target editing efficiency *in vitro*, it remained to be answered whether hybrid gRNAs could function as effectively *in vivo*. We formulated LNPs with ABE8.8 mRNA and PAH1_hybA gRNA, exactly paralleling LNPs with ABE8.8 mRNA and the standard PAH1 gRNA that we used in a recently published study[4]. We administered the ABE8.8/PAH1_hybA LNPs to homozygous humanized P281L PKU mice at a dose of 2.5 mg/kg so that we could directly compare to mice treated with ABE8.8/PAH1 LNPs at the same 2.5 mg/kg dose. We observed that both LNP treatments resulted in complete normalization of blood Phe levels in 48 hours, with superimposable trends (**Fig. 3a**). With ABE8.8/PAH1 LNPs, there was mean 40%

whole-liver P281L corrective editing; with ABE8.8/PAH1_hybA LNPs, there was mean 52% editing, establishing that the use of the hybrid gRNA did not compromise and in fact actually improved on-target editing (**Fig. 3b**). To our surprise, the increased on-target editing was accompanied by reduced bystander editing (mean 0.3% editing with ABE8.8/PAH1_hybA LNPs versus 0.8% editing with ABE8.8/PAH1 LNPs), pointing to a potential unanticipated advantage of hybrid gRNAs when employed with base editing.

We performed a dose-response study of ABE8.8/PAH1_hybA LNPs, using homozygous humanized P281L PKU mice as well as compound heterozygous humanized P281L PKU mice (with only half the editable P281L alleles). Across the dose range, there was minimal bystander editing compared to the desired corrective editing (**Fig. 3d**). We found that LNP doses down to 0.25 mg/kg resulted in complete to near normalization of blood Phe levels in both homozygous and compound heterozygous PKU mice (**Fig. 3e**, **3f**).

Screening of hybrid guide RNAs for reduction of bystander editing

Now cognizant of the potential of hybrid gRNAs to reduce bystander editing, we revisited our analyses of the editing outcomes with the full series of PAH1 hybrid gRNAs in mRNA/gRNA-transfected P281L HuH-7 hepatocytes (**Fig. 4a**, **4b**). For ABE8.8/PAH1, bystander editing is limited to the adenine base in position 3 of the protospacer sequence, two positions upstream of the target P281L variant adenine base. Examining the single-substitution PAH1 hybrid gRNAs (PAH1_hyb1 through PAH1_hyb8), it was evident that, as was the case with off-target editing, certain positions in the spacer sequence have more influence than other positions on bystander editing. A DNA nucleotide substitution in position 3 substantially reduced bystander editing, and a substitution in either position 5 or position 8 reduced bystander editing to a lesser degree. Dual-

substitution (PAH1_hyb9 through PAH1_hyb15) and triple-substitution PAH1 hybrid gRNAs (PAH1_hyb16 through PAH1_hyb21) generally had even greater reductions of bystander editing, especially when positions 3, 5, and/or 8 were involved. PAH1_hybA, with substitutions in positions 4, 5, 6, 9, and 10, had less bystander editing that all of the single/double/triple-substituted hybrid gRNAs. Thus, the effects of PAH1 hybrid gRNAs on bystander edits reflect both the positioning of substitutions but also the overall number of substitutions.

Because PAH1_hybA did not include a substitution in position 3, which our analyses indicated was the position that most influenced bystander editing, we designed a few additional PAH1 hybrid gRNAs beyond our original series (**Fig. 2a**). PAH1_hybB had a triple substitution shifted by one position in the 5' direction in order to incorporate position 3: substitutions in positions 3, 4, 5, 9, and 10. PAH1_hybC added a substitution in position 11 to PAH1_hybA, and PAH1_hybD added a substitution in position 11 to PAH1_hybB. We found that PAH1_hybB indeed had reduced bystander editing compared to PAH1_hybA, PAH1_hybC had similar bystander editing to PAH1_hybA, and PAH1_hybD had the least bystander editing of all (**Fig. 4b**). In light of the favorable effects on bystander editing, we speculated that PAH1_hybB, PAH1_hybC, and PAH1_hybD might have reduced off-target editing as well. Because PAH1_hybA already had undetectable editing at the seven verified PAH1 off-target sites, we instead performed ONE-seq with ABE8.8 and each of the four newer PAH1 hybrid gRNAs (**Fig. 4c**). We confirmed the highly favorable off-target profile of PAH1_hybA compared to the standard PAH1 gRNA—there were far fewer genomic sites with ONE-seq scores greater than 0.01—but the other hybrid gRNAs were even more favorable, with PAH1_hybB and PAH1_hybC having far fewer sites than PAH1_hybA, and PAH1_hybD having no sites at all, matching the pattern of favorable effects seen with bystander editing. Finally, we formulated

ABE8.8/PAH1_hybB and ABE8.8/PAH1_hybD LNPs and administered them to homozygous humanized P281L PKU mice at a dose of 2.5 mg/kg, and they achieved even higher levels of whole-liver P281L corrective editing (61%, 59%) than observed with ABE8.8/PAH1_hybA (52%) and with standard ABE8.8/PAH1 (40%), with even less bystander editing (0.2% vs. 0.3% vs. 0.08%) (**Fig. 3a**, **3b**).

We then assessed the full series of HPD20 hybrid gRNAs (HPD20_hyb1 through HPD20_hyb21), via ABE 8.8 mRNA/gRNA transfection in wild-type HuH-7 hepatocytes, for both editing of the target splice site adenine—in position 6 of the protospacer sequence, TCTGCAGAAAGCACGGGAAC—and bystander editing of positions 8, 9, and/or 10 (**Fig. 4d**). All of the hybrid gRNAs had similar or greater levels of target adenine editing as the standard HPD20 gRNA, except HPD20_hyb20. There was substantially more bystander editing with the HPD20 gRNAs compared with the PAH1 gRNAs (though, as stated above, bystander editing at the HPD20 site would not be counterproductive for the inactivation of *HPD*). Single DNA nucleotide substitutions had little effect on bystander editing, whereas double substitutions involving positions 3 through 6 had modestly reduced bystander editing, and triple substitutions involving positions 3 through 6 showed even greater reductions, especially with HPD20_hyb17. Based on these results, we designed additional hybrid gRNAs: HPD20_hybA (positions 3, 4, 5, 8, and 9), HPD20_hybB (positions 4, 5, 6, 8, and 9), and HPD20_hybC (positions 3, 4, 5, 7, and 9) (**Fig. 2e**). These additional hybrid gRNAs maintained on-target editing, reduced bystander editing further, and had even fewer sites with ONE-seq scores greater than 0.01 (**Fig. 4e**, **4f**), concordant with the results observed with the analogous PAH1 hybrid gRNAs.

Variant-aware assessment of off-target editing and reduction with hybrid guide RNAs

All standard off-target assessment techniques share a critical limitation: each is tied to the specific individual genome represented by the cells or the genomic DNA sample used for analysis. For this reason, most off-target analyses have simply ignored the potential for naturally occurring human genetic variation to create novel off-target editing sites in some patients. A cautionary tale is provided by the finding that the gRNA used in the recently approved CRISPR-based therapy for sickle cell disease, exa-cel, has substantial off-target editing in hematopoietic stem cells at a genomic site created by a genetic variant present in ≈10% of individuals of African ancestry, resulting in both indel mutations and chromosomal rearrangements[23]. The ONE-seq technique is uniquely capable of performing a variant-aware off-target analysis, accommodating naturally occurring human genetic variation by using oligonucleotide libraries designed not just using the reference human genome but also incorporating data from the 1000 Genomes Project, the Human Genome Diversity Project, etc. We used the bioinformatic tool CRISPRme[23] to design a variant-aware ONE-seq library for the PAH1 protospacer with ≈7,000 non-reference-genome oligonucleotides. In performing a variant-aware ONE-seq experiment with the ABE8.8/PAH1 combination, we identified 40 variant sites with ONE-seq scores greater than 0.01 (**Fig. 5a**). One of the top variant sites, with a ONE-seq score of 0.19, is a rare singleton 1-bp deletion that alters a reference genomic site that has 7 mismatches with the on-target protospacer, with an NTG PAM, into a site that has 3 mismatches, with an NGG PAM (**Fig. 5b**). Another top variant site, with a ONE-seq score of 0.11, is common enough to have been previously cataloged as rs76813758, a single-nucleotide variant that alters a reference genomic site that has 4 mismatches with the on-target protospacer, with an NGG PAM, into a site that has 3 mismatches, with an NGG PAM; the variant is present in ≈8% of individuals of African ancestry and ≈3% of individuals of European ancestry.

One limitation of variant-aware off-target analysis is that upon identifying candidate variant sites, it can be challenging to verify whether editing actually occurs at any of those sites in the therapeutically relevant cells (e.g., hepatocytes) if there is no way to obtain such cells from individuals with those variants. We reasoned that the use of hybrid gRNAs should reduce the potential for off-target editing not only at reference genomic sites but also at variant sites, mitigating the need to evaluate variant sites. Accordingly, we performed variant-aware ONE-seq experiments for the ABE8.8/PAH1_hybA and ABE8.8/PAH1_hybD combinations (**Fig. 5a**). With PAH1_hybA, there were just 3 variant sites with ONE-seq scores greater than 0.01, and with PAH1_hybD, there were no variant sites with ONE-seq scores greater than 0.01. For rs76813758, the ONE-seq score dropped from 0.11 to 0.004 with PAH1_hybA and to 0.0001 with PAH1_hybD (one-ten thousandth of the *in vitro* editing activity of the on-target site, within the background of the assay).

Favorable effects of hybrid guide RNAs on editing by a PAM-relaxed adenine base editor

Editors based on the SpRY variant of *Streptococcus pyogenes* Cas9 are near-PAMless in their ability to engage genomic sites[19], greatly expanding their targeting range but also incurring an increased potential for off-target editing. To evaluate the SpRY-ABE8.8/PAH4 combination for the correction of the *PAH* R408W variant, we used a ONE-seq library of ≈37,000 oligonucleotides with up to 5 protospacer mismatches, or up to 3 mismatches plus up to 2 bulges, relative to the reference human genome. Rather than interrogate candidate off-target sites nominated by ONE-seq with the standard PAH4 gRNA, we immediately tested a limited series of hybrid gRNAs (double/triple substitutions, “PAH4_hyb9” through “PAH4_hyb21” matching the designs of the analogous PAH1 and HPD20 hybrid gRNAs) with the goal of reducing the potential of SpRY-ABE8.8 for off-target editing and prospectively winnowing the list of

candidate off-target sites for verification (**Fig. 6a**). Several of the PAH4 hybrid gRNAs displayed increased on-target editing and/or decreased bystander editing at protospacer position 10 (nonsynonymous variant) (**Fig. 6b**). ONE-seq demonstrated reduced off-target potential with some of these hybrid gRNAs despite the use of a PAM-relaxed adenine base editor (**Fig. 6c**).

Discussion

PKU, HT1, and MPSI are autosomal recessive disorders for which the most advanced treatment options have significant limitations. In PKU, a bacteria-derived injectable enzyme (pegvaliase) is associated with a substantial risk of anaphylaxis and has a black box warning from the U.S. Food and Drug Administration on its label for that reason, and the dosing regimen is complex, starting with once-weekly injections and slowly up-titrating to once-daily injections; it is available in the U.S. only through a Risk Evaluation and Mitigation Strategy program. In HT1, patients must adhere to twice-daily drug dosing of a repurposed herbicide (nitisinone) and a restrictive diet, leading to noncompliance rates of >50% and to cases in which treatment failure results in the need for liver transplantation[24]. In MPSI, hematopoietic stem cell transplantation carries a high risk of morbidity and mortality, whereas intravenous enzyme replacement therapy induces antidrug antibodies in up to 80% of patients[25]. In each case, "one-and-done" somatic genome-editing therapies would offer substantial advantages over current therapies, and for the relevant patients, could become a new standard of care.

In this study, we investigated the off-target editing profiles of therapeutic lead gRNAs in combination with adenine base editors for the correction of pathogenic variants in PKU and MPSI and for inactivation of the *HPD* gene, a modifier for HT1. Leveraging previously identified lead gRNAs for direct correction of the *PAH* P281L, *PAH* R408W, and *IDUA* W402X

variants, and a lentiviral screen to identify an optimal lead gRNA for *HPD* knockout to treat HT1, we assessed the ability of hybrid gRNAs to mitigate the potential for off-target editing by the standard versions of the gRNAs and found that some hybrid gRNAs dramatically reduce off-target editing, although other hybrid gRNAs have little effect on or even worsen off-target editing. We further discovered that some hybrid gRNAs can also reduce bystander editing while maintaining or even enhancing on-target editing efficiency *in vitro* and *in vivo*. Our findings suggest that the use of hybrid gRNAs to reduce off-target and bystander editing is generalizable across different target loci and underscores its potential as a broadly applicable strategy to improve the safety and efficacy of base-editing therapies for various genetic disorders. Our findings also suggest that there will not be a single set of hybrid gRNA modifications that will optimize off-target and on-target editing across all loci, but that empirical testing might be needed for each new lead gRNA being considered for therapeutic use.

Notably, we observed a very wide range of specificities for our therapeutic lead gRNAs, as ascertained by ONE-seq: from hundreds of nominated sites with scores > 0.01, with 7 verified sites of off-target editing (PAH1), to just 2 sites with scores > 0.01 and both sites being just above 0.01 (IDUA1). Our work suggests that some lead gRNAs can be used in the standard format (all RNA nucleotides) without concern for off-target editing, but most lead gRNAs will incur off-target editing that can be mitigated by the use of hybrid gRNAs—if mRNA-LNPs or some other non-viral delivery method that entails the use of synthetic gRNAs is being used. There might be scenarios where decreased bystander editing and/or increased on-target efficiency is reason enough to use a hybrid gRNA even if off-target editing is not an issue. Although all of the work in this study focused on base editing, it is possible that the advantages

we observed with the use of hybrid gRNAs are relevant to other editing modalities that rely on the use of gRNAs, such as nuclease editing, prime editing, and epigenome editing.

We additionally note the utility of ONE-seq as a method that can serve multiple purposes related to the optimization of a therapeutic lead gRNA. It is well suited to the evaluation of off-target base editing since it can directly read out the nickase and deamination activities intrinsic to Cas9 base editors, even those that are PAM-altered or PAM-relaxed (as demonstrated here with SpRY-ABE8.8/PAH4). It can be employed for variant-aware analysis that uniquely accounts for the effects of natural genetic variation on off-target editing (as demonstrated here with PAH1), unlike almost all other experimental off-target nomination techniques. It can provide an assessment of the potential of a gRNA for off-target editing, i.e., the number of sites with ONE-seq scores above a specified threshold, which could bear some correlation with the magnitude of off-target editing at individual verified sites (as demonstrated here with PAH1). In that same vein, it can provide head-to-head comparisons of the off-target potential of different configurations of the same gRNA (as amply demonstrated here with a wide variety of hybrid gRNAs), which can be useful for rapid screening especially if no individual verified sites have yet been identified.

Indeed, ONE-seq enables a novel strategy for de-risking of editing therapies: rather than the usual approach of assessing a standard gRNA by techniques such as ONE-seq, GUIDE-seq, CHANGE-seq, etc., compiling a large list of nominated candidate genomic sites, extensively analyzing the candidate sites for verified off-target editing, and then retrospectively making changes to the therapeutic lead to mitigate that off-target editing site-by-site (as we initially did here with PAH1), one can instead prospectively use ONE-seq to screen hybrid gRNAs and identify a hybrid gRNA that has preserved or improved on-target editing while winnowing the

set of nominated candidate off-target sites requiring further evaluation—including variant genomic sites that would be challenging to test—to a small number (as shown here with HPD20 and PAH4). From a regulatory perspective, such a strategy could greatly streamline the development of editing therapies, which will be essential if the field is to move towards just-in-time, bespoke treatments for individual patients with inborn errors of metabolism and other serious conditions with unmet medical need, particularly conditions in which there is limited time from diagnosis to progression to irreversible pathology causing significant morbidity and mortality.

Methods

The research described here complied with all relevant regulations. All recombinant DNA research was approved by the Institutional Biosafety Committee at the University of Pennsylvania, where the studies were performed, and were consistent with local, state, and federal regulations as applicable, including the National Institutes of Health Guidelines for Research Involving Recombinant of Synthetic Nucleic Acid Molecules. All procedures used in animal studies were approved by the Institutional Animal Care and Use Committee at the University of Pennsylvania (protocol #805887), where the studies were performed, and were consistent with local, state, and federal regulations as applicable, including the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

RNA production. 100-mer gRNAs were chemically synthesized under solid phase synthesis conditions by a commercial supplier (Agilent or Integrated DNA Technologies) with end-

modifications as well as heavy 2'-O-methylribosugar modification as previously described[26]: for cellular studies, 5'-mX*mX*mX*XXXXXXXXXXXXXXXXXXGUUUUAGAGCUAGAAAUAGCAAGUUAAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCU*U*U*U-3'; for mouse studies, 5'-mX*mX*mX*XXXXXXXXXXXXXXXXXXGUUUUAGAmGmCmUmAmGmAmAmAmUmAmGmCAAGUUAAAAUAAGGCUAGUCCGUUAUCAmAmCmUmUmGmAmAmAmAmAmGmUmGmGmCmAmCmCmGmAmGmUmCmGmGmUmGmCmU*mU*mU*mU-3'; where the Xs indicate the spacer sequence positions (specified in Fig. 2a, Fig. 2e, and Fig. 6a), and "m" and * respectively indicate 2'-O-methylation and phosphorothioate linkage. ABE8.8 mRNA and SpRY-ABE8.8 mRNA were produced via in vitro transcription (IVT) and purification as previously described[4,5]. In brief, a plasmid DNA template containing a codon-optimized ABE8.8 or SpRY-ABE8.8 coding sequence and a 3' polyadenylate sequence was linearized. An IVT reaction containing linearized DNA template, T7 RNA polymerase, NTPs, and cap analog was performed to produce mRNA containing N1-methylpseudouridine. After digestion of the DNA template with DNase I, the mRNA product underwent purification and buffer exchange, and the purity of the final mRNA product was assessed with spectrophotometry and capillary gel electrophoresis. Elimination of double-stranded RNA contaminants was assessed using dot blots and transfection into human dendritic cells. Endotoxin content was measured using a chromogenic Limulus amebocyte lysate (LAL) assay; all assays were negative.

