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Supplemental information

Editing of homologous globin genes by nickase-deficient base editor mitigates large intergenic deletions in HSPCs

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Materials and Methods

Plasmid Constructs and gRNAs:

pLenti-ABE8e-puro vector was constructed as previously described[1] by inserting the base editor sequence from Addgene #138495 (Gift from David Liu) into lenti viral vector (Addgene #112675- Gift from Lucas Dow). pLenti-dCas9-ABE8e-puro vector was constructed by introducing H840A mutation in the Cas9 domain of pLenti-ABE8e-puro vector. dCas9 ABE8e was similarly constructed from Addgene #138495. lentiCas9n(D10A)-Blast (Addgene #63593- gift from Feng Zhang) was obtained from Addgene. All gRNAs were designed and cloned in pLKO5.sgRNA.EFS.GFP vector (Addgene #57822) as described previously[2]. Plasmids were isolated using NucleoBond Xtra Midi EF kit (Macherey-Nagel) according to the manufacturer's instruction. The synthetic gRNAs were purchased from Synthego. Details of gRNAs used in this study are listed in Supplemental Table 1

Cell Culture:

HEK 293T cells were cultured in Dulbecco's modified Eagle medium (DMEM, Gibco) supplemented with 10% (v/v) FBS and 1× Pen-Strep. HUDEP 2 cells were cultured in Stemspan Media (SFEM II - STEMCELL Technologies) with 50ng/ml SCF (ImmunoTools), 1 µM dexamethasone (Alfa Aesar), 1 µg/ml doxycycline (Sigma-Aldrich), 1× L-glutamine 200 mM (Gibco), 3 U/ml EPO (Zyrop 4000 IU injection) and 1× Pen-Strep (Gibco) as per established protocols. After 8 days of editing, cells were set up for erythroid differentiation. Erythroid differentiation of HUDEP cells were carried out in a two phase protocol as described previously[3]. The peripheral blood mononuclear cells (PBMNCs) were obtained from a healthy donor according to the clinical protocols approved by the Intuitional Review Boards of Christian Medical College, Vellore. The PBMNCs were purified by density gradient centrifugation (Lymphoprep Density Gradient Medium|STEMCELL Technologies). CD34+

cells were isolated from the purified PBMNCs by EasySep Human CD34 positive selection kit II (STEMCELL Technologies) and expanded in HSC expansion media and further subjected to erythroid differentiation as per the 3 phase protocol described previously[4].

Lenti-virus production and transduction

Lenti-virus for stable cell lines and for gRNAs were prepared in HEK293T cells using Fugene HD as described previously[2]. The second generation packaging constructs pMD2.G and psPAX2 (Addgene #12259, 11260) were a gift from Didier Trono. The viral supernatant at 48hrs and 72hrs from 10 cm dish was collected, concentrated, and resuspend in 240ul of 1x PBS separately. From this 30ul was used to transduce 0.1 million HUDEP-2 cells taken in 24 well plate with 1% HEPES and 3µg polybrene (Sigma-Aldrich). Spinfection was carried out at 800G for 30 minutes at room temperature. Stable cell lines were prepared by maintaining the transduced cells in Puromycin or Blasticidine as appropriate. gRNA transduction efficiency was evaluated by measuring GFP expression using flow cytometry.

In Vitro transcription of base editor mRNA

nABE8e and dABE8e were synthesised using the plasmids described above using Takara IVTpro™ mRNA Synthesis System as per the manufacturer's protocol. Complete substitution of N-methyl pseudouridine (Trilink) was done and co-transcriptional capping was performed using ARCA (Jena Biosciences). mRNA was made into aliquots and stored in -80 until use.

Nucleofection of Base editing components

5ug of base editor mRNA along with 100pmol of gRNA was electroporated in 0.5 million cells using Maxcyte GtX electroporation system using the program HSC3 (HSPCs/HUDEP). The mRNA:gRNA:cells ratio was maintained while scaling up or down except for the dose dependency study where the cells number was maintained at 0.5 million and the cargo was

varied. Cells were allowed to recover at 37 degree Celsius for 15 minutes before transferring to prewarmed media.

