

INDUCTION OF FETAL HAEMOGLOBIN USING PAIRED CAS9 NICKASES

TECHNICAL FIELD

[001] The present disclosure relates generally to the field of biotechnology. More particularly, the present disclosure relates to the field of gene therapy.

BACKGROUND

[002] The human globin gene clusters on chromosome 11 controls the expression of β -subunit, which together with α -subunit, produces the function haemoglobin (Hb). Several β -subunit exists, including the predominantly fetal γ chain (HBG gene) and the adult δ (HBD gene; minor) and β (HBB gene; major) chains. Individuals that inherit mutations that disrupt β -chain, a condition known as β -hemoglobinopathies, fail to produce functional haemoglobin (adult HbA: $\alpha_2\beta_2$).

[003] Conventional methods of treating β -hemoglobinopathies which use allogenic hematopoietic cells transplantation are limited by the availability of compatible donors. Furthermore, other conventional methods of gene therapy using CRISPR/Cas9 for β -hemoglobinopathies face issues such as off-target effects, which cause detrimental health issues to the subject. The only approved gene therapy product for β -hemoglobinopathies which is based on lentiviral transfer of β -chain (β -^{A-T87Q} mutant) causes random insertional mutagenesis in hematopoietic stem cells.

[004] Therefore, there is a need for a gene therapy for treating β -hemoglobinopathies that addresses some of the above-mentioned problems.

SUMMARY

[005] In one aspect, the present disclosure provides a kit comprising: a CRISPR-Cas9 nickase; a pair of guide RNAs (gRNA); wherein the pair of guide RNAs binds to target sequences, wherein the binding of the guide RNAs to target sequences will result in a disruption of binding sites of repressor LRF of a promoter of a hemoglobin gamma (HbG) locus.

[006] In another aspect, the present disclosure provides a method for modifying a hemoglobin gamma (HbG) locus in a eukaryotic cell, the method comprising introducing into the eukaryotic cell a kit comprising: a CRISPR-Cas9 nickase; a pair of guide RNAs (gRNA), wherein the pair of guide RNAs binds to target sequences, wherein the binding will result in a disruption of binding sites of repressor LRF of a promoter of a hemoglobin gamma (HbG) locus.

[007] In another aspect, the present disclosure provides a composition comprising the kit of the disclosure.

[008] In another aspect, the present disclosure provides a method of treating β -hemoglobinopathy in a subject, wherein the method comprises administering the composition of the disclosure to a subject with a β -hemoglobinopathy.

[009] In another aspect, the present disclosure provides a gene therapy for preventing β -hemoglobinopathy in a subject, wherein the method comprises administering the composition of the disclosure to a subject susceptible to β -hemoglobinopathy.

BRIEF DESCRIPTION OF DRAWINGS

[0010] Disrupting HBG promoters restore gamma haemoglobin expression.

[0011] Fig. 1a is a schematic diagram of the human beta-globin loci on chromosome 11. Expression of beta-globin genes (HBB, HBD, HBG1, HBG2, and HBE) are modulated by the locus control region and is highly coordinated with the stage of development. Normally the β - and δ -chains are expressed in adult when the expression of fetal γ - and α -chains are repressed. The two HBG genes are highly homologous, in both promoter and the coding regions.

[0012] Fig. 1b is a schematic diagram showing the identical promoter sequences of HBG1 and HBG2. The binding sites of the repressors BCL11A and LRF are underlined. Naturally occurring heredity persistence of fetal haemoglobin (HPFH) mutations that resulted in elevated HbF level are in bold. The position and orientation of the gRNAs used in this study are illustrated.

[0013] Examination of individual gRNAs, and in combinations.

[0014] Fig. 2a is a schematic showing the T7E1 assay results of individual gRNA on cord blood (CB) 34^+ cells. The gRNAs were combined with wildtype Cas9 nucleases (Cas9) or Cas9 nickase (Cas9n) to produce the ribonucleoprotein complex (RNP), before electroporation into cord blood (CB) 34^+ cells. Genomic DNA was harvested 4 days post electroporation and the extent of editing was evaluated by T7E1 assay. The percentages of cleavage were shown below the lanes, which represented the extent of editing.

[0015] Fig. 2b is a schematic showing the T7E1 assay results of initial testing of gRNA A1 with paired nickases on cord blood (CB) 34^+ cells.

[0016] Fig. 2c is a schematic showing the T7E1 assay results of comprehensive testing of all combinations with paired nickases on cord blood (CB) 34^+ cells.

[0017] Fig. 2d is a schematic showing the T7E1 assay results of the most efficient nickase pairs (A2 + S3 and A2 + S4) in adult CD 34^+ cells under the same experimental conditions.

[0018] Testing of paired nickases in adult hematopoietic cells.

[0019] Fig. 3a is a schematic showing the T7E1 assay results comparing the efficiency of Cas9 nickase and nuclease in inducing HbF re-expression. The RNPs (Cas9n with A2 +S4, and Cas9 with A2) was electroporated into adult CD 34^+ cells, and the editing efficiency was evaluated as before on day 4 post-electroporation.

[0020] Fig. 3b is a flow plot showing the functional consequence of editing of HBG promoter. The edited cells were expanded and differentiated into erythrocytes for 17-20 days. The cells were then harvested for both RNA extraction (RT-PCR assay) and intracellular staining (flow analysis). The percentage of HbF expressing CD235 $^+$ erythrocyte (F cells) was determined by flow cytometry, shown by the representative flow plots (cells in Q2, which were CD235 $^+$ and HbF $^+$)).

[0021] Fig. 3c is a schematic showing the T7E1 assay results of the effect of editing on adult CD 34^+ hematopoietic cells. Results of T7E1 assay (for editing efficiency), flow cytometry (percentage of F cells) and RT-PCR (level of gamma transcript) are summarized (n = 3).

[0022] Paired nickases induce HbF on hematopoietic cells from individuals with Thalassemia.

[0023] Fig. 4a is a schematic showing the T7E1 assay results of the editing efficiency of selected gRNA pairs. Selected gRNAs and Cas9 nickases and nucleases were introduced into CD34⁺ thalassemia cells. Experimental details are the same as described for adult CD34⁺ cells in Fig. 3.

5 **[0024] Fig. 4b** is a representative flow plot for samples Thal_#1 showing the induction of HbF upon editing.

[0025] Fig. 4c is a schematic showing the T7E1 assay results of editing on individual thalassemia samples was shown, with exact data shown for each sample (n = 3).

[0026] Paired Cas9 nickases greatly reduced off-target editing of the Cas9 nucleases.

10 **[0027] Fig. 5a** is a schematic showing the targeted amplicon sequencing analysis of the specificity of Cas9 editing. The genomic DNA was extracted from the edited cells 4-6 days post-electroporation. A total of 54 specific targets, including the editing site on HBG promoter and the potential off-target sites (OFT), were included in the amplicon panel for targeted sequencing. Editing by Cas9 nuclease or nickase, presented by Indels (insertions/deletions) created through non-homologous end joining
15 (NHEJ), were analysed for the targets. Only targets with indels above the level of control (Cas9 or Cas9n alone) are shown, for adult CD34⁺ samples.

[0028] Fig. 5b is a schematic showing the targeted amplicon sequencing analysis of the specificity of Cas9 editing. Only targets with indels above the level of control (Cas9 or Cas9n alone) are shown, for thalassemia CD34⁺ samples.

20 **[0029] On target editing of HBG promoters resulted in deletion of the inter-promoters region.**

[0030] Fig. 6a is a schematic showing the gRNA targeting regions on HBG1 and HBG2 gene, and the position of primer probes (half-arrows) for detection of deletion. PCR was performed to detect the presence of ~1.5 kb fragment resulting from the deletion. Single gRNA with wildtype Cas9 were introduced as before but the harvested genomic DNA was used for the deletion specific PCR instead.

25 **[0031] Fig. 6b** is a schematic showing the PCR results for testing of individual gRNA as well as the most efficient combinations in cord blood CD34⁺ cells.

[0032] Fig. 6c is a schematic showing the PCR results for testing of individual gRNA as well as the most efficient combinations in thalassemia samples.

[0033] Fig. 6d is a schematic showing the digital PCR results to quantify the deletion created by editing
30 using paired or single gRNAs. The change in copy number for the inter-promoters region was normalized to that of the control region (CD44), for both adult 34⁺ and thalassemia samples (n = 3 each).

DEFINITIONS

35 **[0034]** As used herein, the terms “gene”, “locus”, and “gene locus”, refer to a region of nucleic acid on a eukaryotic chromosome which comprises the enhancer, silencer, promoter, 5’ untranslated region, open reading frame, 3’ untranslated region, and terminator.

[0035] As used herein, the term “target sequence” and “target site” are used interchangeably and refer to a section of a target molecule which is to be recognised using the gene editing method as disclosed herein. Such target(s) can be, but are not limited to DNA, RNA, single nucleotide polymorphism (SNP), microRNA (miRNA), genomic DNA, viral DNA, protein, post-translational modified proteins, cell surface receptors, metabolites, lipids, carbohydrates and small molecules.

[0036] As used herein, the term “nuclease” refers to an enzyme capable of cleaving phosphodiester bonds between nucleotides of nucleic acids. The cleaving of phosphodiester bonds between nucleotides of nucleic acids can occur on, but are not limited to, a DNA or RNA. The cleaving of phosphodiester bonds can occur on one or more sites on a DNA or RNA. These sites can comprise a nucleotide sequence which is complementary to the enzyme. The cleaving of phosphodiester bonds can also occur at sites which are not complementary to the enzyme. When the cleavage of phosphodiester bonds occurs on a DNA or RNA, the DNA or RNA may be single or double stranded. Thus, the cleavage of phosphodiester bonds can occur on a single strand of the DNA or RNA or on both strands of the DNA or RNA. The cleavage of phosphodiester bonds by a nuclease can occur at a phosphodiester bond within a DNA or RNA, or at a phosphodiester bond on an exposed end of a DNA or RNA.

[0037] As used herein, the term “nickase” refers to a nuclease which cleaves phosphodiester bonds between nucleotides of nucleic acids which can be, but are not limited to, on a single strand of a DNA or RNA. The cleaving of phosphodiester bonds can occur on one or more sites on a single strand of a DNA or RNA. These sites can comprise a nucleotide sequence which is complementary to the enzyme. The cleaving of phosphodiester bonds can also occur at sites which are not complementary to the enzyme.

[0038] As used herein, the terms “gRNA” and “guide RNA” are used interchangeably and refer to a RNA strand that is complementary to a target sequence which can be, but are not limited to, on a DNA, or another RNA. Thus, in one example, the “gRNA” and “guide RNA” used herein can be, but are not limited to, a CRISPR RNA (crRNA), a trans-activating CRISPR RNA (tracrRNA), or a single guide RNA (sgRNA). In a further example, the “gRNA” and “guide RNA” used herein is a crRNA;tracrRNA. The size of the “gRNA” and “guide RNA” used herein can be, but is not limited to, 30 to 150 nucleotides. In a specific example, the size of the “gRNA” and “guide RNA” used herein is about 37 nucleotides, about 67 nucleotides, or about 100 nucleotides. The “gRNA” or “guide RNA” as used herein can recognise and bind to a target sequence on a DNA, or another RNA which the “gRNA” or “guide RNA” is complementary to. The “gRNA” or “guide RNA” as used herein can be bound to an enzyme, where the enzyme can be a nuclease or nickase. The “gRNA” or “guide RNA” as used herein, when bound to an enzyme, can guide the enzyme to a target sequence which the “gRNA” or “guide RNA” recognises. Upon guidance of the enzyme to the target sequence, the enzyme can perform its enzymatic activity such as the cleavage of phosphodiester bonds if the enzyme is a nuclease or nickase.

[0039] As used herein, the term “mutant” refers to a molecule which comprises a structure different from its native form. Such molecules can be, but are not limited to DNA, RNA, genomic DNA,

messenger RNA (mRNA), and proteins. Such molecules, when comprising a structure different from its native form, can have properties different from its native form. Such differences in properties, when occurring on an enzyme, can be, but are not limited to, a change in enzymatic activity, and a change in the recognition of complementary binding sites. Such differences in properties, when occurring on a nucleic sequence located on a DNA or RNA, can be caused by, but are not limited to, a change in structure, a change in the number of bases, a change in the number of nucleotides, and a change in the modifications present on the sequences. When such changes occur on a nucleic sequence located on a DNA or RNA, target sequences can be altered in ways such as, but are not limited to, a change in structure, a change in the number of bases, a change in the number of nucleotides, and a change in the modifications present on the target sequences. Accordingly, a change in nucleic sequence located on a DNA or RNA can result in the loss of sequences which are recognised and bound by complementary enzymes. The loss of such sequences results in complementary enzymes failing to bind at such sequences.