LNP formulation. LNPs were formulated as previously described[4,5,27], with the lipid components (SM-102, 1,2-distearoyl-sn-glycero-3-phosphocholine, cholesterol, and PEG-2000

at molar ratios of 50:10:38.5:1.5) being rapidly mixed with an aqueous buffer solution containing ABE8.8 or SpRY-ABE8.8 mRNA and either PAH1 gRNA or PAH2 gRNA in a 1:1 ratio by weight in 25 mM sodium acetate (pH 4.0), with an N:P ratio of 5.6. The resulting LNP formulations were subsequently dialyzed against sucrose-containing buffer, concentrated using Amicon Ultra-15 mL Centrifugal Filter Units (Millipore Sigma), sterile-filtered using 0.2-µm filters, and frozen until use. The LNPs underwent quality control for particle size (Z-Ave, hydrodynamic diameter), polydispersity index as determined by dynamic light scattering (Malvern NanoZS Zetasizer) and total RNA encapsulation as measured by the Quant-iT Ribogreen Assay (Thermo Fisher Scientific).

Generation of an *IDUA* W402X homozygous HuH-7 cell line with prime editing. HuH-7 cells in a well of a 6-well plate were transfected with 9 µL TransIT®-LT1 Transfection Reagent, 1.5 µg PEmax plasmid, 0.75 µg epegRNA-expressing plasmid, and 0.75 µg ngRNA plasmid as previously described[4] (epegRNA spacer: 5'-CGCAGAUGAGGAGCAGCUCU-3'; RTT sequence: 5'-UUCGGCCuAGA-3'; PBS sequence: 5'-GCUGCUCCUCAU-3'; ngRNA spacer: 5'-uAGAGCUGCUCCUCAUCUGC-3'). Cells were dissociated with trypsin 48 hours post-transfection and replated onto 10-cm plates (5,000 cells/plate) with conditioned medium to facilitate recovery, and genomic DNA was isolated from the remainder of the cells as a pool to perform PCR and Sanger sequencing of the *IDUA* W402X site. Single cells were permitted to expand for 7-14 days to establish clonal populations. Colonies were manually picked and replated into individual wells of a 96-well plate. Genomic DNA was isolated from individual clones, and PCR and Sanger sequencing was performed to identify W402X homozygous HuH-7 clones. One representative clone was expanded for use in subsequent studies. This W402X

homozygous HuH-7 cell line is readily available from the authors via a Transfer of Research Material agreement with the University of Pennsylvania.

Culture and transfection of HuH-7 cells. Wild-type HuH-7 cells, *PAH* P281L homozygous HuH-7 cells[4], *PAH* R408W homozygous HuH-7 cells[5], and *IDUA* W402X homozygous HuH-7 cells were used for this study. HuH-7 cells were maintained in Dulbecco's modified Eagle's medium (containing 4 mM L-glutamine and 1 g/L glucose) with 10% fetal bovine serum and 1% penicillin/streptomycin at 37°C with 5% CO_2. HuH-7 cells were seeded on 6-well plates (Corning) at 3.5×10^5 cells per well. For plasmid transfections, at 16–24 hours after seeding, cells were transfected at approximately 80–90% confluency with 9 µL TransIT®-LT1 Transfection Reagent (MIR2300, Mirus), 2 µg base editor plasmid, and 1 µg gRNA plasmid per well according to the manufacturer's instructions. An ABE8.8 (ABE8.8-m) expression plasmid[10] was used as previously described[4], and HPD3, HPD4, HPD5, and HPD20 gRNA expression plasmids were newly generated for this study. For mRNA/gRNA transfections, in one tube, 7.5 µL Lipofectamine MessengerMax (LMRNA008, Thermo Fisher Scientific) was added to 125 µL of Opti-MEM and incubated for 10 minutes; in another tube, 1.25 µg mRNA and 1.25 µg gRNA was suspended in a total of 125 µL of Opti-MEM. The diluted RNA was added to the tube of diluted MessengerMAX, and the subsequent RNA/MessengerMax/Opti-MEM mixture was incubated for 5 minutes at room temperature and then added dropwise onto the cells. For each type of transfection, cells were cultured for 72 hours after transfection, and then media were removed, cells were washed with 1× DPBS (Corning), and genomic DNA was isolated using the DNeasy Blood and Tissue Kit (QIAGEN) according to the manufacturer's instructions.

ONE-seq. ONE-seq was performed as previously described[4,8,22]. The human ONE-seq libraries for the PAH1, PAH4, HPD3, HPD4, HPD5, HPD20, and IDUA1 gRNAs were designed using the GRCh38 Ensembl v98 reference genome (ftp://ftp.ensembl.org/pub/release-98/fasta/homo_sapiens/dna/Homo_sapiens.GRCh38.dna.chromosome.{1-22,X,Y,MT}.fa, ftp://ftp.ensembl.org/pub/release-98/fasta/homo_sapiens/dna/Homo_sapiens.GRCh38.dna.nonchromosomal.fa). Sites with up to 6 mismatches and sites with up to 4 mismatches plus up to 2 DNA or RNA bulges, compared to the on-target site, were identified with Cas-Designer v1.2[28]. The variant-aware ONE-seq library for the PAH1 gRNA was designed using CRISPRme[23], with similar mismatch criteria. The final oligonucleotide sequences were generated with a script[22], and the oligonucleotide libraries were synthesized by Twist Biosciences. Recombinant ABE8.8 protein and SpRY-ABE8.8 protein were produced by GenScript. Duplicate ONE-seq experiments were previously performed for ABE8.8/PAH1[4]; mean ONE-seq scores were used for this study. All other ONE-seq experiments were performed specifically for this study as follows. Each library was PCR-amplified and subjected to 1.25× AMPure XP bead purification. After incubation at 25°C for 10 minutes in CutSmart buffer, RNP comprising 769 nM recombinant ABE8.8-m protein and 1.54 μM gRNA was mixed with 100 ng of the purified library and incubated at 37°C for 8 hours. Proteinase K was added to quench the reaction at 37°C for 45 minutes, followed by 2× AMPure XP bead purification. The reaction was then serially incubated with EndoV at 37°C for 30 minutes, Klenow Fragment (New England Biolabs) at 37°C for 30 minutes, and NEBNext Ultra II End Prep Enzyme Mix (New England Biolabs) at 20°C for 30 minutes followed by 65°C for 30 minutes, with 2× AMPure XP bead purification after each incubation. The reaction was ligated with an annealed adaptor oligonucleotide duplex at 20°C for 1 hour to facilitate PCR

amplification of the cleaved library products, followed by 2× AMPure XP bead purification. Size selection of the ligated reaction was performed on a BluePippin system (Sage Science) to isolate DNA of 150-200 bp on a 3% agarose gel cassette, followed by two rounds of PCR amplification to generate a barcoded library, which underwent paired-end sequencing on an Illumina MiSeq System as described below. The analysis pipeline[22] used for processing the data assigned a score quantifying the editing efficiency with respect to the on-target site to each potential off-target site. Sites were ranked based on this ONE-seq score, which was used for site prioritization.

Next-generation sequencing (NGS). NGS was performed as previously described[4]. For targeted amplicon sequencing, PCR reactions were performed using NEBNext Polymerase (NEB) using the primer sets listed in Supplementary Table 7, designed with Primer3 v4.1.0 (https://primer3.ut.ee/). The following program was used for all genomic DNA PCRs: 98°C for 20 seconds, 35× (98°C for 20 seconds, 57°C for 30 seconds, 72°C for 10 seconds), 72°C for 2 minutes. PCR products were visualized via capillary electrophoresis (QIAxcel, QIAGEN) and then purified and normalized via an NGS Normalization 96-Well Kit (Norgen Biotek Corporation). A secondary barcoding PCR was conducted to add Illumina barcodes (Nextera XT Index Kit V2 Set A and/or Nextera XT Index Kit V2 Set D) using ≈15 ng of first-round PCR product as template, followed by purification and normalization. Final pooled libraries were quantified using a Qubit 3.0 Fluorometer (Thermo Fisher Scientific) and then after denaturation, dilution to 10 pM, and supplementation with 15% PhiX, underwent single-end or paired-end sequencing on an Illumina MiSeq System. The amplicon sequencing data were analyzed with CRISPResso2 v2[29] and scripts to quantify editing. For on-target editing, A-to-G editing was quantified at the site of the P281L variant (position 5 of the PAH1 protospacer sequence,

position 4 of the PAH2 protospacer sequence) and at the site of the potential bystander adenine (position 3 of the PAH1 protospacer sequence, position 2 of the PAH2 protospacer sequence), with no other adenines present in positions 1 to 10 of either protospacer sequence. For candidate off-target sites, A-to-G editing was quantified throughout the editing window (positions 1 to 10 of the protospacer sequence). In some cases, PCR amplicons were subjected to confirmatory Sanger sequencing, performed by GENEWIZ.

Hybrid capture sequencing. The PAH1 probe library was generated to cover the 280 ONE-seq-nominated genomic sites with ONE-seq scores greater than 0.01, as well as additional sites. The probe library was generated serially using Agilent SureDesign, where probes were created using three rounds of generation settings. In the initial round, probes were generated with 2× tiling density, moderately stringent masking, and optimized performance with 90 minutes hybridization boosting. Regions that were not covered with the initial settings had probes generated with 2× tiling density, least stringent masking, and optimized performance with 90 minutes hybridization boosting. The remaining uncovered regions had probes generated with 1× tiling density, no masking, and no boosting. The hybrid capture procedure was conducted using the SureSelect XT HS2 DNA Reagent Kit (Agilent) as per the manufacturer's instructions. Briefly, 200 ng of genomic DNA from ABE8.8/PAH1 mRNA/gRNA-treated or control untreated HuH-7 cells, quantified on the TapeStation Genomic DNA ScreenTape platform (Agilent), were prepared and enzymatically fragmented with modifications for 2×150 reads. The DNA fragments were then processed for library construction using molecular barcodes. Adaptors were ligated to the DNA fragments, followed by repair and dA-tailing and then purification using AMPure XP beads. The libraries were amplified (8 cycles), indexed, and purified with AMPure XP beads,

and their quality was assessed via the TapeStation D1000 ScreenTape platform (Agilent); 1000 ng of each library was prepared and hybridized to the PAH1 probe library, captured using streptavidin beads, amplified (16 cycles), and purified using AMPure XP beads. The final libraries were purified and quantified via the TapeStation High Sensitivity D1000 ScreenTape platform (Agilent). The libraries were pooled and sequenced on an Illumina NovaSeq, performed by Novogene. The resulting reads were processed and analyzed using the Agilent Alissa Reporter software with the following settings: normal/tumor pair analysis mode for treated samples, and tumor only for control samples, molecular barcode deduplication mode, duplex MBC deduplication consensus mode, 2 read pairs per MBC minimum, 10 reads minimum coverage depth, 3 reads minimum supporting variant allele, and 0.001 minimum variant allele frequency. For each of the 280 sites, A-to-G editing was quantified throughout the editing window (Supplementary Table 1). For sites with any position displaying a mean net A-to-G editing level ≥ 0.1% in treated versus control samples, the sites were confirmed with targeted amplicon sequencing, as described above. Only 2 out of the 280 sites failed to be captured and sequenced, with both sites being on chromosome Y, which is absent from HuH-7 cells.

***HPD* lentiviral base editing screen.** A pool of oligonucleotides spanning guide RNA sequences and target sequences, along with unique barcodes, was synthesized by Twist Bioscience and was amplified using KAPA HiFi HotStart ReadyMix (Roche), using various conditions to minimize template switching. After determining the optimal PCR strategy (48 parallel PCR reactions with 0.5 ng of the oligonucleotide pool per reaction, with 15 cycles), the amplified pool was cloned into a lentiviral vector using In-Fusion (Takara Bio) per the manufacturer's instructions. Lentiviruses with a gene encoding ABE8.20 (ABE8.20-m[10]) and with the cloned

gRNA/barcode/target sequences were generated using the Broad Institute's RNAi Consortium protocol (https://portals.broadinstitute.org/gpp/public/dir/download?dirpath=protocols/production&filename=TRC%20shRNA%20sgRNA%20ORF%20Low%20Throughput%20Viral%20Production%20201506.pdf) for low-throughput lentiviral production and then concentrated using PEG-it virus precipitation solution (System Biosciences) per the manufacturer's instructions. Primary human hepatocytes (Gibco/Thermo Fisher Scientific) were thawed into a 24-well Collagen I coated plate (Gibco/Thermo Fisher Scientific) and transduced with the lentiviruses 6 hours later. The cells were treated at 3 different virus concentrations: low (1 μL), medium (5 μL), and high (25 μL), with 8 biological replicates per condition. Four days after transduction, each group was pooled and then harvested using the DNeasy Blood and Tissue Kit (QIAGEN). NGS library preparation was performed with KAPA HiFi HotStart ReadyMix (Roche), with 35 PCR cycles using a primer pair spanning the barcode and the target sequence. Following a secondary indexing PCR round and sequencing on an Illumina MiSeq system, as described above, target sequences were binned via the unique barcodes, and A-to-G editing was quantified in the editing window of each target sequence as described above.

Mouse studies. Mouse studies were performed as previously described[4]. Mice were maintained on a 12-hour light/12-hour dark cycle, with a temperature range of 65°F to 75°F and a humidity range of 40% to 60%, and were fed ad libitum with a chow diet (LabDiet, Laboratory Autoclavable Rodent Diet 5010). Humanized PKU mice homozygous for the *PAH* P281L allele[4] or compound heterozygous for the *PAH* P281L allele and the *PAH* R408W allele[5] were generated as littermates/colonymates. Genotyping was performed using PCR amplification from

genomic DNA samples (prepared from clipped tails/ears) followed by Sanger sequencing or NGS. Roughly equal numbers of female and male littermates/colonymates were used for experiments at ages ranging from two to eight months, with random assignment of animals to various experimental groups when applicable, and with collection and analysis of data performed in a blinded fashion when possible. LNPs were administered to the mice at one of a range of doses (2.5 mg/kg, 1.0 mg/kg, 0.5 mg/kg, or 0.25 mg/kg) via retro-orbital injection under anesthesia with 1%-2% inhaled isoflurane. Mice were euthanized at 1 to 2 weeks after treatment, and 8 liver samples (2 from each lobe) were obtained on necropsy and processed with the DNeasy Blood and Tissue Kit (QIAGEN) as per the manufacturer's instructions to isolate genomic DNA. Euthanasia in all instances was achieved via terminal inhalation of carbon dioxide followed by secondary euthanasia through cervical dislocation or decapitation, consistent with the 2020 American Veterinary Medical Association Guidelines on Euthanasia. NGS results from the liver samples were averaged to provide quantification of whole-liver editing. In both short-term and long-term studies, blood samples were collected via the tail tip at various timepoints (pre-treatment, day 1, day 2, day 3, and/or day 7), in the early afternoon to account for diurnal variation in blood phenylalanine levels.

Measurement of blood phenylalanine. The blood phenylalanine levels were measured by an enzymatic method using the Phenylalanine Assay Kit (MAK005, Millipore Sigma) according to the manufacturers' instructions. Briefly, plasma samples were deproteinized with a 10 kDa MWCO spin filter (CLS431478-25EA, Millipore Sigma) and pre-treated with 5 μL of tyrosinase for 10 minutes at room temperature prior to start of the assay. Reaction mixes were made

according to the manufacturers' instructions, and the fluorescence intensity of each sample was measured (λ_{ex} = 535/λ_{em} = 587 nm).

Data analysis. Sequencing data were analyzed as described above. Other data were collected and analyzed using GraphPad Prism v10.2.2.

Data availability

DNA sequencing data that support the findings of this study will be deposited in the NCBI Sequence Read Archive upon publication of this manuscript. Other data supporting the findings of this study are available within the manuscript and its Supplementary Information. The GRCh38 Ensembl v98 reference genome (ftp://ftp.ensembl.org/pub/release-98/fasta/homo_sapiens/dna/Homo_sapiens.GRCh38.dna.chromosome.{1-22,X,Y,MT}.fa, ftp://ftp.ensembl.org/pub/release-98/fasta/homo_sapiens/dna/Homo_sapiens.GRCh38.dna.nonchromosomal.fa) annotation was used.

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Author contributions

K.M., M.-G.A., W.H.P., and X.W. supervised the work with intellectual input on experimental design and data analysis from J.Z.W. and R.C.A.-N. M.N.W., D.L.B., A.Q., I.J., P.Q., and X.W. contributed to web laboratory experiments. M.N.W. and K.M. contributed to bioinformatic

analyses. M.-G.A. contributed to mRNA production and LNP formulation. M.N.W., K.M., and X.W. drafted the manuscript, and all authors contributed to the editing of the manuscript.

