

STRATEGIES FOR PRECISION EDITING OF HOMOLOGOUS REGIONS

FIELD OF INVENTION

The invention generally relates to genetic engineering for therapeutic benefits. More particularly, the invention relates to methods for precision
5 editing of highly homologous regions without causing double strand breakage.

BACKGROUND

An array of mutation occurs in the coding region of the genes leading to several hereditary disorders. Certain conditions can be treated with genetic
10 engineering techniques such as CRISPR Cas9. However, since these techniques rely on DNA strand breakage, they may result in large deletions, especially in homologous regions in some cases causing off-target edits. Hence, effective gene editing approaches that offer high editing efficiency in the target region without nicking the DNA preventing a
15 double stand breakage or deletion of homologous region is crucial.

In one such known approach, gene editing involves base editing or amino acid substitution for creating precise mutations without double strand break in the DNA. The technique is gaining importance as a gene therapy tool because of its versatility and ease of use. However, while editing the globin
20 locus with base editors, there is occurrence of large deletion. The sequence homology between A-gamma and G-gamma genes results in deletion of intervening region between the two gRNA binding sites along with and simultaneous DNA nicking. The said approach exhibits low editing efficiency and deletion of homologous regions. Therefore, there is need for
25 a gene editing approach that can mediate base substitution or mutations in the homologous regions without DNA nicking, and deletion of homologous regions.

SUMMARY

The invention provides a gene editing strategy to overcome genetic disorders resulting due to genetic mutations in the coding regions of the genes having highly homologous regions.

- 5 One aspect of the invention discloses a method for precision editing of a highly homologous region. The method includes identification of a region of interest for precision editing of the homologous regions. A vector construct for expression of a fusion protein specific to the region of interest is obtained. The fusion protein is a gene construct having a sequence for
10 expressing deadCas9 nuclease and a ABE8e base editor for editing at the identified region of interest. One or more vector constructs are prepared for expression of guide RNA complementary to the region of interest. The gRNA vector constructs along with the expression vector for the fusion protein is transformed into a suitable cell line for precision editing of the
15 highly homologous regions.

- Another aspect of the invention relates to a method for precision editing of highly homologous regions. The method includes obtaining one or more truncated guide RNA (deadgRNA/ dgRNA) specific to a region of interest. One or more vector constructs are obtained for carrying a fusion complex
20 specifically generated for binding at the region of interest. Each fusion complex includes a dgRNA, a Cas9 nickase and a deaminase for binding at the region of interest. The generated vector construct is transduced into a suitable cell line for precision editing of the homologous regions.

- The methods disclosed herein prevents double strand breakage, off-target
25 edits and reduced Insertion–deletion mutations (InDels) while creating point mutations. The method averts deletion of intervening regions while nicking at homologous target regions.

BRIEF DESCRIPTION OF DRAWINGS

So that the manner in which the recited features of the invention can be understood in detail, some of the embodiments are illustrated in the appended drawings. It is to be noted, however, that the appended drawings
5 illustrate only typical embodiments of the invention and are therefore not to be considered limiting of its scope, for the invention may admit to other equally effective embodiments.

FIG. 1a shows the deletion frequency among gRNA targeting different regions in the gamma globin promoter, according to an embodiment of the
10 invention.

Fig. 1b shows the deletion frequency of different base editor with gRNA 2, according to an embodiment of the invention.

FIG. 1c shows the comparison of editing efficiency of base editors with different gRNAs, according to an embodiment of the invention.

15 FIG. 1d shows the editing efficiency of gRNA 2, gRNA 3 and gRNA 11, according to an embodiment of the invention.

FIG. 2a shows the deletion frequency with different truncated gRNAs using the base editor nCas9, according to an example of the invention.

FIG. 2b shows the deletion frequency with different truncated gRNAs using
20 the base editor dCas9, according to an example of the invention.

FIG. 2c shows the deletion frequency with different truncated gRNAs using the base editor ABE8e, according to an example of the invention.

