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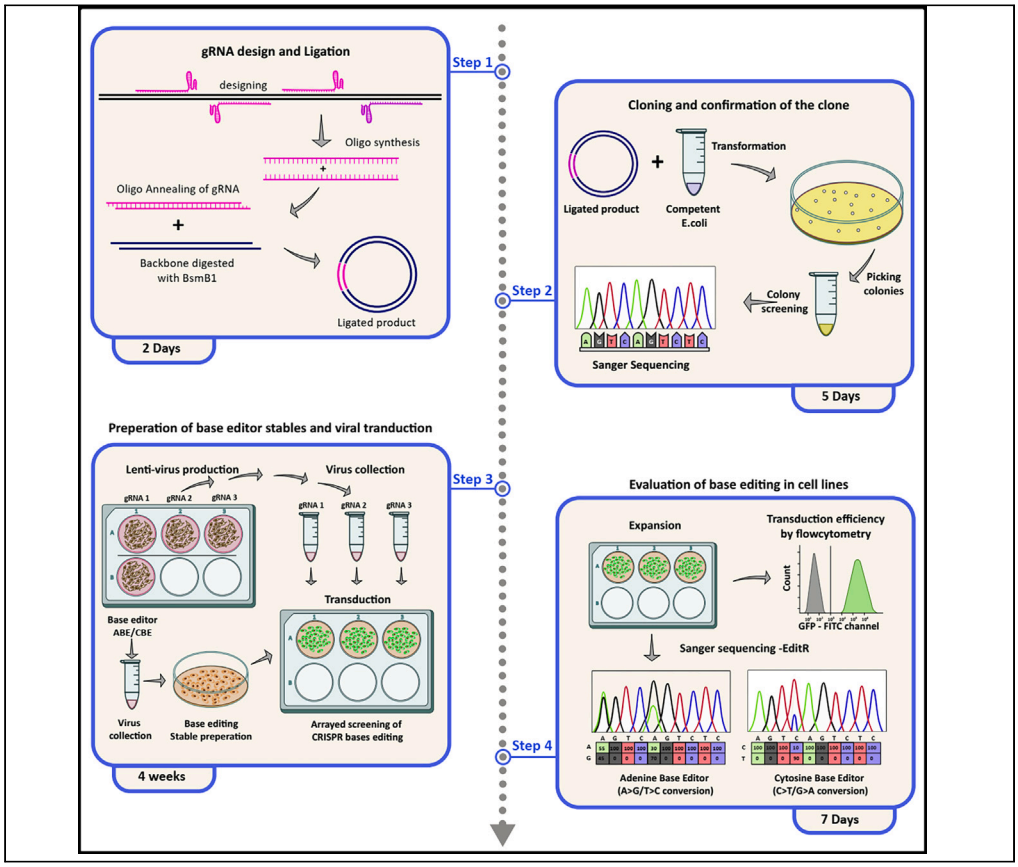
Protocol

Protocol for arrayed gRNA screening by base editors in mammalian cell lines using lentiviral system



Base editing, a CRISPR-based genome engineering technique, enables precise single-nucleotide modifications while minimizing double-strand breaks. Here, we present a protocol for arrayed mutagenesis using base editors to identify regulatory elements within the gamma-globin locus. We describe steps for guide RNA (gRNA) cloning into lentiviral vectors, establishing stable cell lines with base editor expression, transducing gRNAs, and assessing editing efficiency. This protocol can be applied to diverse genomic regions and cell lines for arrayed screening, facilitating genetic research, and target discovery.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Highlights

CRISPR-based base editing for precise single-nucleotide modification

Arrayed mutagenesis using base editors can identify regulatory elements

Protocol for genomic arrayed screening for cell lines at any locus

Instructions on gRNA design, stable preparation, and evaluating gene editing

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Protocol for arrayed gRNA screening by base editors in mammalian cell lines using lentiviral system

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SUMMARY

Base editing, a CRISPR-based genome engineering technique, enables precise single-nucleotide modifications while minimizing double-strand breaks. Here, we present a protocol for arrayed mutagenesis using base editors to identify regulatory elements within the gamma-globin locus. We describe steps for guide RNA (gRNA) cloning into lentiviral vectors, establishing stable cell lines with base editor expression, transducing gRNAs, and assessing editing efficiency. This protocol can be applied to diverse genomic regions and cell lines for arrayed screening, facilitating genetic research, and target discovery.

For complete details on the use and execution of this protocol, please refer to Ravi et al. (2022)[1]

BEFORE YOU BEGIN

This protocol delineates the procedure for conducting arrayed mutagenesis within the specific genomic loci using adenine and cytosine base editing techniques. Specifically, we focused on screening a 300 bp region upstream of the transcription start site at the gamma globin promoter in human erythroid cell line. The objective was to identify targets that could enhance fetal hemoglobin expression which will offer potential therapeutic applications for beta hemoglobinopathies. Importantly, this protocol could be applied to explore other genomic regions of any size, enabling the identification of regulatory elements and facilitating the development of therapeutic gene editing strategies. It is worth noting that screening a large number of candidate guide RNAs (gRNAs) simultaneously by a single researcher may compromise the quality of the results. For the purpose of achieving consistent and reproducible outcomes, it is recommended to limit the number of gRNAs to approximately 50 per screening. Especially when conducting screening in multiple batches, it is essential to include appropriate internal controls and replicates during the screening process. The strict adherence to good laboratory practices is crucial to prevent microbial or cellular cross-contamination.