Competing interests

M.N.W. is a fellow with Artis Ventures. J.Z.W. is an employee of Agilent Technologies. R.C.A.-N. is an advisor to Latus Bio. K.M. is an advisor to and holds equity in Verve Therapeutics and Variant Bio, is an advisor to LEXEO Therapeutics, and receives research funding from Nava Therapeutics. M.-G.A. is a co-founder of and an advisor to AexeRNA Therapeutics. The University of Pennsylvania and Children's Hospital of Philadelphia have filed patent applications related to the use of base editing for the treatment of phenylketonuria and tyrosinemia (inventors include M.N.W., D.L.B., R.C.A.-N., K.M., W.H.P., and X.W.). The remaining authors declare no competing interests.

Correspondence and requests for materials should be addressed to K.M., M.-G.A., W.H.P., or X.W.

Figure legends

Fig. 1 | Assessment of on-target and off-target editing by candidate therapeutic lead guide RNAs. a, Lentiviral screening for adenine base editing of heterologous *HPD* splice site target sequences by various gRNAs in combination with ABE8.20; target adenines are in bold and underlined. MIT score = off-target/specificity score indicated by UCSC Genome Browser. Primary human hepatocytes were treated with the lentiviral library at three doses (high:medium:low ratio of 25:5:1). Four candidate gRNAs (HPD3, HPD4, HPD5, and HPD20) were taken forward for off-target analyses. **b**, Targeted amplicon sequencing of ONE-seq-nominated sites in plasmid-treated vs. control wild-type HuH-7 cells with HPD3, HPD4, HPD5, or HPD20 gRNA in combination with ABE8.8 (n = 2 treated and 2 untreated biological replicates). Sites with unsuccessful sequencing are omitted. Arrows indicate verified sites of off-target editing. **c**, Targeted amplicon sequencing of ONE-seq-nominated sites in mRNA-treated vs. control *IDUA* W402X HuH-7 cells with IDUA gRNA in combination with ABE8.8 (n = 3 treated and 3 untreated biological replicates). **d**, Hybrid capture sequencing of ONE-seq-nominated sites in mRNA-treated vs. control *PAH* P281L HuH-7 cells with PAH1 gRNA in combination with ABE8.8 (n = 3 treated and 3 untreated biological replicates), with seven sites verified to have off-target editing. Mean ± standard deviation shown where applicable.

Fig. 2 | Reduction of off-target editing by PAH1 and HPD20 hybrid guide RNAs. a, Spacer sequences of PAH1 standard and hybrid gRNAs. "rN" = ribonucleotide; "dN" = deoxyribonucleotide; "m" = 2'-O-methylation; * = phosphorothioate linkage. DNA substitutions are in red bold and underlined. **b**, Targeted amplicon sequencing of *PAH* on-target site and verified PAH1_OT3 off-target site in mRNA-treated vs. control *PAH* P281L HuH-7 cells with PAH1 standard or hybrid gRNA in combination with ABE8.8. **c**, Number of sites with ONE-seq score > 0.01 for each PAH1 standard or hybrid gRNA. **d**, Targeted amplicon sequencing of *PAH* on-target site and seven verified PAH1 off-target sites in mRNA-treated vs. control *PAH* P281L HuH-7 cells with PAH1 or PAH1_hybA gRNA in combination with ABE8.8 (n = 12 biological replicates per group). **e**, Spacer sequences of HPD20 standard and hybrid gRNAs. **f**, Number of sites with ONE-seq score > 0.01 for each HPD20 standard or hybrid gRNA.

Fig. 3 | Improved on-target editing in humanized PKU mice with PAH1 hybrid guide RNAs. a, Blood phenylalanine levels in homozygous P281L mice (n = 4-5 animals per group) before and after treatment with 2.5 mg/kg dose of LNPs with PAH1 standard or hybrid gRNA in combination with ABE8.8 mRNA, comparing levels at various timepoints up to 7 days following treatment (1 blood sample per timepoint). The dotted line marks the guideline-directed goal of <360 µmol/L recommended for PKU patients. **b**, **c**, A-to-G editing (correction only, or correction plus bystander editing) of *Pah* on-target site in the liver (each point reflects the mean of 8 samples taken from an individual liver) in homozygous P281L or compound heterozygous mice (n = 3-6 animals per group), assessed ≥1 week following treatment with indicated dose of LNPs with PAH1 standard or hybrid gRNA in combination with ABE8.8 mRNA. For compound heterozygous mice, each displayed number is % edited P281L alleles (editable alleles) divided by two. **d**, **e**, Blood phenylalanine levels in homozygous P281L or compound heterozygous mice (n = 4-8 animals per group) before and after treatment with indicated dose of LNPs with PAH1_hybA gRNA in combination with ABE8.8 mRNA (1 blood sample per timepoint). Mean ± standard deviation shown where applicable.

Fig. 4 | Reduction of off-target editing by PAH1 and HPD20 hybrid guide RNAs. a, **b**, A-to-G editing (correction only, or correction plus bystander editing) of *PAH* on-target site in mRNA-treated vs. control *PAH* P281L HuH-7 cells with PAH1 standard or hybrid gRNA in combination with ABE8.8. **c**, Number of sites with ONE-seq score > 0.01 for each indicated PAH1 standard or hybrid gRNA. **d**, **e**, A-to-G editing (splice site disruption only, or splice site plus bystander editing) of *HPD* on-target site in mRNA-treated vs. control wild-type HuH-7 cells with HPD20 standard or hybrid gRNA in combination with ABE8.8. **f**, Number of sites with ONE-seq score > 0.01 for each indicated HPD20 standard or hybrid gRNA.

Fig. 5 | Reduction of variant-aware off-target editing by PAH1 hybrid guide RNAs. a, Number of variant (non-reference-genome) sites with ONE-seq score > 0.01 for each indicated PAH1 standard or hybrid gRNA. **b**, Examples of variant sites nominated by ONE-seq, with reductions in ONE-seq scores observed with PAH1 hybrid gRNAs. Differences between reference and variant versions of nominated sites are in bold and underlined.

Fig. 6 | Reduction of off-target editing by PAH4 hybrid guide RNAs. a, Spacer sequences of PAH1 standard and hybrid gRNAs. "rN" = ribonucleotide; "dN" = deoxyribonucleotide; "m" = 2'-O-methylation; * = phosphorothioate linkage. DNA substitutions are in red bold and underlined. **b**, A-to-G editing (correction with or without synonymous bystander editing, or correction plus unwanted nonsynonymous bystander editing) of *PAH* on-target site in mRNA-treated vs. control *PAH* R408W HuH-7 cells with PAH4 standard or hybrid gRNA in combination with SpRY-ABE8.8. **c**, Number of sites with ONE-seq score > 0.01 for each indicated PAH4 standard or hybrid gRNA.

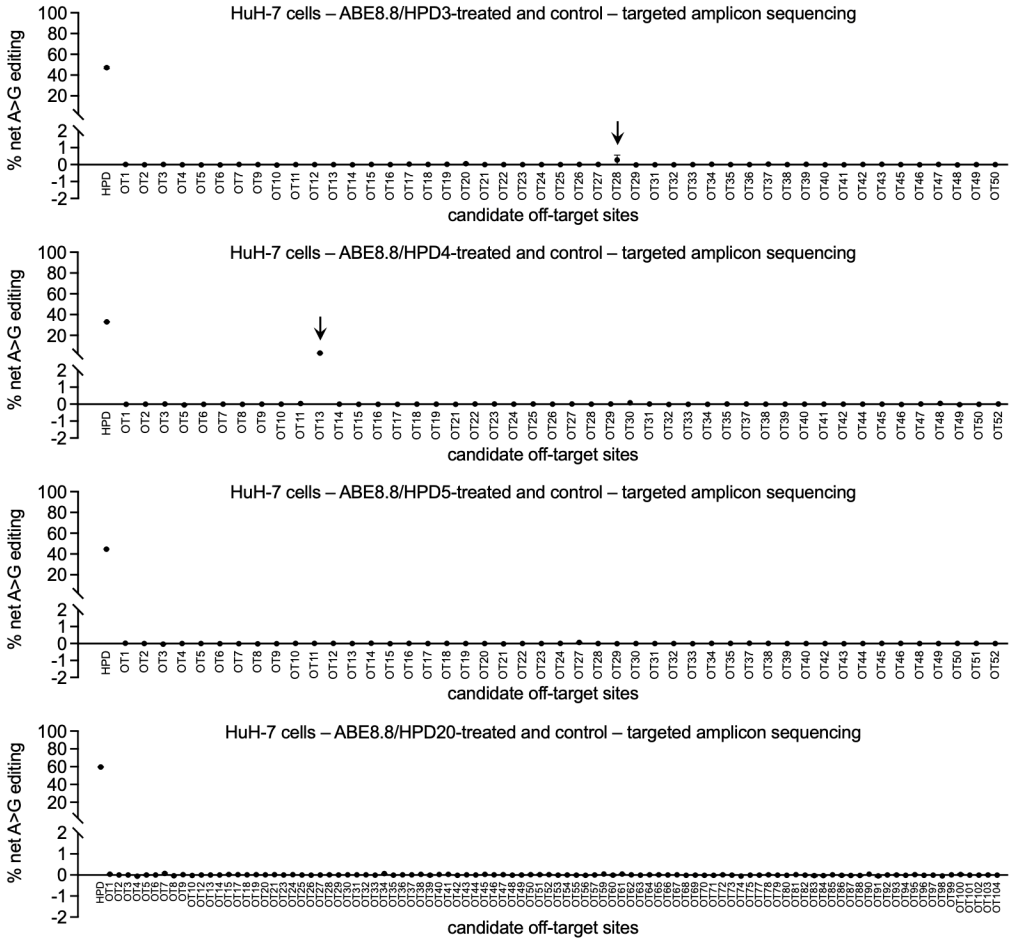
Figure 1

a

ABE8.20 editing of human *HPD* sequences following lentiviral insertion into primary human hepatocytes

Label	Protospacer	PAM	Target Site	MIT Score	High dose Desired A>G edit%	High dose Any A>G edit%	Medium dose Desired A>G edit%	Medium dose Any A>G edit%	Low dose Desired A>G edit%	Low dose Any A>G edit%
A	GCACCTC**A**CCTTTGTTCCAG	GGG	exon 5 splice donor	75	13%	16%	12%	15%	12%	12%
B	CAC**A**GAGATGGGCGATCACC	TGG	exon 6 splice acceptor	76	14%	18%	13%	17%	13%	18%
C	CTCTGC**A**GAAAGCACGGGAA	CGG	exon 7 splice acceptor	60	26%	26%	25%	26%	21%	23%
D (HPD20)	TCTGC**A**GAAAGCACGGGAAC	GGG	exon 7 splice acceptor	83	40%	41%	33%	33%	32%	34%
E	CTGC**A**GAAAGCACGGGAACG	GGG	exon 7 splice acceptor	85	43%	44%	35%	37%	24%	25%
F (HPD3)	ACACTC**A**CAGTTTAGGAAGT	AGG	exon 8 splice donor	65	52%	53%	47%	49%	45%	48%
G (HPD4)	CACTC**A**CAGTTTAGGAAGTA	GGG	exon 8 splice donor	72	40%	42%	35%	38%	33%	34%
H (HPD5)	ACTC**A**CAGTTTAGGAAGTAG	GGG	exon 8 splice donor	65	38%	40%	37%	38%	32%	33%
I	CTC**A**CAGTTTAGGAAGTAGG	GGG	exon 8 splice donor	66	41%	43%	39%	42%	40%	42%
J	TCCC**A**GGCCCAAATGCAGTC	TGG	exon 9 splice acceptor	66	28%	28%	24%	24%	19%	19%
K	AAGGTCTC**A**CCATTCGGAGG	CGG	exon 9 splice donor	90	16%	16%	16%	16%	11%	11%
L	CGCCT**A**GGAGCTGAAAATCC	TGG	exon 13 splice acceptor	79	15%	21%	12%	16%	18%	24%
M	CT**A**GGAGCTGAAAATCCTGG	TGG	exon 13 splice acceptor	66	14%	20%	16%	20%	11%	16%
N	GT**A**CCTGGTGGTTGTGGCGC	TGG	exon 13 splice donor	82	29%	29%	23%	23%	21%	21%
O	CAAGCAGT**A**CCTGGTGGTTG	TGG	exon 13 splice donor	67	34%	36%	31%	34%	28%	31%
P	CCGCCAC**A**GGGTTTTGGAGC	CGG	exon 14 splice acceptor	79	9%	13%	9%	11%	7%	9%

b



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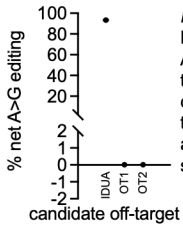

IDUA W402X HuH-7 cells – ABE8.8/IDUA1-treated and control – targeted amplicon sequencing

d

Verified PAH1 off-target sites in *PAH* P281L HuH-7 cells – hybrid capture

Site	ONE-seq score	Protospacer/PAM	Chromosome:Position	% net A>G editing
PAH P281L	1.00	TCACAGTTCGGGGGTATACATGG	chr12:102852811+102852833	94.6%
PAH1_OT1	0.74	TCACAGcTCGGtGGaATACAGGG	chr1:118122604-118122582	0.40%
PAH1_OT2	0.10	gCcttaTTCGGGGGTATACAAGA	chr1:167096363-167096341	0.41%
PAH1_OT3	0.08	TCACAG--aGaGGGTATACAAGG	chr3:106907233-106907213	1.26%
PAH1_OT4	0.05	gtAgtaTTCaGGGGTATACAAGG	chr13:35840575-35840553	0.95%
PAH1_OT5	0.03	TaACAGTaCaGaGGTATACAGGG	chr14:23897564-23897542	0.62%
PAH1_OT6	0.02	TttaAGTTCaGGGGTATACAAGG	chrX:75341785+75341807	0.22%
PAH1_OT7	0.01	gCACAG--aaaGGGTATACATGG	chr1:160067049-160067029	1.22%

Figure 2

a PAH1 hybrid guide RNA designs

gRNA	Spacer sequence
PAH1	mU*mC*mA*rCrArGrUrUrCrGrGrGrGrGrUrArUrArCrA
PAH1_hyb1	mU*mC*dA*rCrArGrUrUrCrGrGrGrGrGrUrArUrArCrA
PAH1_hyb2	mU*mC*mA*dCrArGrUrUrCrGrGrGrGrGrUrArUrArCrA
PAH1_hyb3	mU*mC*mA*rCdArGrUrUrCrGrGrGrGrGrUrArUrArCrA
PAH1_hyb4	mU*mC*mA*rCrAdGrUrUrCrGrGrGrGrGrUrArUrArCrA
PAH1_hyb5	mU*mC*mA*rCrArGdTrUrCrGrGrGrGrGrUrArUrArCrA
PAH1_hyb6	mU*mC*mA*rCrArGrUdTrCrGrGrGrGrGrUrArUrArCrA
PAH1_hyb7	mU*mC*mA*rCrArGrUrUdCrGrGrGrGrGrUrArUrArCrA
PAH1_hyb8	mU*mC*mA*rCrArGrUrUrCdGrGrGrGrGrUrArUrArCrA
PAH1_hyb9	mU*mC*dA*dCrArGrUrUrCrGrGrGrGrGrUrArUrArCrA
PAH1_hyb10	mU*mC*mA*dCdArGrUrUrCrGrGrGrGrGrUrArUrArCrA
PAH1_hyb11	mU*mC*mA*rCdAdGrUrUrCrGrGrGrGrGrUrArUrArCrA
PAH1_hyb12	mU*mC*mA*rCrAdGdTrUrCrGrGrGrGrGrUrArUrArCrA
PAH1_hyb13	mU*mC*mA*rCrArGdTdTrUrCrGrGrGrGrGrUrArUrArCrA
PAH1_hyb14	mU*mC*mA*rCrArGrUdTdCrGrGrGrGrGrUrArUrArCrA
PAH1_hyb15	mU*mC*mA*rCrArGrUrUdCdGrGrGrGrGrUrArUrArCrA
PAH1_hyb16	mU*mC*dA*dCdArGrUrUrCrGrGrGrGrGrUrArUrArCrA
PAH1_hyb17	mU*mC*mA*dCdAdGrUrUrCrGrGrGrGrGrUrArUrArCrA
PAH1_hyb18	mU*mC*mA*rCdAdGdTrUrUrCrGrGrGrGrGrUrArUrArCrA
PAH1_hyb19	mU*mC*mA*rCrAdGdTdTrUrCrGrGrGrGrGrUrArUrArCrA
PAH1_hyb20	mU*mC*mA*rCrArGdTdTdCrGrGrGrGrGrUrArUrArCrA
PAH1_hyb21	mU*mC*mA*rCrArGrUdTdCdGrGrGrGrGrUrArUrArCrA
PAH1_hybA	mU*mC*mA*dCdAdGrUrUdCdGrGrGrGrGrUrArUrArCrA
PAH1_hybB	mU*mC*dA*dCdArGrUrUdCdGrGrGrGrGrUrArUrArCrA
PAH1_hybC	mU*mC*mA*dCdAdGrUrUdCdGdGrGrGrGrUrArUrArCrA
PAH1_hybD	mU*mC*dA*dCdArGrUrUdCdGdGrGrGrGrUrArUrArCrA