Genomic DNA isolation

DNA was isolated from the cells on day 8 after transduction for lentiviral experiments or on day 5 after electroporation (unless specified) of HSPCs using QiaAMP DNA blood mini kit (Qiagen) as per the manufacturer's protocol and eluted in nuclease free water. For experiments requiring repeated sampling DNA was extracted using QuickExtract (LGC Biosearch Technologies).

Analysis of editing efficiency

The target region was amplified using primers listed in Supplemental Table 2. Sanger sequencing or next generation sequencing (Illumina Nova seq 6000) was performed as appropriate. Sanger sequencing data was analysed using EditR[5] or ICE(Synthego)[6] and NGS data was analysed using CRISPResso[7].

Evaluation of 4.9 kb deletion using qRT PCR

Primers were designed targeting deleted intervening region (on target) as well as the locus control (Supplemental table2). qRT PCR was performed as described previously using QuantStudio 6 Flex Real-Time PCR System (Applied Biosystems). SsoFast EvaGreen Supermix (Takara) was used along with 5 pmol each of forward and reverse primer and 50ng of DNA for the PCR.

Evaluation of 4.9kb deletion using ddPCR

BioRad ddPCR system was used for quantification of 4.9kb deletion using ddPCR supermix for probes. The primers and probes used are listed in supplemental table 2. The frequency of

deletion was calculated by normalizing the ratio of the deletion probe with locus control probe against the average ratio observed in unedited control samples

Evaluation of large deletion using PCR

Forward primer specific to HBG2/HBD and reverse primer specific to HBG1/HBB was designed to specifically amplify the fusion gene product generated after large deletion. 100 ng of DNA was amplified using the appropriate primer set using GoTaq Hotstart master mix (Promega) as per the manufacturer's protocol.

Intracellular HbF staining

0.5 million cells were processed and stained for intracellular HbF as described previously[8] using 2ul of antibody (Fetal Hemoglobin Monoclonal Antibody APC Invitrogen) per sample. The F positive cells were evaluated by Flow Cytometry (CytoFLEX LX Flow Cytometer – BC).

RP-HPLC

HUDEP2 cells were collected after erythroid differentiation, washed with 1x PBS, and resuspended in sterile water. The cells were lysed by sonication and the supernatant was used for reverse phase HPLC (Shimadzu Corporation-Phenomenex) to quantify globin chains as described earlier[3].

CD90 sorting

CD34+ve cells were stained using Antihuman CD90 antibody (BD)(5ul/10^6 cells), washed and taken for sorting using BD Aria III.

CFU assay

Colony forming assay was performed using Methocult Medium (Stem Cell technologies). 500 cells per sample were seeded in the medium as per the manufacturer's protocol. Colonies were enumerated after 14 days.

RNA isolation

RNA was isolated from the cultured cells using NucleoSpin RNA XS Kit (MN) as per the manufacturer's protocol. For Reverse transcription 1ug of RNA was used with iScript cDNA synthesis kit (BioRad).

qRT PCR

Relative gene expression (ΔΔCT) was measured using primers listed in supplemental table 2. GAPDH was used as internal control for normalising the gene expression. Commercially available primers targeting GAPDH and CDKN1a were purchased from Qiagen (QuantiTect primer assay) for the assay and used with TB green premix (Takara) for quantifying gene expression.

Statistical Analysis

All experiments are performed in triplicates unless specified otherwise. HUDEP2 lenti-virus experiments were performed as biological duplicates. Statistical analysis was performed using GraphPad Prism 8.1. The details of statistical methods are provided in the respective figure legends.

Supplemental Note 1

To test if the frequency of large deletion is in any way associated with the gRNA efficiency, we evaluated the efficiency score of each gRNA predicted by 2 different tools- E CRISP [12], a hypothesis driven tool and DeepSpCas9 [13], a machine learning based tool. Interestingly we

found that G3 had the lowest score in both tools while G11 had very high efficiency score and this observation is in line with the deletion frequency of each gRNA (Figure 1A). We also evaluated the microhomology score for the three gRNAs using CRISPR RGEN tool as MMEJ pathway was reported to reduce the NHEJ frequency while increasing the frequency of large deletion. Interestingly, G11 exhibited a very high MMEJ score compared to both G2 and G3 (Figure S1 E), suggesting that the presence of microhomology could be a contributing factor for increased deletion frequency. However, further evaluation with multiple gRNAs targeting different loci is required to reach any conclusion regarding the association of gRNA properties with frequency of deletion

