Achieve high specificity and on-target efficiency with TrueCut HiFi Cas9 Protein

CRISPR-Cas9 genome editing is one of the most popular gene therapy tools due to its simplicity and low cost, but its successful application depends on many factors. We have years of experience refining and optimizing each factor to help ensure maximum editing efficiency across a broad spectrum of cell types, including induced pluripotent stem cells (iPSCs) and primary cells. While refinements in the delivery and localization of nucleases have steadily improved on-target editing efficiency, off-target effects continue to be a problem. This is extremely relevant for therapeutic applications in which low on-target editing efficiency and off-target edits can have undesirable effects. To address these issues, we set out to identify a Cas9 variant that would limit the number of off-target edits.

We applied a target-enriched GUIDE-seq (TEG-seq) designed to detect off targets in cell genomes [1]. We then screened several Cas9 candidates that were either published or engineered in-house to identify a high-fidelity Cas9 protein. We optimized editing efficiency by modifying the N- and C-termini of a high-fidelity Cas9 protein, which resulted in Invitrogen™ TrueCut™ High-Fidelity (HiFi) Cas9 Protein (TrueCut HiFi Cas9). The data presented here show that the fidelity of TrueCut HiFi Cas9 in HEK293 cells, primary T cells, and iPSCs is higher than that of wild type Invitrogen™ TrueCut™ Cas9 Protein v2 (wt-Cas9) and other commercially available high-fidelity Cas9 proteins.

Table 1 shows the results of genome-wide screening for off targets in HEK293 cells transfected with an HEK4 gRNA and either wt-Cas9, high-fidelity Sniper Cas9 [2], Supplier I high-fidelity Cas9, or TrueCut HiFi Cas9. TrueCut HiFi Cas9 saw fewer off targets than the Sniper and Supplier I proteins.

To better understand the high fidelity of TrueCut HiFi Cas9, we evaluated additional genes in the HEK293 genome. More genome-wide screening was performed using TEG-seq to detect off targets in the *HEK1*, *HEK4*, *VEG1*, and *VEG3* genes. The data showed that TrueCut HiFi Cas9 generated fewer off-target edits than wt-Cas9 and Supplier I high-fidelity Cas9 protein (Figure 1). The percentage of off-target edits at each editing site was compared to the percentage of on-target edits to determine an off-target/on-target probability ratio for the respective site. Each editing event was plotted against its probability ratio (Figure 1A), and the total number of off targets was grouped according to probability (Figure 1B). The results demonstrated that TrueCut HiFi Cas9 generated significantly fewer off-target edits than the wt-Cas9 and Supplier I high-fidelity Cas9. Only one off-target edit for TrueCut HiFi Cas9 had a probability of >10%. In contrast, the wt-Cas9 and Supplier I high-fidelity Cas9 had 16 and 6 off targets, respectively (Figure 1B).

Table 1. Off-target reads per million (RPM) detected by TEG-seq with an HEK4 gRNA in HEK293 cells.