[0040] As used herein, the term “disruption” when used in the context of a gene, refers to a loss of a whole or part thereof of the gene, which can occur due to mutations that result from, but are not limited to, additions, deletions, substitutions, homologous recombination, inversions, frameshift, missense, and nonsense. The loss of the whole or part thereof of the gene can result in a mutant gene. A mutant gene can have changes in properties such as, but are not limited to, a change in structure, a change in the number of bases, a change in the number of nucleotides, and a change in the modifications present on the sequences. Such changes in properties can result in the loss of sequences which are recognised and bound by complementary enzymes. Accordingly, the expression of sequences located downstream or upstream of the disrupted gene can be altered.

[0041] As used herein, the term “unmutated” has the same meaning as the term “non-mutated” and when used in the context of a nucleic acid, refers to the absence of mutations in the nucleic acid sequence relative to a reference nucleic acid sequence. When used in the context of a protein, the terms “unmutated” and “non-mutated” refer to the absence of differences in the protein’s corresponding nucleic acid sequence relative to a reference protein’s corresponding nucleic acid sequence, such that the protein is folded into the native structure of the protein, and not a mutant form. Thus, for example, an unmutated Cas9 nuclease refers to a Cas9 nuclease without mutations in the nucleic acid sequence and can refer to a wild-type Cas9 nuclease. In another example, an unmutated Cas9 nuclease can serve as a reference protein to which a mutant is compared, and refers to a Cas9 nuclease which lacks the mutations of the mutant. In a further example, an unmutated Cas9 nuclease can refer to a Cas9 nuclease which comprises a nucleic acid sequence that does not deviate from a nucleic acid sequence defined by a SEQ ID NO. In a more specific example, an unmutated Cas9 nuclease can refer to a Cas9 nuclease which comprises a nucleic acid sequence that does not deviate from SEQ ID NO: 9.

DETAILED DESCRIPTION

[0042] The human globin gene clusters on chromosome 11 controls the expression of β -subunit, which together with α -subunit, produces the functional haemoglobin (Hb). Several β -subunit exists, including the predominantly fetal γ chain (HBG gene) and the adult δ (HBD gene; minor) and β (HBB gene; major) chains. Expression of specific β -like globin chains during development follows the same order in which they are genetically arranged, a process known as “globin switching”. The ϵ -chain (HBE) which is expressed during the embryonic gestation is replaced by the two γ -chains ($G\gamma$ and $A\gamma$, from HBG2 and HBG1 respectively) during fetal development. The γ chains are then gradually downregulated after birth, whereas δ (HBD; minor) and β (HBB; major) chains become the predominant forms thereafter (Figure 1A).

[0043] Individuals who inherit mutations that disrupt β -chain, a condition known as β -hemoglobinopathies (Mendelian disorders such as thalassemia and sickle cell disease), start to develop symptoms few months after birth, due to a failure in an increased production of normal adult haemoglobin (HbA; $\alpha_2\beta_2$) with a reciprocal reduction of fetal haemoglobin (HbF; $\alpha_2\gamma_2$). Such individuals develop a condition known as β -hemoglobinopathies, (such as thalassemia and sickle cell disease), and fail to produce functional haemoglobin (adult HbA: $\alpha_2\beta_2$). Approximately 300,000–400,000 children with β -hemoglobinopathies are born annually.

[0044] Rare individuals inherit a benign condition known as heredity persistence of fetal haemoglobin (HPFH), at which the silencing of HBG genes is retarded by mutations of the cis-regulatory elements, leading to persistently high production of γ -chains and fetal haemoglobin (HbF; $\alpha_2\gamma_2$). The re-expression of HbF alleviated the clinical severity of β -hemoglobinopathies by rescuing the defective HbA. The clinical severity of β -hemoglobinopathies can be ameliorated by naturally occurring mutations in the HBG promoter, namely hereditary persistence of fetal haemoglobin (HPFH), which revert haemoglobin synthesis from the defective adult β -globin to functional fetal γ -globin well into adulthood.

[0045] Conventional methods of treating β -hemoglobinopathies using allogenic hematopoietic cells transplantation are limited by the availability of compatible donors or transplant-related toxicities. Furthermore, emerging methods of gene therapy with CRISPR/Cas9 still face issues such as off-target effects, which cause detrimental health issues to the subject. The only approved gene therapy product for β -hemoglobinopathies which is based on lentiviral transfer of β -chain (β^{A-T87Q} mutant) causes random insertional mutagenesis in hematopoietic stem cells.

[0046] Therefore, there is a need for a gene therapy for treating β -hemoglobinopathies with minimal off-target effect.

Several gene therapy approaches had been adopted for β -hemoglobinopathies. A direct way is to express a functional or anti-sickling copy of β -chain in hemopoietic cells. As disclosed herein, the present disclosure adopts the use of heredity persistence of fetal haemoglobin (HPFH) for disrupting control elements of the HbF gene to induce HbF production. Noticeably, many HPFH mutations are single

nucleotide substitutions on HBG promoters that disrupt the binding sites of repressors LRF or BCL11A (Figure 1B), which are potent inducers of HbF (up to 40% of Hb in the HPFH individuals). Current gene therapy methods which induce HPFH mutations by small insertions and deletions (indels) using CRISPR/Cas9 nucleases can only result in HbF induction to a small extent.

5 [0047] The canonical CRISPR/Cas9 nuclease (Cas9), which induces double strand break (DSB) and small indels on target DNA, can be converted into a nickase (Cas9n) that generates single strand nick instead. A pair of Cas9 nickases, when placed on opposite strands at a distance apart, can generate DSB and longer indels (tens to hundreds bps) to cover more heredity persistence of fetal haemoglobin (HPFH) mutation sites simultaneously. As discussed herein, paired Cas9 nickases (the D10A mutant)
10 was used to target the cluster of heredity persistence of fetal haemoglobin (HPFH) mutations around the LRF binding site on HBG promoters.

Paired Cas9 nickases are more efficient and highly specific as compared to single Cas9 nuclease for HBG promoters editing

[0048] While Cas9 nuclease generates double stranded DNA breaks to specific sites when guided by
15 a guide RNA (gRNA), the mutant Cas9 nickase (Cas9n) generates single stranded DNA breaks. When used with a pair of guide RNA (gRNA) with proper spacing and orientation, the Cas9n (D10A mutant) can generate double strand DNA break with high specificity. Pairs of gRNAs that couple with Cas9n (gRNA + Cas9n) were designed, demonstrating that these combinations can specifically gene-edit the HBG promoters. The gene editing results in the induction of HbF in hematopoietic progenitors obtained
20 both from healthy donors and from individuals with thalassemia.

[0049] Accordingly, in one example, disclosed herein is a gene editing method with low off-target effect using paired Cas9 nickases. As disclosed herein, it is shown that paired Cas9 nickases are more efficient than the respective Cas9 nucleases in editing and causes better HbF induction. Mechanistically, editing of the HBG1 and HBG2 promoters simultaneously leads to the deletion of HBG2 containing
25 inter-promoter region. Compared with Cas9 nuclease, the Cas9 nickases significantly reduce off-target mutagenesis in primary hematopoietic cells.

Pairs of gRNA + Cas9n having high editing efficiency on HBG promoters were identified

[0050] Pairs of guide RNAs (gRNAs) for testing with nickase or nuclease were identified. The paired nickases are more efficient than the respective nucleases in editing and inducing HbF re-expression, as
30 demonstrated in hematopoietic progenitors obtained from both healthy donors and thalassemia patients. In addition, the intrinsic off-target activity of the nuclease was eliminated by the paired nickases in the primary cells.

[0051] Accordingly, in another example, described herein is a gene editing method to induce HbF expression by targeting the cluster of heredity persistence of fetal haemoglobin (HPFH) mutations
35 around the LRF binding site on HBG promoters. In one example, the target sequences of the guide RNAs (gRNAs) overlap. In another example, the target sequences of the guide RNAs (gRNAs) do not overlap. In a further example, the target sequences of the individual guide RNAs (gRNAs) in a pair of

selected guide RNAs (gRNAs) do not overlap. As used herein, the term “do not overlap” when used in the context of individual guide RNAs (gRNAs) in a pair of selected guide RNAs (gRNAs) would thus exclude, for example, a combination of SEQ ID NO: 2 and SEQ ID NO: 5, or SEQ ID NO: 4 and SEQ ID NO: 5, but not a combination of SEQ ID NO: 2 and SEQ ID NO: 7, SEQ ID NO: 2 and SEQ ID NO: 8, or SEQ ID NO: 3 and SEQ ID NO: 8.

[0052] As disclosed herein, gRNA + Cas9n pairs are more efficient than the respective gRNA + Cas9 in editing and inducing HbF. In particular, the pair A2+S4 shows the best efficiency, followed by the pairs A3+S4 and A4+S4. The pair gRNAs + Cas9n approach is more specific than the respective single gRNA + Cas9 (wildtype), minimizing potential off-target editing.

[0053] Thus, in another example, described herein is a gene editing method to induce HbF which demonstrates low off-target effect.

EXAMPLES

Paired Cas9 nickases are more efficient than Cas9 nuclease in editing HBG promoters

[0054] The sequences of HBG1 and HBG2 promoters are identical from position -223 to the transcription start site +1. The region around the LRF binding site contains multiple hereditary persistence of fetal haemoglobin (HPFH) mutations, including one (-196 C > T) that leads to high level (~40%) of A γ expression. This cluster of hereditary persistence of fetal haemoglobin (HPFH) mutations can be targeted at once by paired nickases, possibly resulted in more prominent γ -chains re-expression. Over the region, 4 pairs of gRNAs were identified, which are in opposite orientation (anti-sense A1 to A4; and sense S1 to S4) (Figure 1B; table 1). Pairing of an anti-sense and a sense gRNAs with Cas9 nickases in a PAM (protospacer-adjacent motif)-out configuration, can generate efficient DSB with various lengths.

[0055] The efficiency of individual gRNAs with Cas9 nuclease in cord blood (CB) CD34⁺ cells was tested. The gRNA was combined with the Cas9 protein (nuclease or nickase) to produce the ribonucleoprotein (RNP) complex, and was electroporated into the cells. In all experiments, the same amount of gRNAs and Cas9 protein was used for electroporation (i.e. 2.5 μ g of Cas9 protein and 4 μ g of gRNA, or 2 μ g each for pair of gRNAs) so that the editing efficiency of nuclease and nickase can be directly compared. Genomic DNA was extracted 4 days post-electroporation, and the percentage of on-target editing was estimated by T7 endonuclease (T7E1) mismatch assay using primers specific to HBG1 promoter. All gRNAs demonstrated a level of editing with Cas9 nuclease, with S4 (54 %) being the most efficient gRNAs (Figure 2A). Two other gRNAs were also shown to result in efficient editing (A2, 32%; A3; 48%). Both HBG1- and HBG2-specific primers gave similar result on the T7E1 assay (data not shown).

[0056] Next, gRNAs were tested with the nickase. The gRNA A1 was able to edit substantially when paired with another gRNA and the nickases (12-22%; Figure 2B), suggesting the nicking approach worked in primary hematopoietic cells. All combinations were then tested using the paired nickases. Surprisingly, most combinations worked quite well, with highest editing efficiency up to ~85 - 86% for

the A2 + S4 and A3 + S4 pairs (A2/A3/A4 + S2/S3, ranged from 62 to 81%; summarized in table 4) (Figure 2C). Notably, the paired nickases were more efficient than the respective nuclease alone (e.g. A2 + S4 pair gave ~86%; A2 and S4 alone gave ~32% and ~54 % respectively). In adult bone marrow (AD) CD34+ cells, the combinations A2 + S4 and A3 + S4 also demonstrated much higher editing efficiency (76% and 69% respectively) than that of the nuclease alone (37%, A2; Figure 2D).

Editing by paired nickases induce stronger HbF re-expression in hematopoietic progenitors

[0057] Although the paired nickases approach worked well in gene editing of cord blood (CB) CD34+ cells, these cord blood cells have yet begun “globin switching” and, upon erythrocyte differentiation, expresses almost exclusively HbF (>95% F cells) even without editing. The consequence of editing in adult bone marrow (AD) CD34+ cells was examined for the most efficient nickases pair A2 + S4 and the respective nuclease A2 for comparison. The edited adult bone marrow (AD) CD34+ cells were differentiated into erythrocytes following the 3-phases expansion protocol. The erythrocytes were harvested at 17-20 day for quantification of γ -chain production by flow cytometry and real-time PCR. The editing efficiency of paired nickases A2 + S4 was higher than that of nuclease A2 (71 ± 4.8 % versus 31 ± 6.3 %; Figure 3A). In line with higher editing efficiency, the paired nickases also outperformed the nuclease by generating more F cells (44.3 ± 5.6 % versus 27 ± 5.7 %; Figure 3B and Figure 3C) and increasing production of γ -chain transcripts (ratio of γ/α chains, 0.33 ± 0.07 % versus 0.22 ± 0.09 %; Figure 3C). The substantial background level of F cells (~21% for the Cas9 and Cas9n controls) is likely intrinsic to the artificial culture condition of the assay. Thus, it was demonstrated that comparing to nuclease, the paired nickases was more efficient in HbF induction in normal hematopoietic cells.