FIG. 2d shows the deletion frequency with different truncated gRNAs using the base editor dCas9 ABE8e, according to an example of the invention.

25 FIG. 3 shows the editing efficiency of the different truncated gRNA targeting at the target gene sequence, according to another embodiment of the invention.

FIG. 4 shows the base editing efficiency of dead gRNA, according to another embodiment of the invention.

FIG. 5 shows the off-target editing at HBD gene with different truncated gRNA, according to another embodiment of the invention.

- 5 FIG. 6 shows the gene editing at off-target sites, according to an example of the invention.

FIG. 7A shows the gene editing efficiency of nCas9ABE8e and dCasABE8e in HBB and HBD regions, according to an embodiment of the invention.

- 10 FIG. 7B shows the comparison of unedited cells, cells transformed with nABE8e and dABE8e for demonstrating fold change in the fusion protein and deletions in HBB and HBD region, according to an embodiment of the invention.

- 15 FIG. 7C shows the comparison of results of nCas9ABE8e and dCas9 ABE8e with nCas9 ABE8e generating a band corresponding to large deletions, according to an embodiment of the invention.

FIG. 7D shows the editing efficiency of dABE8e and nABE8e, before differentiation and after differentiation along with the identification of InDels, according to an embodiment of the invention.

- 20 FIG. 7E shows the elevation in the HbF+ve cells, according to an embodiment of the invention.

FIG. 7F shows the comparison of frequency of large deletion by nABE8e and dABE8e measured by qRT PCR, according to an embodiment of the invention.

- 25 FIG. 7G shows the comparison of frequency of large deletion by nABE8e and dABE8e measured by ddPCR, according to an embodiment of the invention.

FIG. 7H shows the generation of large deletions by nABE8e and dABE8e evaluated by PCR, according to an embodiment of the invention.

FIG. 8A shows the editing percentage of the nABE8e and dABE8e in CD90+ve cells, according to an embodiment of the invention.

- 5 FIG. 8B shows the effect of editing percentage of the nABE8e and dABE8e in CD90+ve cells by increasing base editor cargo, according to an embodiment of the invention.

FIG. 8C shows the editing percentage of the nABE8e and dABE8e in HBB region, according to an embodiment of the invention.

- 10 FIG. 8D shows the editing percentage of the nABE8e and dABE8e in HBG region, according to an embodiment of the invention.

FIG. 8E shows the results of the clonogenic assay for samples including cells transformed with nABE8e, dABE8e and unedited cells, according to an embodiment of the invention.

- 15 FIG. 8F shows the levels of DNA damage markers, P21 and GADD45 in cells transformed with nABE8e and dABE8e, according to an embodiment of the invention.

DETAILED DESCRIPTION OF THE INVENTION

- 20 The definitions, terms and terminology adopted in the disclosure have their usual meaning and interpretations, unless otherwise specified.

The term “**Cas9**” is a bacterial RNA-guided endonuclease that uses base pairing to recognize and cleave target DNAs complementary to the guide RNA.

- 25 The term “**deadCas9 or dCas9**” is a mutant form of Cas9 whose endonuclease activity is removed through point mutations in its endonuclease domains.

The term “**Guide RNA (gRNA)**” refers to a piece of RNA that functions as a guide for RNA- or DNA-targeting enzymes, with which it forms complexes to delete, insert or otherwise alter the targeted RNA or DNA.

The term “**dead Guide RNA (dgRNA)**” refers to a truncated gRNA having
5 a nucleotide length ranging between 10-15 base pairs.

The invention provides gene editing strategies which prevent deletion of homologous regions in a gene sequence and double strand breakage of the gene sequence.