Prior to commencing the screening process, ensure the availability of the required lentiviral constructs and target gRNA oligos. In our study, we obtained the lentiviral constructs for the cloning of gRNAs from Addgene, and the respective identifiers are provided in the key resources table. Several web-based tools are available for gRNA design, many of which consider the specific base editor variant being used. Reliable options include BE-Designer,[2] DeepBaseEditor,[3] ACEofBASEs,[4] and PnB designer.[5] The links for all these tools are provided in the key resources table. Alternatively, gRNAs can be designed manually or using general gRNA design software such as Benchling or CHOPCHOP.[6] In order to achieve the desired nucleotide conversions with higher

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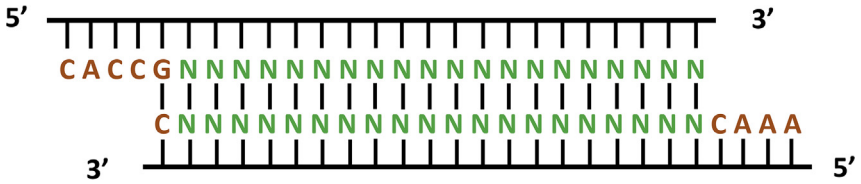

Figure 1. Representative image of the gRNA oligos to be synthesized

efficiency, it is important to carefully select the gRNA with optimal editing windows for the specific base editor variant being used. It is recommended to include a gRNA targeting a safe harbor locus (e.g., AAVS1, CCR5) or a scramble control as a reference. The gRNAs should be synthesized with appropriate overhangs (Figure 1) to facilitate their cloning into the lentiviral vector, and desalted oligos are sufficient for this purpose. During the design of sequencing primers, careful consideration of the sequencing platform is essential, whether it is Next-Generation Sequencing (NGS) or Sanger sequencing. This critical assessment ensures that the primers are customized to meet the specific requirements of the chosen sequencing platform, thereby producing dependable and accurate sequencing data. The primers used in our study are provided in the key resources table.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Bacterial and virus strains		
DH10B competent cells	ECOS, Yeastern Biotech	CAT # FYE507-10VL
Chemicals, peptides, and recombinant proteins		
GoTaq G2 Hot Start Master Mix	Promega	CAT # M7422
T4 polynucleotide kinase	NEB	CAT # M0201
T4 DNA ligase	NEB	CAT # M0202
BsmB1	NEB	CAT # R0580
Polyethylene glycol	Sigma-Aldrich	CAT # 89510
Lenti-X Concentrator	Takara	CAT # 631232
SCF	Immuno Tools	CAT # 11343325
EPO	Zydus Nephrosciences	Zyrop 4000 IU injection
Penstrep	Gibco	CAT # 15140122
Dexamethasone	Alfa Aesar	CAS # 1177-87-3
Doxycycline	Sigma-Aldrich	CAS # 24390-14-5
Glutamine	Gibco	CAT # 25030081
FBS	Gibco	CAT # 10270106
PBS	HyClone	CAT # SH30256.02
Polybrene	Sigma-Aldrich	CAS # 28728-55-4
HEPES	Gibco	CAT # 15630080
Puromycin	Gibco	CAT # A1113803
StemSpan SFEM-II	STEMCELL Technologies	CAT # 09655
DMEM	HyClone	CAT # SH30243.01
Opti-MEM reduced serum media	Gibco	CAT # 31985062
FuGENE HD	Promega	CAT # E2312
LB agar	HiMedia	CAT # M1151
LB broth	HiMedia	CAT # M1245
Ampicillin sodium salt	SRL	CAT # 61314
QuickExtract DNA extraction solution	Epicentre	CAT # QE09050
HighPrep PCR	MagBio	REF # AC-60005
HighPrep DTR	MagBio	REF # DT-70005
Critical commercial assays		
Zymoclean Gel DNA Recovery Kit	Zymo Research	CAT # D4001

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
NucleoBond Xtra Midi	Macherey-Nagel	REF # 740410
NucleoSpin Blood – DNA kit	Macherey-Nagel	REF # 740951
NucleoSpin plasmid	Macherey-Nagel	REF # 740588
BigDye Terminator v3.1 Cycle Sequencing Kit	Applied Biosystems	CAT # 4337458
Experimental models: Cell lines		
HEK293T	ATCC	
HUDEP-2	Cell Engineering Division, RIKEN BioResource Center	
Oligonucleotides		
gRNAs cloned in #57822	Ravi et al.[1]	Check Table S1
Sequencing F: gagggcctatttcccatgat	Ravi et al.[1]	Sanger sequencing primer for colony PCR
Sequencing R: tggatctctgctgtccctgt	Ravi et al.[1]	
HBF 1 F: acaaaagaagtcctggtatc	Ravi et al.[1]	
HBF 2 F: ttactgcgctgaaactgtgg	Ravi et al.[1]	
HBF 1 R: cttcccagggtttctcctcc	Ravi et al.[1]	
NGS 2 F: gctcttccgatct tgaatcggaacaaggcaaagg	Ravi et al.[1]	
NGS 2 R: gctcttccgatct gtgaaatgacccatggcgtc	Ravi et al.[1]	
NGS 3 F: gctcttccgatct cctggacctatgcctaaaaca	Ravi et al.[1]	
NGS 3 R: gctcttccgatct agtttagccagggaccgttt	Ravi et al.[1]	
Recombinant DNA		
pLKO5.sgRNA.EFS.GFP	Addgene	#57822
pLenti-FNLS-P2A-Puro	Addgene	#110841
pLenti-ABERA-P2A-Puro	Addgene	#112675
pMD2.G	Addgene	#12259
psPAX2	Addgene	#12260
Software and algorithms		
Synthego ICE	Synthego	
EditR	EditR: Edit Deconvolution by Inference of Traces in R (shinyapps.io)	
CRISPResso2	CRISPResso2 (partners.org)	
Benchling	CRISPR gRNA Design Tool: Benchling	
SnapGene	SnapGene: software for everyday molecular biology	
BE-Designer	http://www.rgenome.net/be-designer	
DeepBaseEditor	http://deepcrispr.info/DeepBaseEditor	
ACEofBASEs	https://aceofbases.cos.uni-heidelberg.de	
PnB designer	https://fgcz-shiny.uzh.ch/PnBDesigner	
CHOPCHOP v3	CHOPCHOP (uib.no)	

STEP-BY-STEP METHOD DETAILS

Designing gRNA and ligation

Timing: 2 days

Here, we outline the process of designing gRNA and performing ligation for CRISPR-based genome editing. This two-day procedure involves the preparation of a lentiviral vector backbone for gRNA cloning and the annealing of oligos, followed by ligation to create a functional construct. This detailed walkthrough will facilitate the precise design and assembly of gRNAs, a fundamental component of CRISPR-based experiments, allowing for targeted genomic modifications.