Target	MM	Align sequence	PAM	wt-Cas9	Sniper	Supplier I	TrueCut HiFi Cas9
On	0	GGCACTGCGGCTGGAGGTGG	GGG	25,950	112,147	57,977	41,848
Off-1	2	. . . . . GA.	GGG	23,050	26,225	6,608	691
Off-2	2	. . . . A . . . . . C . .	AGG	20,196	37,895	21,393	497
Off-3	2	. . . G. . . . . G.	AGG	18,843	8,898	1,074	7
Off-4	3	A. . T. . . . . A. .	GGG	16,942	3,890	24	0
Off-5	3	A. . . G. . . . . A.	TGG	10,310	5,654	629	0
Off-6	3	T. . . . . C. . . . . A. .	TGG	9,697	13,852	12,438	10
Off-7	3	A .G. . . . . G.	TGG	8,763	4,072	881	5
Off-8	4	. A. . . CA. . . . . A . . .	TGG	6,934	619	0	0
Off-9	3	. . . . TCA.	AGG	5,215	0	0	2
Off-10	2	. . . . . T. . . . . C.	AGG	3,113	976	0	2
Off-11	2	. . . . . G . . T	GGG	2,988	0	2,180	0
Off-12	2	. . . . . T . . . . . G	TGG	1,984	172	0	0
Off-13	2	. . . T . . . . . G . .	TGG	1,386	1,987	208	0
Off-14	2	. . . . – . . . . . g	AGG	1,272	0	0	0
Off-15	3	A . A . . . . . T	TGG	1,182	0	0	0
Off-16	3	CC . . . . . G	GGG	1,128	0	0	0
Off-17	3	T . . . . . T . . . . . A . . .	GGG	1,014	2	2	0
Off-18	3	. . T . . . CT	TGG	908	0	0	0
Off-19	3	. C . . . . . A . . . A	AGG	869	0	0	0
Off-20	3	. . . . . g . . A . . C	TGG	800	344	718	0
Off-21	3	. . . T . C . . A	GGG	744	0	0	0
Off-22	3	. . . . . A . A . . . . G	GGG	628	0	0	0
Off-23	4	A . . . . . A . . . . . GA	AGG	609	676	0	0
Off-24	3	. A . . . . . A . . . A	GGG	550	0	0	0
Off-25	3	T . G . . . . . a . . .	AGG	511	271	114	0
Off-26	2	. . . . . G . . . . . C . .	GGG	498	2,145	0	0
Off-27	4	. A . . . C.T.A	AGG	414	182	0	0
Off-28	3	. . . . . G . . . . . G . . A . .	GGG	320	0	353	0
Off-29	2	. . . . . A . . G	GGG	216	0	0	0
Off-30	4	. . . TG . . . . . CA	AGG	211	0	0	0
Off-31	2	. . A . . . . T	CAG	194	287	0	0
Off-32	3	. . . . . G . A . . . . –	TGG	135	0	0	0
Off-33	3	. C . . . . . G . . . . . G	GGG	80	0	0	0
Off-34	3	. . A . . . . G . . . . . G	GGG	42	0	0	0