The paired nickases approach work efficiently on β -thalassemia cells

[0058] Upon confirmation of the high efficiency of paired nickases in normal CD34+ cells, it was examined whether the same approach will work for those with defective β -chain. CD34+ cells were collected from three individuals with β -thalassemia having the following genotypes: Thal_#1 (IVS II-654 C>T; CD 41/42 -CTTT, β 0/ β +), Thal_#2 (41/42 -CTTT; Codon 43 G to T) and Thal_#3 (homozygous 41/42 -CTTT). Besides the previously examined pair of A2 + S4, the A3/A4 + S4 pairs were also included for evaluation in β -thalassemia cells. The editing efficient was higher with paired nickases (A2 + S4, 60.7 ± 2.7 %; A3 + S4, 64 ± 1 %; A4 + S4, 59.7 ± 7.2 %) than with the respective nucleases (A2, 36 ± 5.2 %; A3, 46 ± 4 %; A4, 41 ± 3 %) (Figure 4A and 4C). Again, all the paired nickases in general produced more F cells (A2 + S4, 68.3 ± 3.9 %; A3 + S4, 69.7 ± 9.1 %; A4 + S4, 70.4 ± 2.8 %) than that of the respective nucleases (A2, 63.9 ± 6.1 %; A3, 56.7 ± 10.2 %; A4, 53.2 ± 8.1 %), and produced more γ -chain transcript (ratio of γ/α chains, A2 + S4, 1.5 ± 0.9 %; A4 + S4, 1.3 ± 0.7 %; A2, 1 ± 0.6 %) (Figure 4C). When compared with the adult bone marrow (AD) cells, the β -thalassemia cells have a much higher background level of F cells (thalassemia versus adult bone marrow (AD) cells, 51.8 ± 9.1 % versus 21.3 ± 5.2 %) and γ -chain expression (ratio of γ/α chains for thalassemia versus adult bone marrow (AD) cells, 0.95 ± 0.55 % versus 0.12 ± 0.04 %) in the artificial *ex vivo* culture system,

with higher variability between samples (shown by individual data points in Figure 4C). For every sample, the paired nickases resulted in higher editing efficiency and HbF/ γ -chain production than the respective nucleases.

Paired nickases minimize off-target mutagenesis created by nuclease

5 [0059] The paired nickases as disclosed herein are efficient for editing the HBG promoters. Furthermore, the editing specificity of the paired nickases as disclosed herein was determined in comparison to that of nuclease. Targeted amplicon deep sequencing was employed to analyse the target site on HBG promoters, as well as a panel of 54 potential off-target sites (OFT) for the gRNAs A2, A3 and S4 (Table 5). These 54 off-target sites included off-target sites for gRNA A2 and A3 (23 and 2 sites
10 respectively), and the predicted OFTs of gRNA S4 (29 sites). Targeted amplicon sequencing was carried out on the adult bone marrow (AD) and β -thalassemia CD34⁺ samples (3 each) to determine the percentage of non-homologous end joining (NHEJ) mediated indels that resulted from Cas9 editing. Consistent with the result of T7E1 assay, the on-target editing by the paired nickase A2 + S4 ($47.6 \pm 4.4\%$) was higher than that of nuclease A2 ($27.8 \pm 0.8\%$) on adult bone marrow (AD) cells (Figure 5A) and on the thalassemia cells (paired nickase A2 + S4, $61.6 \pm 6.5\%$; nuclease A2, $44.8 \pm 11.7\%$) (Figure
15 5B). Other nickase pairs (A3 + S4) and nucleases (A3, S4) were also included for some experiment, which generated on-target editing as well (Figure 5B).

[0060] For the off-target sites, no significant indels were found for the no gRNAs controls (above the background level of ~ 0.1 - 0.2% , variable between OFTs). Also, an increase of indels for most of the
20 OFTs was not found, except for the three gRNA A2 OFTs (OFT-13, OFT-25 and OFT-40). For the adult bone marrow (AD) CD 34⁺ samples, indels were found exclusively over these sites for editing by A2 and nuclease (OFT-25, $0.4 \pm 0.06\%$; OFT-40, $0.68 \pm 0.1\%$; OFT-13, $0.31 \pm 0.04\%$) but not for editing by the paired nickases A2 + S4 (all at background level of ~ 0.1 - 0.2% , same as level of control) (Figure 5A). Consistently for the β -thalassemia cells, indels on these three sites were found only for
25 editing by A2 and nuclease (OFT-25, $14.7 \pm 7.2\%$; OFT-40, $1.25 \pm 0.5\%$; OFT-13, $0.94 \pm 0.4\%$; Figure 5B). The paired nickases were able to eliminate off-target caused by the nuclease, demonstrating the greater specificity and enhanced efficiency of a paired nickase editing approach.

Editing of HBG promoters lead to deletion of inter-HBGs region

[0061] The nearly identical HBG1 and HBG2 promoters can be recognized and edited by Cas9 RNPs
30 simultaneously, as suggested by the results of the T7E1 assay as disclosed herein. The two closely distanced DSBs in a highly homologous context can result in the loss of intervening HBG2 gene and promoter regions, fusing the HBG1 gene to the promoter of HBG2, thus resulting in the upregulation of HBG1 expression. To demonstrate the potential loss of intervening genome region, two different assays were employed (Figure 6A). The first assay is a standard PCR reaction that aim to amplifies a
35 unique deletion-specific ~ 1.5 kb fragment. And second, a digital PCR assay that quantifies the absolute copy number (CN) of the inter-HBG region, with reference to another control region on the same arm of chromosome 11. On the cord blood (CB) CD34⁺ cells, the detection of the deletion-specific fragments

by PCR in all samples edited by nuclease Cas9 RNPs was achieved, regardless of the gRNAs used and the editing efficiency (Figure 6B; the same samples for T7E1 assay of Figure 2A). The same deletion-specific fragments were also detected for the Cas9 RNPs (paired nuclease A2 + S4 and A4 + S4; nuclease A2 and S4) treated β -thalassemia CD34⁺ cells (Figure 6C; the same samples for T7E1 assay of Figure 4A). After screening by the semi-quantitative standard PCR assay, the extent of intervening HBG2 deletion on the Cas9 edited adult bone marrow (AD) and β -thalassemia CD34⁺ cells was confirmed and quantified (Figure 6D). When the copy number (CN) of the inter-HBG region of the no gRNAs controls was set to 2, the copy number (CN) was reduced significantly for cells edited by nuclease A2 (adult bone marrow (AD), 1.46 ± 0.07 ; β -thalassemia, 1.1 ± 0.13) and reduced further for the cells edited by paired nickases A2 + S4 (AD, 1.05 ± 0.01 ; β -thalassemia, 0.85 ± 0.1), confirming the deletion of inter-HBG region proportionally upon editing of the HBG promoters. Taken together, the above assays confirmed that the loss of HBG2 gene was common to all hematopoietic cells edited by Cas9 nuclease or paired nickases, and suggested that most of the γ -chain expression in edited cells were mechanistically derived from de-suppressed HBG1 rather than HBG2.

[0062] The Cas9 mutant, the D10A nickase, was tested herein on its ability to reactivate γ -chain expression by disrupting the binding sites of repressor LRF on HBG promoters. PAM-out Cas9 nickase pairs was shown herein to be more efficient and specific than the respective Cas9 nucleases in editing the HBG promoters, leading to greater HbF re-expression in adult and β -thalassemia hematopoietic cells.

[0063] Heredity persistence of fetal haemoglobin (HPFH) comprises of diverse mutations, including deletion forms that could span from a few to hundreds kb that often cause the loss of HBB gene, and non-deletion form that only affect single base in the promoters. Mechanistically, it is clear that the beta-like goblin gene promoters (HBB, HBD and HBG1/2) are competing for the upstream locus control region (LCR), the master controller for expression of this gene cluster. As such, disrupting either the active HBB promoter or the repressed HBG promoters can favour the expression of HBG genes. Alternatively, the paired nickase approach, as demonstrated herein, mediates even higher editing efficiency and specificity as compared to the single gRNA nuclease.

[0064] Since development, CRISPR/Cas9 nuclease is the most common choice for editing experiments, owing to its great versatility and accessibility. However, Cas9 nuclease is prone to off-targeting cleavages, which represents a major drawback for clinical application. Cas9 can tolerate some mismatch over 5' end of the target sequence, creating source of off target editing. It was shown herein that the paired nickases can remarkably reduce the off-target effect of nuclease.

[0065] Double nicking by paired nickase is not widely employed for editing experiment because of the "presumed" low efficiency. In addition, to plan for paired nickases experiment, one must identify the rightly aligned gRNA pairs (i.e. in PAM-out) and evaluate the efficient of these different pairs, which present extra hurdles. The findings as shown herein on primary hematopoietic cells demonstrate that paired nickase can outmatch Cas9 nuclease on both editing efficient and specificity.

[0066] Simultaneous editing of HBG promoters leads to deletion of HBG2 containing inter-promoter region, fusing the promoter of HBG2 to that of HBG1 (Figure 6). The same deletion was observed previously with Cas9-mediated editing of LCF or BCL11A binding sites. In fact, the highly homologous HBG promoters are subjected to natural gene rearrangements that produced a number of novel γ -chain variants in human, including duplications, triplication and even quadruplication of γ -globin genes of various G γ and A γ combinations. One of these rearrangements, resembling the editing-induced HBG2 deletion, is a 5 kb deletion over the HBG promoter that fuses the first and second exons of HBG2 to third exon of HBG1, generating unique G γ -A γ hybrid chain. Individuals carrying this variant are normal phenotypically, except having reduced G γ to A γ ratio. Considering the absence of noticeable deleterious effect on erythrocytes differentiation and the marked increase in HbF expression and F cells with our gene-editing approach, it shows that re-expression of HBG1 alone can sufficiently correct β -hemoglobinopathies.

[0067] Cas9 nuclease produces blunt DSB that predominantly repaired by non-homologous end joining (NHEJ); in contrast, the PAM-out paired D10A nickases produce 5'-overhangs that favours HDR. It appears that since Cas9 nuclease and nickases create different type of DNA lesions particular lesion can favour the formation of HBG2 deletion. Nonetheless the HBG2 deletion is found in cells edited either by nuclease and nickase in this study, indicating the deletion is not driven by the difference in DNA repair mechanism. As such, the difference in the extent of HBG2 deletion probably reflects the difference in editing efficiency. The results shown herein show that, due to high sequence homology, targeting any element on the HBG promoters generates the specific HGB2 deletion. The deletion of the entire HBG2 containing inter-promoter region in turn resulted in upregulation of HBG1 expression and F cells.

[0068] In summary, the present disclosure illustrates the feasibility of achieving efficient and specific editing of the HBG promoter by deploying paired Cas9 nickases. Potential non-specific editing brought by Cas9 nuclease can be eliminated, rendering a much safer editing approach for clinical translation and the treatment of β -hemoglobinopathies.

EXPERIMENTAL SECTION

Study approval, cell processing and culture

[0069] The study was approved by Singhealth centralised institutional review board (CIRB 2019/2866; CIRB 2021/2541) and IRB of HKU. All donors provided informed consents. Cord bloods (CB) were obtained from Singapore Cord Blood Bank. Adult hemopoietic cells were obtained from KKH. Thalassemia samples were obtained from KKH and HKU. Mononuclear cells were separated by Ficoll-paque (cat. 17544203, GE Healthcare) and CD34⁺ cells were enriched by magnetic microbeads (cat. 130-046-702, Miltenyi Biotec). The CD34⁺ cells were cultured in completed StemPro-34 medium (with nutrient supplement, glutamine and Pen-Strep) (cat. 10639011, ThermoFisher) supplemented with 300 ng/ml SCF, 300 ng/ml FLT3L, 100 ng/ml TPO and 60 ng/ml IL-3 (cat. 300-07, 300-19, 300-18 and

200-03, all from Peprotech) for 48 hours before electroporation. Sequence of the primers used are summarized in Table 3.

Guide RNA, Cas9 RNP and electroporation

[0070] Guide RNAs (gRNAs) were designed using GPP sgRNA Designer (and currently updated to CRISPick, <https://portals.broadinstitute.org/gppx/crispick/public>) and are summarized in Table 1. The gRNA was produced by hybridization of crRNA;tracrRNA duplex by mixing equal molarity of crRNA and tracrRNA, heated up to 94°C for 2mins, and cooled to 60°C for 1min, and further cooled to room temperature. The gRNA was then reconstituted to 2 µg/µl in duplex buffer (all from Integrated DNA Technologies, IDT). The Cas9 ribonucleoproteins (Cas9 RNP) complex was formed by mixing 2.5 µg of either the nuclease Cas9 (Alt-R S.p. Cas9 Nuclease V3, cat. 1081059, IDT) or the nickase Cas9n (Alt-R S.p. Cas9 D10A nickase, cat 1081063, IDT) with 4 µg of gRNAs in 6 µl of buffer R for 15 mins at room temperature, in the present of 1 µg/ul electroporation enhancer (cat. 1075916, IDT). The target cells were washed thrice with PBS (without Ca²⁺/Mg²⁺), and resuspended in the desired volume of buffer R. Typically 0.1-0.2x10⁶ cells were resuspended in 6µl of buffer R, combined with 6 µl of Cas9 RNP complex, and electroporated using the 10 µl Neon tips with the pre-set program no. 24 of the Neon system (1600V, 10ms pulse, 3 pulses) (all from ThermoFisher, cat. MPK5000). Electroporated cells were released immediately to pre-warmed complete StemPro 34 medium (Phase 1 medium for erythrocyte expansion) for recovery and continuous culture. For 1-2 x10⁶ starting cells, the reagents were scaled up and 100 µl Neon® tips were used instead.