Day 1

1. Preparation of backbone for cloning gRNAs:
   a. Digest the lentiviral vector (Addgene #57822)[7] using the BsmB1 enzyme.

i. Assemble the reaction mixture as follows.

Reagent	Volume
Addgene # 57822 (500 ng)	8 μL
NEB buffer 3.1 (10×)	5 μL
BsmB1 enzyme (NEB) 10 U/μL	2 μL
Nuclease free water	Upto 50 μL
Total	**50 μL**

ii. Incubate at 55°C for 3 h to ensure complete digestion.

b. Perform gel electrophoresis of the digested product on a 0.8% agarose gel.
   i. Ensure that the samples are run for at least 90 min to achieve sufficient separation.
   ii. To serve as a control for digestion, load the undigested plasmid in the adjacent well.
c. Carefully excise the desired digested backbone (~8000 bp) using a clean blade and purify it using the Zymo gel purification Kit as per the manufacturer's protocol.
d. Elute the purified product in 25 μL of nuclease-free water and adjust the concentration to 50 ng/μL.

Note: The digestion and gel purification of 4 μg of plasmid will yield approximately 2000–2500 ng, which is adequate for cloning 40–50 gRNAs. It is essential to digest an adequate amount of plasmid, considering the total number of gRNAs to be cloned.

Day 2

2. Annealing of gRNA oligos:
   a. Reconstitute the lyophilized forward and reverse oligos to 100 pm concentration.
   b. Assemble the oligo annealing reaction as follow in a PCR tube:

Reagent	Volume
Forward Oligo (100 pm)	1 μL
Reverse Oligo (100 pm)	1 μL
NEB ligase buffer 10×	1 μL
T4 polynucleotide kinase 10 U/μL (NEB)	0.5 μL
Nuclease free water	6.5 μL
Total	**10 μL**

c. Set up the following reaction in a thermocycler.

Cycles	1 Cycle	1 Cycle	1 Cycle	1 Cycle
Temperature (°C)	37°C	95°C	25°C	4°C
Time (min)	45 min	5 min	1 min	∞
Ramp rate	Ramp 100%	Ramp 100%	Ramp 3%	

d. Take 1 μL of annealed product and dilute it (1:200) using sterile water. Store at 4°C until use.

Note: The steps described are for a single gRNA. Perform similar reactions for all gRNAs.

3. Ligation:
   a. Set up the reaction as follows.

Reagent	Volume
Vector backbone (50 ng/μL)	1 μL
Oligo product (1:200 diluted in H_2O) (Insert)	6 μL
NEB ligase buffer 10×	2 μL
NEB ligase	0.5 μL
Nuclease free water	10.5 μL
Total	**20 μL**

b. Mix thoroughly and incubate at 16°C for 12–16 h, then store at 4°C until transformation.

Cloning and confirmation of clones

Timing: 5 days

This protocol outlines the step-by-step process for cloning and validating the guide RNAs (gRNAs) for use in downstream editing experiments. The protocol spans five days and includes key procedures such as transformation, colony picking, glycerol stock preparation, colony PCR, Sanger sequencing, and plasmid isolation. These critical steps ensure the generation of plasmids containing accurately cloned gRNAs, crucial for successful editing.

Day 1

4. Transformation and plating:
   a. Prepare LB Agar plate with ampicillin (100 μg/mL) as per the requirement.
   b. Thaw 50 μL of DH10B competent cells on ice.
   c. Add 6 μL of ligated product to the cells and mix by flicking the tubes.
   d. As a control add 50 ng of digested backbone to another vial of competent cells.
   e. Keep on ice for 30 min. Set up water bath at 42°C.
   f. After 30 min, give heat shock for 45 s at 42°C.
   g. Rapidly transfer to an ice bath and incubate for 5 min.
   h. Plate the whole volume on LB Ampicillin plate and incubate at 37°C for 12–14 h.

Day 2

5. Picking colonies and preparation of glycerol stock:
   a. Check the control plate and ensure that there are no colonies.
   b. Prepare LB broth (3 mL for each gRNA).
   c. Arrange 2 mL tubes containing one mL of LB broth (with ampicillin), 3 tubes for each gRNA.
   d. Pick and inoculate 3 isolated colonies into this LB broth from the plate.
   e. The plates can be stored at 4°C for about 1 week as a backup.
   f. Keep the inoculum in a shaker incubator for 6 h at 37°C.
   g. The inoculum should be visibly turbid by then.
   h. From one mL of the inoculum take 500 μL and prepare glycerol stock by adding 500 μL of 50% glycerol, and store immediately at −80°C.
   i. Centrifuge the remaining inoculum at 2700 g for 5 min.
   j. Remove the supernatant and add 500 μL of Sterile water.
   k. Centrifuge again, discard the supernatant, and add 500 μL of sterile water.
   l. Mix well and store at 4°C.
   m. Use this as a template for the colony PCR as described below.