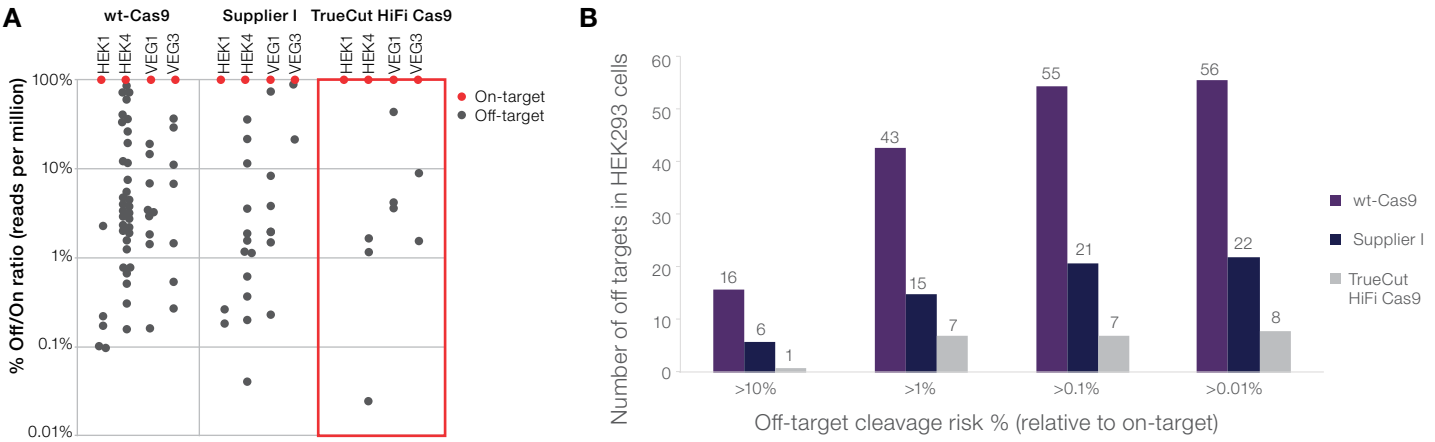

Figure 1. Off targets detected in HEK293 cells by TEG-seq and the corresponding off/on ratios. (A) Each off/on ratio was calculated based on the reads per million (RPM) for an individual off target divided by the RPM for the corresponding on target. The RPMs were derived from an Ion Torrent™ next-generation sequencing (NGS) TEG-seq assay. The red dots represent on targets and are set at 100%. The gray dots represent off targets plotted according to the corresponding off/on ratios. **(B)** Off-target cleavage risk (%) relative to on-target.

To test the effectiveness of TrueCut HiFi Cas9, wt-Cas9, and Supplier I high-fidelity Cas9 in a more diverse cell set, we performed genome-wide screening for off targets with 21 gRNAs that targeted the therapeutically relevant genes *CD52*, *TRAC*, *TRBC*, and *PD1* in primary T cells (Figure 2). Some of these gRNAs have also been used for CAR T cell gene therapy [2,3]. To identify differences between the fidelities of the three Cas9 proteins, we intentionally included TRBC4, PD1-4, and PD1-5 gRNAs that had a large number of *in silico* off targets. TrueCut HiFi Cas9 generated fewer off-target edits and had lower off/on ratios for individual off targets than the wt-Cas9 and Supplier I high-fidelity Cas9 (Figure 2A). As expected, we detected several off targets with the TRBC4, PD1-4, and PD1-5 gRNAs. A total of 22 and 8 off-target edits were detected for wt-Cas9 and Supplier I high-fidelity Cas9, respectively (Figure 2B). Each off-target edit had read numbers that exceeded the corresponding on-target read numbers, resulting in an off/on ratio >100%, while TrueCut HiFi Cas9 had no off-target edits at the same ratio. The wt-Cas9 and Supplier I high-fidelity Cas9 had 203 and 63 off targets, respectively, with probabilities in the >10% percentile. This was significantly more than the 13 off targets identified for TrueCut HiFi Cas9 (Figure 2B).

The on-target activity in T cells, including indels and homology-directed repairs (HDRs), was further evaluated using a subset of 11 gRNAs that had the fewest off-target edits. Single-stranded oligodeoxynucleotides were used to evaluate HDR, and targeted NGS-based amplicon-seq was used for screening. The percentages of indels and HDRs are shown in Table 2A. The percentages of indels and HDRs normalized to wt-Cas9 (100%) are summarized in Table 2B. The normalized percentages of indels occurring with Supplier I high-fidelity Cas9 and TrueCut HiFi Cas9 were 80% and 84%, respectively. The HDR percentages for the proteins were 79% and 88%, respectively (Figure 3). On average, the percentages of indels and HDRs observed with TrueCut HiFi Cas9 were on par with those observed with Supplier I high-fidelity Cas9.