T7E1 endonuclease mismatch cleavage assay

[0071] The T7E1 assays were performed using Alt-R® Genome Editing Detection Kit (cat. 1075932, IDT). Genomic DNA was extracted by Dneasy Blood & Tissue Kits (cat 69504, Qiagen). A 20 µl PCR reaction was performed using Gotaq master mix (cat M7122, Promega) with the following parameters: 95°C for 10 mins, and 40 cycles of 95°C for 30 sec, 55°C for 30 sec and 72°C for 30 sec, and final amplification of 72°C for 7 mins. After that, 1 µl of the 10X T7E1 reaction buffer was added to 8 µl of the PCR product and the mixture was denatured in a thermal cycler for 10 mins at 95°C, and cooled to 85°C at the ramp rate of 2°C/sec, and further cooled to 25°C at the ramp rate of 0.3°C/sec. The reannealed product was mixed with 1 µl of T7 endonuclease I (1 unit/µl) and incubated at 37°C for 1 hour. The digested products were resolved on 2% agarose gel. The intensity of the bands on the captured gel image was then quantified by ImageJ to calculate the percentage of intact and cleaved products.

Three phases serum-free expansion of erythrocyte

[0072] The 3 phases erythrocyte expansion culture was performed as described previously (Lee, de Vasconcellos et al. 2013). The StemPro-34 complete medium was used throughout, with specific supplements added each phase. The phase 1 medium (day 2 to 7 for the electroporated cells) was supplemented with 50 ng/mL SCF, 50 ng/mL FLT3 Ligand and 10 ng/mL IL-3. Phase 2 medium (day 7 to day 14) was supplemented with 4 U/mL of erythropoietin (EPO) (cat. 100-64, Peprotech), 10 ng/mL SCF, 10 µg/mL insulin (cat. I9278-5ML, Sigma-Aldrich), 3 U/mL of heparin (cat. H3149-250KU,

Sigma-Aldrich), and 0.8 mg/mL of holo Transferrin (cat. T0665, Sigma-Aldrich). Phase 3 medium (day 14 to day 21) was supplemented with 4 U/mL EPO, 3 μ M RU486 (cat. M8046, Sigma-Aldrich), 10 μ g/mL insulin, 3 U/mL heparin, and 0.8 mg/mL holo Transferrin. At the end of phase 1 expansion, some cells were collected for genomic DNA extraction. The expanded erythrocytes were then harvested for flow cytometry and RNA extraction between day 17 and day 21.

Real-time quantitative PCR

[0073] RNA was extracted from the RBC using the Rneasy extraction kit (cat. 74004, Qiagen). The cDNA was synthesised using Superscript VILO cDNA kit (cat. 11754050, Thermo). The real-time PCR reaction was setup by mixing the cDNA with 2x iQ SYBR Green Supermix (cat. 1708880, Bio-rad) and 0.2 μ M final of both forward and reverse primers, at 25 μ l per well in the CFX96 Real-Time PCR Detection System. The condition of PCR reaction was: association at 95°C for 3mins, followed by 40 cycles of 95°C 10sec, annealing at 63°C for 30sec and extension at 72°C for 20sec.

Intracellular staining and flow cytometry

[0074] Intracellular staining of RBC was performed based on the protocol from Inside Stain Kit (130-090-477, Miltenyi Biotec). Briefly, about 0.1×10^6 RBCs were fixed for 20 mins with 0.5 ml of Inside Fix. Cells were then washed once with autoMCAS rinsing buffer (cat. 130-091-222, Miltenyi Biotec), followed by 0.5 ml of Inside Perm. Afterward the cells were resuspended in 90 μ l of Inside Perm and co-stained with anti-CD235 (1:100; cat. 349106, Biolegend) and anti-HbF (1:10; cat. 130-108-241, Miltenyi Biotec) antibodies in a total volume of 100 μ l for 10 mins in dark. The cells were then washed once with 0.5 ml of Inside Perm, and resuspend in 0.25 ml of autoMACS buffer for flow acquisition on BD LSRII. The percentage of HbF expressing erythrocyte (F cells) was analysed by Flowjo (BD biosciences).

CRISPR target amplicon sequencing

[0075] The amplification primers for amplicon panel were designed and produced by the rhAmpSeq CRISPR analysis system (IDT). The amplicon panel was designed to include the targeted locus (the HBG promoter 5249974 to 5250057, hg38) and a total of 54 validated/ predicted off-target loci (of the guides A2, A3 and S4; table 5). Amplicon libraries were constructed using 10-50 ng of genomic DNA per sample and were amplified using rhAmpSeq CRISPR library kit (cat. 10007317), following the instructions on protocol. The libraries were then indexed, pooled, and cleaned up by AMPure XP beads before sending for sequencing on the NovaSeq platform (Novogene), aiming for coverage of 10000x per target. The sequencing data was analysed on the cloud-based rhAmpSeq CRISPR analysis platform (BlueBee, IDT).

Detection of inter-HBG region deletion

[0076] The deletion of inter-HBG region was screened by PCR and further quantified by digital PCR. **For PCR:** the PCR was performed using the GoTaq PCR master mix, under the same condition used for T7E1 assay. The amplified product was then resolve on 1% gel agarose. **For digital PCR:** digital PCR was performed on QIAcuity One (Qiagen). Primer pairs targeting the inter-HBG region (upstream

of the HBG1 promoter) or the control region (targeting CD44 on chr.11, same arm of HBG genes) (CNV assay; cat. PCH111-0175806A, Qiagen) were used to quantify the exact copy number. Each reaction consisted of the genomic DNA, 4 µl of 3x evagreen mix (cat. 250111 Qiagen) and 0.8 µM primers in a total volume of 12 µl, was loaded onto a well on the 8.5K 24 wells plate (cat. 250011, Qiagen). The quantification reactions were run in duplicate or triplicate using the program: activation at 95°C for 2 mins, followed by 40 cycles of denaturation at 95°C for 15 sec, annealing at 60°C for 15 sec and extension at 72°C for 15 sec, and with final extension at 40°C for 5 mins. Data analysis was done on QIAcuity Software Suite.

Statistical analysis

- 10 [0077] Data were reported as mean $\pm$ standard error of the mean (SEM). Statistical significance was determined by two tailed Student t test.

Table 1: gRNA sequences of the present disclosure

SEQ ID NO	gRNA	5' to 3' sequence	PAM	Orientation	Strand	Target sequence location on chromosome	Start (HBG1/HBG2)	End (HBG1/HBG2)
1	A1	GTGGGGAAGGGGCCCCCAAG	AGG	Antisense	+	Chr11	5250049/5254973	5250068/5254992
2	A2	ATTGAGATAGTGTGGGGAAG	GGG	Antisense	+	Chr11	5250038/5254962	5250057/5254981
3	A3	GCATTGAGATAGTGTGGGGA	AGG	Antisense	+	Chr11	5250036/5254960	5250055/5254979
4	A4	ATTTGCATTGAGATAGTGTG	GGG	Antisense	+	Chr11	5250032/5254956	5250051/5254975
5	S1	ATGCAAATATCTGTCTGAAA	CGG	Sense	-	Chr11	5250020/5254944	5250039/5254963
6	S2	TATCTGTCTGAAACGGTCCC	TGG	Sense	-	Chr11	5250013/5254937	5250032/5254956
7	S3	CCCTGGCTAAACTCCACCCA	TGG	Sense	-	Chr11	5249996/5254920	5250015/5254939
8	S4	CCTGGCTAAACTCCACCCAT	GGG	Sense	-	Chr11	5249995/5254919	5250014/5254938

Table 2: Nickase sequences of the present disclosure

SEQ ID NO	Sequence name	5' to 3' sequence
9	Wild-type Cas9 (Cas9 Nickase V3 of IDT)	ATGGATAAGAAATACTCAATAGGCTTAGATATCGGCACAAATAGCGTCGGATGGGCGGTGATCACTG ATGAATATAAGGTTCCGTCTAAAAAGTTCAAGGTTCTGGGAAATACAGACCGCCACAGTATCAAAAA AAATCTTATAGGGGCTCTTTTATTTGACAGTGGAGAGACAGCGGAAGCGACTCGTCTCAAACGGACA GCTCGTAGAAGGTATACACGTCGGAAGAATCGTATTTGTTATCTACAGGAGATTTTTTCAAATGAGAT GGCGAAAGTAGATGATAGTTTCTTTCATCGACTTGAAGAGTCTTTTTTGGTGGAAGAAGACAAGAAG CATGAACGTCATCCTATTTTTGGAAATATAGTAGATGAAGTTGCTTATCATGAGAAATATCCAACAT CTATCATCTGCGAAAAAAATTGGTAGATTCTACTGATAAAGCGGATTTGCGCTTAATCTATTTGGCCT TAGCGCATATGATTAAGTTTTCGTGGTCATTTTTTGATTGAGGGAGATTTAAATCCTGATAATAGTGAT GTGGACAAACTATTTATCCAGTTGGTACAAACCTACAATCAATTATTTGAAGAAAACCTATTAACGC AAGTGGAGTAGATGCTAAAGCGATTCTTCTGCACGATTGAGTAAATCAAGACGATTAGAAAATCTC ATTGCTCAGCTCCCCGGTGAGAAGAAAAATGGCTTATTTGGGAATCTCATTGCTTTGTCATTGGGTTT GACCCCTAATTTTAAATCAAATTTTGATTTGGCAGAAGATGCTAAATTACAGCTTTCAAAAAGATACTT ACGATGATGATTTAGATAATTTATTGGCGCAAATTGGGAGATCAATATGCTGATTTGTTTTTGGCAGCT AAGAATTTATCAGATGCTATTTTACTTTAGATATCCTAAGAGTAAATACTGAAATAACTAAGGCTCC CCTATCAGCTTCAATGATTAACGCTACGATGAACATCATCAAGACTTGACTCTTTTAAAAGCTTTAG TTCGACAACAACCTTCCAGAAAAGTATAAAGAAATCTTTTTTGATCAATCAAAAAACGGATATGCAGG TTATATTGATGGGGGAGCTAGCCAAGAAGAATTTTATAAATTTATCAAACCAATTTTAGAAAAAATG GATGGTACTGAGGAATTATTGGTGAAACTAAATCGTGAAGATTTGCTGCGCAAGCAACGGACCTTTG ACAACGGCTCTATTCCCATCAAATTCACCTGGGTGAGCTGCATGCTATTTTGAGAAGACAAGAAGA CTTTTATCCATTTTAAAAGACAATCGTGAGAAGATTGAAAAAATCTTGACTTTTCGAATTCCTTATTA TGTTGGTCCATTGGCGCGTGGCAATAGTCGTTTTGCATGGATGACTCGGAAGTCTGAAGAAACAATTA CCCCATGGAATTTTGAAGAAGTTGTGATAAAGGTGCTTCAGCTCAATCATTTATTGAACGCATGACA AACTTTGATAAAAAATCTTCCAAATGAAAAAGTACTACCAAAACATAGTTTGCTTTATGAGTATTTTAC GGTTTATAACGAATTGACAAAGGTCAAATATGTTACTGAAGGAATGCGAAAACAGCATTTCCTTTCA GGTGAACAGAAGAAAGCCATTGTTGATTTACTCTTCAAAACAAATCGAAAAGTAACCGTTAAGCAAT TAAAAGAAGATTATTTCAAAAAAATAGAATGTTTTGATAGTGTTGAAATTTAGAGATTGAAGATAG ATTTAATGCTTCATTAGGTACCTACCATGATTTGCTAAAAAATTATTAAGATAAAGATTTTTTGGATA ATGAAGAAAATGAAGATATCTTAGAGGATATTGTTTTAACATTGACCTTATTGAAGATAGGGAGAT GATTGAGGAAAGACTTAAAACATATGCTCACCTCTTTGATGATAAGGTGATGAAACAGCTTAAACGT CGCCGTTATACTGGTTGGGGACGTTTGTCTCGAAAATTGATTAATGGTATTAGGGATAAGCAATCTGG CAAAACAATATTAGATTTTTTGAATCAGATGGTTTTGCCAATCGCAATTTTATGCAGCTGATCCATG ATGATAGTTTGACATTTAAAGAAGACATTCAAAAAGCACAAGTGTCTGGACAAGGCGATAGTTTACA