e HPD20 hybrid guide RNA designs

gRNA	Spacer sequence
HPD20	mU*mC*mU*rGrCrArGrArArArGrCrArCrGrGrGrArArC
HPD20_hyb1	mU*mC*dT*rGrCrArGrArArArGrCrArCrGrGrGrArArC
HPD20_hyb2	mU*mC*mU*dGrCrArGrArArArGrCrArCrGrGrGrArArC
HPD20_hyb3	mU*mC*mU*rGdCrArGrArArArGrCrArCrGrGrGrArArC
HPD20_hyb4	mU*mC*mU*rGrCdArGrArArArGrCrArCrGrGrGrArArC
HPD20_hyb5	mU*mC*mU*rGrCrAdGrArArArGrCrArCrGrGrGrArArC
HPD20_hyb6	mU*mC*mU*rGrCrArGdArArArGrCrArCrGrGrGrArArC
HPD20_hyb7	mU*mC*mU*rGrCrArGrAdArArGrCrArCrGrGrGrArArC
HPD20_hyb8	mU*mC*mU*rGrCrArGrArAdArGrCrArCrGrGrGrArArC
HPD20_hyb9	mU*mC*dT*dGrCrArGrArArArGrCrArCrGrGrGrArArC
HPD20_hyb10	mU*mC*mU*dGdCrArGrArArArGrCrArCrGrGrGrArArC
HPD20_hyb11	mU*mC*mU*rGdCdArGrArArArGrCrArCrGrGrGrArArC
HPD20_hyb12	mU*mC*mU*rGrCdAdGrArArArGrCrArCrGrGrGrArArC
HPD20_hyb13	mU*mC*mU*rGrCrAdGdArArArGrCrArCrGrGrGrArArC
HPD20_hyb14	mU*mC*mU*rGrCrArGdAdArArGrCrArCrGrGrGrArArC
HPD20_hyb15	mU*mC*mU*rGrCrArGrAdAdArGrCrArCrGrGrGrArArC
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HPD20_hybB	mU*mC*mU*dGdCdArGdAdArArGrCrArCrGrGrGrArArC
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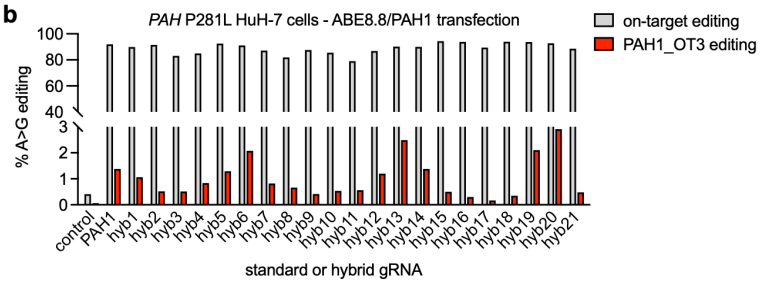
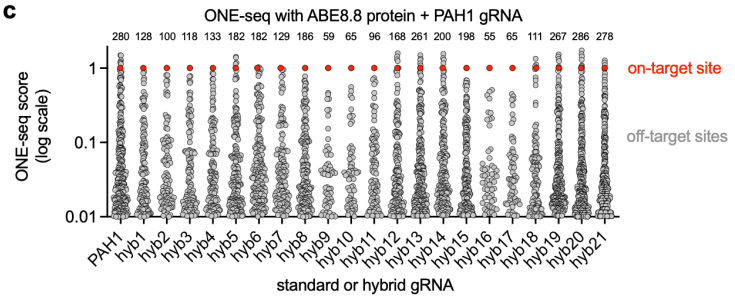
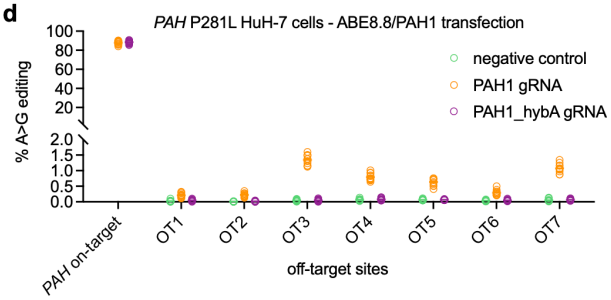






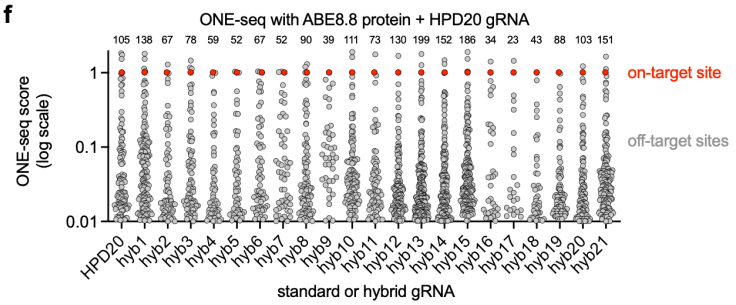
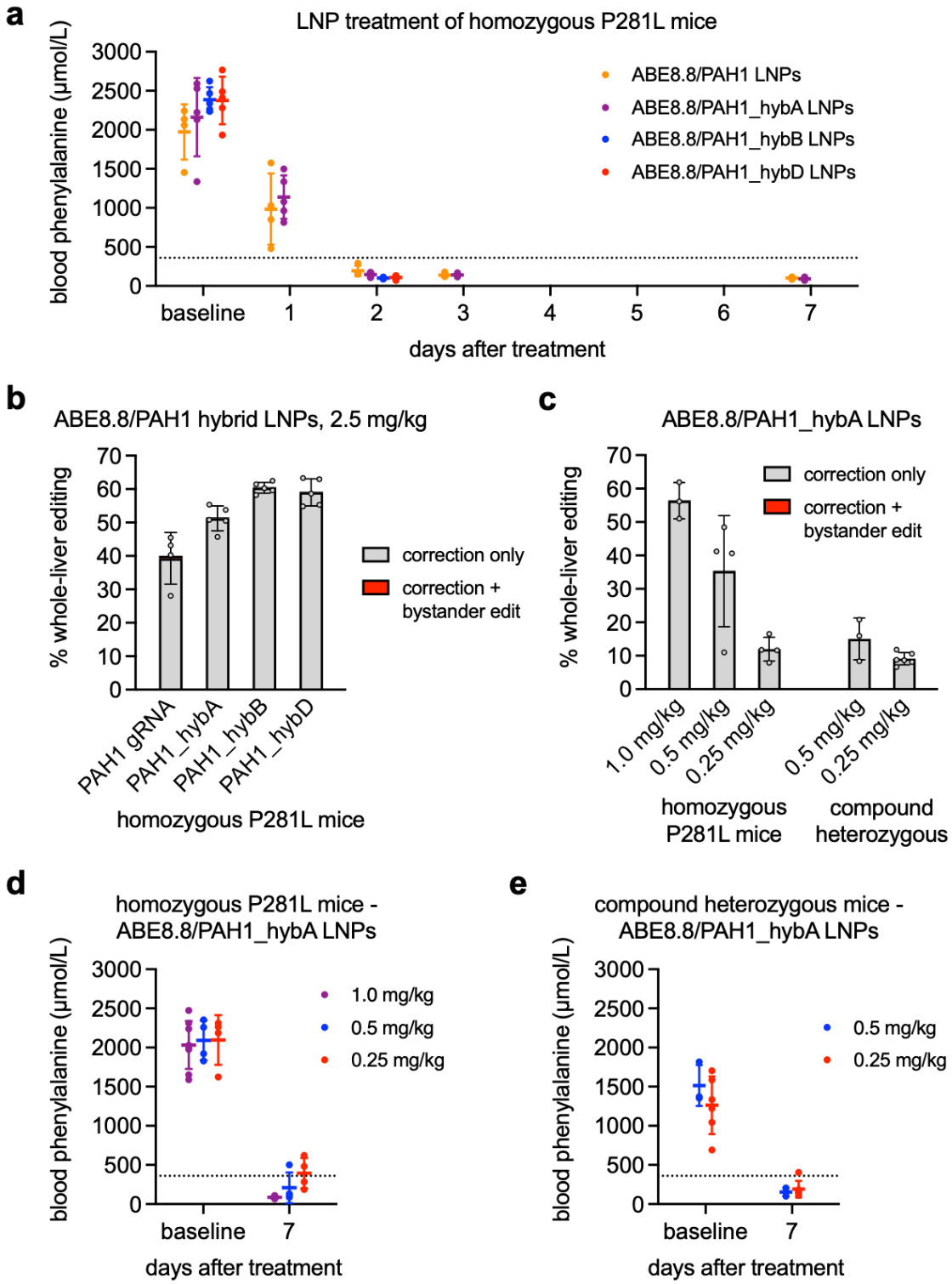


Figure 3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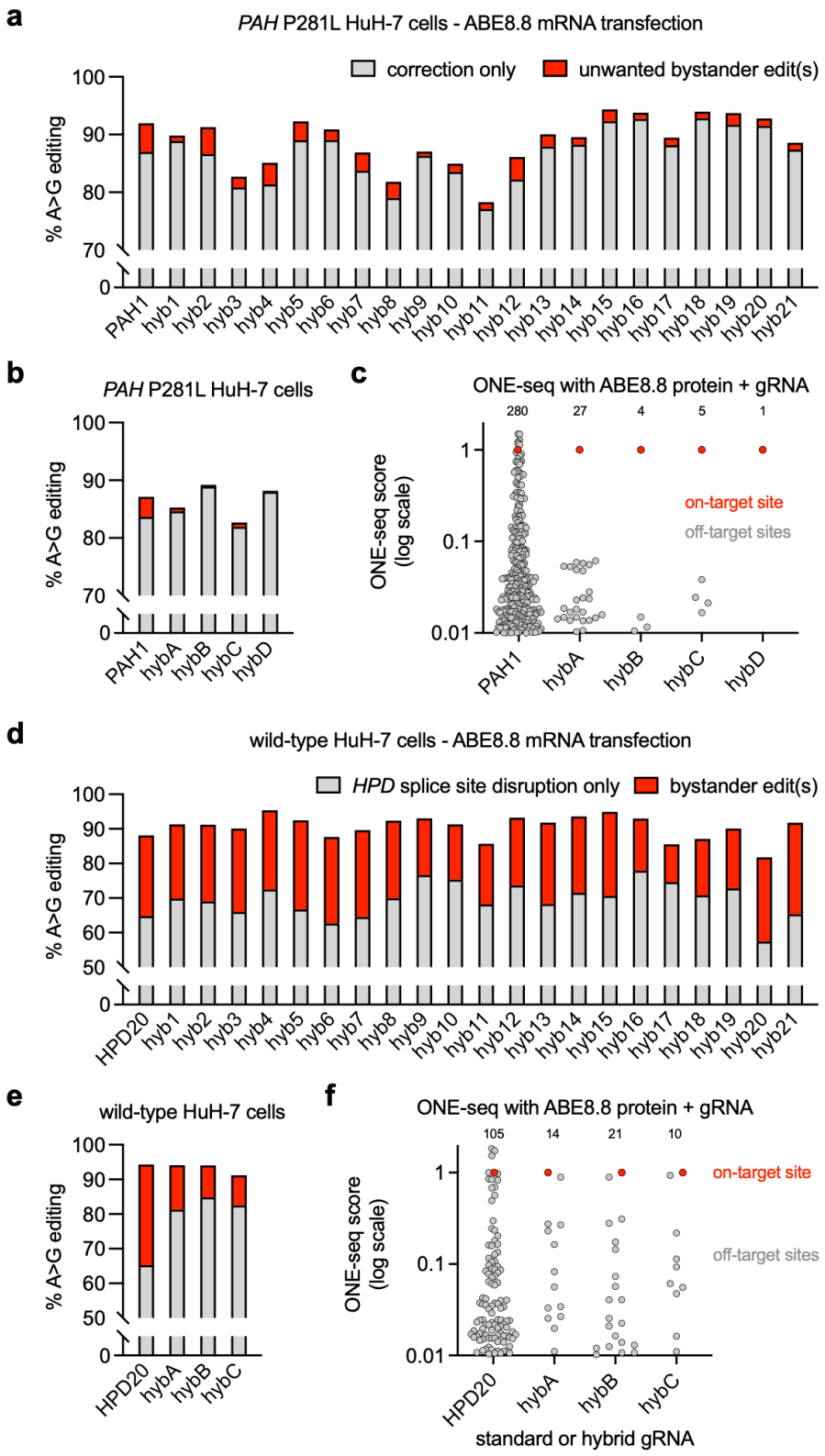
LNP treatment of homozygous P281L mice
blood phenylalanine (µmol/L)
3000
2500
2000
1500
1000
500
0
baseline
1
2
3
4
5
6
7
days after treatment
ABE8.8/PAH1 LNPs
ABE8.8/PAH1_hybA LNPs
ABE8.8/PAH1_hybB LNPs
ABE8.8/PAH1_hybD LNPs
b
ABE8.8/PAH1 hybrid LNPs, 2.5 mg/kg
% whole-liver editing
70
60
50
40
30
20
10
0
PAH1 gRNA
PAH1_hybA
PAH1_hybB
PAH1_hybD
homozygous P281L mice
correction only
correction + bystander edit
c
ABE8.8/PAH1_hybA LNPs
% whole-liver editing
1.0 mg/kg
0.5 mg/kg
0.25 mg/kg
0.5 mg/kg
0.25 mg/kg
homozygous P281L mice
compound heterozygous
correction only
correction + bystander edit
d
homozygous P281L mice - ABE8.8/PAH1_hybA LNPs
blood phenylalanine (µmol/L)
baseline
7
days after treatment
1.0 mg/kg
0.5 mg/kg
0.25 mg/kg
e
compound heterozygous mice - ABE8.8/PAH1_hybA LNPs
blood phenylalanine (µmol/L)
baseline
7
days after treatment
0.5 mg/kg
0.25 mg/kg

Figure 4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b
PAH P281L HuH-7 cells
% A>G editing
PAH1 hybA hybB hybC hybD
c
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off-target sites
PAH1 hybA hybB hybC hybD
d
wild-type HuH-7 cells - ABE8.8 mRNA transfection
HPD splice site disruption only
bystander edit(s)
% A>G editing
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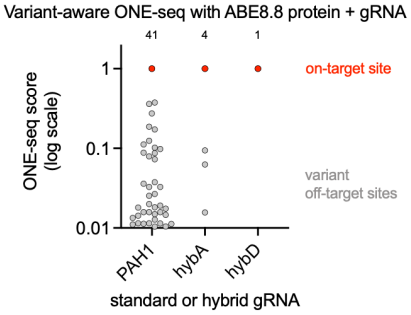
e
wild-type HuH-7 cells
% A>G editing
HPD20 hybA hybB hybC
f
ONE-seq with ABE8.8 protein + gRNA
105 14 21 10
ONE-seq score (log scale)
on-target site
off-target sites
HPD20 hybA hybB hybC
standard or hybrid gRNA

Figure 5

a



b

On-target site	TCACAGTTCGGGGGTATACA|TGG
Candidate site, reference version	TCACAGTcCGGGGGg**g**agac|AtGG
Candidate site, variant version (1-bp deletion)	TCACAGTcCGGGGGgAgACA|TGG
Minor allele frequency = 0.00001	
ONE-seq score: PAH1 gRNA = 0.19 / PAH1_hybA = 0.06 / PAH1_hybD = 0.00996	

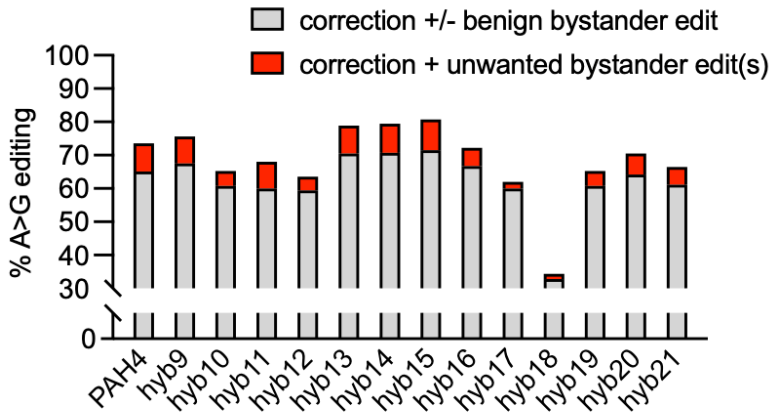
On-target site	TCACAGTTCGGGGGTATACA|TGG
Candidate site, reference version	aCACAGTTCtaGGGTA**c**AgA|TGG
Candidate site, variant version (substitution)	aCACAGTTCtaGGGTA**T**AgA|TGG
Minor allele frequency = 0.02	
ONE-seq score: PAH1 gRNA = 0.11 / PAH1_hybA = 0.004 / PAH1_hybD = 0.0001	

Figure 6

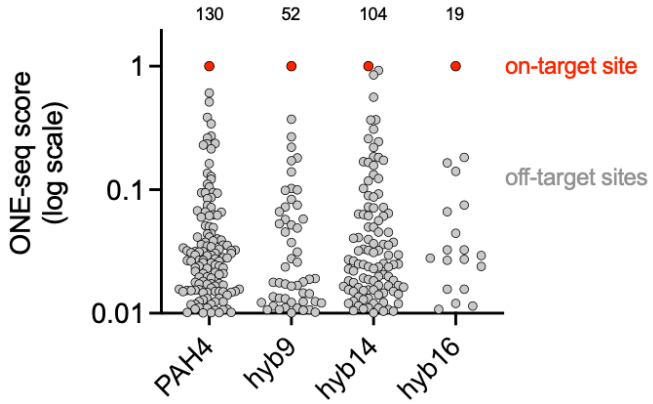
a PAH4 hybrid guide RNA designs

gRNA	Spacer sequence
PAH4	mU*mC*mA*rCrArGrUrUrCrGrGrGrGrGrUrArUrArCrA
PAH4_hyb9	mU*mC*dA*dCrArGrUrUrCrGrGrGrGrGrUrArUrArCrA
PAH4_hyb10	mU*mC*mA*dCdArGrUrUrCrGrGrGrGrGrUrArUrArCrA
PAH4_hyb11	mU*mC*mA*rCdAdGrUrUrCrGrGrGrGrGrUrArUrArCrA
PAH4_hyb12	mU*mC*mA*rCrAdGdTrUrCrGrGrGrGrGrUrArUrArCrA
PAH4_hyb13	mU*mC*mA*rCrArGdTdTrCrGrGrGrGrGrUrArUrArCrA
PAH4_hyb14	mU*mC*mA*rCrArGrUdTdCrGrGrGrGrGrUrArUrArCrA
PAH4_hyb15	mU*mC*mA*rCrArGrUrUdCdGrGrGrGrGrUrArUrArCrA
PAH4_hyb16	mU*mC*dA*dCdArGrUrUrCrGrGrGrGrGrUrArUrArCrA
PAH4_hyb17	mU*mC*mA*dCdAdGrUrUrCrGrGrGrGrGrUrArUrArCrA
PAH4_hyb18	mU*mC*mA*rCdAdGdTrUrCrGrGrGrGrGrUrArUrArCrA
PAH4_hyb19	mU*mC*mA*rCrAdGdTdTrCrGrGrGrGrGrUrArUrArCrA
PAH4_hyb20	mU*mC*mA*rCrArGdTdTdCrGrGrGrGrGrUrArUrArCrA
PAH4_hyb21	mU*mC*mA*rCrArGrUdTdCdGrGrGrGrGrUrArUrArCrA

b *PAH* R408W HuH-7 cells - SpRY-ABE8.8 mRNA transfection



c ONE-seq with SpRY-ABE8.8 protein + gRNA



Supplementary Table 1. Assessment of off-target editing with ABE8.8/PAH1 with mRNA delivery in HuH-7 cells via hybrid capture sequencing of all sites with ONE-seq score > 0.01.