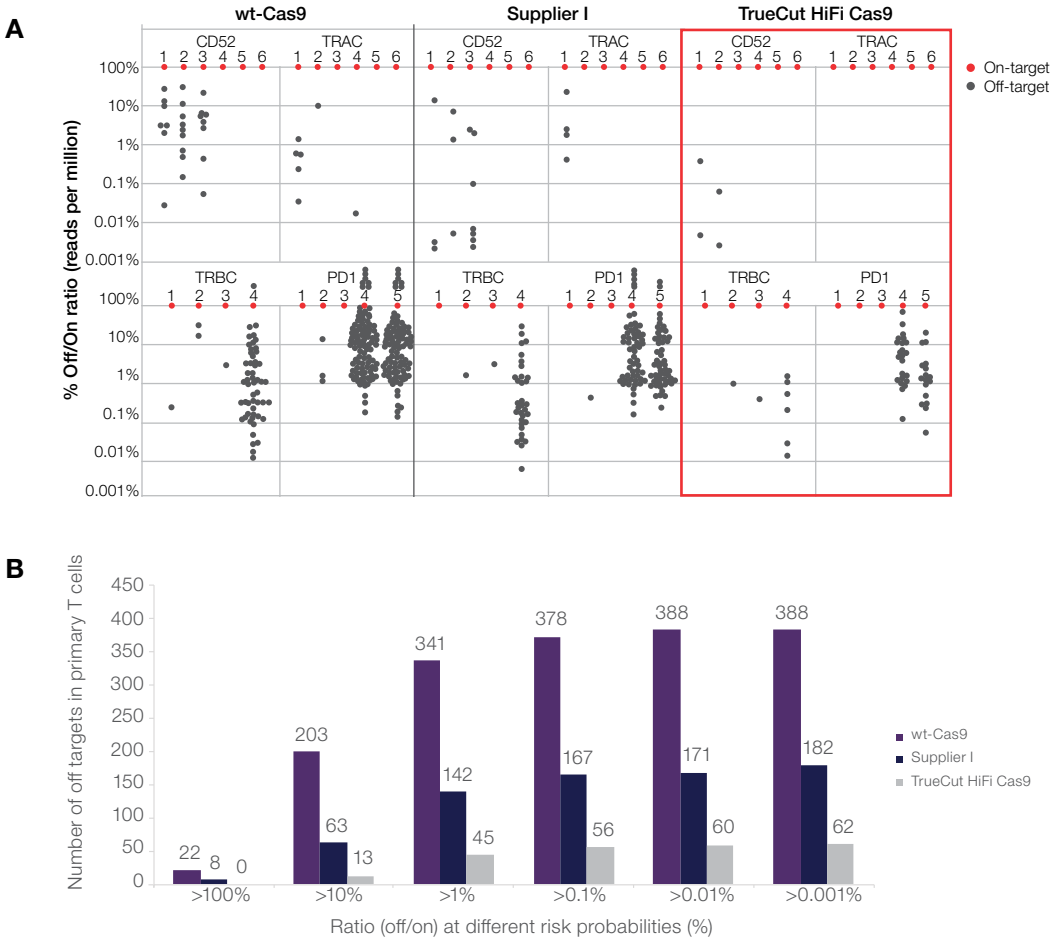

Figure 2. Off targets detected in primary T cells by TEG-seq and the corresponding off/on ratios. (A) 21 gRNAs targeting the *CD52*, *TRAC*, *TRBC*, and *PD1* genes were co-transfected with wt-Cas9, Supplier I high-fidelity Cas9, and TrueCut HiFi Cas9. The numbers listed under the gene names are the gRNA ID numbers. The off/on ratios were calculated based on the RPMs for individual off targets divided by the RPMs for the corresponding on targets. The red dots represent on targets and are set at 100%. The gray dots represent off targets and are plotted according to off/on ratio. Gray dots appearing above the red dots are off targets with reads that exceed those of the on targets. **(B)** Total number of off targets grouped according to risk probability.

Table 2. On-target indel and HDR activity of wt-Cas9, Supplier I high-fidelity Cas9, and TrueCut HiFi Cas9.