Table S1: List of gRNAs used in the study

Sl No	Name	Sequence	Source
1	HBG G2	CTTGACCAATAGCCTTGACA	Ravi et al[1]
2	HBG G3	ATATTTGCATTGAGATAGTG	Ravi et al
3	HBG G4	GTGGGGAAGGGGCCCCCAAG	Ravi et al
4	HBG G11	GGCAAGGCTGGCCAACCCAT	Ravi et al
5	AAVS1	GGGGCCACTAGGGACAGGAT	Ravi et al
6	HBB1	CATGGTGCATCTGACTCCTG	This Study
7	HBB2	GTAACGGCAGACTTCTCCTC	This Study
8	HBB3	CCACGTTCACCTTGCCCCACA	Prasad et al[9]
9	HBB4	CGTGGATGAAGTTGGTGGTG	Venkatesan et al[10]

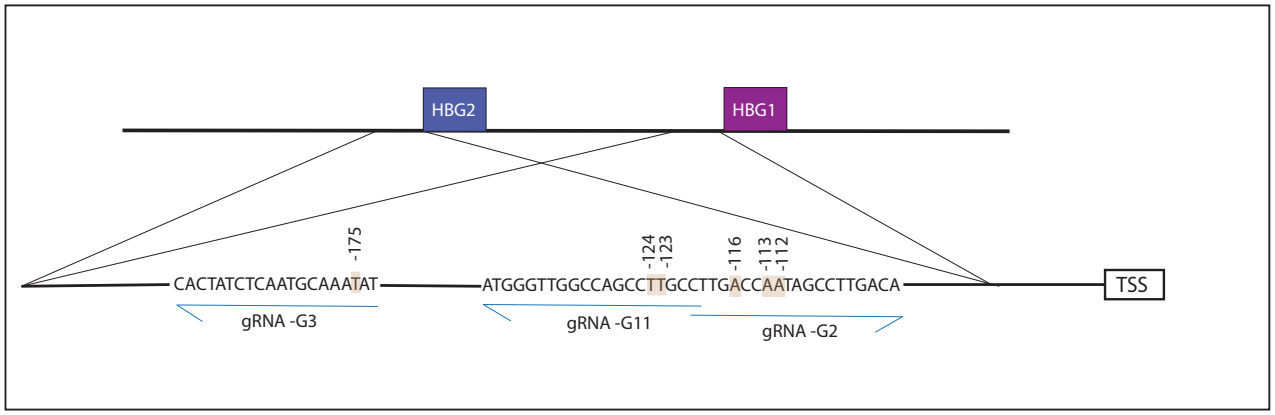
Table S2: List of primers used in the study

	Name	Sequence
1	HBG_NGS_F	TACACGACGCTCTTCCGATCTGGAATGACTGAATCG GAACAA
2	HBG_NGS_R	AGACGTGTGCTCTTCCGATCTGACGTTCCAGAAGCG AGTGT
3	HBG_SAN_F	ACAAAAGAAGTCCTGGTATC
4	HBG_SAN_R	CTTCCCAGGGTTTCTCCTCC
5	HBB_SAN_F	GTCATCACTTAGACCTCACC
6	HBB_SAN_R	CTGTACCCTGTTACTTATCC

7	HBD_SAN_F	ATGTAGAGGAGAACAGGGTT
8	HBD_SAN_R	CCATCACTAAAGGCACCTAG
9	AAVS1_SAN_F	GAGATGGCTCCAGGAAATGG
10	AAVS1_SAN_R	ACCTCTCACTCCTTTCATTTGG
11	HBG_RT_DEL_F	AGGGGCTCAACGAAGAAAAAGTGT
12	HBG_RT_DEL_R	CACTTCATTGTAGTTACCGTGGAAAGA
13	HBG_RT_LC_F	AAATGAATCAGCAGAGGCTCAC
14	HBG_RT_LC_R	ATGCACTAACATCCAACTATACAAAA
15	HBB_RT_DEL_F	CACTTTCTTCTGACATAACA
16	HBB_RT_DEL_R	CACAGGGCAGTAACGGCAGA
17	dd_HBG_DEL_F	ACGGATAAGTAGATATTGAGGTAAGC
18	dd_HBG_DEL_R	GTCTCTTTCAGTTAGCAGTGG
19	RT_GADD45_F[11]	GAGAGCAGAAGACCGAAAGGA
20	RT_GADD45_R	CAGTGATCGTGCGCTGACT