Day 3

6. Colony PCR:
   a. Set up the PCR reaction as follows.

Reagent	Volume
Forward Primer (10 pm)	1 μL
Reverse Primer (10 pm)	1 μL
Processed inoculum	2 μL
Master mix 2× (Promega)	10 μL
Nuclease free water	6 μL
Total	**20 μL**

   b. Set up the following reaction in a thermocycler.
      i. The primers used are mentioned in the key resources table.

Steps	Temperature	Time	Cycles
Initial Denaturation	95°C	10 min	1
Denaturation	95°C	20 s	35 cycles
Annealing	55°C	20 s	
Extension	72°C	45 s	
Final extension	72°C	10 min	1
Hold	16°C	Forever	

      ii. Run 5 μL of the PCR product on 1% agarose gel.
      iii. Positive colonies are expected to exhibit a band at ~500 bp (based on the primers designed).
7. Sanger sequencing:
   a. Proceed for Sanger sequencing with one positive colony from each gRNA group.
   b. Use the 15 μL of PCR product that was remaining after the gel run for sequencing.

Day 4

8. Analyzing Sanger sequencing data:
   a. Using the Sanger sequencing data, confirm whether the gRNA sequence has been cloned correctly.
   b. Make sure that the sequencing data obtained matches exactly with the gRNA sequence (Figure 2).

   Note: If any clone yields a negative result, proceed to the sequencing stage for the subsequent clones.

9. Inoculation of positive clones:
   a. After confirming the clones, proceed to plasmid isolation by inoculating the positive clones.

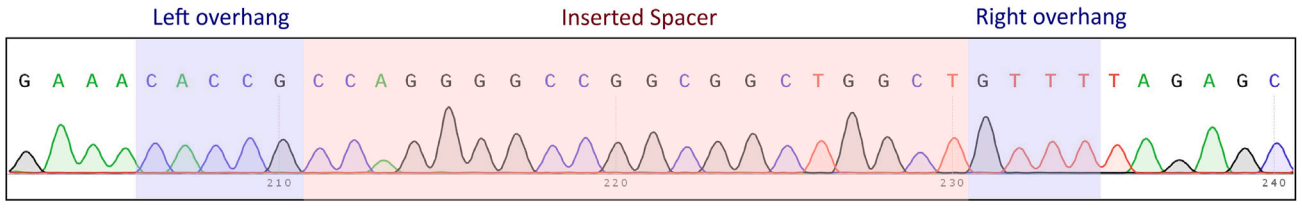

Figure 2. Representative Sanger sequencing data for successful cloning of gRNA into lentiviral backbone

b. For each gRNA, prepare 10 mL of LB broth (with ampicillin).
c. Using a sterile loop, transfer a small volume of the glycerol stock from the positive clone into the LB broth.
d. Keep in a shaker incubator for 16 h at 37°C.

Note: ensuring that the glycerol stock does not thaw completely during the process.

Day 5

10. Isolation of plasmid DNA from the inoculum:
    a. Isolate the plasmid using MN mini plasmid kit as per the manufacturer's protocol.
    b. Subsequently, dilute the isolated plasmids to achieve a final concentration of 500 ng/μL. Store the plasmid at −20°C until use.

Lenti-virus production for gRNA

Timing: 4–5 days

This section outlines the steps involved in the production and collection of lentiviral particles packaged with gRNA. The focus here is to culture healthy and actively dividing HEK293T cells, followed by the transfection of gRNA plasmids along with lentiviral packaging vectors to generate lentivirus. The protocol spans 4–5 days and includes critical procedures such as cell seeding, transfection, verification of successful transfection through GFP expression, and virus collection. These steps are essential for obtaining high-quality viral particles.

Day 1

11. Culturing HEK293T cells:
    a. Seed healthy and actively dividing HEK293T cells in one well of 6 well plate so that it would reach 70–80% confluency in 24 h.
    b. Increase the number of wells based on the number of gRNAs.

Day 2

12. Transfection:
    a. Ensure that HEK293T cells are approximately 80% confluent and healthy.
    b. Bring the transfection reagent (FuGENE HD) to 25–30°C.
    c. Prepare the transfection mixture as given below:

Reagent	Final concentration	Volume
OptiMEM		100 μL
Addgene # 12259 (500 ng/μL)	500 ng	1 μL
Addgene # 12260 (500 ng/μL)	700 ng	1.4 μL
57822 plasmid with gRNA (500 ng/μL)	800 ng	1.6 μL
FuGENE HD	3:1 (reagent (μL): DNA (μg))	6 μL
Total		**110 μL**

    d. Incubate the mixture in 25–30°C for 15–20 min.
    e. Remove the cell culture dish with HEK293T cells from the incubator and place it inside the biosafety cabinet.
    f. Add the transfection reagent dropwise to the cells.

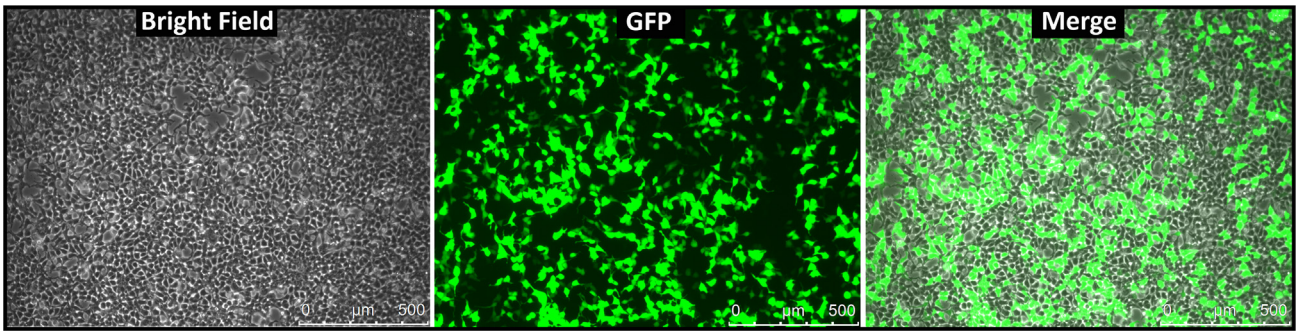

Figure 3. GFP expression in HEK293T cells 48 h after transfection

g. Gently swirl the dish to ensure even distribution of the mixture, then return the dishes and incubate for 48 h.