A	Indel average (%)			HDR average (%)		
gRNA	wt-Cas9	Supplier I	TrueCut HiFi Cas9	wt-Cas9	Supplier I	TrueCut HiFi Cas9
CD52-4	47.67	44.39	43.33	41.83	42.95	41.13
CD52-5	56.70	56.29	55.43	35.67	34.39	35.25
CD52-6	42.66	40.20	23.16	49.49	51.27	38.43
TRA-2	21.53	21.63	22.94	21.75	19.95	15.79
TRA-4	73.94	72.20	34.55	20.93	22.79	17.41
TRC-6	49.12	30.10	38.67	4.70	3.04	6.53
TRB-1	47.41	40.84	44.92	23.14	18.12	23.29
TRB-2	36.06	30.21	31.92	29.24	21.17	16.35
PD1-1	13.70	9.19	12.21	4.63	2.92	3.23
PD1-2	32.09	20.63	30.05	14.94	9.54	16.23
PD1-3	15.00	4.98	12.99	3.77	0.97	2.54
Average (%)	**39.62**	**33.69**	**31.83**	**22.73**	**20.64**	**19.65**

B	Indels normalized to wt-Cas9 (%)			HDRs normalized to wt-Cas9 (%)		
gRNA	wt-Cas9	Supplier I	TrueCut HiFi Cas9	wt-Cas9	Supplier I	TrueCut HiFi Cas9
CD52-4	100%	93%	91%	100%	103%	98%
CD52-5	100%	99%	98%	100%	96%	99%
CD52-6	100%	94%	54%	100%	104%	78%
TRA-2	100%	100%	107%	100%	92%	73%
TRA-4	100%	98%	47%	100%	109%	83%
TRC-6	100%	61%	79%	100%	65%	139%
TRB-1	100%	86%	95%	100%	78%	101%
TRB-2	100%	84%	89%	100%	72%	56%
PD1-1	100%	67%	89%	100%	63%	70%
PD1-2	100%	64%	94%	100%	64%	109%
PD1-3	100%	33%	87%	100%	26%	67%
Average (%)	**100%**	**80%**	**84%**	**100%**	**79%**	**88%**

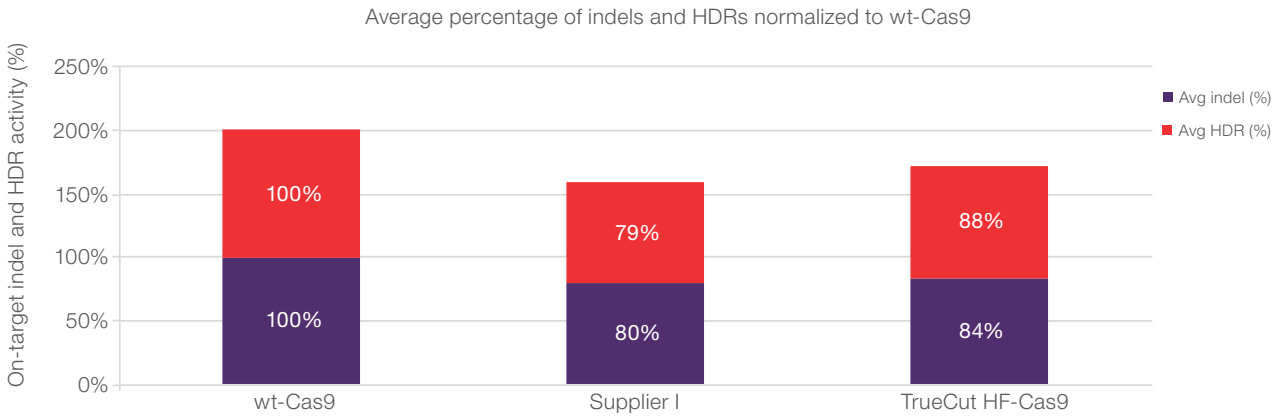

Figure 3. Normalized on-target activity of wt-Cas9, Supplier I high-fidelity Cas9, and TrueCut HiFi Cas9 for indels and HDRs in T cells. The average normalized percentages of indels and HDRs from Table 2B are shown.