See accompanying Excel spreadsheet.

Supplementary Table 2. Assessment of off-target editing with ABE8.8/HPD3 with plasmid delivery in HuH-7 cells via targeted amplicon sequencing.

Genomic site	Protospacer/PAM sequence*	Amplicon (chromosome: position range)†	ONE-seq score‡	Treated sample 1§	Treated sample 2	Control sample 1	Control sample 2	Mean net A→G editing %
HPD	CACTCACAGTTTAGGAAGTAGGG	chr12:121849613+121849785	1.00	46.56	47.71			
OT1	CACaCACAGTTTAGatAGTAGGC	chr9:71133899-71134050	6.72	0.07	0.12	0.06	0.11	+0.01
OT2	CACaCACAGTTTAG--AGTtGGC	chr8:129535012-129535209	4.12	0.06	0.06	0.08	0.05	–0.01
OT3	CACTCTACAGTTTAGGAAtgtGGA	chr11:34141552-34141694	1.15	0.02	0.05	0.03	0.03	+0.01
OT4	CAtaCACAGTTTAGaAAtTtGGT	chr1:179641516+179641660	0.90	0.02	0.03	0.04	0.02	–0.01
OT5	tcCTCACAGTTcAGGAgGTAGGA	chr5:13257359+13257546	0.59	0.02	0.01	0.03	0.03	–0.02
OT6	CACTCtCAGTTTAGGgAGagGAG	chr15:52222324+52222506	0.57	0.00	0.02	0.04	0.01	–0.02
OT7	CAtTCACAGTTTAGaAAGTgAGG	chr14:20158742-20158893	0.56	0.04	0.04	0.02	0.04	+0.01
OT8	CACaCACAGTTTgGaAAtTgGGC	chr3:164629339+164629484	0.56	n.d.	n.d.	n.d.	n.d.	n.d.
OT9	aACcCACAGTTTAGG-AGTgGGA	chr2:240663681+240663791	0.43	0.05	0.04	n.d.	0.04	+0.00
OT10	CACTCACAcTcTAGGAAcTgGGA	chr1:153768158+153768304	0.43	0.03	0.02	0.06	0.04	–0.03
OT11	tcCTCACAGTccAGGAgGTgGGG	chr19:10914311+10914483	0.41	0.05	0.04	n.d.	0.04	+0.00
OT12	CACaCACAGTTTAGGtctggGGG	chr17:65841698+65841939	0.39	0.12	0.08	0.06	0.13	+0.00
OT13	CAtTCACAGTTTAGaAtGTgGGG	chr12:100653598+100653785	0.37	0.05	0.05	0.06	0.03	+0.00
OT14	CtaTCACAGTTcAGGAAGTgTGC	chr12:61254986-61255170	0.36	0.03	0.04	0.05	0.05	–0.01
OT15	CACTCACAGGTcTAGGAtGTtGGT	chr1:184773598-184773733	0.36	0.04	0.03	0.03	0.04	+0.01
OT16	ttCTCACAGTTcAGGAAGctGGG	chr8:125682758+125682876	0.35	0.03	0.02	0.03	0.02	+0.00
OT17	gACTCACAGTTcAGGAgGTtGGG	chr11:101382779-101382932	0.31	0.07	0.03	0.03	0.03	+0.02
OT18	CACatACAGTTTAGaAAGTAAGT	chr5:156260561+156260739	0.28	0.06	0.05	0.06	0.04	+0.01
OT19	tACTCACAGaaTAGGAAa-AGGG	chr9:104188183+104188347	0.28	0.05	0.07	0.05	0.04	+0.02
OT20	agCTCACAGTTTAGGAAGgAGGC	chr17:62097919-62098019	0.27	0.08	0.13	0.06	0.05	+0.05
OT21	CACTCACAGTTTA-GAAGagGGA	chr21:37868854+37869027	0.26	0.04	0.05	0.05	0.04	+0.00
OT22	ttCTCACAGTTcAGGAgGTtGGA	chr13:87903905+87904103	0.26	0.03	0.03	0.04	0.02	+0.00
OT23	CACTCACAGTaaAGGAgGTgGGA	chr1:208850269+208850445	0.26	0.05	0.02	0.02	0.04	+0.00
OT24	ttCTCACAGTTcAGGAgGTtGGA	chr2:196572793-196572973	0.26	0.02	0.04	0.04	0.03	+0.00
OT25	tACaCACAGTTTAGG-AGTtGGA	chr5:28033147+28033325	0.24	0.05	0.06	0.05	0.07	+0.00
OT26	CACcCACAGTTTAGGAAGcAGCT	chr5:6741426-6741552	0.23	0.03	0.05	0.04	0.03	+0.01
OT27	CACTCACAa-TTAGGAAGgtCGG	chr2:101723864+101723991	0.22	0.05	0.07	0.06	0.04	+0.01
OT28	tgCTCACAGTTTAGGAAGctGGG	chr12:32901606-32901769	0.22	0.10	0.50	0.03	0.03	**+0.27**
OT29	CAtTCACAGTTTAGGAAcTcAGA	chr13:108113244-108113356	0.19	0.05	0.03	0.06	0.06	–0.02
OT30	ttCTCACAGTTTAGGAtGTgGGG	chrX:79848611-79848728	0.19	n.d.	n.d.	n.d.	n.d.	n.d.
OT31	CACTCACAtTTTAGGgAaTtGGC	chr4:144387480-144387659	0.19	0.03	0.03	0.03	0.04	0.00
OT32	ACACcCACAG-TcAGGAAcTGGG	chr3:11222120+11222285	0.18	0.02	0.04	0.03	0.06	–0.01
OT33	CACTCACAGagTAGGAAtatGGC	chr2:213478565-213478798	0.18	0.08	0.05	0.07	0.06	+0.00
OT34	gtCTCACAGTTT-GGAAGTtGGG	chr9:90910874-90911035	0.17	0.05	0.06	0.04	0.04	+0.02
OT35	ttCTCACAGTTcAGGAAGTtAGG	chr15:76465133+76465249	0.16	0.03	0.00	0.02	0.01	+0.00
OT36	CACTCACAGTTgAGGAAtagGGT	chr5:75926694+75926854	0.16	0.04	0.04	0.04	0.04	+0.00
OT37	ACACT-ACAGaTTAGGAAGaGGA	chr19:49249583-49249695	0.16	0.05	0.09	0.05	0.03	+0.03
OT38	tgCTCACAGTTcAGGAgGTtGGG	chr3:107403236-107403382	0.15	0.03	0.02	0.03	0.03	+0.00
OT39	aACTCACAGTTcAGaAAcTgGGG	chr16:49598950-49599074	0.15	0.06	0.05	0.03	0.03	+0.02
OT40	CgCTCACAGTTTAGGAAaTtGGT	chr7:45486206+45486388	0.14	0.03	0.03	0.04	0.03	–0.01
OT41	CACTCAgAGTcTAaGAAGTcAGC	chr6:120673970+120674124	0.14	0.04	0.05	0.06	0.05	–0.01
OT42	aACaCACAGTTTAGGtAGTtGGC	chr5:73985188+73985369	0.14	0.09	0.10	0.10	0.06	+0.01
OT43	gtCTCACAGTTcAGGAAGTtAGA	chr2:226416848+226416985	0.13	0.05	0.03	0.03	0.02	+0.01
OT44	ttCTCACAGTTTAGGAtGcAGGT	chr14:41204728+41204884	0.13	n.d.	n.d.	n.d.	n.d.	n.d.
OT45	aACTCACAG-TTAGGAAcTtGGA	chr4:59970165+59970299	0.12	0.04	0.05	0.05	0.03	+0.00
OT46	CtCTCACAGTTcAGGAAGccGGA	chr11:71457536+71457667	0.12	0.02	0.02	0.02	0.03	+0.00
OT47	gtCTCACAGTTTAGGAgGgtAGG	chr13:21460941+21461098	0.12	0.05	0.04	0.04	0.03	+0.01
OT48	CACaCACAGTTT-GGtAGTtGGC	chr8:96405209-96405328	0.12	0.03	0.03	0.03	0.07	–0.02
OT49	tACTCACAcTTTAaGAAGTAGGA	chr10:74459927+74460112	0.12	0.04	0.03	0.03	0.04	+0.00
OT50	ttCTCACAGTTcAGGAAGctGGG	chr10:14951533+14951651	0.11	0.02	0.02	0.02	0.03	+0.00

*The *HPD* on-target sequence and top ONE-seq-nominated candidate off-target sequences with mismatched positions and bulges indicated by lower case letters and dashes.

†Coordinates in the GRCh38/hg38 assembly of the human genome; "+" and "-" indicate forward or reverse orientation of protospacer/PAM sequence.

‡This table includes the top 51 ONE-seq-nominated sites, all of which have ONE-seq score > 0 01.

§n.d. = PCR was unsuccessful for the genomic site, or PCR was not attempted.

Supplementary Table 3. Assessment of off-target editing with ABE8.8/HPD4 with plasmid delivery in HuH-7 cells via targeted amplicon sequencing.

Genomic site	Protospacer/PAM sequence*	Amplicon (chromosome: position range)†	ONE-seq score‡	Treated sample 1§	Treated sample 2	Control sample 1	Control sample 2	Mean net A→G editing %
HPD	CACTCACAGTTTAGGAAGTAGGG	chr12:121849585+121849753	1.00	32.80	33.23			
OT1	tACaCACAG-aTAGGAAGTAAGG	chr21:20182393-20182543	1.72	0.06	0.07	0.08	0.08	–0.01
OT2	CACTCACAGaTcAGGAAcTATGG	chr3:9092925+9093061	1.44	0.05	0.06	0.04	0.06	+0.00
OT3	CAtTCACAGTTTAGaAAGTgAGG	chr14:20158742-20158893	0.46	0.06	0.05	0.06	0.04	+0.01
OT4	CACTtACAGTTTAGGAAtacAGC	not assessed	0.46	n.d.	n.d.	n.d.	n.d.	n.d.
OT5	CACaCACAGTTTAGatAGTAGGC	chr9:71133899-71134050	0.41	0.03	0.07	0.07	0.14	–0.05
OT6	CACTCTACAGTTTAGGAAtgtGGA	chr11:34141552-34141694	0.41	0.05	0.03	0.05	0.04	–0.01
OT7	CACTCACt-TTcAGGAAGTAAAG	chr16:86145755-86145933	0.34	0.03	0.02	0.03	0.03	+0.00
OT8	tgCTCACAG-TTAGGAAGTtTGG	chr12:119662566+119662714	0.34	0.03	0.03	0.04	0.05	–0.01
OT9	tACTCACAG-aTAGGAAGcAAGG	chr1:105956790-105956927	0.33	0.07	0.05	0.06	0.05	+0.00
OT10	CACcCACAGTTTAGGAgGTgAGG	chr8:123436530+123436719	0.32	0.03	0.04	0.04	0.04	+0.00
OT11	aACTCACAG-aaAGGAAGTAGGG	chr3:126880143+126880286	0.31	0.12	0.12	0.08	0.08	+0.04
OT12	CACTCACAG-aaAGGAAGccAGG	chr13:111545332+111545529	0.24	n.d.	n.d.	n.d.	n.d.	n.d.
OT13	CACTCACAG-aTAGGAAGTgGGG	chr15:96510560+96510735	0.23	3.15	2.82	0.07	0.07	+**2.92**
OT14	agCTCACAG-TTAGGAAGcAAGG	chr1:234082875-234083074	0.22	0.04	0.05	0.04	0.04	+0.00
OT15	aACaCACAGTTTAGGtAGTtGGC	chr5:73985188+73985369	0.20	0.07	0.06	0.07	0.07	–0.01
OT16	CACTCACAGagTAGGAAtatGGC	chr2:213478565-213478798	0.19	0.06	0.06	0.07	0.06	–0.01
OT17	tACTtACAG-aTAGGAAGcAAGG	chr8:53729339+53729495	0.18	0.06	0.03	0.06	0.05	–0.01
OT18	aAgTCACAGTTcAGGAAaTgAGG	chr6:28301059+28301330	0.17	0.21	0.14	0.20	0.15	+0.00
OT19	tcCTCACAGTTgAGGAAGTAGGG	chr10:34725514-34725668	0.16	0.03	0.03	0.03	0.03	+0.00
OT20	CACTCACAG-aaAGGAAGcgAGG	chr13:111545396+111545569	0.15	n.d.	n.d.	n.d.	n.d.	n.d.
OT21	CACTCA-tcTTTAGGAAGTATGA	chr6:102884454-102884607	0.14	0.02	0.02	0.03	0.03	–0.01
OT22	tACTCACAcTTTAaGAAGTAGGA	chr10:74459927+74460112	0.13	0.04	0.05	0.03	0.04	+0.01
OT23	aACcCACAGTTcAGGAAtTATGG	chr15:59358774-59358929	0.13	0.05	0.05	0.04	0.04	+0.01
OT24	agCTCACA-TTTAaGAAGTgGGG	chr8:98563913-98564062	0.12	0.05	0.03	0.04	0.03	+0.00
OT25	CACTCACAaTTT-GGAAGTATGG	chr5:62633698+62633838	0.11	0.08	0.06	0.05	0.06	+0.01
OT26	tACTtACAG-aTAGGAAGcAAGG	chr4:2363523-2363691	0.11	0.06	0.07	0.07	0.07	+0.00
OT27	aACTCACt-TTTAaGAAGTtGGG	chr11:58388631-58388793	0.11	0.05	0.04	0.03	0.03	+0.01
OT28	tACTCACA-TTcAGGAAGgACGG	chr20:23706230-23706385	0.11	0.05	0.02	0.04	0.04	+0.00
OT29	aACTCACAG-aaAGGAAGTAGTG	chr19:5249437+5249599	0.10	0.10	0.07	0.08	0.09	+0.01
OT30	gtaTCACAGTTTA-GAAGTAGAG	chr15:59025979+59026237	0.09	0.16	0.12	0.07	0.04	+0.08
OT31	CACT-ACAGTccAGGAAaTATGG	chr1:174531664+174531804	0.09	0.05	0.05	0.05	0.03	+0.01
OT32	tACaCACAGTTcAaGAAGTAAGG	chr9:97753663+97753822	0.09	0.05	0.04	0.06	0.07	–0.02
OT33	CgCTCACAGTTTAGGAAaTtGGT	chr7:45486206+45486388	0.09	0.03	0.03	0.03	0.04	–0.01
OT34	ttCTCACAGTTcAGGAAGTtAGG	chr15:76465133+76465249	0.09	0.03	0.02	0.03	0.04	–0.01
OT35	tACaCACAG-aTAGGAAGaAAGG	chr6:106865425+106865557	0.09	0.10	0.08	0.07	0.09	+0.01
OT36	aACTCAtAGTTTAGGAAGagGGC	chr3:171833685-171833914	0.08	n.d.	n.d.	n.d.	n.d.	n.d.
OT37	tACTCACA-TgaAGGAAGTAAGG	chr6:64294296-64294544	0.08	0.06	0.05	0.06	0.03	+0.01
OT38	gtCTCACAGTTT-GGAAGTtGGG	chr9:90910874-90911035	0.08	0.03	0.05	0.04	0.03	+0.00
OT39	agCTCACAGTcTA-GAAGTgGGG	chr1:58937014+58937174	0.08	0.05	0.04	0.05	0.05	+0.00
OT40	ataTCACAGTTTAaGAAaTATGG	chrX:129100077+129100226	0.08	0.06	0.06	0.07	0.05	+0.00
OT41	CACTCAC-GTaTtGGAAGTAGAG	chr2:218276927+218277070	0.07	0.06	0.05	0.05	0.05	+0.00
OT42	CACTCACAGTTgAGGAAtagGGT	chr5:75926694+75926854	0.07	0.05	0.04	0.04	0.04	+0.00
OT43	gACTCACAa-TTAGaAAGTAGGG	chrX:83541561+83541739	0.07	n.d.	n.d.	n.d.	n.d.	n.d.
OT44	ttCTCACAGTTTtGGAAGTgGGG	chrX:108926545-108926663	0.07	0.03	0.04	0.04	0.03	+0.00
OT45	attTCACAGTTTA-GAAGTAGAG	chr5:102250459+102250630	0.06	0.06	0.07	0.07	0.06	+0.00
OT46	CctTCACAGTTTAGGAAGcAAGG	chr13:30173299+30173455	0.06	0.03	0.04	0.06	0.05	–0.02
OT47	tACTCACAGaaTAGGAAa-AGGG	chr9:104188183+104188416	0.06	0.06	0.05	0.06	0.04	+0.01
OT48	tgCTCACAGTTTAGGAAGctGGG	chr12:32901606-32901769	0.06	0.06	0.08	0.03	0.03	+0.04
OT49	gACTCACAGTTaAGGgAagtAGG	chr9:82247483+82247626	0.06	0.04	0.07	0.11	0.07	–0.03
OT50	tACTCACAG-aTAGGgAGcAGGG	chr15:26019155-26019299	0.06	0.06	0.06	0.06	0.07	–0.01
OT51	tACTCACAGTTctGGAAGTAGGG	chr14:26888839+26889031	0.06	n.d.	n.d.	n.d.	n.d.	n.d.
OT52	CACTCACAa-TTAGGAAGgtCGG	chr2:101723864+101723991	0.06	0.06	0.06	0.04	0.05	+0.01

*The *HPD* on-target sequence and top ONE-seq-nominated candidate off-target sequences with mismatched positions and bulges indicated by lower case letters and dashes.