Day 4

13. Confirmation of transfection:
    a. Before proceeding with virus collection ensure that the transfection was successful and gRNA plasmids are expressing GFP by using fluorescence microscopy.
    b. Successful transfection should result in over 75% of the HEK cells expressing GFP within 48 h (Figure 3).
14. Virus collection:
    a. Collect the supernatant from each well separately without disturbing the cells.
    b. Clarify by spinning the supernatant at 400 g for 5 min at 25–30°C.
    c. Transfer the 2 mL of clarified supernatant to another tube containing 700 µL of virus concentrator.
    d. Mix well and incubate at 4°C for 1 h.
    e. Centrifuge at 2700 g for 30 min at 4°C.
    f. Discard the supernatant and resuspend the pellet in 100 µL of cold 1× PBS.
    g. Store immediately at −80°C and do not thaw until use.
    h. The HEK293T cells can be topped up with fresh media and the virus was collected again after 24 h.
    i. This virus can be stored as a backup or used for trial transduction experiments if needed.

Preparation of base editor cell lines

Timing: 3 weeks

This protocol outlines steps essential for the production of lentiviral vectors and the establishment of stable cell lines harboring base editor plasmids. The protocol encompasses several key phases, starting with the preparation of endotoxin-free plasmids, followed by the production of lentivirus, and culminating in the creation of stable cell lines.

In this protocol, we have used codon optimized ABE and CBE max constructs with puromycin selectable marker from Lucas Dow (pLenti-FNLS-P2A-Puro (Addgene #110841-CBE) and pLenti-ABERA-P2A-Puro (Addgene #112675-ABE)).[8] There are newer versions of base editors with increased processivity, modified PAMs, and editing windows, which can be obtained from plasmid repositories such as Addgene. To ensure consistent and reproducible results in downstream processes, we recommend using BE lentiviral constructs that incorporate antibiotic selection markers. These markers enable the isolation of pure transduced populations by conferring resistance to specific

antibiotics, allowing the generation of stable cell lines with the desired characteristics. We have used second generation lentiviral packaging constructs in this study (Addgene #12259 and #12260), a gift from Didier Trono. Readers are directed to the review article by Anzalone et al. for a detailed comparison of various base editors available that would aid in choosing the BE for your application.[9]

Day 1

15. Endotoxin free plasmid preparation:
    a. Inoculate the bacterial stock in LB broth with appropriate antibiotic (Ampicillin 100 μg/mL) and culture for 16 h.
    b. Harvest the bacteria by centrifuging the inoculum at 2700 g for 10 min at 25–30°C in a 50 mL Falcon tube.
    c. After centrifugation, carefully collect the pellet for plasmid isolation, and discard the supernatant.
    d. Isolate the plasmid using NucleoBond Xtra Midi EF (Macherey-Nagel) kit.

Note: Ensure that the final concentration of plasmid preparation is not below 500 ng/μL.

Day 2–5

16. Lentivirus production:

Note: The steps described here are for preparing lentivirus with one of the above-mentioned constructs. Ensure the availability of reagents as per your need.

    a. Day 2: Seeding cells.
        i. Seed low passage HEK293T cells in a 10 cm cell culture dish that will result in 70–80% confluent in 24 h.
    b. Day 3: Transfection.
        i. Make sure HEK293T cells are ~80% confluent and are healthy.
        ii. Bring the transfection reagent (FuGENE HD) to 25–30°C.
        iii. Prepare the transfection mixture as given below in 1 mL Eppendorf tube:

Reagent	Final concentration	Volume
OptiMEM		500 μL
Addgene # 12259 (500 ng/μL)	2500 ng	5 μL
Addgene # 12260 (500 ng/μL)	3500 ng	7 μL
Addgene # 112675 (ABE7.10) (500 ng/μL) or Addgene # 110841 (BE 3) (500 ng/μL)	4000 ng	8 μL
FuGENE HD	3:1 (reagent (μL): DNA (μg))	30 μL
Total		550

        iv. Vortex well and incubate at 25–30°C for 15 min.
        v. Take out the cell culture dish from the incubator and gently add the transfection reagent mixture dropwise to the cell culture medium inside the Bio Safety Cabinet (BSC).
        vi. Swirl the dish to spread the mixture evenly and return the dishes to the incubator.
    c. Day 5: Harvesting the lentivirus.
        i. 48 h after transfection, collect the supernatant without disturbing the cells in a 15 mL falcon tube.
        ii. Clarify by centrifugation at 400 g for 5 min to remove any unadhered cells and debris.
        iii. Carefully transfer the supernatant to a falcon tube containing 4× Lenti X concentrator (Takara) or 4× PEG viral concentrator.

iv. The ratio of concentrator to media should be 1:3.
v. Mix well and incubate at 4°C for 1 h.

Note: The HEK293T cells can be topped up with fresh media and the virus can be collected again after 24 h and will be processed as above. After collecting the virus bleach and discard the HEK293T cells.

vi. Composition for preparation of PEG virus concentrator.

Reagent	Amount
PEG-8000 (sigma)	200 g
NaCl	35 g
Milli Q water	200 mL
10x PBS (pH 7.4) HyClone	50 mL
Dissolve by placing on a magnetic stirrer, with low heat if needed	
Makeup with MilliQ water	**500 mL**
Vacuum filter using 500 mL 0.2 μ filter (Thermo) and store in 4°C	

vii. Centrifuge the incubated mixture at 2700 g for 30 min at 4°C.
viii. Remove the supernatant and resuspend the viral pellet in 200 μL of 1x PBS.
ix. Make two aliquots of 100 μL and store the virus at −80°C for the downstream process.