Various embodiments of the invention provide a method for gene editing.
10 The method involves creating a deadCas 9 (dCas 9). The protein is capable of binding to the region of interest for gene modification, however, does not cause double strand breakage in the DNA. The obtained deadCas9 is fused with a base editor for expression as a fusion protein. In one example of the invention, the base editor is an adenosine base editor,
15 particularly, ABE8e. The fusion protein is transformed into a suitable cell line for gene editing. In one example of the invention, the cell lines are mostly cells carrying a genetic mutation, and a fusion protein is used to correct the genetic mutation or edit the genes to ameliorate various genetic disorders. The transformed cells are over expressed and verified to confirm
20 successful gene editing for rectifying the genetic mutation. The method described herein shall be described in detail below.

A catalytically dead Cas9 nuclease capable of binding to the precise region of interest is generated, however, unlike the Cas9 nuclease, they do not cause double strand breakage. In one example, a Cas9 D10A is used,
25 which nicks only a non-edited DNA strand, and does not cause double strand breakage. Subsequently, one or more base editors are screened to introduce point mutations in the target region. In one example, ABE8e, adenosine deaminase is used as the base editor to create point mutation. Later, the screened base editor, ABE8e is fused with the deadCas9. The
30 fusion protein is effective in base editing without causing double stand

breakage and prevents deletion in homologous regions, while retaining the editing efficiency. The method thus ensures a safe and a reliable approach for gene editing. In one example, the fusion protein of dCas9 and ABE8e is obtained by introducing a H840A mutation in HNH-like nuclease domain of the Cas9 nuclease. The obtained gene construct is transformed into an ABE8e vector to obtain a Lentiviral construct. The construction of the fusion protein described herein shall be described in detail below.

The vector carrying the fusion protein is constructed by introducing the base editor sequence of adenosine deaminase into a suitable vector. The vector is selected from a listing comprising Lenti viral vector and Adeno Associated Virus (AAV) vector. In one example, a lentiviral vector is used for creating base editor constructs. An Abe8e vector construct is obtained by inserting ABE8e sequence into the lentiviral vector. In one example, a pLenti-ABE8e-puro vector is obtained by inserting the ABE8e sequence in the lentiviral vector. Similarly, the lentiviral vector carrying the fusion protein (dCas9 and ABE8e) is obtained by introducing H840A mutation in the Cas9 domain of pLenti-ABE8e-puro vector. Subsequently, plurality of gRNAs complementary to the target region are designed. Each of the gRNAs are transformed into a vector to obtain a plurality of vector constructs carrying the gRNAs. The vectors for creating the gRNA constructs are selected from a list comprising Lenti viral vector, u6 vector.

In one example, the gRNAs are constructed in a pLKO5 vector to obtain a gRNA vector construct specific to the promoter region of hemoglobin subunit gamma 1 and hemoglobin subunit gamma 2 (HBG1 and HBG2). The vector constructs carrying the gene sequence for deadCas9 ABE8e along with a plurality of vector constructs for each of the gRNAs are transformed into a suitable cell. The cells are cultured in a suitable media for expansion and over expressions of the vector in the cell lines. In one example, the pLenti-dCas9-ABE8e-puro vector is transformed into a suitable cell line. The cell lines are selected from a list comprising of limited to HUDEP-2 cells, K562 cells, HEL cells, HEK cells, HL60 cells, Jurkat

cells. In one example the obtained vector construct is transduced in HUDEP-2 cell line along with the vector carrying the gRNA for target identification and gene editing in the transformed cell line. The edited cells are cultured in a suitable media and over expressed. The obtained edited
5 cells are analyzed to identify the rectification of the genetic mutation.

In one example, plurality of gRNA constructs selected from a group comprising guide RNAs having a SEQ ID. No. gRNA2, SEQ ID No. gRNA3 and SEQ ID No. gRNA11, specifically targeting the gamma globin locus G2, G3 and G11 respectively are obtained. The gRNAs are complementary
10 to the promoter region of HBG1 and HBG2. Initially the gRNAs are evaluated to identify relationship between the deletion frequency and the role of the base editor. The guide RNA sequence used in the study is listed in the table below:

Table 1:

Sl. No.	Sequence ID No.	Sequence
1	gRNA 2	CTTGACCAATAGCCTTGACA
2	gRNA 3	ATATTTGCATTGAGATAGTG
3	gRNA 11	GGCAAGGCTGGCCAACCCAT