Figure S1

A)

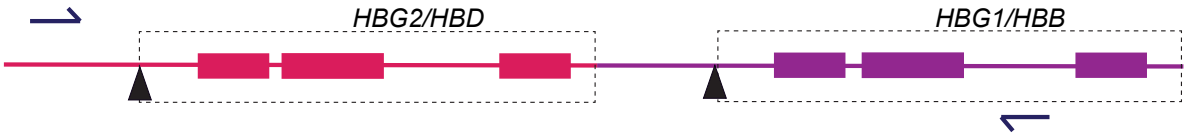


B) Strategy for detection of 4.9 kb deletion by qRT PCR

C) Strategy for detection of 4.9 kb deletion by probe based ddPCR

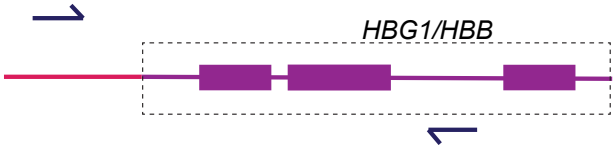
D) Strategy for deletion specific PCR

Intact Genes



No amplification

Fused Gene after interstitial deletion



Amplicon ~1690 bp (HBG2/HBG1)
~ 800 bp (HBD/HBB)
qRT PCR - ~96 bp

Figure S1: Strategies for detection of interstitial deletion

A) Schematic representation of gRNAs targeting the gamma globin promoter used in the study showing the position and target bases. **B)** Schematic representation of the method for detection of 4.9kb deletion in the gamma globin locus by qRT PCR. The black triangles represent the gRNA cut site. Green arrows represent the primers specific to the deleted region, the amplification of which will be reduced proportionate to deletion. Orange arrows represent the locus control primers. **C)** Schematic representation of the method for detection of 4.9kb deletion in the gamma globin locus by probe based ddPCR. The black triangles represent the gRNA cut site. Blue arrows represent the primers specific to the deleted region and green circle represents the probe (FAM) signal of which will be lost in DNA with 4.9kb deletion. Yellow arrows represent the locus control primers and red circle indicates the corresponding probe (HEX) **D)** Representation of the approach for PCR amplification of fusion gene formed by 4.9kb/7.4 kb deletion. If the gene is intact there will be no amplification with an extension time of 2 minutes while the presence of deletion will give a band at ~1690 bp in gamma globin locus and at ~800 bp in beta globin locus which can be detected by agarose gel electrophoresis.