Note: ensure that the lentivirus is transported and maintained on ice to prevent any drop in efficiency.

Day 6–26

17. Transduction of relevant cell line to prepare base editor stables:

Note: This protocol describes the utilization of the HUDEP-2 cell line. However, it is important to note that the protocol can be readily customized and applied to different cell lines as needed.

a. Day 6: Revive approximately 0.5 million cells (1 million cells are needed on the day of transduction).
b. Day 8: Passage the cells and make sure they are healthy.
c. Day 10: Transduction.
   i. Seed 0.5 million cells per well in 2 wells of a 6 well plate.
   ii. To each well add 20 μL of 1 M HEPES buffer and 2 μL of polybrene (6 mg/mL).
   iii. Carefully thaw the stored virus (100 μL) and add it into one well.
   iv. The other well would serve as the non-transduced control.
   v. Spinfection is carried out by centrifugation of the plate at 800 g for 30 min.
   vi. Make sure the acceleration and brake are low (5 and 5).
   vii. Keep the plates back in the incubator.

Note: Use parafilm to seal the plate during centrifugation to avoid any accidental spillage as the culture contains lentivirus.

d. Day 12 & 14: Selection of the stables.
   i. Make sure that the transduced cells are viable.
   ii. Reseed the cells in a fresh 6 well plate.

iii. Add 2 μL puromycin per mL of media (1 mg/mL) to both controls as well as the transduced samples.
iv. After two days check the cells under a microscope.
v. All cells in the control well must be dead and there would be ~60%–80% cell death in the transduced well.
vi. Perform a media change and reseed the viable cells with the same concentration of puromycin.

Note: If viable cells are persisting in the control well, increase the puromycin concentration until complete cell death is observed. Ensure that the same concentration is used for the transduced cells as well. Performing a literature search will provide insights into the suitable puromycin concentration for each specific cell line.

e. Day 16–26: Stable cells maintenance and expansion.
   i. Do media change on alternate days and maintain the cells in puromycin.
   ii. Ensure the cells are healthy and expand enough.
   iii. After 10 days of puromycin selection, the cells are ready for gRNA transduction.
   iv. Freeze cells in multiple vials as backup and store them in liquid nitrogen (−196°C).

Note: Transcriptome profiling of the stable cells can be performed and compared with wild type cells to ensure that during stable preparation the intrinsic properties of the cells are not altered significantly.

Transduction of gRNAs in base editor stables

Timing: 10 days

This comprehensive 10-day protocol focuses on transducing a base editing stable cell line with lentivirus carrying gRNA. The primary objective is to confirm the transduction and the editing obtained in the stable cells. The protocol encompasses crucial steps like maintaining optimal cell density, conducting lentiviral transduction, and assessing transduction efficiency through GFP expression via flow cytometry. Precise execution of these steps is imperative to achieve a transduction efficiency exceeding 95%, maximizing the potential for base substitutions across most cells. The protocol culminates in DNA isolation, target region amplification, and the assessment of base editing efficiency using data obtained from Sanger sequencing or NGS with appropriate software.

Day 1

18. Reviving stable cell line:
    a. Revive and expand the cryopreserved base editor stable cells by culturing them in a growth medium supplemented with puromycin prior.
    b. It is crucial to have approximately 0.2 million cells per guide RNA (gRNA) to facilitate simultaneous screening of the gRNAs.
    c. Therefore, ensure an adequate quantity of cells to accommodate the screening of the gRNAs concurrently.
19. Transduction:
    a. Upon reaching the desired cell quantity, carefully distribute 0.2 million cells per well in a 12-well plate, utilizing 1 mL of media for each well.
    b. To each well, add 10 μL of 1 M HEPES buffer and 1 μL of polybrene (6 mg/mL).
    c. Thaw the stored lentivirus collected at 48 h and add it individually to each well.