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FIG. 1a shows the deletion frequency among gRNA targeting different regions in the gamma globin promoter, according to an embodiment of the invention. The ABE7.10 base editor is used along with each of the guide RNAs. The gRNA 2, gRNA 3 and gRNA 11 and transduced into HUDEP-2
20 cell lines for evaluating the deletion frequency of the three gRNAs. gRNA 3 (specific to G3 locus) exhibits less editing compared to gRNA 2 (specific to G2 locus), and gRNA 11 (specific to G11 locus). FIG. 1b shows the deletion frequency of different base editor with gRNA 2, according to an embodiment of the invention. The efficiency of adenosine base editors and
25 cytosine base editor is evaluated. The fold change in the 4.9 kb intergenic region compared to locus control with both adenosine base editor (ABE7.10) and cytosine base editor (CBE) is evaluated. It is observed that even with the use of same gRNA, the deletion frequency varies between

the base editors and the editing efficiency is inversely correlating with the deletion frequency.

Further gRNA 3 and gRNA 11 are used to identify the deletion frequency of both ABE7.10 and ABE8e. FIG. 1c shows the comparison of editing efficiency of base editors with different gRNAs, according to an example of the invention. The base editor ABE8e and ABE7.10 are both used to compare the effect of base editor with respect to the guide RNA sequences complementary to the HBG1 and HBG2, specifically gRNA 3 and gRNA 11. gRNA 11 (G11) exhibited very few deletions with both the base editors.

Based on the above study the gRNAs are further evaluated to assess the variation in 4.9kb deletion among different gRNAs irrespective of the base editors. In one example, the gRNAs are transduced as Lenti-virus to D10A nickase HUDEP-2 stable cells without any fusion of the base editor to evaluate the genomic alterations and the functional consequences. FIG. 1d shows the GC content of gRNA 2, gRNA 3 and gRNA 11, according to an example of the invention. The evaluation of the GC content does not correlate with the deletion frequency. The gRNA 2, 3, and 11 are transduced with more than 90% efficiency. The use of D10A nickase Cas9 did not exhibit base conversions or InDels except in G11, which exhibited a small percentage of InDels suggesting that nicking by itself doesn't cause generation of InDels.

In one example, reactivation of fetal hemoglobin expression to compensate the lack of functional beta globin to ameliorate the beta hemoglobinopathies is studied by using the gene editing method described herein above. The fusion protein deadCas9 and ABE8e, along with one or more guide RNA specific to the HBG1 and HBG2 genes are used for base editing. However, to validate the effective gene editing approach and to identify the frequency of large deletion in the promoter region of HBG1 and HBG2 genes, different base editors are used in the study. The promoter region of the HBG1 and HBG2 genes are edited using dCas9, nCas9, ABE8e and dCas9-ABE8e. FIGs. 2a-2d shows the deletion frequency with

different variants of base editors, according to an example of the invention. The panel (a) of FIG. 2 displays the fold change in 4.9kb intergenic region compared to locus control to identify the deletion frequency with various gRNAs using the base editor nCas9. A simple nicking is sufficient to generate large deletion in the homologous regions. The panel (b) of FIG. 2 displays the fold change in 4.9kb intergenic region compared to locus control to identify the deletion frequency with various gRNAs using the base editor dCas9. The cells transformed with nuclease deficient dCas9 exhibits prevention of large deletions in the homologous regions. The panel (c) of FIG. 2 displays the fold change in 4.9kb intergenic region compared to locus control to identify the deletion frequency with various gRNAs using the base editor ABE8e. The study determined that the hyperactive variant ABE8e increases editing efficiency and reduces large deletions to a greater extent. The panel (d) of FIG. 2 displays the fold change in 4.9kb intergenic region compared to locus control to identify the deletion frequency with various gRNAs using the base editor dCas9-ABE8e. The study establishes that the use of nuclease deficient version of ABE8e (dCas9 ABE8e) can evade large deletion and give sufficient editing to elevate fetal hemoglobin expression to a therapeutically significant level. The use of nickase deficient variants of base editor can abolish large deletions in the homologous gamma globin region.