The performance of TrueCut HiFi Cas9 was also evaluated in iPSCs. Genome-wide screening for off targets was performed with iPSCs using 4 gRNAs. One of the gRNAs targeted a commonly studied *HEK4* target, and two of the gRNAs targeted SNPs in the hemoglobin β subunit gene (*HBB*) associated with sickle cell anemia. The remaining gRNA has been used to knock out the *BCL11A* gene to cure sickle cell anemia. As shown in Figure 4, off targets were detected with the HBB1, HBB2, and HEK4 gRNAs. No off targets were detected with the BCL11A gRNA (data not shown). In accordance with its efficiency in other cell types, TrueCut HiFi Cas9 generated fewer off-target edits and had lower off/on ratios for individual off targets than wt-Cas9 and Supplier I high-fidelity Cas9 in iPSCs.

On-target activity in the iPSCs, including the percentages of indels and HDRs, was evaluated with the BCL11A and HBB1 gRNAs. TrueCut HiFi Cas9 maintained high on-target activity with both gRNAs (Figure 5).

A

HBB1			Reads per million (RPM)		
Target	Align sequence	PAM	wt-Cas9	Supplier I	TrueCut HiFi Cas9
On	CTTGCCCCCACAGGGGCAGTAA	CGG	141,922	258,580	284,917
Off1	TCA.....	GGG	126,583	970	132
Off2	T.....T.G.	CAG	13,100	15,871	1,229

HBB2			Reads per million (RPM)		
Target	Align sequence	PAM	wt-Cas9	Supplier I	TrueCut HiFi Cas9
On	CTTGCCCCCACAGGGGCAGTAA	AGG	431,212	356,556	452,904
Off1	...AA.....	TGG	246,927	3,153	319
Off2	..A..a.....C.....C.....	GGG	929	228	7
Off3	G.....A.	AGG	118	2,197	0

HEK4			Reads per million (RPM)		
Target	Align sequence	PAM	wt-Cas9	Supplier I	TrueCut HiFi Cas9
On	GGCACTGCGGCTGGAGGTGG	GGG	82,924	147,851	159,782
Off1	...G.....G.....	AGG	149,096	848,833	8,695
Off2	.....GA.....	GGG	151,950	390	105
Off3	A.G.....G.....	TGG	246,887	927	124
Off4	.....T.....C.....	AGG	118,633	119	65
Off5	.....A.....C...	AGG	12,949	8	0
Off6	.....G.....C...	GGG	3,005	21	0
Off7	T.....C.....A...	TGG	1,734	0	0
Off8	.....–G.....g.....	AGG	99	0	0
Off9	.....T.....G...	TGG	52	0	0