Figure S2

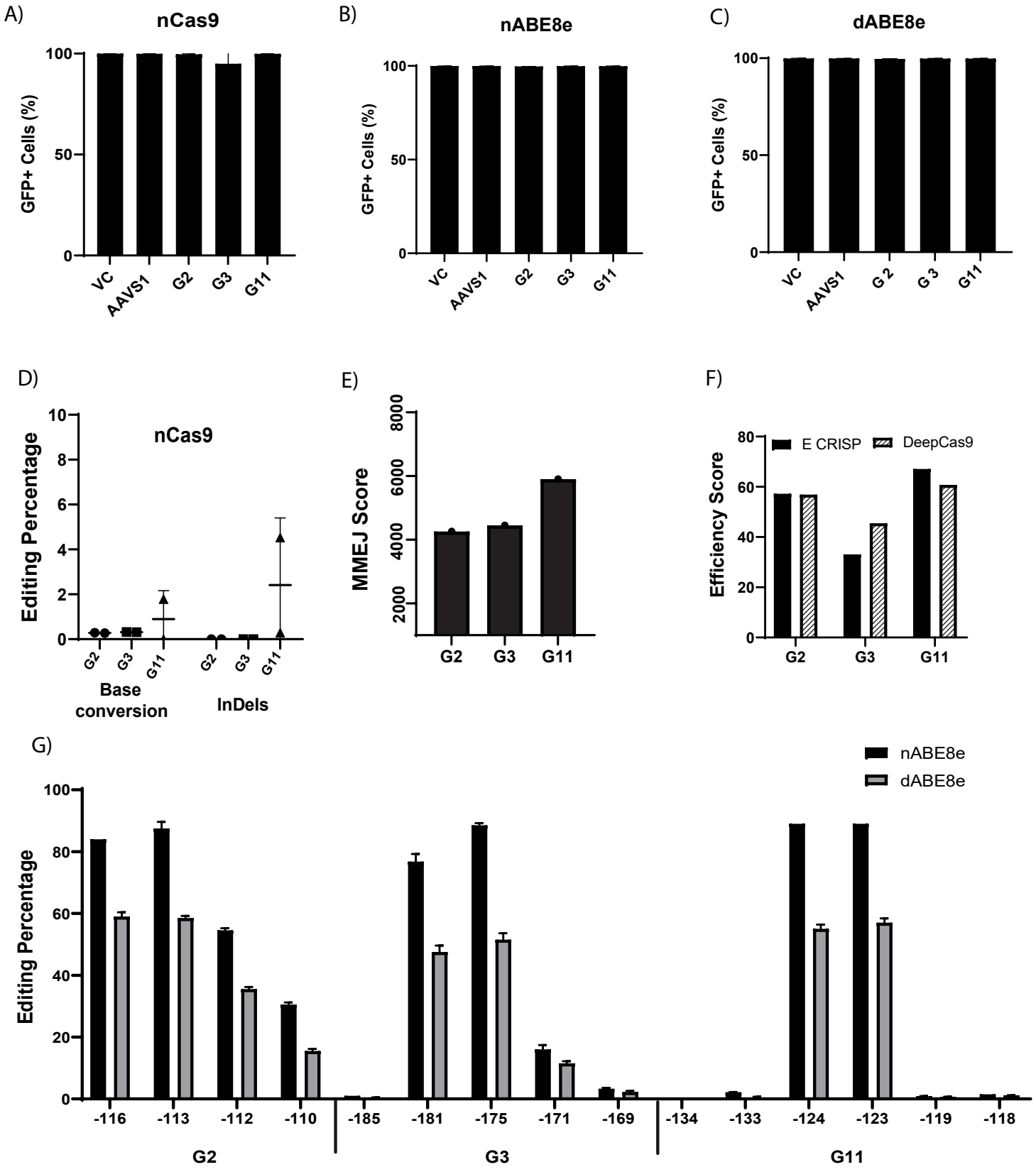


Figure S2: Evaluation of dABE8e in HUDEP2 cells

A-C) Percentage of GFP positive cells as a measure of transduction efficiency evaluated by flow cytometry in HUDEP nCas9 stables (A), nABE8e stables (B) and dABE8e stables (C). **D)** Base conversions and InDels obtained while editing in the nCas9 stables with respective gRNAs evaluated by NGS **E)** MMEJ score of the gRNAs predicted by CRISPR RGEN tool **F)** gRNA efficiency score predicted by ECRISP and deepSpCas9 algorithms **G)** Percentage of individual base conversions at the gamma globin promoter with each gRNA measured by NGS in nABE8e and dABE8e edited samples. (Data represented as mean± SD. n=2 for all samples)