In one example, the editing efficiency of the base editor dCas9-ABE8e are evaluated in human CD34+ Hematopoietic Stem and Progenitor Cells (HSPCs). The objective of the study is to determine if the base editor dCas9 ABE8e prevents large deletions or interstitial deletions in HSPCs during base editing in the homologous regions of gamma globin. Editing efficiency is first evaluated in CD34+ HSPCs in a therapeutically relevant Hemoglobin subunit beta (HBB) gene using gRNAs targeting exon 1 with and without homology to hemoglobin subunit delta (HBD) gene. The cells are initially nucleofected with nCas9 ABE8e mRNA and dCas9 ABE8e mRNA and guide RNA (sgRNA) complementary to the exon 1 regions.

Editing efficiencies are evaluated in both HBB and HBD regions. The editing efficiency is found to be slightly reduced while targeting with dCas9 ABE8e (dABE8e) compared to cells targeted with nCas9 ABEe (nABE8e) in both HBB and HBD regions, as shown in panel A of FIG. 7. The base conversion in HBD and large deletions with nABE8e is observed only in Hemoglobin subunit beta 1 (HBB1) gRNA having binding sites at Hemoglobin subunit beta (HBB) and Hemoglobin subunit gamma (HBG) gene and not with Hemoglobin subunit beta 2 (HBB2) having a binding site only in HBB. The samples for the study are obtained from Healthy HSPC donors. Donor 1, 2, 3 indicates HSPCs obtained from Healthy donor samples. The fold change in fusion genes determines deletions in homologous regions. Real-Time Quantitative Reverse Transcription PCR (qRT PCR) with primers specific to large deletion between HBD and HBB showed that ABE8e shows a 10-fold increase in amplicons with large deletion compared to unedited control and dABE8e edited samples as shown in panel B of FIG. 7. Large deletions are absent even in cells exhibiting high editing at HBB and HBG genes using HBB1 specific gRNA as shown in panel C of FIG. 7. Generation of large deletion causes the formation of a smaller size fusion gene that can be picked up by the PCR. A further comparison of both the editors, nABE8e and dABE8e in HBG promoter reveals that the editing efficiently is above 90% for the base editor nABE8e. The editing is observed in A4 and A5 base position within the gRNA. However, the editing efficiency of dABE8e is found to be slightly lower compared to ABE8e as shown in Panel D of FIG. 7. The elevation of HbF+ve cells are identified in differentiated cells by comparing with percentage increase in unedited cells and a control sample using a AAVS1 integrated with nABE8e and dABE8e. The edited cell upon differentiation shows similar elevation in HbF reaching close to 80% in both ABE8e and dABE8e integrated with gRNA 11, suggesting that editing by dABE8e is sufficient for therapeutically relevant HbF+ve cells elevation as shown in panel E of FIG. 7. AAVS1 indicates sgRNA targeting AAVS1 locus while

G11 indicated sgRNA targeting gamma globin promoter. Further, it is identified that large deletions are observed in cells edited with nABE8e, but deletions are not observed in cells edited with dABE8e. The 4.9kb deletion is observed with the base editor ABE8e evaluated by gRT PCR as shown in panel F of FIG. 7. The comparison of the results for 4.9kb deletion in cells edited by ABE8e and nABE8e are evaluated by ddPCR as shown in panel G of FIG. 7. The 4.9kb deletion is observed in samples edited with the base editor ABE8e, however deletions are absent in unedited sample and dABE8e evaluated by PCR as shown in panel H of FIG. 7.