		TGAACATATTGCAAATTTAGCTGGTAGCCCTGCTATTA AAAAAGGTATTTTACAGACTGTAAAAGTTG TTGATGAATTGGTCAAAGTAATGGGGCGGCATAAGCCAGAAAATATCGTTATTGAAATGGCACGTGA AAATCAGACAAC TCAAAGGGCCAGAAAAATTCGCGAGAGCGTATGAAACGAATCGAAGAAGGTAT CAAAGAATTAGGAAGTCAGATTCTTAAAGAGCATCCTGTTGAAAATACTCAATTGCAAAATGAAAAG CTCTATCTCTATTATCTCCAAAATGGAAGAGACATGTATGTGGACCAAGAATTAGATATTAATCGTTT AAGTGATTATGATGTCGATCACATTGTTCCACAAAGTTTCCTTAAAGACGATTCAATAGACAATAAGG TCTTAACGCGTTCTGATAAAAATCGTGGTAAATCGGATAACGTTCCAAGTGAAGAAGTAGTCAAAAA GATGAAAAACTATTGGAGACAAC TCTAAACGCCAAGTTAATCACTCAACGTAAGTTTGATAATTTA ACGAAAGCTGAACGTGGAGGTTTGAGTGAAC TTGATAAAGCTGGTTTTATCAAACGCCAATTGGTTG AAACTCGCCAAATCACTAAGCATGTGGCACAAATTTTGGATAGTCGCATGAATACTAAATACGATGA AAATGATAAACTTATTCGAGAGGTTAAAGTGATTACCTTAAATCTAAATTAGTTTCTGACTTCCGAA AAGATTTCCAATTCTATAAAGTACGTGAGATTAACAATTACCATCATGCCCATGATGCGTATCTAAAT GCCGTCGTTGGAAGTGTCTTTGATTAAAGAAATATCCAAAAC TTGAATCGGAGTTTGTCTATGGTGATTA TAAAGTTTATGATGTTCTGTA AAAATGATTGCTAAGTCTGAGCAAGAAATAGGCAAAGCAACCGCAAAA TATTTCTTTTACTCTAATATCATGAAC TCTTCAAAAACAGAAATTACACTTGCAAAATGGAGAGATTCTG CAAACGCCCTCTAATCGAAACTAATGGGGAAACTGGAGAAATTGTCTGGGATAAAGGGCGAGATTTT GCCACAGTGCGCAAAGTATTGTCCATGCCCCAAGTCAATATTGTCAAGAAAACAGAAAGTACAGACAG GCGGATTCTCCAAGGAGTCAATTTTACCAAAAAGAAATTCGGACAAGCTTATTGCTCGTAAAAAAGA CTGGGATCCAAAAAAATATGGTGGTTTTGATAGTCCAACGGTAGCTTATTCAGTCCTAGTGGTTGCTA AGGTGGAAAAAAGGGAAATCGAAGAAGTTAAATCCGTTAAAGAGTTACTAGGGATCACAATTATGG AAAGAAGTTCCTTTGAAAAAAATCCGATTGACTTTTTTAGAAGCTAAAGGATATAAGGAAGTTAAAAA AGACTTAATCATTAAACTACCTAAATATAGTCTTTTTTGAGTTAGAAAACGGTCGTAAACGGATGCTGG CTAGTGCCGGAGAATTACAAAAAGGAAATGAGCTGGCTCTGCCAAGCAAATATGTGAATTTTTTATA TTTAGCTAGTCATTATGAAAAGTTGAAGGGTAGTCCAGAAGATAACGAACAAAAACAATTGTTTGTG GAGCAGCATAAGCATTATTTAGATGAGATTATTGAGCAAATCAGTGAATTTTCTAAGCGTGTTATTTT AGCAGATGCCAATTTAGATAAAGTTCTTAGTGCATATAACAAACATAGAGACAAACCAATACGTGAA CAAGCAGAAAATATTATTCATTTATTTACGTTGACGAATCTTGGAGCTCCCGCTGCTTTTAAATATTTT GATACAACAATTGATCGTAAACGATATACGTCTACAAAAGAAGTTTTAGATGCCACTCTTATCCATCA ATCCATCACTGGTCTTTATGAAACACGCATTGATTTGAGTCAGCTAGGAGGTGACCCCAAGAAGAAG AGGAAGGTGATGGATAAGCATCACCACCACCATCACTAA
10	Mutant Cas9 (Cas9 Nickase V3 of IDT with D10A mutation (Cas9n))	ATGGATAAGAAATACTCAATAGGCTTAGCTATCGGCACAAATAGCGTCGGATGGGGCGGTGATCACTG ATGAATATAAGGTTCCGTCTAAAAAGTTCAAGGTTCTGGGAAATACAGACCGCCACAGTATCAAAAA AAATCTTATAGGGGCTCTTTTATTTGACAGTGGAGAGACAGCGGAAGCGACTCGTCTCAAACGGACA GCTCGTAGAAGGTATACACGTCCGAAGAATCGTATTTGTTATCTACAGGAGATTTTTTCAAATGAGAT GGCGAAAGTAGATGATAGTTTCTTTTCATCGACTTGAAGAGTCTTTTTTGGTGGAAGAAGACAAGAAG

		CATGAACGTCATCCTATTTTTGGAAATATAGTAGATGAAGTTGCTTATCATGAGAAATATCCAACATAT CTATCATCTGCGAAAAAATTGGTAGATTCTACTGATAAAGCGGATTTGCGCTTAATCTATTTGGCCT TAGCGCATATGATTAAGTTTCGTGGTCATTTTTTGATTGAGGGAGATTTAAATCCTGATAAAGTGAT GTGGACAACTATTTATCCAGTTGGTACAAACCTACAATCAATTATTTGAAGAAAACCTATTAACGC AAGTGGAGTAGATGCTAAAGCGATTCTTTCTGCACGATTGAGTAAATCAAGACGATTAGAAAATCTC ATTGCTCAGCTCCCCGGTGAGAAGAAAAATGGCTTATTTGGGAATCTCATTGCTTTGTCATTGGGTTT GACCCCTAATTTAAATCAAATTTTGATTTGGCAGAAGATGCTAAATTACAGCTTTCAAAAGATACTT ACGATGATGATTTAGATAATTTATTGGCGCAAATTTGGAGATCAATATGCTGATTTGTTTTTGGCAGCT AAGAATTTATCAGATGCTATTTTACTTTTCAGATATCCTAAGAGTAAATACTGAAATAACTAAGGCTCC CCTATCAGCTTCAATGATTAACGCTACGATGAACATCATCAAGACTTGACTCTTTTAAAAGCTTTAG TTCGACAACAACTTCCAGAAAAGTATAAAGAAATCTTTTTTGATCAATCAAAAAACGGATATGCAGG TTATATTGATGGGGGAGCTAGCCAAGAAGAATTTTATAAATTTATCAAACCAATTTTAGAAAAAATG GATGGTACTGAGGAATTATTGGTGAAACTAAATCGTGAAAGATTGCTGCGCAAGCAACGGACCTTTG ACAACGGCTCTATTCCCATCAAATTCCTTGGGTGAGCTGCATGCTATTTTGAGAAGACAAGAAGA CTTTTATCCATTTTAAAAGACAATCGTGAGAAGATTGAAAAAATCTTGACTTTTCGAATTCCTTATTA TGTTGGTCCATTGGCGCGTGGCAATAGTCGTTTTGCATGGATGACTCGGAAGTCTGAAGAAACAATTA CCCCATGGAATTTTGAAGAAGTTGTGATAAAGGTGCTTCAGCTCAATCATTTATTGAACGCATGACA AACTTTGATAAAAAATCTTCCAAATGAAAAAGTACTACCAAAACATAGTTTGCTTTATGAGTATTTTAC GGTTTATAACGAATTGACAAAGGTCAAATATGTTACTGAAGGAATGCGAAAACCAGCATTCTTTTCA GGTGAACAGAAGAAAGCCATTGTTGATTTACTCTTCAAAACAAATCGAAAAGTAACCGTTAAGCAAT TAAAAGAAGATTATTTCAAAAAAATAGAATGTTTTGATAGTGTTGAAATTTTCAGGAGTTGAAGATAG ATTTAATGCTTCATTAGGTACCTACCATGATTTGCTAAAAATTATTAAAGATAAAGATTTTTTGGATA ATGAAGAAAATGAAGATATCTTAGAGGATATTGTTTTAACATTGACCTTATTTGAAGATAGGGAGAT GATTGAGGAAAGACTTAAAACATATGCTCACCTCTTTGATGATAAGGTGATGAAACAGCTTAAACGT CGCCGTTATACTGGTTGGGGACGTTTGTCTCGAAAATTGATTAATGGTATTAGGGATAAGCAATCTGG CAAAACAATATTAGATTTTTTGAATCAGATGGTTTTGCCAATCGCAATTTTATGCAGCTGATCCATG ATGATAGTTTGACATTTAAAGAAGACATTCAAAAAGCACAAAGTGTCTGGACAAGGCGATAGTTTACA TGAACATATTGCAAATTTAGCTGGTAGCCCTGCTATTAAAAAAGGTATTTTACAGACTGTAAAAGTTG TTGATGAATTGGTCAAAGTAATGGGGCGGCATAAGCCAGAAAATATCGTTATTGAAATGGCACGTGA AAATCAGACAACTCAAAGGGCCAGAAAAATTCGCGAGAGCGTATGAAACGAATCGAAGAAGGTAT CAAAGAATTAGGAAGTCAGATTCTTAAAGAGCATCTGTGAAAATACTCAATTGCAAAATGAAAAG CTCTATCTCTATTATCTCCAAAATGGAAGAGACATGTATGTGGACCAAGAATTAGATATTAATCGTTT AAGTGATTATGATGTCGATCACATTGTTCCACAAAGTTTCCTTAAAGACGATTCAATAGACAATAAGG TCTTAACGCGTTCTGATAAAAAATCGTGGTAAATCGGATAACGTTCCAAGTGAAGAAGTAGTCAAAAA GATGAAAAACTATTGGAGACAACCTCTAAACGCCAAGTTAATCACTCAACGTAAGTTTGATAATTTA
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		ACGAAAGCTGAACGTGGAGGTTTGAGTGAACCTTGATAAAGCTGGTTTTATCAAACGCCAATTGGTTG AAACTCGCCAAATCACTAAGCATGTGGCACAAATTTTGGATAGTCGCATGAATACTAAATACGATGA AAATGATAAACTTATTCGAGAGGTTAAAGTGATTACCTTAAATCTAAATTAGTTTCTGACTTCCGAA AAGATTTCCAATTCTATAAAGTACGTGAGATTAAACAATTACCATCATGCCCATGATGCGTATCTAAAT GCCGTCGTTGGAACGTCTTTGATTAAGAAATATCCAAAACCTGAATCGGAGTTTGTCTATGGTGATTA TAAAGTTTATGATGTTTCGTAAAATGATTGCTAAGTCTGAGCAAGAAATAGGCAAAGCAACCGCAAAA TATTTCTTTTACTCTAATATCATGAACTTCTTCAAAACAGAAATTACACTTGCAAATGGAGAGATTCTG CAAACGCCCTCTAATCGAACTAATGGGGAACTGGAGAAATTGTCTGGGATAAAGGGCGAGATTTT GCCACAGTGCGCAAAGTATTGTCCATGCCCCAAGTCAATATTGTCAAGAAAACAGAAAGTACAGACAG GCGGATTCTCCAAGGAGTCAATTTTACCAAAAAGAAATTCGGACAAGCTTATTGCTCGTAAAAAAGA CTGGGATCCAAAAAATATGGTGGTTTTGATAGTCCAACGGTAGCTTATTCAGTCCTAGTGGTTGCTA AGGTGGAAAAAGGGAAATCGAAGAAGTTAAATCCGTTAAAGAGTTACTAGGGATCACAATTATGG AAAGAAGTTCCTTTGAAAAAATCCGATTGACTTTTTAGAAAGCTAAAGGATATAAGGAAGTTAAAAA AGACTTAATCATTAACTACCTAAATATAGTCTTTTTTGAGTTAGAAAACGGTCGTAAACGGATGCTGG CTAGTGCCGGAGAATTACAAAAAGGAAATGAGCTGGCTCTGCCAAGCAAATATGTGAATTTTTTATA TTTAGCTAGTCATTATGAAAAGTTGAAGGGTAGTCCAGAAGATAACGAACAAAAACAATTGTTTGTG GAGCAGCATAAGCATTATTTAGATGAGATTATTGAGCAAATCAGTGAATTTTCTAAGCGTGTTATTTT AGCAGATGCCAATTTAGATAAAGTTCTTAGTGCATATAACAAACATAGAGACAAACCAATACGTGAA CAAGCAGAAAATATTATTCATTTATTTACGTTGACGAATCTTGGAGCTCCCGCTGCTTTTAAATATTTT GATACAACAATTGATCGTAAACGATATACGTCTACAAAAGAAGTTTTAGATGCCACTCTTATCCATCA ATCCATCACTGGTCTTTATGAAACACGCATTGATTTGAGTCAGCTAGGAGGTGACCCCAAGAAGAAG AGGAAGGTGATGGATAAGCATCACCACCACCATCACTAA
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Table 3: Primer sequences used in the present disclosure