B

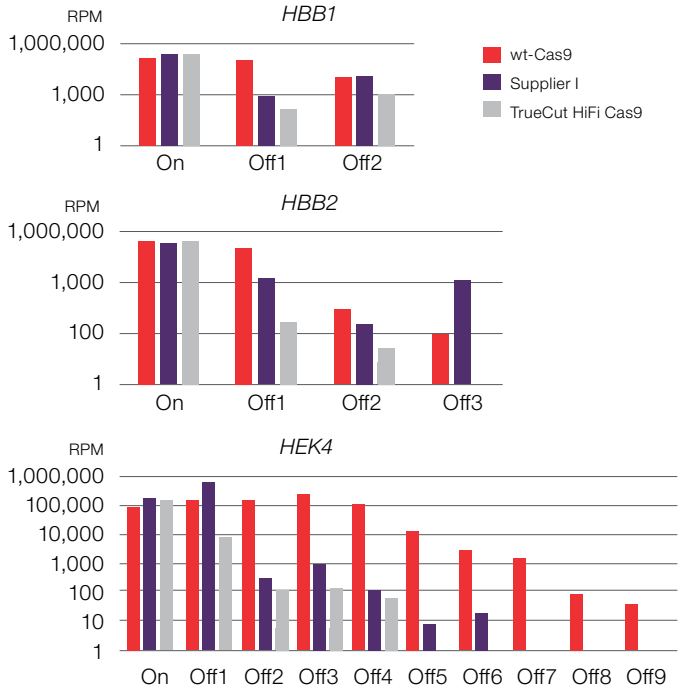

Figure 4. Off targets detected in iPSCs. Two gRNAs targeted SNPs in the hemoglobin β subunit gene (*HBB*) associated with sickle cell anemia. Another gRNA targeted a commonly studied off-target site in *HEK4*. **(A)** Sequences and RPMs for all on and off targets. **(B)** RPM bar graphs for on and off targets listed in **A**. The fidelity of TrueCut HiFi Cas9 was higher than that of wt-Cas9 and Supplier I high-fidelity Cas9.

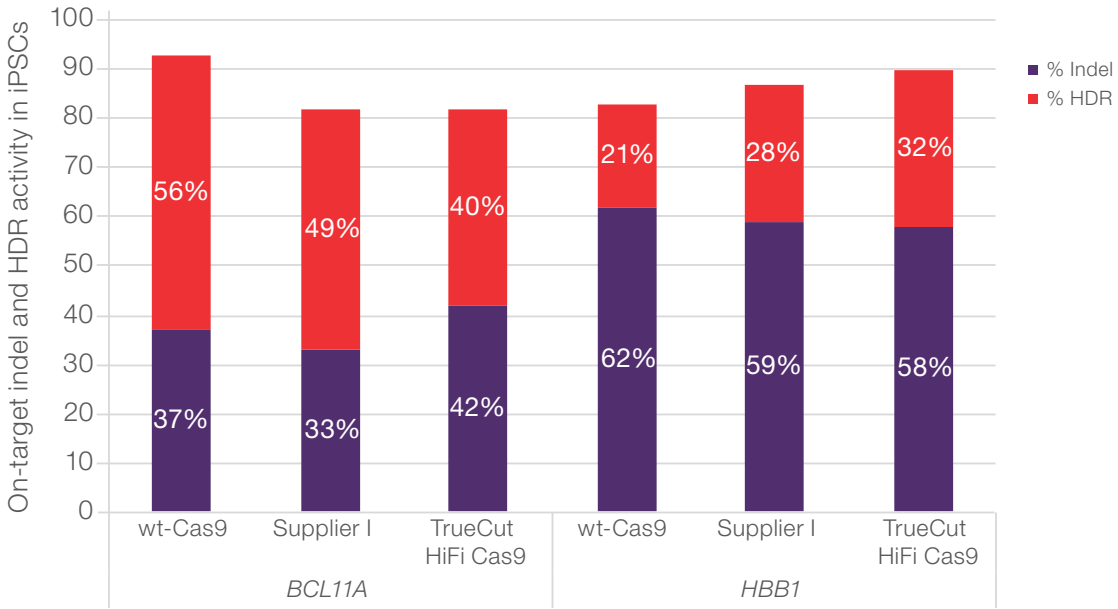

Figure 5. On-target indel and HDR activity of TrueCut HiFi Cas9 with three gRNAs in iPSCs. One gRNA targeted SNPs in the hemoglobin β subunit gene (*HBB1*) and another one targeted the *BCL11A* gene. TrueCut HiFi Cas9 maintained high on-target activity.

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Conclusion

High fidelity and editing efficiency are especially important when working with difficult-to-edit and valuable cells, such as primary cells, immune cells, and stem cells. In this work, we showed that the editing performance of TrueCut High-Fidelity (HiFi) Cas9 Protein was superior to that of wild type TrueCut Cas9 Protein v2 and Supplier I high-fidelity Cas9 protein. TrueCut HiFi Cas9 displayed higher fidelity and comparable on-target editing efficiency for multiple targets across different cell types, including HEK293 cells, primary T cells, and iPSCs. TrueCut HiFi Cas9 Protein provides a streamlined, end-to-end solution to maximize editing efficiency in primary T cells and iPSCs.

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