Further, the dABE8e activity in the HSPCs are characterized for identifying the extent of editing efficiency and reduction in InDel formation for therapeutic applications. The editing efficiency of dABE8e is observed to be slightly reduced compared to nABE8e in the homologous regions. A further evaluation is performed to identify if the limited editing efficiency of dABE8e is more in primitive quiescent cells. The editing efficiency of nABE8e and dABE8e is compared in CD90+ve primitive HSPCs with bulk edited cells. No significant difference is observed as shown in base position sites A4 and A5 in panel A of FIG. 8. Subsequently, a further analysis is conducted to identify improvement in the editing efficiency in the homologous regions by increasing a base editor cargo. Moderate editing efficiency is observed, however, no improvement in editing percentage despite a fourfold increase in the cargo is noticed, thereby suggesting that reagent availability is not the limiting factor for editing at the homologous region as shown in panel B of FIG. 8.

The base editors nABE8e and dABE8e start editing within three hours of nucleofection, however, the editing kinetics is identified to be much faster in case of nABE8e for editing in HBB region (panel C of FIG. 8) and the editing in HBG region (panel D of FIG. 8). A clonogenic assay is performed to identify the colony formation potential between sample of nABE8e cells, DABE8e cells and Unedited cells (UE). The edited cells are subjected to clonogenic assay and no difference in colony forming potential between

- each of the samples is noticed as shown in panel E of FIG. 8. Further, markers of DNA damage P21 and GADD45 levels are determined for percentage of deletions. The levels of P21 and GADD45, markers of DNA damage response in each of the cells nucleofected with nABE8e and dABE8e. nABE8e showed slight elevation in RNA levels, dABE8e and mock electroporated sample showed similar levels as shown in panel F of FIG. 8. Thus, dABE8e exhibits to be a better approach in terms of purity of outcomes for editing regions that are highly homologous and during multiplexed editing for therapeutic applications.
- 10 According to one other embodiment of the invention, the invention provides a gene editing strategy. The strategy involves a method for base editing at the homologous regions in a target gene sequence and prevention of large deletions while off-target base conversion. Additionally, the invention prevents editing of unintended nucleotides. The gene editing strategy
- 15 involves obtaining one or more truncated guide RNA (deadgRNA) specific to a region of interest. Subsequently, obtaining a vector construct for carrying a fusion complex capable of binding at the region of interest. The vector for carrying the fusion complex is selected from a list comprising Lenti viral vector and Adeno Associated Virus (AAV) vector. In one
- 20 example, the vector construct is a Lenti viral vector carrying a fusion complex comprising, truncated or dead gRNA construct, a Cas9 nickase and a deaminase. The obtained vector construct is transformed into a suitable cell line. The cell lines are selected from a list comprising of limited to HUDEP-2 cells, K562 cells, HEL cells, HEK cells, HL60 cells, Jurkat
- 25 cells. In one example the obtained vector construct is transformed in HUDEP-2 cells for over expression and evaluating the editing efficiency. The truncated gRNAs are delivered as Lentivirus into the cells for over expressing the base editor. The editing efficiency is evaluated after eight days using Sanger sequencing.
- 30 In one example, the editing efficiency of the truncated gRNA of varying lengths is evaluated. The truncated gRNA for the study includes a dgRNA

2, with a varying length between 20 nucleotides to 10 nucleotides. The 20 nucleotides dgRNA 2 is represented by a Sequence ID. No. dgRNA2.20 (G2.20), the 15 nucleotides dgRNA 2 is represented by a Sequence ID. No. dgRNA 2.15 (G2.15), the 14 nucleotides dgRNA 2 is represented by a sequence ID. No. dgRNA 2.14 (G2.14), the 13 nucleotides dgRNA 2 is represented by a Sequence ID. No. dgRNA 2.13 (G2.13), the 12 nucleotides dgRNA 2 is represented by a Sequence ID. No. dgRNA 2.12 (G2.12), the 11 nucleotides dgRNA 2 is represented by a Sequence ID. No. dgRNA 2.11 (G2.11), 10 nucleotides of dgRNA 2 is represented by a Sequence ID. No. dgRNA 2.10 (G2.10). The guide RNA sequences used for the study are provided in the table below:

Table 2:

Sl. No.	Sequence ID No.	Sequence
1	dgRNA 2.20	CTTGACCAATAGCCTTGACA
2	dgRNA 2.10	AGCCTTGACA
3	dgRNA 2.11	TAGCCTTGACA
4	dgRNA 2.12	ATAGCCTTGACA
5	dgRNA 2.13	AATAGCCTTGACA
6	dgRNA 2.14	CAATAGCCTTGACA
7	dgRNA 2.15	CCAATAGCCTTGACA
8	HBB dsgrNA.20	TTGGTGGTGAGGCCCTGGGC
9	HBB dsgrNA.10	GGCCCTGGGC
10	HBB dsgrNA.11	AGGCCCTGGGC
11	HBB dsgrNA.12	GAGGCCCTGGGC
12	HBB dsgrNA.13	TGAGGCCCTGGGC
13	HBB dsgrNA.14	GTGAGGCCCTGGGC
14	HBB dsgrNA.15	GGTGAGGCCCTGGGC

Fig. 3 shows the editing efficiency of the different truncated gRNA targeting at the target gene sequence, according to an embodiment of the invention. The gRNA 2, of varying length of nucleotides between 20-10 are used in the study. The guide RNA 2 having a length of 20 nucleotides (G2.20) is used as control. The gRNAs of length less than or equal to 14 nucleotides did not show any significant editing, while gRNA of 15 nucleotides (G2.15)

exhibits editing although with lesser efficiency. A5, A8, A9 and A11 denote base positions within the gRNA that shows the possibility of base editing.

The truncated gRNA are further studies to evaluate the base editing efficiency in the beta globin gene for correcting HBE mutation. Fig. 4 shows the base editing efficiency of dead gRNA, according to another embodiment of the invention. The HBB guide RNA having a Sequence ID No. HBB dsgrNA.20, and its truncated versions are used in the study. The sequence of the same are provided in table 2. HBB dsgrNA of 14 nucleotides length (HBB dsgrNA 14) is effective in base editing at the target, however the editing efficiency is reduced to half compared to the editing efficiency of gRNA of 20 nucleotides length (HBB dsgrNA 20).

Subsequently, the dead gRNAs are evaluated to assess the editing efficiency at HBD (Hemoglobin Subunit delta) and HBB (Hemoglobin Subunit Beta) regions. The HbE gRNA has a homology at HBD gene except a single nucleotide mismatch. Many base editing approaches have resulted in deletion of the HBD/HBB intergenic region and cause off target editing at HBD. The gRNA also shows bystander editing at the adjacent nucleotide similar to the intended nucleotide. In one example, the truncated gRNAs, didn't exhibit deletion at the HBD gene. Thereby, elucidating that the truncated gRNAs are target specific and does not cause off-target base conversion. Fig. 5 shows the off-target editing at HBD gene with different truncated gRNA, according to another embodiment of the invention. The HBB guide RNA having a Sequence ID No. HBB dsgrNA.20, and its truncated versions are used in the study. The sequence of the same are provided in table 2. The truncated gRNAs with nucleotides ranging between 10 to 15 did not exhibit editing at the HBD gene. Thus, confirming the target specificity of the truncated gRNA.

The study is further extended to identify the gene editing in off-target region at other genomic sites (such as Chr8 -GDF6 gene, Chr 5 SALC25A gene etc.), specifically with truncated gRNA having a length of 14 nucleotides, 15 nucleotides and 20 nucleotides. Fig. 6 shows the gene editing at off-target

sites, according to another example of the invention. The HBB guide RNA has a Sequence ID No. HBB dsgrNA 20, and its truncated versions having a Sequence ID. No. HBB dsgrNA 14, and Sequence ID. No. HBB dsgrNA 15 are used for the analysis. The sequence of the same are provided in
5 table 2. The analysis revealed that a substantial reduction is observed in off-target editing at other genomic sites (such as Chr8 -GDF6 gene, Chr 5 SALC25A gene etc.) when edited with truncated gRNAs. Thus, providing an effective strategy for preventing off-target base conversions.