Figure S3

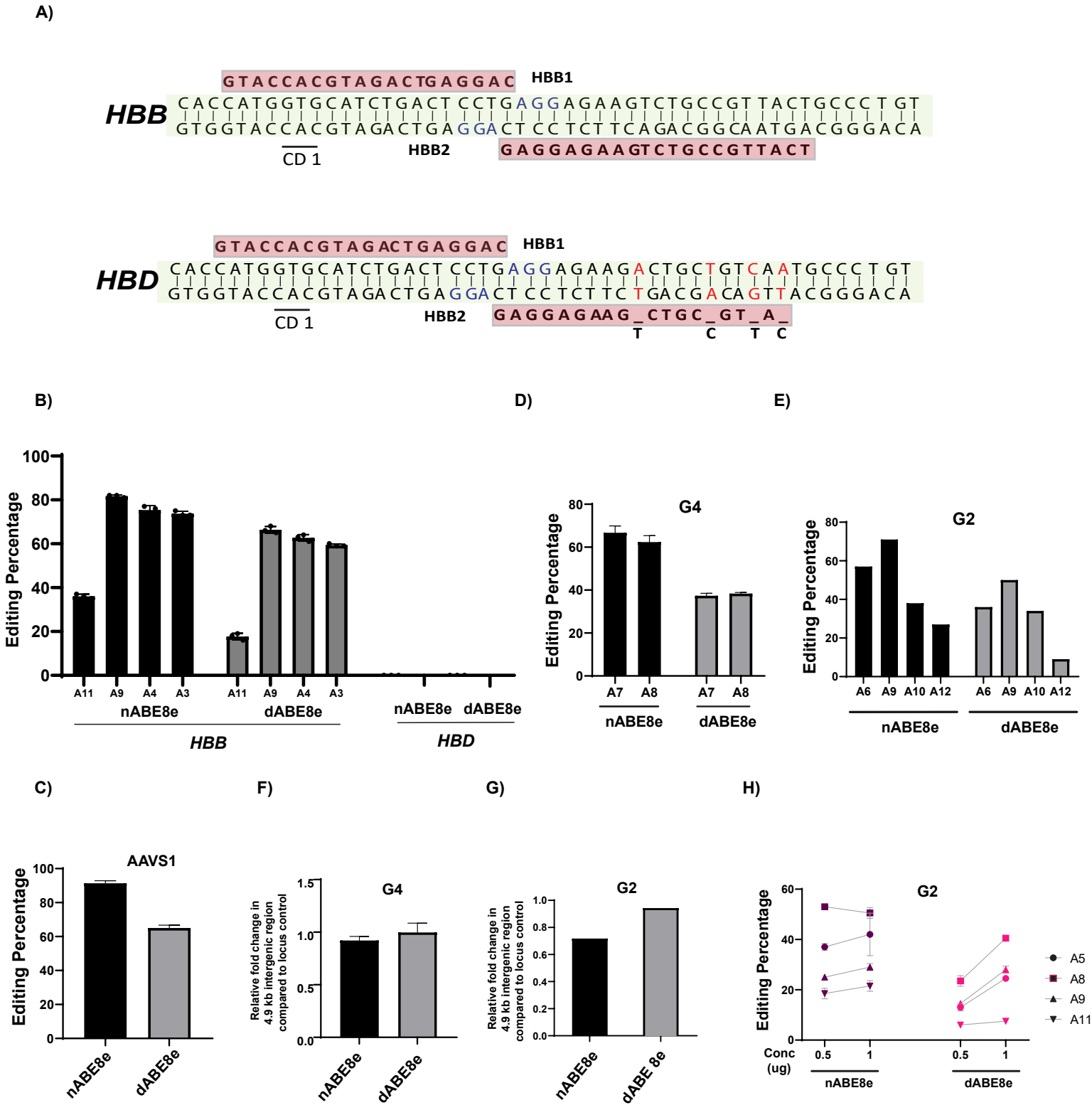


Figure S3: Evaluation of dABE8e in CD34+ve HSPCs

A) Schematic representation of the position of gRNAs in the beta globin and delta globin genes. Red colour indicating bases that differ in *HBD* gene and dashes representing the mismatches of HBB2 in *HBD*. **B-E)** Editing efficiency in HSPCs using HBB2 gRNA (B), AAVS1 gRNA (C), G4 (D) and G2 (E) measured by sanger sequencing. (n=3 for all data except E where n=1) **F-G)** Reduction in 4.9kb intergenic region caused by interstitial deletions while targeting the gamma globin promoter using G4 (F) and G2 (G) measured as relative fold change compared to locus control by qRT PCR and normalised using unedited control. **H**) Editing efficiency in HSPCs at 48 hrs after electroporation with varying concentrations of mRNA(nABE8e or dABE8e) evaluated using Sanger sequencing(n=2).

Figure S4

A)

B)

Figure S4: Multiplexed base editing in CD34+ve HSPCs

A) Schematic representation of the position of gRNAs in the beta globin gene used for multiplexed editing.