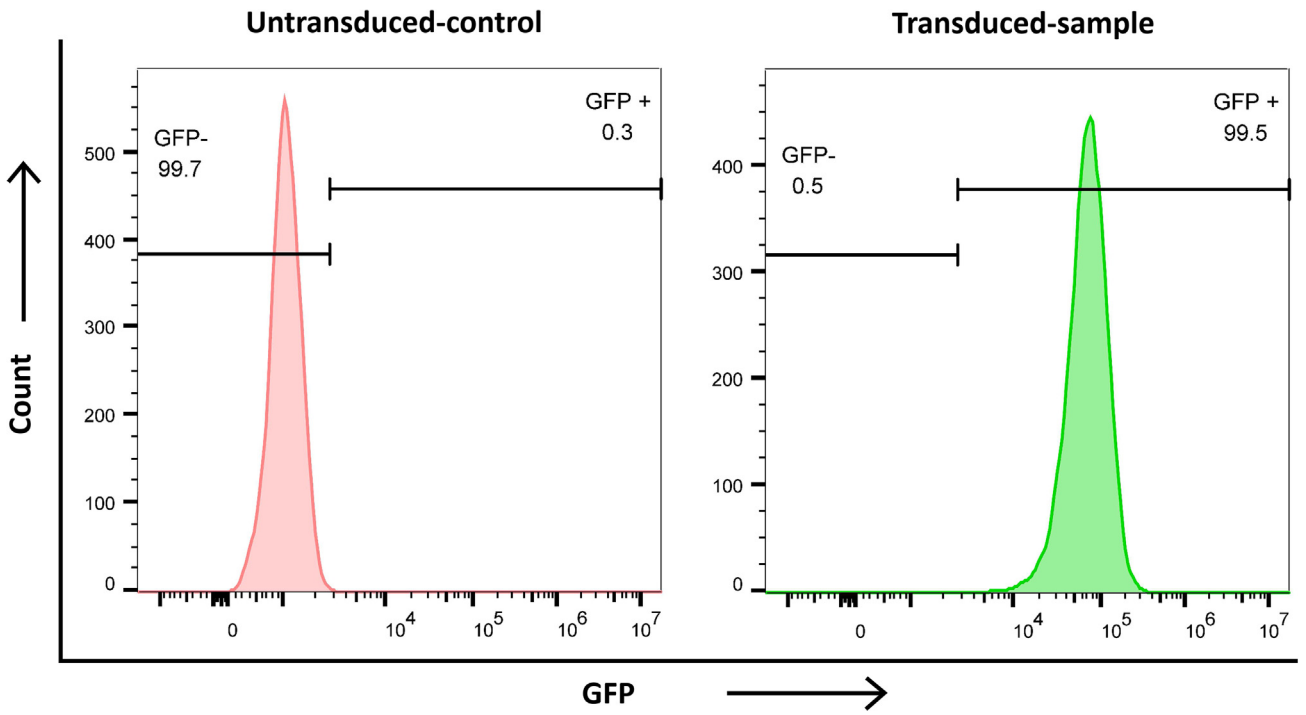

Figure 4. Representative flow cytometry data for evaluating transduction efficiency using intracellular GFP

d. Mix gently and perform spinfection by centrifuging at 800 g for 30 min with minimum acceleration and brake.
e. Incubate the cells at 37°C with 5% CO2 for 48 h.

Note: The lentiviral transduction protocol mentioned above was optimized specifically for the HUDEP-2 cell line. For other cell lines, the viral titer and polybrene concentration may need to be adjusted accordingly to achieve a transduction efficiency exceeding 95%.

Note: Polybrene, HEPES buffer, and spinfection enhance lentivirus transduction efficiency by facilitating better viral entry, maintaining stable conditions, and increasing contact between viral particles and target cells, respectively.

Note: Measuring MOI values for each gRNA in the screening would be time consuming and incur additional cost. This is the reason why we recommend optimizing the transduction protocol to achieve maximum transduction efficiency.

Day 2

20. Evaluation of transduction efficiency:
    a. Utilize approximately 150 µL of the cell culture for analysis.
    b. Prior to analysis, wash the cells with 1x PBS.
    c. Evaluate transduction efficiency using flow cytometry by measuring the percentage of GFP expression (Figure 4).
    d. Include a control group consisting of un-transduced cells for comparison.
    e. Under ideal conditions and with careful execution of all protocol steps, it is anticipated that more than 95% of the cells will exhibit GFP expression.
    f. If all cells are transduced efficiently with respective gRNA, transfer the cells to fresh media in a 6 well plate.

Day 4

21. Maintenance and cryopreservation:
    a. Perform media change by centrifuging the culture at 200 g and resuspending the cell pellet in fresh media.
    b. Cryopreserve adequate cells in appropriate freezing media for backup storage prior to media change.

Day 6–8

22. Maintenance:
    a. Perform media change every 2 days to maintain the cells healthy and discard any excess cells.

Note: If your functional assay requires more number of cells, expand the cells rather than discarding them.

Day 10

23. Evaluation of base editing in the stable cell line:
    a. Isolate DNA from 0.5 million cells using NucleoSpin Blood – DNA kit (MN) as per the manufacturer's protocol.
    b. You can freeze the remaining cells or continue to culture them depending on the functional assays that need to be performed.

Note: The protocol for evaluation of HbF expression can be found in Ravi et al.[1] The control edited sample can be used as a base line to evaluate the HbF elevation induced by the gRNA.

    c. Amplify the target region and perform Sanger sequencing/ targeted NGS using the primer mentioned in the key resources table.
    d. Evaluate base editing efficiency using EditR (Sanger sequencing data) (Figures 5A and 5B)/ CRISPResso2 (NGS reads) software (Figures 5C and 5D).

Note: The use of variants like ABE 8e allows cells to be edited within 48 h, so there is no need to expand the cells for 10 days

EXPECTED OUTCOMES

The successful completion of this protocol will enable arrayed mutagenesis in specific genomic loci using adenine and cytosine base editing techniques. Our study focuses on screening a 300 bp region upstream of the gamma globin promoter in a human erythroid cell line to identify targets for enhancing fetal hemoglobin expression, potentially benefiting beta hemoglobinopathies and identifying new transcription regulators. By utilizing this approach, base substitutions can be introduced at any desired genomic sites, enabling the investigation of regulatory elements and the development of therapeutic gene editing strategies. This method serves both basic science research and translational applications. However, it is essential to ensure that the target region complies with the specific rules governing base editors, such as the availability of PAM sequences and the presence of target bases within the editing window region. The protocol outlines steps including gRNA design and cloning, lentivirus production, stable cell line generation, and assessment of editing efficiency using Sanger sequencing or NGS. The editing efficiency of gRNAs at a genomic locus varies depending on factors such as the target region, the position of the base within the target region, and the specific variants of base editors utilized. Consequently, different gRNAs may exhibit diverse editing efficiencies and functional outcomes. To obtain a more comprehensive insight into the editing and functional outcomes, we recommend referring to Ravi et al.[1]