†Coordinates in the GRCh38/hg38 assembly of the human genome; "+" and "-" indicate forward or reverse orientation of protospacer/PAM sequence.

‡This table includes the top 53 ONE-seq-nominated sites, all of which have ONE-seq score > 0 01.

§n.d. = PCR was unsuccessful for the genomic site, or PCR was not attempted.

Supplementary Table 4. Assessment of off-target editing with ABE8.8/HPD5 with plasmid delivery in HuH-7 cells via targeted amplicon sequencing.

Genomic site	Protospacer/PAM sequence*	Amplicon (chromosome: position range)†	ONE-seq score‡	Treated sample 1§	Treated sample 2	Control sample 1	Control sample 2	Mean net A→G editing %
HPD	CACTCACAGTTTAGGAAGTAGGG	chr12:121849585+121849753	1.00	44.77	44.62			
OT1	aACTtACAGTTTAGGAAGaAGAA	chr2:149730234-149730433	0.75	0.05	0.06	0.02	0.06	+0.02
OT2	tACTtACAGTTTAGGAAaaAGAG	chr7:7216857-7217047	0.62	0.04	0.03	0.04	0.03	+0.00
OT3	tACTCACAcTTTAaGAAGTAGGA	chr10:74459927+74460112	0.60	0.02	0.04	0.03	0.09	–0.04
OT4	CACcCACAGTTgAGGAAGcAGAG	chr5:134574934+134575076	0.55	0.03	0.03	0.03	0.04	+0.00
OT5	aACTCACAGTTaAGGgAGTAGTG	chr14:42104487+42104676	0.53	0.07	0.04	0.04	0.06	+0.00
OT6	gACTCAgAGTTTAGGAAGaACAG	chr13:67489114+67489255	0.48	0.04	0.02	0.03	0.04	–0.01
OT7	tCTCACAG-TaAGGAAGcAGAGG	chr20:34952014+34952156	0.43	0.02	0.03	0.03	0.04	–0.01
OT8	gAaTCACAGTTTAaGAAGaAGAG	chr2:63499942-63500082	0.38	0.04	0.02	0.03	0.07	–0.02
OT9	CctTCACAGTTTAGGAAGcAAGG	chr13:30173296+30173430	0.37	0.01	0.02	0.02	0.02	–0.01
OT10	atCTCACAG--aAGGAAGTAGGG	chr3:105980524-105980663	0.36	0.05	0.06	0.04	0.05	+0.01
OT11	ACTCACAG-aaAGGAAaTAGGTG	chr5:114806740+114806991	0.34	0.13	0.09	0.12	0.08	+0.01
OT12	tACTCACAG-aTAGGtAGTAAGG	chr1:217473036-217473177	0.31	0.04	0.04	0.03	0.03	+0.01
OT13	tACTCACAGaTTAGGAtGTACAG	chr4:100586150+100586282	0.26	0.02	0.03	0.03	0.03	+0.00
OT14	CACTCACAGgTTAGGAgGaAGAG	chr1:222073075+222073242	0.25	0.05	0.07	0.05	0.04	+0.02
OT15	tACTCACAG--TAGGAAGTAGCA	chr5:150433268-150433412	0.23	0.01	0.03	0.02	0.04	-0.01
OT16	tACTCACAG-TcAGaAAGTAGAG	chr2:46394123+46394265	0.21	0.03	0.05	0.02	0.04	+0.01
OT17	agCTCACAGTTTAGGAAGgAGGC	chr17:62097919-62098019	0.20	0.03	0.03	0.03	0.03	+0.00
OT18	aACTCACAGacTAGGAAGcATAG	chr6:148367572-148367728	0.19	0.04	0.04	0.03	0.03	+0.01
OT19	tAagCACAGTTTAGGAAGgtGAG	chr6:71011412-71011565	0.18	0.06	0.05	0.05	0.05	+0.00
OT20	aACTtACAGTTaAGaAAGTAGAG	chr2:216658842+216659027	0.17	0.03	0.03	0.04	0.03	+0.00
OT21	aACTCACAG-aaAGGAAGTAGGG	chr3:126880143+126880286	0.17	0.03	0.04	0.06	0.04	–0.02
OT22	aACTCACAGTTTgGGAAGaAAGA	chr3:188702412-188702578	0.16	0.04	0.04	0.04	0.04	+0.00
OT23	CAtTCACAGTTcAGGgAGaAGAG	chr12:13031726-13031879	0.16	0.04	0.04	0.04	0.03	+0.00
OT24	aACaCACAGTcTAGGAAGaAAAG	chr2:179266080-179266250	0.15	0.06	0.06	0.05	0.05	+0.01
OT25	gAaaCACAGTTTAGGAAGTtTTG	chr9:39981045+39981248	0.15	n.d.	n.d.	n.d.	n.d.	n.d.
OT26	gACTCACAGagAGaAAGaAGGG	chr8:59224924+59225128	0.13	n.d.	n.d.	n.d.	n.d.	n.d.
OT27	tACTCACAGTTctGGAAGTAGGG	chr14:26888839+26889031	0.12	0.06	0.08	0.00	0.00	+0.07
OT28	tcCTCACAGTTgAGGAAGTAGGG	chr10:34725514-34725668	0.11	0.02	0.03	0.02	0.03	+0.00
OT29	ACTCACAG--TcaGgAaTAGGGG	chr1:98462417-98462579	0.11	0.03	0.03	0.03	0.04	–0.01
OT30	CACTCACAG-aTAGGAAGTgGGG	chr15:96510560+96510735	0.11	0.05	0.05	0.06	0.04	+0.00
OT31	tACTCACAG-aTAGGgAGcAGGG	chr15:26019155-26019299	0.10	0.04	0.04	0.03	0.05	+0.00
OT32	ttCTCACAGTTTAGGgAtTACAG	chr9:114758066-114758199	0.10	0.03	0.03	0.02	0.04	+0.00
OT33	gAaaCACAGTTTAGGAAGTtTTG	chr9:41482259+41482469	0.09	0.11	0.10	0.12	0.12	–0.01
OT34	ttTCACAGaTTAGGAAGctGAGG	chr1:204565742-204565921	0.09	0.04	0.05	0.03	0.03	+0.01
OT35	tACaCACAGTTcAaGAAGTAAGG	chr9:97753663+97753822	0.09	0.04	0.04	0.03	0.03	+0.01
OT36	gAaaCACAGTTTAGGAAGTtTTG	chr9:62269477+62269674	0.09	n.d.	n.d.	n.d.	n.d.	n.d.
OT37	tACTCACAG-aTAGGAAGcAAGG	chr1:105956790-105956927	0.09	n.d.	0.05	0.04	0.03	+0.01
OT38	CAtTCACAGTTTAGaAAGTgAGG	chr14:20158742-20158893	0.08	0.04	0.04	0.03	0.04	+0.01
OT39	gAaTCACAGTTTAGGgAGagTGG	chr5:143910524-143910667	0.08	0.04	0.05	0.05	0.04	+0.00
OT40	cCTCACAG-TTAGGAAaTAGTTG	chr14:51982054-51982213	0.08	0.03	0.03	0.02	0.02	+0.00
OT41	gtCaCACAGTTTAGGAAGTAGCT	chr20:42662345-42662525	0.08	n.d.	n.d.	n.d.	n.d.	n.d.
OT42	gACTCACA-TTcAGGAAGgtAGG	chr2:81325426+81325576	0.08	0.02	0.02	0.02	0.03	+0.00
OT43	gtCTCACAGTTcAGGAAGTtAGA	chr2:226416848+226416985	0.08	0.02	0.01	0.03	0.03	–0.01
OT44	aAtTCACAGTTTAGGAAGTAGCA	chr14:32471376-32471550	0.08	0.02	0.04	0.02	0.04	+0.00
OT45	tACTCACtG--TAGcAAGTAGAG	chr12:33782613-33782771	0.07	0.03	0.01	0.01	0.02	+0.01
OT46	CACTgACAGTTTAGGAAGaAGTG	chr19:4998447-4998567	0.07	0.03	0.04	0.03	0.02	+0.01
OT47	ACTCACAGTaagGGAAaTAGAAG	chr17:12794963-12795150	0.07	n.d.	n.d.	n.d.	n.d.	n.d.
OT48	tACcCACAGTTTAGGAgGctGAG	chr2:31854443-31854570	0.07	0.03	0.03	0.03	0.04	+0.00
OT49	aACTCACAGaTaAGGAAtTAGCA	chr2:147463226-147463390	0.06	0.05	0.03	0.04	0.03	+0.00
OT50	tCTCACAG--TAGGgAGaAGTGG	chr3:46953117+46953280	0.06	0.04	0.02	0.01	0.02	+0.01
OT51	tAtTCACAG--TAGGAAGTAGGA	chr2:4649666+4649776	0.06	0.02	0.04	0.02	0.03	+0.01
OT52	gACTCACA-TTTAGGgAGTAGCA	chr5:153457501+153457622	0.06	0.04	0.04	0.06	0.01	+0.00

*The *HPD* on-target sequence and top ONE-seq-nominated candidate off-target sequences with mismatched positions and bulges indicated by lower case letters and dashes.

†Coordinates in the GRCh38/hg38 assembly of the human genome; "+" and "-" indicate forward or reverse orientation of protospacer/PAM sequence.

‡This table includes the top 53 ONE-seq-nominated sites, all of which have ONE-seq score > 0 01.

§n.d. = PCR was unsuccessful for the genomic site, or PCR was not attempted.

Supplementary Table 5. Assessment of off-target editing with ABE8.8/HPD20 with plasmid delivery in HuH-7 cells via targeted amplicon sequencing.

Genomic site	Protospacer/PAM sequence*	Amplicon (chromosome: position range)†	ONE-seq score‡	Treated sample 1§	Treated sample 2	Control sample 1	Control sample 2	Mean net A→G editing %
HPD	TCTGCAGAAAGCACGGGAACGGG	chr12:121854709-121854850	1.00	59.38	59.98			
OT1	gCTGCAGAAAGCACaGGtcCAGG	chr1:24300946+24301142	1.82	0.11	0.08	0.06	0.06	+0.03
OT2	TCTGCAGAAAGCACGGcAcaCAG	chr3:46293912+46294063	1.73	0.05	0.05	0.06	0.04	+0.00
OT3	aCTGCAGAAAGCtCaGGAAtCAG	chr11:119970623-119970822	1.53	0.06	0.07	0.07	0.06	+0.00
OT4	gaaGCAGAAAGCACGGGAgtCGG	chr13:42673670+42673866	0.98	0.05	0.08	0.14	0.11	–0.06
OT5	TCTGCAGtAAGCACtGGgAaCAG	chr5:35954904+35955063	0.85	0.03	0.05	0.06	0.03	+0.00
OT6	cCTGCAGAAAaCAtGGGAcCAGG	chr8:25687286-25687419	0.84	0.06	0.04	0.05	0.04	+0.00
OT7	TCTcCAGAAAGCACGGGAcCTGG	chr15:41845393-41845492	0.83	0.08	0.12	0.02	0.03	+0.07
OT8	cCTGCAGAAAGCAtGGaAACAAG	chr3:78206254-78206486	0.69	0.02	0.04	0.08	0.05	–0.03
OT9	gtaGCAGAAAGCACaGGAAtTGG	chr1:165659845+165660004	0.68	0.07	0.09	0.05	0.08	+0.01
OT10	TaTGCAGAAAGCACaGGgcaCAG	chr12:57481823-57481952	0.56	n.d.	0.05	0.06	0.09	–0.02
OT11	caTGCAGAAAGCAaGGGAgCGGG	chr19:10984549-10984826	0.49	n.d.	n.d.	n.d.	n.d.	n.d.
OT12	aCTGCAGAAAGCAgGGagACGGG	chr11:69529236-69529341	0.30	0.08	0.05	0.07	0.05	+0.00
OT13	ggTGCAGAAAaCACaGGAtCTGG	chr22:45315767-45315879	0.24	0.05	0.03	0.04	0.05	+0.00
OT14	cCTGCAG-AAGCACGGGctCTGG	chr15:89333349-89333503	0.23	0.04	0.01	0.02	0.03	+0.00
OT15	ctgGCAGAAAGCAtGGGAACAGG	chr11:6002392+6002543	0.20	0.12	0.06	n.d.	0.07	+0.02
OT16	TCTGCAGAAAGCACGGtccaGGG	chr20:772807+772980	0.18	n.d.	n.d.	n.d.	n.d.	n.d.
OT17	TaTGCAGAAAaCAtGGaAACAGG	chr1:208900627-208900791	0.17	0.08	0.08	0.07	0.07	+0.01
OT18	TgTGCAGAAAGCACGGGAcCAGC	chr7:27496094+27496275	0.16	0.05	0.05	0.04	0.05	+0.00
OT19	aCTGCAGAgAGCACaGGActCTG	chr12:3473405-3473596	0.16	0.09	0.06	0.06	0.07	+0.02
OT20	TCTaCAGAAAaCA-GGaAACAGG	chr11:94882630-94882780	0.13	0.05	0.06	0.04	0.05	+0.01
OT21	TCTaCAGgAAGCACaGGctCAGG	chr5:80631206+80631387	0.13	0.05	0.03	0.02	0.04	+0.01
OT22	TCTGCAGAtAGCACaGGAAaCAG	chr14:93434139-93434369	0.12	n.d.	n.d.	n.d.	n.d.	n.d.
OT23	cCTGCAG-AAGCACaGGAtCTGG	chr2:85318510+85318630	0.11	0.05	0.03	0.02	0.05	+0.00
OT24	cCaGCAGAAAGCACtGGgAaCAG	chr20:44128949+44129094	0.11	0.06	0.06	0.05	0.04	+0.02
OT25	TCTGCAGgcAGCACaaGAACAGG	chr3:131568483+131568700	0.10	0.04	0.02	0.02	0.04	+0.00
OT26	aCTGCAtgAAGCAtGaGAACGGG	chr2:45775271-45775462	0.09	0.05	0.06	0.09	0.04	–0.01
OT27	aCTGCAGAAAGacCGGGgACGGG	chr19:52962559+52962740	0.09	0.08	0.07	0.08	0.07	+0.00
OT28	TCTGCAGAcAGCACaGGtAaCAG	chr10:2911412-2911569	0.09	0.03	0.04	0.02	0.03	+0.01
OT29	TCaGCAcAAAGCAaGGGAtCAGG	chr7:126635688-126635920	0.09	0.09	0.06	0.09	0.06	+0.00
OT30	aCTGCAG-AAGCACtGGgACTGG	chr20:45497078-45497239	0.08	0.05	0.05	0.03	0.06	+0.01
OT31	caTGCAGAAAGCA-GGGcACAGG	chr3:52371719+52371838	0.08	0.06	0.07	0.07	0.06	+0.00
OT32	TCTGCAGttAaCACaGGAACAAG	chr1:230774607-230774718	0.08	0.02	0.03	0.01	0.02	+0.01
OT33	TCTGCAGAcAGCACtGGAACAAG	chr14:104387236-104387343	0.07	0.03	0.03	0.03	0.06	–0.01
OT34	gaTaCAGAAAGCACaGGAACAGG	chr2:100149130+100149266	0.07	0.13	0.14	0.06	0.07	+0.07
OT35	TCTGCAGAAAGCAgGGaccCTGG	chr7:157785580-157785682	0.06	0.08	0.04	0.04	0.10	–0.01
OT36	TCTGCAGgAAGCACaGGcACATG	chr17:32929801+32929935	0.06	0.05	0.04	0.03	0.04	+0.01
OT37	TCTGCAGAAAGCAtGGGtgaTGG	chr11:126901459+126901631	0.06	0.05	0.06	0.06	0.05	+0.01
OT38	aCTaCAGAAAGtA-GGGAACAGG	chr10:46703078-46703275	0.06	0.12	0.13	0.06	0.13	+0.03
OT39	aCTGCAGgAAGCAtGGGAgtGGG	chrX:47225699-47225859	0.04	0.08	0.00	0.04	0.06	–0.01
OT40	TCTGCAGAAAGCACaGtAgaCAG	chr16:60622413+60622573	0.04	0.05	0.05	0.04	0.03	+0.02
OT41	aCTaCAGAAAGtA-GGGAACAGG	chr10:47637113+47637310	0.04	0.12	0.13	0.06	0.13	+0.03
OT42	TgTaCAGAAAGCAgGGaAAtGGC	chr5:137991546+137991760	0.04	0.03	0.06	0.07	0.05	–0.02
OT43	TCTGCAGAgAGaAaGGGAACTGG	chr16:5870906-5871083	0.04	0.04	0.05	0.03	0.04	+0.01
OT44	gtTGCAcAAAGCACaGGAACTGG	chr14:62033276+62033525	0.04	0.04	0.04	0.04	0.05	+0.00
OT45	aCTaCAGAAAGtA-GGGAACAGG	chr10:46856878-46857075	0.04	0.12	0.13	0.06	0.13	+0.03
OT46	TCTGtAGAAAGCAgGGaAAgGGG	chr17:50179782-50179903	0.04	0.06	0.04	0.04	0.05	+0.01
OT47	TCTGCAG-gAGCACaGGAcCCGG	chr20:57125614+57125735	0.04	0.08	0.05	0.03	0.06	+0.01
OT48	ctTGCAGAAAcCACaGGAtCGGG	chr12:69091620-69091827	0.03	0.02	0.03	0.03	0.04	–0.01
OT49	aCTGCAGAAAGCA-GaaAACAGG	chr5:170436647-170436760	0.03	0.07	0.07	0.09	0.05	+0.00
OT50	TCTGCAGtAAtCAtGGGAtCAGG	chr8:37073890+37074041	0.03	0.04	0.05	0.04	0.03	+0.01
OT51	TCTGCAGAAtGCACaGGAAaCAG	chr8:109854275-109854473	0.03	0.07	0.05	0.02	0.06	+0.01
OT52	TCTGCAGgAAGCACaGagAaCTG	chr18:66087503+66087640	0.03	0.06	0.04	0.04	0.03	+0.02
OT53	agaGCAGAAAGCACGGGtAgGGG	chr15:101961283+101961394	0.02	0.06	0.08	0.08	0.08	–0.01
OT54	cgTGCAGgAAGCACGGGAAaGGG	chr16:1525089+1525237	0.02	0.05	0.06	0.07	0.03	+0.00
OT55	agaGCAGAAAGCACGGGtAgGGG	chr9:29346-29457	0.02	0.06	0.08	0.08	0.08	–0.01
OT56	TCTGCAGAAAGCtaGGGActTGG	chr11:74019360+74019555	0.02	0.04	0.06	0.07	0.07	–0.02
OT57	TCTGCA-AAAGCACGGGAtaAGG	chr16:12159771-12159942	0.02	0.05	0.04	0.05	0.05	+0.00
OT58	TgTaCAGAAAGC-CaGGAACAGG	chr3:125754761-125754903	0.02	n.d.	n.d.	n.d.	n.d.	n.d.
OT59	TgTaCAGAAAGCA-GGGAAtGGG	chr2:122750544+122750732	0.02	0.08	0.08	0.04	n.d.	+0.05