The present invention provides a method for effecting gene editing. The
10 gene editing approach prevents double strand breakage of the DNA. The method also ensures the prevention of deletion of large homologous regions while gene editing as well as off-target base conversion and by stander editing. The invention provides an effective strategy by designing a fusion protein of dead Cas9-ABE8e or fusion of deaminase (Cytosine
15 deaminase/ Adenosine deaminase) with other programmable nucleases with inactivated nuclease domain in the form of DNA or mRNA or protein along with gRNA.

The invention also provides another strategy for gene editing by using a fusion complex which includes a truncated gRNA (deadgRNA or dgRNA) of
20 varying lengths along with base editors in the form of DNA or mRNA or protein.

The fusion protein or the fusion complex is delivered by adopting non-viral or viral based systems. The delivery systems include but are not limited to electroporation, Lenti viral vectors, AAV vector, or lipid-based delivery for
25 the alteration of target gene sequence for gene therapy and other applications.

The foregoing description of the invention has been set for merely to illustrate the invention and is not intended to be limiting. Since modifications of the disclosed embodiments incorporating the scope and
30 substance of the invention may occur to person skilled in the art, the

invention should be construed to include everything within the scope of the appended claims and equivalents thereof.

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WE CLAIM:

1. A method for precision editing of a highly homologous regions, the method comprising of:
 - identifying a region of interest;
 - 5 creating one or more vector constructs complementary to the region of interest;
 - expressing the vector constructs to obtain at least one of a guide RNA, a deadCas9 nickase, and an ABE8e base editor.
 - transforming each of the expressed vectors into a suitable cell
 - 10 line for precision editing of the highly homologous regions of a gamma globin;
 - wherein the method prevents double strand breakage, off-target edits and reduced InDels while creating point mutations.
- 15 2. The method as claimed in claim 1, wherein the region of interest is selected from at least one of a gamma globin locus of HBG2(HBD) and HBG1(HBB) genes.
3. The method as claimed in claim 1, wherein the vector construct for creating gene constructs is selected from a list comprising of
- 20 a Lenti viral vector, an adeno associated viral vector, an u6 vector.
4. The method as claimed in claim 1, wherein the suitable cell line is at least one of a HUDEP-2 cell, a K562 cell, a HEL cell, a HEK cell, a HL60, a Jurkat cell.
- 25 5. The method as claimed in claim 1, wherein the highly homologous regions include but not limited to a promoter region of hemoglobin subunit gamma 1 (HBG1) and hemoglobin subunit gamma 2 (HBG2).

6. A method for precision editing of highly homologous regions, the method comprising of:
- identifying a region of interest;
- creating one or more vector constructs complementary to the region of interest;
- expressing the vector constructs to obtain at least one of a truncated guideRNA(dead gRNA), a Cas9 nickase, and a deaminase;
- transforming the expressed vector constructs in a suitable cell line for precision editing of the homologous regions of a gamma globin;
- wherein the method prevents double strand breakage, off-target edits and reduced InDels while creating point mutations.
7. The method as claimed in claim 6, wherein the region of interest is selected from at least one of a gamma globin locus of HBG2 (Hemoglobin subunit delta/HBD) and gamma globin locus of HBG1 (Hemoglobin subunit beta/HBB) genes.
8. The method as claimed in claim 6, wherein the dgRNA are truncated gRNA having a base pair in the range of 10 nucleotides to 15 nucleotides.
9. The method as claimed in claim 6, wherein the vector is selected from a listing comprising of Lenti viral vector and Adeno Associated Virus (AAV) vector.
10. The method as claimed in claim 6, wherein the suitable cell line is at least one of a HUDEP-2 cell, a K562 cell, a HEL cell, a HEK cell, a HL60, and a Jurkat cell.
11. The method as claimed in claim 6, wherein the highly homologous regions include but are not limited to a promoter

region of hemoglobin subunit gamma 1 (HBG1) and hemoglobin subunit gamma 2 (HBG2).

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