SEQ ID NO:	Name	Primer purpose	Sequence	Sequence location on chromosome	Start	End
11	HBG1pT7E1_F	Forward primer for T7E1 assay (HBG1)	TACTGCGCTGA AACTGTGGC	Chr11	5250463	5250482
12	HBG2pT7E1_F	Forward primer for T7E1 assay (HBG2)	GCACTGAAACT GTTGCTTTATAG GAT	Chr11	5255379	5255404
13	HBGpT7E1_R	reverse primer for T7E1 assay (HBG1/2)	GCGCTCTGGAC TAGGAGCTTATT G	Chr11	5249805/5254729	5249828/5254752
14	FlankHBG1-2_F	Detection of HBG2 deletion (PCR)	GTTTTAAAACA ACAAAAATGAG GGAAAGA	Chr11	5249058	5249086
15	FlankHBG1-2_R	Detection of HBG2 deletion (PCR)	GTTGCTTTATAG GATTTTTCAC CAC	Chr11	5255367	5255393
16	HBG1_del_F	Detection of HBG2 deletion (digital PCR)	ACGGATAAGTA GATATTGAGGT AAGC	Chr11	5250515	5250540
17	HBG1_del_R	Detection of HBG2 deletion (digital PCR)	GTCTCTTTCAGT TAGCAGTGG	Chr11	5250339	5250359
18	HBA_F	Quantitative PCR (HBA)	CGGTCAACTTC AAGCTCCTAA			
19	HBA_R	Quantitative PCR (HBA)	ACAGAAGCCAG GAACTTGTC			
20	HBB_F	Quantitative PCR (HBB)	GCAAGGTGAAC GTGGATGAAGT			

21	HBB_R	Quantitative PCR (HBB)	TAACAGCATCA GGAGTGGACAG A			
22	HBG_F	Quantitative PCR (HBG)	CCTGTCCTCTGC CTCTGCC			
23	HBG_R	Quantitative PCR (HBG)	GGATTGCCAAA ACGGTCAC			

Table 4: Editing efficiency of gRNA pairs

gRNA-antisense	Nick position	gRNA-sense	Nick position (HBG1)	Distance between nicks (bps)	Editing efficiency (%)
A1	5250064	S1	5250024	40	24
	5250064	S2	5250017	47	16
	5250064	S3	5250000	64	19
	5250064	S4	5249999	65	22
A2	5250053	S1	5250024	29	32
	5250053	S2	5250017	36	62
	5250053	S3	5250000	53	68
	5250053	S4	5249999	54	86
A3	5250051	S1	5250024	27	2
	5250051	S2	5250017	34	72
	5250051	S3	5250000	51	81
	5250051	S4	5249999	52	85
A4	5250047	S1	5250024	23	0
	5250047	S2	5250017	30	23
	5250047	S3	5250000	47	71
	5250047	S4	5249999	48	77

Table 5: Targeted sites on HBG promoters

SEQ ID NO	ID	target of	Target	Sequence	PAM	#MisMatch	Chr	Start	End	Sign
24	On-Target A2/A3	A2/A3/S4 on-target	chr11_5250038_5250057RH.1031E5BACE314C2Z0Z	ATTGAG ATAGTGT GGGGAA G	GGG		chr11	5250037	5250057	+
25	On-Target S4	A2/A3/S4 on-target	chr11_5250038_5250057RH.1031E5BACE314C2Z0Z	CCTGGCT AAACTCC ACCCAT	GGG		chr11	5249974	5249994	+
26	Off-1	A2 GUIDE-seq off-target sites	chr1_20109231_20109250RH.A3A9E18079E741DZ0Z	ACTGAG ATAGGG TGGGGA GG	GGG		chr1	20109231	20109250	+
27	Off-2	S4 predicted off-target sites	chr1_30247687_30247707RH.F2B8949D00FC49DZ0Z	CCAGGC AAGACT CCACCCA T	CAG	3	chr1	30247687	30247707	-
28	Off-3	A2 GUIDE-seq off-target sites	chr1_94461289_94461308RH.73B9181E2B594A1Z0Z	ATGGAG ATAGGG TGGGGA GA	GGC		chr1	94461289	94461308	+
29	Off-4	S4 predicted off-target sites	chr1_105396952_105396972RH.CBEF315AC1C746EZ0Z	CCCAGCT AAACTCC ACCCTT	AAG	3	chr1	1.05E+08	1.05E+08	-

30	Off-5	S4 predicted off-target sites	chr1_20482 5344_2048 25364RH.4 F1424365F 5948CZ0Z	CCCGGCT AAACCC CACCTT	AAG	3	chr1	2.05E+08	2.05E+08	-
31	Off-6	S4 predicted off-target sites	chr1_20913 3372_2091 33392RH.8 9C589C94 996403Z0Z	CCTGGCT AAACCC CACCTT	AAG	2	chr1	2.09E+08	2.09E+08	-
32	Off-7	S4 predicted off-target sites	chr1_24617 6252_2461 76272RH. A1A083A D3516490 Z0Z	CCTTCCT CAACTAC ACCCAT	AAG	4	chr1	2.46E+08	2.46E+08	+
33	Off-8	A2 GUIDE-seq off-target sites	chr2_60709 386_60709 405RH.CB 89B580FE 7C4EAZ0Z	CAGGAG ATAGTAT GGGGAA T	GGG		chr2	60709386	60709405	-
34	Off-9	S4 predicted off-target sites	chr2_61933 476_61933 496RH.CC 2A17758A 904FFZ0Z	TCCGGCT AAACCC CACCTT	AAG	4	chr2	61933476	61933496	-
35	Off-10	S4 predicted off-target sites	chr2_10717 7412_1071 77432RH.7 6DA30E5A 9EB46AZ0 Z	GCTAGC AAATCTC CACCCAT	GGG	4	chr2	1.07E+08	1.07E+08	-
36	Off-11	A2 GUIDE-seq	chr2_15796 2996_1579 63015RH.9	AAAGAG ACAGTG	GGG		chr2	1.58E+08	1.58E+08	+

		off-target sites	E3A880C8 8FC4D3Z0 Z	AAGGGA AG						
37	Off-12	S4 predicted off-target sites	chr3_40067 275_40067 295RH.88F FD08795F D463Z0Z	CTTTGCT CAAATCC ACCCAT	GGG	4	chr3	40067275	40067295	-
38	Off-13	A2 GUIDE-seq off-target sites	chr3_12727 9487_1272 79507RH.1 6EF2DD10 CD6472Z0 Z	GGGGAG ATAGTGT GGGGAA G	GGG		chr3	1.27E+08	1.27E+08	+
39	Off-14	S4 predicted off-target sites	chr3_19312 1904_1931 21924RH.1 B150E3C3 EA3453Z0 Z	CCCGGCT AAACCC CACCCCTT	AAG	3	chr3	1.93E+08	1.93E+08	-
40	Off-15	S4 predicted off-target sites	chr4_17742 443_17742 463RH.AD F49D18261 7425Z0Z	CCCAGCT AAACCC CACCCCTT	AAG	4	chr4	17742443	17742463	+
41	Off-16	S4 predicted off-target sites	chr4_10768 1576_1076 81596RH. AA7808F0 C05240CZ 0Z	CCTCTCT AGAATC CACCCAT	AAG	4	chr4	1.08E+08	1.08E+08	+
42	Off-17	S4 predicted off-target sites	chr5_11731 871_11731 891RH.ED	TCTGCCA AAACTTC ACCCAT	AGG	4	chr5	11731871	11731891	+

			1F154E54 C5448Z0Z							
43	Off-18	A2 GUIDE-seq off-target sites	chr5_13409 9929_1340 99948RH.1 2C890F4B 4BB45CZ0 Z	GTTGAG ACAGAG TGGGAA GG	GGT		chr5	1.34E+08	1.34E+08	+
44	Off-19	A3 GUIDE-seq off-target sites	chr5_14957 3509_1495 73528RH.5 956F05EC 43E437Z0 Z	GCATTGA GTTAGTA TGGGTA	AGG		chr5	1.5E+08	1.5E+08	-
45	Off-20	A2 GUIDE-seq off-target sites	chr5_15107 2692_1510 72711RH.8 B23F31985 994E0Z0Z	CTAGAG ATAGTGT GGGGTA G	GGG		chr5	1.51E+08	1.51E+08	-
46	Off-21	S4 predicted off-target sites	chr6_16516 1646_1651 61666RH.0 0691F653E 2E447Z0Z	CTTGCCT TCACTCC ACCCAT	GGG	4	chr6	1.65E+08	1.65E+08	-
47	Off-22	A2 GUIDE-seq off-target sites	chr7_80389 634_80389 653RH.B7 C03A9332 454D2Z0Z	AATGAG ACAGTGT GAGGAA A	GGG		chr7	80389634	80389653	-
48	Off-23	A2 GUIDE-seq off-target sites	chr7_10651 2170_1065 12189RH.3 754A10E2 9D0495Z0 Z	GCTGAG ACAGTCT GGGGAA G	GGG		chr7	1.07E+08	1.07E+08	-

49	Off-24	A2 GUIDE-seq off-target sites	chr8_24932 621_24932 640RH.7E CDB1D71 EA944BZ0 Z	GAAGAG ACAGTGT GGGGAA G	GTG		chr8	24932621	24932640	+
50	Off-25	A2 GUIDE-seq off-target sites	chr9_51087 01_510872 0RH.01B3 349C207C 48BZ0Z	ATAGGG ATAGTGT GAGGAA G	GGG		chr9	5108701	5108720	-
51	Off-26	S4 predicted off-target sites	chr9_38876 805_38876 825RH.C0 F63D02F0 BD4DFZ0 Z	GCTGTCT TAAATCC ACCCAT	TAG	4	chr9	38876805	38876825	-
52	Off-27	S4 predicted off-target sites	chr9_67487 131_67487 151RH.565 DDA3EAE 264D8Z0Z	GCTGTCT TAAATCC ACCCAT	TAG	4	chr9	67487131	67487151	+
53	Off-28	S4 predicted off-target sites	chr9_73065 115_73065 135RH.84 A519B688 2C499Z0Z	CCTGCCT GAACCC CACCCCTT	AAG	4	chr9	73065115	73065135	+
54	Off-29	A2 GUIDE-seq off-target sites	chr9_89010 748_89010 767RH.1D 5EA75334 1B457Z0Z	CCTGAG AAAGTG TGGGGA AG	GGG		chr9	89010748	89010767	+
55	Off-30	A2 GUIDE-seq	chr9_12398 6455_1239 86474RH.8	CATGAG ACAGTGT	GAG		chr9	1.24E+08	1.24E+08	-

		off-target sites	FBC1EFD1 FD6420Z0 Z	GGAGAA G						
56	Off-31	A2 GUIDE-seq off-target sites	chr9_12559 4700_1255 94719RH.1 A02CAC6 A87B408Z 0Z	AATGAG AGAGTG TGGGGA AG	GAG		chr9	1.26E+08	1.26E+08	-
57	Off-32	A2 GUIDE-seq off-target sites	chr10_1897 9812_1897 9831RH.D B10848503 F84E5Z0Z	ATTATGA TAGTGTG GGGAAG	GGG		chr10	18979812	18979831	+
58	Off-33	S4 predicted off-target sites	chr10_3363 7006_3363 7026RH.65 5111C3E50 24DBZ0Z	GCCGGCT AAACCC CACCTT	AAG	4	chr10	33637006	33637026	+
59	Off-34	S4 predicted off-target sites	chr10_4393 3678_4393 3698RH.20 07BD5981 CC446Z0Z	CCTGGCT AAACCC CACCTT	AAG	2	chr10	43933678	43933698	+
60	Off-35	A2 GUIDE-seq off-target sites	chr10_6158 8826_6158 8845RH.7F 10E7BDA2 534B6Z0Z	TCAGAG ATAGTCT GGGGAA T	GGG		chr10	61588826	61588845	+
61	Off-36	S4 predicted	chr11_1034 94746_103 494766RH.	CCTGCCT AAACTCC ACCCTT	CAG	2	chr11	1.03E+08	1.03E+08	+

		off-target sites	763764F1A 65342CZ0 Z							
62	Off-37	S4 predicted off-target sites	chr11_1265 54722_126 554742RH. D382429A A973422Z 0Z	CCCAGCT AAACCC CACCTT	AAG	4	chr11	1.27E+08	1.27E+08	+
63	Off-38	S4 predicted off-target sites	chr12_3414 995_34150 15RH.65B 73EB3B14 64B2Z0Z	GCTGGCT AAACCC CACCTT	AAG	3	chr12	3414995	3415015	-
64	Off-39	S4 predicted off-target sites	chr12_2280 4544_2280 4564RH.69 58355AD6 3648CZ0Z	CCCAGCT AAACCC CACCTT	AAG	4	chr12	22804544	22804564	-
65	Off-40	A2 GUIDE-seq off-target sites	chr12_6818 7682_6818 7701RH.4F 1CD285CE 9E4E7Z0Z	ATTGAG ATAATGT GGGAA T	GGG		chr12	68187682	68187701	+
66	Off-41	S4 predicted off-target sites	chr12_9546 9409_9546 9429RH.F7 66E5612B F6483Z0Z	CCCAGCT AAACCC CACCTT	AAG	4	chr12	95469409	95469429	-
67	Off-42	A2 GUIDE-seq off-target sites	chr13_7066 5755_7066 5774RH.60 403342A00 0419Z0Z	CATGAG ATAGTAT GGAGAA T	GGG		chr13	70665755	70665774	+