OT60	ctgGCAGAAAaCACGGGAAaGGG	chr6:6918026+6918222	0.02	0.02	0.06	0.05	0.04	+0.00
OT61	TCaGCAGAgAGCACaGGAAtGGG	chr13:21004935+21005116	0.02	0.09	0.09	0.06	0.04	+0.04
OT62	TCTGCAGcAAGCACaGGgACACA	chr16:29941570-29941696	0.02	0.04	0.04	0.05	0.02	+0.00
OT63	aCTGCAGAAAGCcaGGGAAgAGG	chr4:140941644+140941750	0.02	0.05	0.05	0.06	0.05	–0.01
OT64	cCTaCAagAAGCACGGGAAaGGG	chr12:38832160+38832334	0.02	0.05	0.05	0.05	0.04	+0.01
OT65	aaaGCAGAAAGCACaGcAACCGG	chr5:148658160+148658297	0.02	0.06	0.09	0.07	0.07	+0.01
OT66	atgGCAGAAAGCA-GGGAgCCGG	chr6:77706756-77706905	0.02	0.08	0.08	0.08	0.07	+0.00
OT67	TCTGCAGAgAGCACGaaAAaTGG	chr18:11223190+11223381	0.02	0.04	0.04	0.02	0.04	+0.01
OT68	gCTGCAGAAAGCAtaGGActTGG	chr5:7514969-7515126	0.02	0.05	0.05	0.06	0.06	–0.01
OT69	agaGCAGAAAGCACGGGtAgGGG	chr1:200084-200195	0.02	0.06	0.08	0.08	0.08	–0.01
OT70	TgTGCAGAAAGCtCaGGAtCAGG	chr20:36414475+36414635	0.02	0.02	0.03	0.04	0.04	–0.01
OT71	cCTGCAG-AAGCACtGGAAtAGG	chr3:186709662+186709845	0.02	0.02	0.04	0.04	0.05	–0.02
OT72	aCTGCAGAAAGCAgGGaAAtGGG	chr3:111673780+111673941	0.02	0.08	0.06	0.06	0.06	+0.01
OT73	agaGCAGAAAGCACGGGtAgGGG	chr2:113583783+113583894	0.02	0.06	0.08	0.08	0.08	–0.01
OT74	TgTGCAGAAAGCA-GGGctCAGG	chr15:22404602-22404777	0.02	0.04	0.03	0.07	0.10	–0.05
OT75	TtaGCAGAAAGCACaaGAcCAGG	chr11:126623596+126623735	0.02	0.07	0.07	0.09	0.08	–0.01
OT76	TCTGCAGAAAaCACGaGAAtTAG	chr10:68737295+68737439	0.02	n.d.	n.d.	n.d.	n.d.	n.d.
OT77	agaGCAGAAAGCACGGGtAgGGG	chr12:31622-31733	0.02	0.06	0.08	0.08	0.08	–0.01
OT78	aggGCAGAAAGCA-GGaAACAGG	chr1:55818798-55818959	0.02	0.08	0.06	0.06	0.06	+0.01
OT79	gCTGCAGAAAGCA-GaGAAtGGG	chr5:55825784-55825928	0.02	0.05	0.06	0.06	0.07	–0.01
OT80	gtaGCAGAAAcCACaGGAACGGG	chr11:62464098+62464250	0.02	0.05	0.05	0.07	0.06	–0.01
OT81	cCTGCAGAAAGCAACaGGAACTGA	chr7:32749854-32749979	0.02	0.06	0.06	0.06	0.04	+0.01
OT82	agaGCAGAAAGCACGGGtAgGGG	chr19:71175-71286	0.02	0.06	0.08	0.08	0.08	–0.01
OT83	TCTGCAGAAAGCAgGGGAAaGGC	chr1:18711522+18711659	0.02	0.05	n.d.	0.05	0.06	+0.00
OT84	TgTGCAGAAAGCA-GGGctCAGG	chr15:20595609-20595784	0.02	0.07	0.05	0.12	0.07	–0.03
OT85	aCTGCAGAGAAaCACGGaAACTAG	chr11:108335836+108336029	0.02	0.05	0.06	0.05	0.04	+0.01
OT86	TtTGCAGAAAaCAaGGGAAaGGG	chr7:26649004+26649176	0.02	0.04	0.07	0.07	0.07	–0.01
OT87	gtTGCAGAAAtCACGGGAgCTGG	chr16:73307950-73308078	0.02	0.04	0.05	0.06	0.08	–0.02
OT88	TaTGCAGAAAGCA--GGAACTGG	chr6:107887698-107887870	0.01	0.07	0.05	0.05	0.11	–0.02
OT89	TgTaCAGAAAGC-CaGGAACAGG	chr11:71901084-71901226	0.01	n.d.	n.d.	n.d.	n.d.	n.d.
OT90	TCaGCAGAAAGCACaGagtCAGG	chr2:109710574+109710842	0.01	0.25	0.08	0.14	0.12	+0.04
OT91	ctTaCAGAAAGaACaGGAACAGG	chr6:13703726-13703885	0.01	0.00	0.05	0.09	0.05	–0.05
OT92	TtaGCAGAAtGCACGGGAAaGGG	chr4:78528872-78529015	0.01	0.04	0.05	0.03	0.07	+0.00
OT93	ctaGCAGAAAGCACaGacACAGG	chr2:96700005+96700195	0.01	0.07	0.05	0.06	0.06	+0.00
OT94	TCTGC--AAAGCACaGGAtCGGG	chr10:79195199-79195302	0.01	0.03	0.04	0.06	0.03	–0.01
OT95	aCTGCAAGgAAaCACGGGAAtGGG	chr3:9010345+9010449	0.01	0.06	0.05	0.04	0.03	+0.02
OT96	TCTGCAGAAg--caGGGAcCTGG	chr17:79426956+79427100	0.01	0.04	0.03	0.05	0.04	–0.01
OT97	aCTGCAtAAAGCACaaGAAaCAG	chr10:47347282-47347458	0.01	0.04	0.05	0.04	0.06	+0.00
OT98	TgaGCAGAAAGCA-GaGAACAGG	chr13:96513276-96513427	0.01	0.07	0.07	0.12	0.11	–0.04
OT99	aCTGtAGAAAGCACGGGccCCAG	chr17:59398252+59398419	0.01	0.06	0.06	0.05	0.05	+0.01
OT100	gCTGCAGAAAaCACGGaAAaGGA	chrX:136061616-136061778	0.01	0.07	0.04	0.03	0.04	+0.02
OT101	TCTGCAGAAAGCAC--aAACAGG	chr8:130448699-130448838	0.01	0.05	0.05	0.08	0.03	–0.01
OT102	agaGCAGAAAGCACGGGtAgGGG	chr1:29568-29679	0.01	0.06	0.08	0.08	0.08	–0.01
OT103	cagGCAGAAAGCAgGGaAcCTGG	chr1:160175195-160175392	0.01	0.07	0.08	0.08	0.07	+0.00
OT104	gCTGCAGAAAGCACaGGggaAGC	chr2:112721634-112721775	0.01	n.d.	0.04	0.05	0.05	–0.01

*The *HPD* on-target sequence and top ONE-seq-nominated candidate off-target sequences with mismatched positions and bulges indicated by lower case letters and dashes.

†Coordinates in the GRCh38/hg38 assembly of the human genome; "+" and "-" indicate forward or reverse orientation of protospacer/PAM sequence.

‡This table includes all sites with ONE-seq score > 0 01.

§n.d. = PCR was unsuccessful for the genomic site, or PCR was not attempted.

Supplementary Table 6. Assessment of off-target editing with ABE8.8/IDUA1 with mRNA delivery in HuH-7 cells via targeted amplicon sequencing.

Genomic site	Protospacer/PAM sequence*	Amplicon (chromosome: position range)†	ONE-seq score‡	Treated sample 1	Treated sample 2	Treated sample 3	Control sample 1	Control sample 2	Control sample 3	Mean net A→G editing %
IDUA	GCTCTAGGCCGAAGTGTCGCAGG	chr4:1002742+1002764	1.00	92.5	93.5	93.9				
OT1	GCTCTAGGCCGAAGaGgCGgGAG	chr1:45625916+45626098	0.02	0.05	0.02	0.05	0.01	0.03	0.04	+0.01
OT2	aCTaTAGGCtGAAGTGTCctGGG	chr14:65720651-65720808	0.01	0.03	0.04	0.04	0.02	0.03	0.02	+0.01

*The *IDUA* on-target sequence and top ONE-seq-nominated candidate off-target sequences with mismatched positions and bulges indicated by lower case letters and dashes.

†Coordinates in the GRCh38/hg38 assembly of the human genome; "+" and "-" indicate forward or reverse orientation of protospacer/PAM sequence.

‡This table includes all sites with ONE-seq score > 0.01.

Supplementary Table 7. PCR primers for next-generation sequencing.

Site*	Forward primer	Reverse primer
HPD3 on-target site	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG ACATGGAGGAAGGGGCATTA	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG GGACACCACACACACCCT
HPD3 OT1	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG AGATGCAAACACAGACACGC	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG TTGAAAACCACCTCTGCTGC
HPD3 OT2	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG GCTTGGGAAACTCTGCTCTG	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG GACAGAACCGAATCCAAACCA
HPD3 OT3	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG GCAGAAGATGAAGGAGCAGC	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG GTGTTCTTCAGCTCTTCCCG
HPD3 OT4	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG AGCGAAACTCCGTCTCAGAA	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG AAAACCCTCGCTCCCAACAT
HPD3 OT5	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG GGGCTCTCTTCTGGTATCCG	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG GACACAGCAAGAAGACAGCC
HPD3 OT6	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG CTACCGTTCCCTGTGTCCAC	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG CCCTCTCTGCCTTGTTCTCT
HPD3 OT7	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG AGCCCTCTCAAGTTCTCCAG	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG AGTGTCTTGTAGCCGGTTGA
HPD3 OT8	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG ACTGTCTCTCACACACACACA	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG GGGACAACTTAAGATTACCTGCT
HPD3 OT9	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG TCCCCTCCCTCCTTTCCATA	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG ATGATGCTACTCTTCCCTCAAG
HPD3 OT10	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG TTGTCCTCTGATGGCTCACC	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG AGAGGAGAAAGGGCCGATAC
HPD3 OT11	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG GTCCCATGTCCTGCTCCTG	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG CCTCCTCACACACGTACACT
HPD3 OT12	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG CCTAATTTTTCTGATTTGATCACAACACTTTGCA AGAATG	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG GTGCCCTAAGTCTCGTGTGTGTGTGTG
HPD3 OT13	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG AGGAGCTTCTGGACATTTGAA	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG ATGCCTCCCACTAAACCCAA
HPD3 OT14	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG GTCAGAATAAGCCACTCATTGA	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG AGCTGTCGTTATAGAGGGAAC
HPD3 OT15	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG CAACCATGTGACTGCAACCA	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG TTCTACTGGATGGTGGGCAA
HPD3 OT16	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG GCTTAGTCGGATTGGGCTTC	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG AAATCGGCCGGCACTTTGAT
HPD3 OT17	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG ACTTGAGACTGGGTAATTTGTC	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG CTTCCCTTTCGCTTCTCTGC
HPD3 OT18	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG TTGCTAAATTTACTTATTAGCTCAAGTAGGTTGT GTATCC	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG CTCAGTGCTTCTATTCAAAATTTTACTGGAGCTCC C
HPD3 OT19	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG TGGCAGTGGGATTTCAGGAT	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG AGCCTGACCTTTTACCACCA
HPD3 OT20	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG ACAATAACACAGCTTTGCCAGT	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG ATGAAATGTCCCATTCTACCTC
HPD3 OT21	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG TTCAGAATCCACAGTACACAAGA	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG CCACTGTGAATTTCTTGCCCA
HPD3 OT22	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG ACTTTCATGTAAGGGAGTGAGAC	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG GTGGGCTCTCACCTGACACTGAATTTGCC
HPD3 OT23	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG GGAAGTGTAATCCCCAGACCT	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG CCTCCCTTCTGTCTGTCTGG
HPD3 OT24	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG ACACGTCTTAGTCAGCTCTG	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG CTACACAGCCACAAGACAGC
HPD3 OT25	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG GCTAGTTTTCCTGGAGCCCT	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG ACAGTGGGTATTAGTGGACAGA
HPD3 OT26	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG ACTCATCAGAAACTGCTTTGGA	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG GGAGAGTGCCAGGAGTGATT

HPD3 OT27	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG TCATGTCAGTCCTTTGCCCT	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG AGAGCAGAAGTTACAGGGAAGA
HPD3 OT28	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG AACAGCCAAACACCGCAAAA	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG AGGCCCTGTCATGTTCTTCT
HPD3 OT29	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG GTGCAGTCTCTCCTCTCACC	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG GCCTACATATCCTTTTCAGTGCT
HPD3 OT30	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG TCTTAGTCTGTTCAGGCTGCT	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG GTCACCAAATCTGCCAGTGT
HPD3 OT31	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG AGCCTCTAAAACTGTGAGAAGT	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG GGTTTCCAGCTTGCTGACTG
HPD3 OT32	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG TCAGATTGTACAGGGGAGCA	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG GTTCCAGCTGTGCACACTTC
HPD3 OT33	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG CCTCCTTGATTTTAGCTTCCCC	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG ACTCTCGAGGATGGTGATTCA
HPD3 OT34	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG GGGGCTTCCTGTCAATACCT	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG AGGCATACACACTGGGAGAC
HPD3 OT35	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG TGACTTATTCCATTTGAGCTGCT	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG CCACTTCTGCCAGCATCTTG
HPD3 OT36	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG GGGCCAGACTGTGCATATGA	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG ACAAGAGATTAAAAGGAACGGGA
HPD3 OT37	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG CCATGCAAACCAATGAGAAAGC	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG TCCTGCTGAATTGGCTCATT
HPD3 OT38	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG TGGGCTGATACAATGAACTGC	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG ACACTGTCTATGAGCCAGAGT
HPD3 OT39	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG AGGGAGGGATTTCTGCTGAG	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG AAGTGGGGTGACTATTGGCT
HPD3 OT40	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG CAGGGAACAGTCTGTCAAACT	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG TGCATTTTCTAGAGCCAAGCA
HPD3 OT41	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG TCCATTGTCTTGACCCTGCT	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG TCCAAATGACTCTAGTACACTCT
HPD3 OT42	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG CCTTCTTGTCAACGTGGAGT	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG TCCACACAGAAAGTACATTGT
HPD3 OT43	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG TGATCACACATTAGCGCATTGT	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG GGAACCAACCCTGACAATACC
HPD3 OT44	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG TACACTACGGTCAGCCTTGA	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG ACACACGTATTAGAAGCTGAA
HPD3 OT45	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG ACCCATGCAGTTGTTTCCTG	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG TGTGGCTTTTGTTCTGAGTGT
HPD3 OT46	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG ATTAATTCCTGTGGCTGCCG	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG CTTCGGAGGGAGCCAGAC
HPD3 OT47	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG CACATTCGTTTCCTAGGGTGAC	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG ACATGGGATTTGGAGGGGAC
HPD3 OT48	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG CCTACTCTCTCAGCACAGCA	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG GGAGTGGGTGTTATTGCTGC
HPD3 OT49	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG CAACCCAAGCCTGTGATCAC	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG TTGGAGCCGAGAAACAGTTG
HPD3 OT50	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG CACCAGCGCTGTAAGGAAAG	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG TGCCCTCTCCAGAAACCAAA
HPD4 on-target site	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG ATTCTGCCCCAACACACATG	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG ACTACATCGGCCAATTCTTGC
HPD4 OT1	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG TATGGTTTGCATGCATCCCC	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG CTGCACAACTGTCCTGAACC
HPD4 OT2	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG TGATTGTGTCCCTGATGTCGA	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG AGCCCTGTCACTTCCCATG
HPD4 OT3	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG AGCCCTCTCAAGTTCTCCAG	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG AGTGTCTTGTAGCCGGTTGA
HPD4 OT5	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG AGATGCAAACACAGACACGC	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG TTGAAAACCACCTCTGCTGC
HPD4 OT6	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG GCAGAAGATGAAGGAGCAGC	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG GTGTTCTTCAGCTCTTCCCG