B) Representative InDel patterns generated after multiplexed base editing using nABE8e measured by sanger sequencing and analysed by Synthego ICE

Figure S5

A)

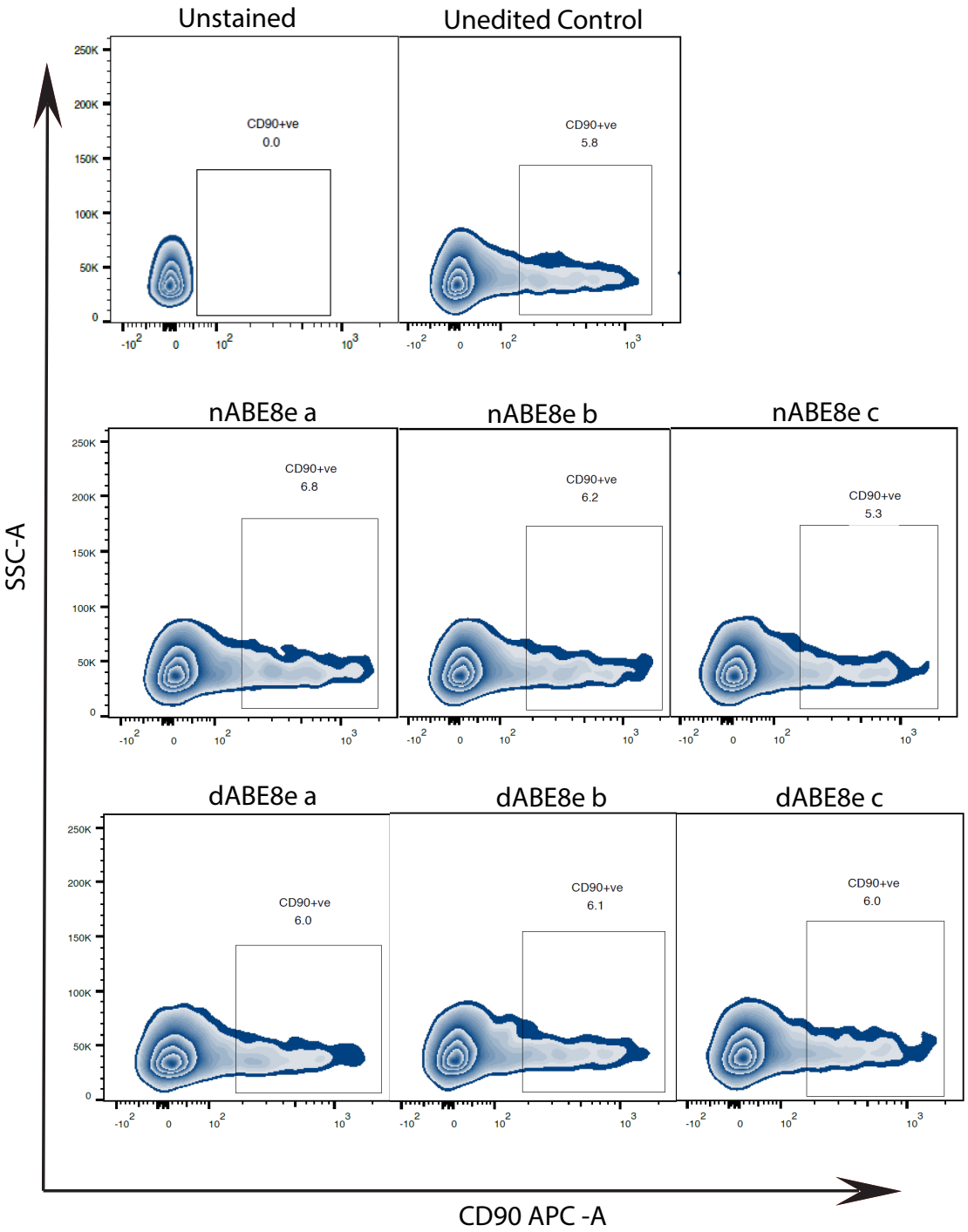


Figure S5: Representation of fluorescence activated cell sorting of HSPCs using CD90 APC conjugate

CD34 +ve HSPCs were edited with nCas9ABE8e /dCas9 ABE8e and editing was evaluated in bulk population and compared with CD90+ve HSPCs which were sorted from bulk population

Supplemental References

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