68	Off-43	S4 predicted off-target sites	chr13_9535 7065_9535 7085RH.F4 860773E6E 749AZ0Z	CCTGGCT AAACCC CACCTT	AAG	2	chr13	95357065	95357085	-
69	Off-44	A2 GUIDE-seq off-target sites	chr14_9278 8849_9278 8868RH.E AC849558 F4F466Z0 Z	ATTGAG ATAGCAT GGAAAA G	GGG		chr14	92788849	92788868	-
70	Off-45	S4 predicted off-target sites	chr15_4000 4051_4000 4071RH.43 0133D0AE 1044AZ0Z	GCTGTCT CAACTTC ACCCAT	CAG	4	chr15	40004051	40004071	+
71	Off-46	S4 predicted off-target sites	chr19_3248 7190_3248 7210RH.44 95FFF0AB C342BZ0Z	CCCGGCT AAACCC CACCTT	AAG	3	chr19	32487190	32487210	+
72	Off-47	A2 GUIDE-seq off-target sites	chr20_7217 53_721772 RH.BD1C1 8FA57EC4 72Z0Z	GCTGAG ATAGTGT GGGGAA G	GTA		chr20	721753	721772	+
73	Off-48	S4 predicted off-target sites	chr20_2169 3357_2169 3377RH.B 752D266E 43D499Z0 Z	CCTGTC- AAACTCC ACCCAT	TGG	2	chr20	21693357	21693377	+
74	Off-49	A3 GUIDE-seq	chr20_4156 6328_4156 6347RH.81	GCAGTG AGATAG	AGG		chr20	41566328	41566347	+

		off-target sites	258A2CA5 71492Z0Z	TGAGGG AA						
75	Off-50	S4 predicted off-target sites	chr21_3408 9743_3408 9763RH.54 F1224D03 D343AZ0Z	TCTGCCT CAACTTC ACCCAT	CAG	4	chr21	34089743	34089763	+
76	Off-51	A2 GUIDE-seq off-target sites	chr22_2200 9255_2200 9274RH.98 CE4DA3B FF04A6Z0 Z	CTTGAGA TAGTGTG GGAGAG	GGG		chr22	22009255	22009274	+
77	Off-52	A2 GUIDE-seq off-target sites	chr22_2364 9392_2364 9411RH.F D503A07D D1A4EEZ0 Z	CTGGAG ACAGCG TGGGGA AG	GGG		chr22	23649392	23649411	+
78	Off-53	A2 GUIDE-seq off-target sites	chrX_6974 4226_6974 4245RH.A 71CD2FD4 2BB4A6Z0 Z	AATGAG ATAATGT GGGGAA A	GGG		chrX	69744226	69744245	-
79	Off-54	S4 predicted off-target sites	chrX_1197 91666_119 791686RH. D0F06BB D67B647C Z0Z	CCTC- CTAAACT CCGCCCA T	CGG	3	chrX	1.2E+08	1.2E+08	-

Table 6: Sequences of HbG locus and promoters

80	HbG locus sequence	AAAACAGTCCCTGCTCTTAAAGAGTTGTTTCCATGCAAAATACATGTCTTATGCTTTAGAATAAGATT CCCTAAGAAGTGAACCTAGCATTTATACAAGATAATTAATTCTAATCCATAGTATCTGGGTAAAGAGCA TTCTACCATCATCTTTACCGAGCATAGAAGAGCTACACCAAAACCCTGGGTCATCAGCCAGCACATA CACTTATCCAGTGATAAATACACATCATCGGGTGCCTACATACATACCTGAATATAAAAAAATACTT TTGCTGAGATGAAACAGGCGTGATTTATTTCAAATAGGTACGGATAAGTAGATATTGAAGTAAGGAT TCAGTCTTATATTATATTACATAACATTAATCTATTCCCTGCACTGAAACTGTTGCTTTATAGGATTTTT CACTACACTAATGAGAACTTAAGAGATAATGGCCTAAAACACAGAGAGTATATTCAAAGATAAGTA TAGCACTTCTTATTTGGAAACCAATGCTTACTAAATGAGACTAAGACGTGTCCCATCAAAAATCCTGG ACCTATGCCTAAAACACATTTTACAATCCCTGAACTTTTCAAAAATTGGTACATGCTTTAACTTTAAA CTACAGGCCTCACTGGAGCTACAGACAAGAAGGTGAAAAACGGCTGACAAAAGAAGTCCTGGTATC TTCTATGGTGGGAGAAGAAAAGTCTAAAGGGAAGAATAAATTAGAGAAAAATTGGAATGACTGA ATCGGAACAAGGCAAAGGCTATAAAAAAAATTAAGCAGCAGTATCCTCTTGGGGGGCCCCCTCCCCAC ACTATCTCAATGCAAATATCTGTCTGAAACGGTCCCTGGCTAAACTCCACCCATGGGTTGGCCAGCCT TGCCTTGACCAATAGCCTTGACAAGGCAAACCTTGACCAATAGTCTTAGAGTATCCAGTGAGGCCAGG GGCCGGCGGCTGGCTAGGGATGAAGAATAAAAGGAAGCACCCCTCAGCAGTTCACACACTCGCTTC TGGAACGTCTGAGGTTATCAATAAGCTCCTAGTCCAGACGCCATGGGTCAATTCACAGAGGAGGACA AGGCTACTATCACAAAGCCTGTGGGGCAAGGTGAATGTGGAAGATGCTGGAGGAGAAACCCTGGGAA GGTAGGCTCTGGTGACCAGGACAAGGGAGGGAAGGAAGGACCCTGTGCCTGGCAAAAAGTCCAGGTC GCTTCTCAGGATTTGTGGCACCTTCTGACTGTCAAACCTGTTCTTGTCATCTCACAGGCTCCTGGTTGT CTACCCATGGACCCAGAGGTTCTTTGACAGCTTTTGCAACCTGTCCTCTGCCTCTGCCATCATGGGCA ACCCCAAAGTCAAGGCACATGGCAAGAAGGTGCTGACTTTCCTTGGGAGATGCCATAAAGCACCTGGA TGATCTCAAGGGCACCTTTGCCCAGCTGAGTGAACCTGCACTGTGACAAGCTGCATGTGGATCCTGAG AACTTCAAGGTGAGTCCAGGAGATGTTTCAGCACTGTTGCCTTTAGTCTCGAGGCAACTTAGACAAC GAGTATTGATCTGAGCACAGCAGGGTGTGAGCTGTTTGAAGATACTGGGGTTGGGAGTGAAGAACT GCAGAGGACTAACTGGGCTGAGACCCAGTGGAATGTTTTAGGGCCTAAGGAGTGCCTCTGAAAATC TAGATGGACAACCTTTGACTTTGAGAAAAGAGAGGTGGAAATGAGGAAAATGACTTTTCTTTATTAGA TTTTCGGTAGAAAGAACTTTACCTTTCCCTATTTTTGTATTTCGTTTTAAACATCTATCTGGAGGCA GGACAAGTATGGTCATTAAAAAGATGCAGGCAGAAGGCATATTGGCTCAGTCAAAGTGGGGAAC TTGGTGGCCAAACATATTGCTAAGGCTATTCTATATCAGCTGGACATATAAAATGCTGCTAAT GCTTCATTACAACTTATATCCTTTAATTCAGATGGGGCAAAGTATGTCAGGGGTGAGGAACAAT TGAAACATTTGGGCTGGAGTAGATTTTGAAAGTCAGCTCTGTGTGTGTGTGTGTGTGTGTGTGCGCGCT GTGTTTGTGTGTGTGTGTGAGAGCGTGTGTTTCTTTTAAACGTTTTTCAGCCTACAGCATACAGGGTTCATGG TGGCAAGAAGATAACAAGATTTAAATTATGGCCAGTGACTAGTGCTGCAAGAAGAACAACCTACCTGC
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		ATTTAATGGGAAAGCAAAATCTCAGGCTTTGAGGGAAGTTAACATAGGCTTGATTCTGGGTGGAAGC TTGGTGTGTAGTTATCTGGAGGCCAGGCTGGAGCTCTCAGCTCACTATGGGTTCATCTTTATTGTCTCC TTTCATCTCAACAGCTCCTGGGAAATGTGCTGGTGACCGTTTTGGCAATCCATTTTCGGCAAAGAATTC ACCCCTGAGGTGCAGGCTTCCTGGCAGAAGATGGTGACTGGAGTGGCCAGTGGCCTGTCTCTCCAGAT ACCACTGAGCTCACTGCCCATGATGCAGAGCTTTCAAGGATAGGCTTTATTCTGCAAGCAATCAAATA ATAAATCTATTCTGCTAAGAGATCACACATGGTTGTCTTCAGTTCTTTTTTTATGTCTTTTTAAATATAT GAGCCACAAAGGGTTTTATGTTGAGGGATGTGTTTATGTGTATTTATACATGGCTATGTGTGTTTGTGT CATGTGCACACTCCACACTTTTTTTGTTTACGTTAGATGTGGGTTTTGATGAGCAAATAAAAGAACTAG GCAATAAAGAAACTTGTACATGGGAGTTCTGCAAGTGGGAGTAAAAGGTGCAGGAGAAATCTGGTTG GAAGAAAGACCTCTATAGGACAGGACTCCTCAGAAACAGATGTTTTGGAAGAGATGGGGAAAGGTT CAGTGAAGGGGGCTGAACCCCTTCCCTGGATTGCAGCACAGCAGCGAGGAAGGGGCTCAACGAAG AAAAAGTGTTCCAAGCTTTAGGAAGTCAAGGTTTAGGCAGGGATAGCCATTCTATTTTATTAGGGGC AATACTATTTCCAACGGCATCTGGCTTTTCTCAGCCCTTGTGAGGCTCTACAGGGAGGTTGAGGTGTT AGAGATCAGAGCAGGAAACAGGTTTTTCTTTCCACGGTAACTACAATGAAGTGATCCTTACTTTACTA AGGAACTTTTCATTTTAAGTGTTGACGCATGCCTAAAGAGGTGAAATTAATCCCATACCCTTAAGTCT ACAGACTGGTCACAGCATTTCAAGGAGGAGACCTCATTGTAAGCTTCTAGGGAGGTGGGGACTTAGG TGAAGGAAATGAGCCAGCAGAAGCTCACAAGTCAGCATCAGCGTGTCTATGTCTCAGCAGCAGAACA GCACGGTCAGATGAAAATATAGTGTGAAGAATTTGTATAACATTAATTGAGAAGGCAGATTCAGTGG AGTTCTTATATAATTGAAAGTTAATGCACGTTAATAAGCAAGAGTTTAGTTTAATGTGATGGTGTTAT GAACTTAACGCTTGTGTCTCCAGAAAATTCACATGCTGAATCCCCAACTCCCAATTGGCTCCATTTGT GGGGGAGGCTTTGGAAAAGTAATCAGGTTTAGAGGAGCTCATGAGAGCAGATCCCCATCATAGAATT ATTTTCCTCATCAGAAGCAGAGAGATTAGCCATTTCTCTTCTTCTGGTGAGGACACAGTGGGAAGTC AGCCACCTGCAACCCAGGAAGAGAGCCCTGACCAGGAACCAGCAGAAAAAGTGAGAAAAAATCCTGT TGTTGAAGTCACCCAGTCTATGCTATTTTGTATAGCACCTTGCACTAAGTAAGGCAGATGAAGAAAG AGAAAAAATAAGCTTCGGTGTTCAAGTGGATTAGAAACCATGTTTATCTCAGGTTTACAAATCTCCAC TTGTCCTCTGTGTTTCAGAATAAAATACCAACTCTACTACTCTCATCTGTAAGATGCAAATAGTAAGC CTGAGCCCTTCTGTCTAACTTTGAATTCTATTTTTTCTTCAACGTACTTTAGGCTTGTAATGTGTTTATA TACAGTGAAATGTCAAGTTCTTTCTTTATATTTCTTTCTTTCTTTTTTTCTCAGCCTCAGAGTTTCC ACATGCCCTTCCTACTTTTCAGGAACCTTTCTTCCAAACGTCTTCTGCCTGGCTCCATCAAATCATAAA GGACCCACTTCAAATGCCATCACTCACTACCATTTTCACAATTCGCACTTTCTTTCTTTGTCTTTTTTT TTTAGTAAAACAAGTTTATAAAAAAATTGAAGGAATAAATGAATGGCTACTTCATAGGCAGAGTAGAC GCAAGGGCTACTGGTTGCCGATTTTTATTGTTATTTTTCAATAGTATGCTAAACAAGGGGTAGATTAT TTATGCTGCCCATTTTTAGACCATAAAAGATAACTTCCTGATGTTGCCATGGCATTTTTTTCTTTTAA TTTTATTTCAATTCATTTTAATTTCAAGGTACATGTGCAGGATGTGCAGGCTTGTTACATGGGTAAAT GTGTGTCTTTCTGGCCTTTTAGCCATCTGTATCAATGAGCAGATATAAGCTTTACACAGGATCATGAA
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		GGATGAAAGAATTTACCAATATTATAATAATTTCAATCAACCTGATAGCTTAGGGGATAAACTAATT TGAAGATACAGCTTGCCTCCGATAAGCCAGAATTCCAGAGCTTCTGGCATTATAATCTAGCAAGGTTA GAGATCATGGATCACTTTCAGAGAAAAACAAAAACAACTAACCCAAAAGCAAAACAGAACCAAAAA ACCACCATAAATACTTCCTACCTGTTAATGGTCCAATATGTCAGAAACAGCACTGTGTTAGAAATAA AGCTGTCTAAAGTACACTAATATTCGAGTTATAATAGTGTGTGGACTATTAGTCAATAAAAAACAACCC TTGCCTCTTTAGAGTTGTTTTCCATGTACACGCACATCTTATGTCTTAGAGTAAGATTCCCTGAGAAGT GAACCTAGCATTTATACAAGATAATTAATTCTAATCCACAGTACCTGCCAAAGAACATTCTACCATCA TCTTTACTGAGCATAGAAGAGCTACGCCAAAACCCTGGGTCATCAGCCAGCACACACACTTATCCAG TGGTAAATACACATCATCTGGTGTATACATACATACCTGAATATGGAATCAAATATTTTTCTAAGATG AAACAGTCATGATTTATTTCAAATAGGTACGGATAAGTAGATATTGAGGTAAGCATTAGGTCTTATAT TATGTAACACTAATCTATTACTGCGCTGAAACTGTGGCTTTATAGAAATTGTTTTCACTGCACTATTGA GAAATTAAGAGATAATGGCAAAAGTCAAAAGAGTATATTCAAAAAGAAGTATAGCACTTTTTCTCTT AGAAACCACTGCTAACTGAAAGAGACTAAGATTTGTCCCGTCAAAAATCCTGGACCTATGCCTAAAA CACATTTCACAATCCCTGAACTTTTCAAAAATTGGTACATGCTTTAGCTTTAACTACAGGCCTCACT GGAGCTAGAGACAAGAAGGTAAAAAACGGCTGACAAAAGAAGTCCTGGTATCCTCTATGATGGGAG AAGGAAACTAGCTAAAGGGAAGAATAAATTAGAGAAAAACTGGAATGACTGAATCGGAACAAGGCA AAGGCTATAAAAAAAATTAGCAGTATCCTCTTGGGGGCCCTTCCCCACACTATCTCAATGCAAATAT CTGTCTGAAACGGTCCCTGGCTAAACTCCACCCATGGGTTGGCCAGCCTTGCCTTGACCAATAGCCTT GACAAGGCAAACTTGACCAATAGTCTTAGAGTATCCAGTGAGGCCAGGGGCCGGCGGCTGGCTAGG GATGAAGAATAAAAGGAAGCACCCCTTCAGCAGTTCCACACACTCGCTTCTGGAACGTCTGAGGTTAT CAATAAGCTCCTAGTCCAGACGCCATGGGTCATTTACAGAGGAGGACAAGGCTACTATCACAAGCC TGTGGGGCAAGGTGAATGTGGAAGATGCTGGAGGAGAAACCCTGGGAAGGTAGGCTCTGGTGACCA GGACAAGGGAGGGAAGGAAGGACCCGTGTGCCTGGCAAAAGTCCAGGTCGCTTCTCAGGATTTGTGGC ACCTTCTGACTGTCAAACGTGTTCTTGTCAATCTCACAGGCTCCTGGTTGTCTACCCATGGACCCAGAG GTTCTTTGACAGCTTTGGCAACCTGTCCTCTGCCTCTGCCATCATGGGCAACCCCAAAGTCAAGGCAC ATGGCAAGAAGGTGCTGACTTCCTTGGGAGATGCCACAAAGCACCTGGATGATCTCAAGGGCACCTT TGCCCAGCTGAGTGAAGTGCCTGTGACAAGCTGCATGTGGATCCTGAGAACTTCAAGGTGAGTCCA GGAGATGTTTCAGCCCTGTTGCCTTTAGTCTCGAGGCAACTTAGACAACGGAGTATTGATCTGAGCAC AGCAGGGTGTGAGCTGTTTGAAGATACTGGGGTTGGGGGTGAAGAACTGCAGAGGACTAACTGGG CTGAGACCCAGTGGTAATGTTTTAGGGCCTAAGGAGTGCCTCTAAAAATCTAGATGGACAATTTTGAC TTTGAGAAAAGAGAGGTGGAAATGAGGAAAATGACTTTTCTTTATTAGATTCCAGTAGAAAGAACTT TCATCTTTCCCTCATTTTTGTTGTTTTAAACATCTATCTGGAGGCAGGACAAGTATGGTCGTTAAAAA GATGCAGGCAGAAGGCATATATTGGCTCAGTCAAAGTGGGGAACCTTGGTGGCCAAACATACATTGC TAAGGCTATTCCTATATCAGCTGGACACATATAAAATGCTGCTAATGCTTCATTACAACTTATATCC TTTAATTCAGATGGGGGCAAAGTATGTCCAGGGGTGAGGAACAATTGAAACATTTGGGCTGGAGTA
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[illegible]