HPD4 OT7	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG TCACACAGACACTACACCTGA	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG GGTATTAATCTTGATCACCTGGC
HPD4 OT8	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG GGCTTCTCTCAGGGTGTCTT	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG ACCATCATACCCCATCTGCA
HPD4 OT9	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG CACCATGCACTAAGTATCGATCC	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG CCTTGGAAATCTGGCTCACC
HPD4 OT10	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG TCCCTGCCTCTTTGACACTT	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG TCAATGGTGATGTCAGACGAGA
HPD4 OT11	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG CTGGGATAAGAGGAGGGCAG	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG CCATCACTCAGTCCCAACCT
HPD4 OT12	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG CCATCCTCAAGTCTCATCCCTCAAATTAACAAAC CAC	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG CAGGCATACAGAAAGTGCTCAGTTAACACTCAC
HPD4 OT13	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG CTGCAGTAACCCAGGAGATG	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG AAAGAGCCAAGCGTGTGAAG
HPD4 OT14	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG TGCCTTCTCTCTCTTATTCTCCT	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG ACCTGGGAATTATGGGAGCC
HPD4 OT15	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG CCTTCTTGTCAACGTGGAGT	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG TCCACACAGAAAGTACATTGT
HPD4 OT16	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG CCTCCTTGATTTTAGCTTCCCC	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG ACTCTCGAGGATGGTGATTCA
HPD4 OT17	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG GAAACAGAACACCCACACCC	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG AGGCATGCACAGATGAGACT
HPD4 OT18	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG GGTCAGCACTGGATTCTGGA	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG TGCATCTCTAACCAGGTTCTG
HPD4 OT19	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG AGAACCACCATGTATTCCCTCA	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG CGTTGCCATTCTGATAAGCCA
HPD4 OT20	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG CCAAGTGATACCCGTGAATGTTAACTGATCAC	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG CATACAGAAAGTGCTCAGTTAACAGTCACGG
HPD4 OT21	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG GATTACTTAGAACTAGTGCCACA	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG TGTTCTCCCTGCATTATTTTACT
HPD4 OT22	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG CAACCCAAGCCTGTGATCAC	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG TTGGAGCCGAGAAACAGTTG
HPD4 OT23	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG AGGTCTCTCAGTTACTTGGCA	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG ACCACTTTGAACCTCTCTTCTAA
HPD4 OT24	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG GGATCACATGACTGAGGTAGC	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG ACTCCATCCTCAAGAAAGTGG
HPD4 OT25	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG AGGAATGCTCAACCAGGTGA	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG GCTTTCTAAAATAGCCAAGTCCC
HPD4 OT26	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG GTTTAGTCCTCCATGCGCTG	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG TAGCCCTGGTGCAAAGAGAA
HPD4 OT27	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG AGACCTGATTTGGGAGATGGA	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG GCAGTGCAATTAGTGGCTAAC
HPD4 OT28	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG TGATTGGTGTAGGGGATGGG	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG ACCAACATCCAGAGGCAGAA
HPD4 OT29	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG TGCAGATGAGGGAACAGAGA	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG ACTTGGCCGTAATCCTCGTT
HPD4 OT30	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG CTTAGCCGCCCAAAGTGC	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG AGTTTATGTGAGCAAAAGGAGGA
HPD4 OT31	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG TTGAGGCTGTAGTGTGCTTT	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG TCCTAGGGTTCCACAATTACTCA
HPD4 OT32	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG CCCTTTCCCCATAGTCTACTCA	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG GGCTTACCTATCCTGCGTCA
HPD4 OT33	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG CAGGGAACAGTCTGTCAAACT	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG TGCATTTTCTAGAGCCAAGCA
HPD4 OT34	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG TGACTTATTCCATTTGAGCTGCT	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG CCACTTCTGCCAGCATCTTG
HPD4 OT35	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG TGGACATACAGCACCTCAGG	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG TTCTTCATATGCCCAGCCCC

HPD4 OT36	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG AACCTTTCTCTCCCCATGGT	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG GAGGACTCACATGTAATAGGGGA
HPD4 OT37	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG GGAAGACGACATGTGATGCA	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG ACTGCAAAGAAGAGGTGAGGA
HPD4 OT38	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG GGGGCTTCCTGTCAATACCT	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG AGGCATACACACTGGGAGAC
HPD4 OT39	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG ACTATGTGCGTGCCATTGTC	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG CTGTTCTGGACCCCTCAAGT
HPD4 OT40	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG TCTCTGCTCACCATCTCAATCA	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG AGGGCTTTCAGTGTCTCAGT
HPD4 OT41	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG GCCTGCGAGACCATGCTATA	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG CTGTGTCCTGGGAATTGTGC
HPD4 OT42	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG GGGCCAGACTGTGCATATGA	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG ACAAGAGATTAAAAGGAACGGGA
HPD4 OT43	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG GCAAGCAGAGCAGGACAAAT	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG ACCTAACTATGCCTGTTCCTGT
HPD4 OT44	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG GTTCTTTCAAACTGCTGTAACA	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG CACTGAATGTACTGGAGCCTTG
HPD4 OT45	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG ACTGCTAGACCCTATGCTGG	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG TGTCCTTCCTTGGTGCTTCT
HPD4 OT46	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG TGCACGCTCACTTCTGACTC	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG TGCAGAGACAGGAAGCGAAT
HPD4 OT47	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG TGGCAGTGGGATTTCAGGAT	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG CTTCCACCTCCTACCCAGTG
HPD4 OT48	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG AACAGCCAAACACCGCAAAA	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG AGGCCCTGTCATGTTCTTCT
HPD4 OT49	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG GGAGTACTGCCAGGCTTTTG	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG TGGATGGCATTTCTGGACCT
HPD4 OT50	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG CCCAAGACGAGTGAGACAGA	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG ATCTGCTCTGGGAAGGCTTC
HPD4 OT51	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG TCTTAGTTTATTCAGTTGCTGCA	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG AGGACCCAGTAAGAAGGCAC
HPD4 OT52	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG TCATGTCAGTCCTTTGCCCT	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG AGAGCAGAAGTTACAGGGAAGA
HPD5 on-target site	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG ATTCTGCCCCAACACACATG	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG ACTACATCGGCCAATTCTTGC
HPD5 OT1	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG CCTGCCTCTGTTGATTGCTC	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG CATGATGTGGTATGGCAGTGT
HPD5 OT2	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG TCTTCCATCTACCCTGTCGC	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG ATGACCATGTGCTTCCCAGA
HPD5 OT3	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG CAACCCAAGCCTGTGATCAC	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG TTGGAGCCGAGAAACAGTTG
HPD5 OT4	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG GCAGTGATGCTCCAACAACA	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG TCTGGAATCCTGTGCCTGAG
HPD5 OT5	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG GCAGTCCTCCATCCCTGAAT	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG TCTCTTTCATCTGGCAGGCT
HPD5 OT6	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG TGCAGCAGATGTACAGATGG	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG CAAGAAAGAAGACCCCGTGC
HPD5 OT7	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG GCTCTTAACAACTTCCATGTGC	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG CTCGGGCAAGTCATTTCACC
HPD5 OT8	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG AAGTCTGGGTAAGGGGTTCC	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG TTTACTTCTGCCTTGCCCCT
HPD5 OT9	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG CATTGCACGCTCACTTCTGA	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG ATCCTGGGCGCATTCTAAGA
HPD5 OT10	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG GGTGGGGATGTTGTGGATTG	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG ATTGCCTTCCCCTCCAAAGA
HPD5 OT11	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG CAGGAAGACAATAAAAATATATGTGCCTCTGGC TCC	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG CACCTTTAAATATATTATTCACATGGCAATCCATA GAGGG

HPD5 OT12	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG TCTCACTAAGGCAGAACACACT	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG CTATCAGTCCCAGGCAGCTT
HPD5 OT13	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG ACACAAGCTCTTAGACTCTTTGT	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG GATGAAGTCGGCCCTGAGAG
HPD5 OT14	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG GCCTCTCTTTACATCATTTTGCT	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG GGGGCTCCTCTGACTCTAAG
HPD5 OT15	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG GTGAGCCAAGATGCCGTTG	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG CTACTTCCTTACACCTCTGATGG
HPD5 OT16	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG GGTAGACCCATCCCCTCTCA	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG TACTCTGGTCAAGCTCAGCC
HPD5 OT17	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG ACAATAACACAGCTTTGCCAGT	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG ATGAAATGTCCCATTCTACCTC
HPD5 OT18	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG ATCCCCACCAACTCCATCAC	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG GACCAGGGTGAAGCAGGAT
HPD5 OT19	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG TGGGACTGTGTAATTGTTGGC	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG TTTTGTTGTGCATTCCTTGACT
HPD5 OT20	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG ACACCCGGCCTAAGTCATC	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG TGTAGCCGGGTTAAAGTCCA
HPD5 OT21	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG CTGGGATAAGAGGAGGGCAG	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG CCATCACTCAGTCCCAACCT
HPD5 OT22	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG GATTACCAAGTGCCAAGAACT	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG CAGCCTTCTTCCGTCTCAGA
HPD5 OT23	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG ATGTGTGATGCAGGAGGGG	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG GCTTGGTTTCAGGGACAACA
HPD5 OT24	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG ACTGTGCTATGTTCTGGGGA	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG TCTCTGCTTTCATAAAATCGTCT
HPD5 OT25	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG CTTCAGTGATGCAAGCCTGG	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG CTGTTCCTGGCCAATGACTG
HPD5 OT26	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG AGAGGGAAGTGGCCAAGATG	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG GTTCCCTTGGCTCCATGTTG
HPD5 OT27	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG TCTTAGTTTATTCAGTTGCTGCA	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG AGGACCCAGTAAGAAGGCAC
HPD5 OT28	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG AGAACCACCATGTATTCCCTCA	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG CGTTGCCATTCTGATAAGCCA
HPD5 OT29	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG GGCACTTGATAAACACTGGG	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG GCTTTGAAAGGGCAGAACTTC
HPD5 OT30	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG CTGCAGTAACCCAGGAGATG	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG AAAGAGCCAAGCGTGTGAAG
HPD5 OT31	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG CCCAAGACGAGTGAGACAGA	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG ATCTGCTCTGGGAAGGCTTC
HPD5 OT32	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG GGTGTCGAATTTCTGGCCTC	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG TTTCTCTGAGCCTAGCCCAC
HPD5 OT33	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG CTGTTCCTGGCCAATGACTG	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG CTTCAGTGATGCAAGCCTGG
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HPD5 OT39	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG CAAGGAAGCATCATGGCCTC	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG CTTTAACATGTAGCTACTTTGGG
HPD5 OT40	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG CCCAATGAGCCCAACTTTCC	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG TCAAAGACTGCCCATCTCCA
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HPD5 OT42	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG ACTCTCCAAACACAGTCTTCCT	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG CCTCCTTTTACCTGCCCCAA
HPD5 OT43	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG TGATCACACATTAGCGCATTGT	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG GGAACCAACCCTGACAATACC
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HPD5 OT46	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG GGGGAAATACACACTGAAGGAG	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG GACATTCCCTGCTTTGCCTT
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HPD5 OT51	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG CCTTTCCATCACCGTTGCAA	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG GGCAAATCACCTCCAACCTC
HPD5 OT52	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG TGTGACCTGCCGAATTCTCT	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG GGTGGAAAGCAACAGGTCAC
HPD20 on-target site	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG GCAAGGGTTCTGGAAGGCTC	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG CAGCACAGCAAACTTCACCT
HPD20 OT1	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG ACTTGGAATGAGGGGCGAG	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG AGGAGAAGGCAGTGTTGTCA
HPD20 OT2	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG TGTTTCTGAGCTGCGTCTTG	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG GCCCCATCCACATCTCACTA
HPD20 OT3	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG CAGAACTAACTGCTCCGCG	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG ATAGGACAGTCAAGGGCTCG
HPD20 OT4	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG CAGTGACAGGGGTGGGATAT	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG CTCCACTCCTCAGCTCTTGT
HPD20 OT5	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG CAAGAAGGTCACTATGCCGC	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG CCCCAACACAAAAGAGTAGAGAG
HPD20 OT6	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG AAGACTCTAGGGCACAGCTG	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG AGGTATAGGTGGGTGGGAGT
HPD20 OT7	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG CCCTGCCTCCTTCCCAAAG	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG GATTGCAGATGTCACACCCA
HPD20 OT8	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG CCATTGGGCTCTGTGTACTT	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG AGGGGCATGGATTTAGAAGGT
HPD20 OT9	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG AGCTGCTAAGTTGGTGGTAATTT	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG GTCTTCCACATCAGCAAGTCTAT
HPD20 OT10	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG AGTCCTTAGCAGCACATGGA	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG CCTTCCTCTGCCTGTTCCTT
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HPD20 OT14	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG CGAGCAAATCTTCGGGCAAG	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG CCAGGTTGTCCCCGTAGAG
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HPD20 OT20	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG GGCTGGGGATGTTACTAAGC	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG TGGAGGGGAAGATGAAAGCC
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HPD20 OT25	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG CAGAGCCACCACGTTTTCAT	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG ACTTGGTGTCATGGAGGGG
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HPD20 OT27	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG GGTGAGGACTTGAAACAGCG	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG ATTGAAACGTCCGGGTGAGA
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HPD20 OT99	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG CGGTGCATCTTTCCTCTTCG	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG GGGGCATGAGGAATCTACCA
HPD20 OT100	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG ACAAGGAGGCTGTGGGAATT	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG CCTCTGATCTGCACGTGATG
HPD20 OT101	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG CCTAGCAAAGCCTCGCATTC	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG AGGAGGAGGAGGGTGGTATT
HPD20 OT102	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG CCTGAGCTTGAGGGGAAGG	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG CATCCGTTGTCTTCCGAGC
HPD20 OT103	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG ACCAAGCAAAGTGTCAGGAT	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG TGAGCCCAATTACACACCCT
HPD20 OT104	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG CCCAGAATCCAAGGCCCAA	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG TTGCCTTCAGACACACATGG
IDUA1 on-target site	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG GGGGACTCCTTCACCAAGGG	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAC TCGCGTAGATCAGCACCG
IDUA1 OT1	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG TGCAACAGTGATAGGCATTCAG	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG GATTCCAAGGCAGTTCAGGG
IDUA1 OT2	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG AAATGTCCATCCCAGCGGTT	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG AATGACTCTCGAACCCAGCA
PAH1 on-target site	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG ACCTCATTCTTGCAGCAGGA	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG TCTCGGGATTTCTTGGGTGG

PAH1 on-target site (human)	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG ACCTCATTCTTGCAGCAGGA	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG TCTCGGGATTTCTTGGGTGG
PAH1 on-target site (humanized mice)	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG TGCTGGCTTACTGTCGTCTC	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG GAGCATCCATTGTGGTTGGC
PAH1_OT1	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG TTCTGTAGTTCTTTCTGTTCTCA	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG ACATCTACAATGGTGGCACT
PAH1_OT2	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG TCACCTATCCCAACCACACA	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG GAAAATTCAGCATTATGGTCCCA
PAH1_OT3	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG AGCTCAAGTCACCAGGCTTA	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG CAAAGCTCACAGTTGACACCA
PAH1_OT4	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG ACAGCCATACCCGTAAGTCT	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG ACTGAAGAAAGAGCTAAAGTGGT
PAH1_OT5	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG GGGCCTGAGGAGAGATGAACTGAG	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG CCCGCTCTCCGAGAAACACCGAAGAATGATC
PAH1_OT6	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG AGTTCCATCCATGTTGTTGC	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG ACCTGGGTGATGAAATAATCTGT
PAH1_OT7	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG GGTGTTGTGCCATCGGAAAA	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG ACTTTGTGGCATGGAGAAGG
PAH4 on-target site	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG ATCTTAAGCTGCTGGGTATTGT	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG GCCCTTCACTCAAGCCTGT
HPD lentiviral screen	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG ATCTTGTGGAAAGGACGAAA	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG CTAGCTAGCGAATTCAAAAC