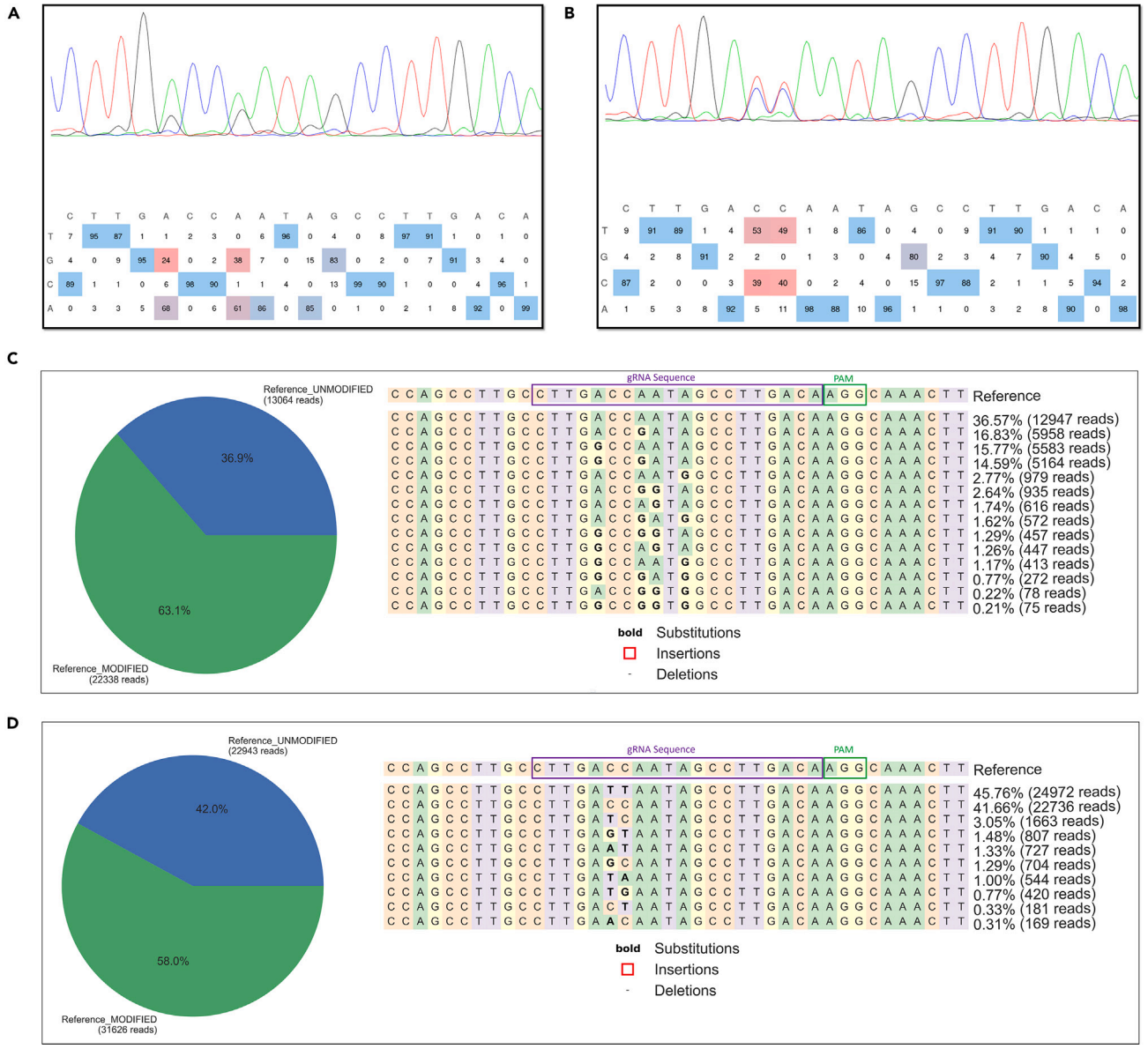

Figure 5. Sequencing output from Sanger sequencing and NGS for Base editor

(A and B) Sanger sequencing data was analyzed for samples edited with ABE(A) and CBE(B)using EditR (the red color boxes indicate the desired converted bases and their substitution percentage). The chromatogram represents a substitution in A>G in ABE and C>T in CBE.

(C and D) Targeted deep sequencing (by NGS) of samples edited with ABE(C) and CBE(D) analyzed by CRISPResso-2. All possible outcomes in base editing are represented in the processed data from NGS.

LIMITATIONS

This protocol is specifically applicable for screening a constrained set of guide RNA (gRNA) targets. To accomplish saturation mutagenesis for more extensive DNA regions, alternative approaches such as pooled lentiviral screening should be employed. Arrayed screening, while providing greater reliability, entails higher costs and demands more labor-intensive procedures.

TROUBLESHOOTING

Problem 1

No GFP is expressed after transfection with one or more gRNA(s).

Potential solution

The lack of GFP expression might have occurred due to the mutation of GFP gene in the plasmid. It is advisable to select an alternate clone, perform sequence confirmation to ensure integrity, and subsequently utilize the plasmid isolated from the selected clone for conducting the experiments.

Problem 2

GFP expression is low in HEK cells 48 h after transfection.

Potential solution

Check the quality of the transfection reagent and optimize the ratio of reagent to DNA, if needed.

Problem 3

GFP expression is low after transduction.

Potential solution

Check the integrity of lentiviral packaging plasmids and perform restriction digestion for all the plasmids used for lentivirus production. Also, ensure that the lentivirus is stored and used properly.

Problem 4

Cells are dying after transduction.

Potential solution

Adjust the lentiviral titer to minimize cell death.

Problem 5

No editing with any gRNAs even after achieving >90% transduction efficiency.

Potential solution

Ensure puromycin selection is complete for cells expressing the base editors. Increase the concentration of puromycin until all the control cells are dead.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Mohankumar KM (mohankumarkm@cmcvellore.ac.in)

Materials availability

This study did not generate new unique reagents. All reagents listed here can be found in the main article Ravi et al. (2022).

Data and code availability

This study did not generate any new data.

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AUTHOR CONTRIBUTIONS

Conceptualization, N.S.R. and K.M.M.; investigation, N.S.R. and A.G.; writing – original draft, N.S.R. and A.G.; writing – review & editing, N.S.R., A.G., and K.M.M.; funding acquisition, K.M.M.; supervision, K.M.M.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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