		TTCTATGGTGGGAGAAGAAAAGCTAGCTAAAGGGAAGAATAAATTAGAGAAAAATTGGAATGACTGA ATCGGAACAAGGCAAAGGCTATAAAAAAATTAAGCAGCAGTATCCTCTTGGGGGCCCCTTCCCCAC ACTATCTCAATGCAAATATCTGTCTGAAACGGTCCCTGGCTAAACTCCACCCATGGGTTGGCCAGCCT TGCCTTGACCAATAGCCTTGACAAGGCAAAGCTTGACCAATAGTCTTAGAGTATCCAGTGAGGCCAGG GGCCGGCGGCTGGCTAGGGATGAAGAATAAAAGGAAGCACCCCTCAGCAGTTCCAC
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CLAIMS

1. A kit comprising:
 - a) a CRISPR-Cas9 nickase;
 - b) a pair of guide RNAs (gRNA);

5 wherein the pair of guide RNAs binds to target sequences, wherein the binding will result in a disruption of binding sites of repressor LRF of a promoter of a hemoglobin gamma (HbG) locus.
- 10 2. The kit of claim 1, wherein the CRISPR-Cas9 nickase is a mutant nickase (Cas9n) comprising a D10A mutation relative to the unmutated Cas9 sequence of SEQ ID NO: 9.
3. The kit of any one of claims 1 to 2, wherein the target sequence of the guide RNA (gRNA) is located 300 nucleotides upstream or downstream relative to a LRF binding site in the promoter of the hemoglobin gamma (HbG) locus.
- 15 4. The kit of any one of claims 1 to 3, wherein the target sequence of the guide RNA (gRNA) comprises a pair of sequences selected from the group consisting of: SEQ ID NOs: 1 to 8.
- 20 5. The kit of any one of claims 1 to 4, wherein the target sequences of each pair of guide RNA (gRNA) do not overlap.
6. The kit of claim 5, wherein the target sequence of the guide RNA (gRNA) comprises a pair of sequences selected from the group consisting of: SEQ ID NO: 2 and 8, SEQ ID NO: 3 and 8, and SEQ ID NO: 4 and 8.
- 25 7. The kit of any one of claims 1 to 6, wherein the disruption of the binding sites of repressor LRF of the target sequence of the hemoglobin gamma (HbG) promoter results in the partial loss of the promoter of a hemoglobin gamma 1 (HbG1) gene and the promoter of a hemoglobin gamma 2 (HbG2) gene.
- 30 8. The kit of any one of claims 1 to 7, wherein the disruption of the binding sites of repressor LRF further results in the loss of a hemoglobin gamma 2 (HbG2) gene.
- 35 9. The kit of any one of claims 7 to 8, wherein the disruption of the binding sites of repressor LRF further results in a fusion of the promoter of the hemoglobin gamma 1 (HbG1) gene to the promoter of the hemoglobin gamma 2 (HbG2) gene.
10. The kit of any one of claims 1 to 9, wherein the disruption of the binding sites of repressor LRF induces expression of the hemoglobin gamma 1 (HbG1) gene.

11. The kit of any one of claims 1 to 10, wherein the pair of guide RNA (gRNA) binds to the target sequences in opposite directions.
- 5 12. The kit of any one of claims 1 to 11, wherein the hemoglobin gamma (HbG) locus is a mammalian or human hemoglobin gamma (HbG) locus.
13. A method for modifying a hemoglobin gamma (HbG) locus in a eukaryotic cell, the method comprising introducing into the eukaryotic cell a kit comprising:
 - 10 a) a CRISPR-Cas9 nickase;
 - b) a pair of guide RNAs (gRNA),
 wherein the pair of guide RNAs binds to target sequences, wherein the binding will result in a disruption of binding sites of repressor LRF of a promotor of a hemoglobin gamma (HbG) locus.
- 15 14. The method of claim 13, wherein the CRISPR-Cas9 nickase is a mutant nickase (Cas9n) comprising a D10A mutation relative to the unmutated Cas9 sequence of SEQ ID NO: 9.
- 20 15. The method of any one of claims 13 to 14, wherein the target sequence of the guide RNA (gRNA) is located 300 nucleotides upstream or downstream relative to a LRF binding site in the promoter of the hemoglobin gamma (HbG) locus.
- 25 16. The method of any one of claims 13 to 15, wherein the target sequence of the guide RNA (gRNA) comprises a pair of sequences selected from the group consisting of: SEQ ID NOs: 1 to 8.
17. The method of any one of claims 13 to 16, wherein the target sequences of each pair of guide RNA (gRNA) do not overlap.
- 30 18. The method of claim 17, wherein the target sequence of the guide RNA (gRNA) comprises a pair of sequences selected from the group consisting of: SEQ ID NO: 2 and 8, SEQ ID NO: 3 and 8, and SEQ ID NO: 4 and 8.
- 35 19. The method of claim 13 to 18, wherein the disruption of the binding sites of repressor LRF results in the partial loss of the promoter of a hemoglobin gamma 1 (HbG1) gene and the promoter of a hemoglobin gamma 2 (HbG2) gene.

20. The method of any one of claims 13 to 19, wherein the disruption of the binding sites of repressor LRF further results in the loss of a hemoglobin gamma 2 (HbG2) gene.
- 5 21. The method of any one of claims 19 to 20, wherein the disruption of the binding sites of repressor LRF further results in a fusion of the promoter of the hemoglobin gamma 1 (HbG1) gene to the promoter of the hemoglobin gamma 2 (HbG2) gene.
22. The method of any one of claims 13 to 21, wherein the disruption of the binding sites of repressor LRF induces expression of the hemoglobin gamma 1 (HbG1) gene.
- 10 23. The method of any one of claims 13 to 22, wherein the pair of guide RNA (gRNA) binds to the target sequences in opposite directions.
- 15 24. The method of any one of claims 13 to 22, wherein the hemoglobin gamma (HbG) locus is a mammalian or human hemoglobin gamma (HbG) locus.
25. The method of any one of claims 13 to 24, wherein the eukaryotic cell is a hemotopoietic stem cell.
- 20 26. The method of claim 25, wherein the hemotopoietic stem cell is a CD34+ or a CD133+ cell.
27. A composition comprising the kit of any one of claims 1 to 12.
- 25 28. A method of treating β -hemoglobinopathy in a subject, wherein the method comprises administering the composition of claim 27 to a subject with a β -hemoglobinopathy.
29. The method of claim 28, wherein the administration induces expression of the hemoglobin gamma 1 (HbG1) gene.
- 30 30. The method of any one of claims 28 to 29 wherein the administration increases γ - globin expressing cells.
31. The method of any one of claims 28 to 30, wherein the β -hemoglobinopathy is selected from the group consisting of: β -thalassemia, and sickle cell disease.
- 35 32. A gene therapy for preventing β -hemoglobinopathy in a subject, wherein the method comprises administering the composition of claim 27 to a subject susceptible to β -hemoglobinopathy.

33. The gene therapy of claim 32, wherein the administration induces expression of the hemoglobin gamma 1 (HbG1) gene.
- 5 34. The gene therapy of any one of claims 32 to 33, wherein the administration increases γ - globin expressing cells.
35. The method of any one of claims 33 to 34, wherein the β -hemoglobinopathy is selected from the group consisting of: β -thalassemia, and sickle cell disease.