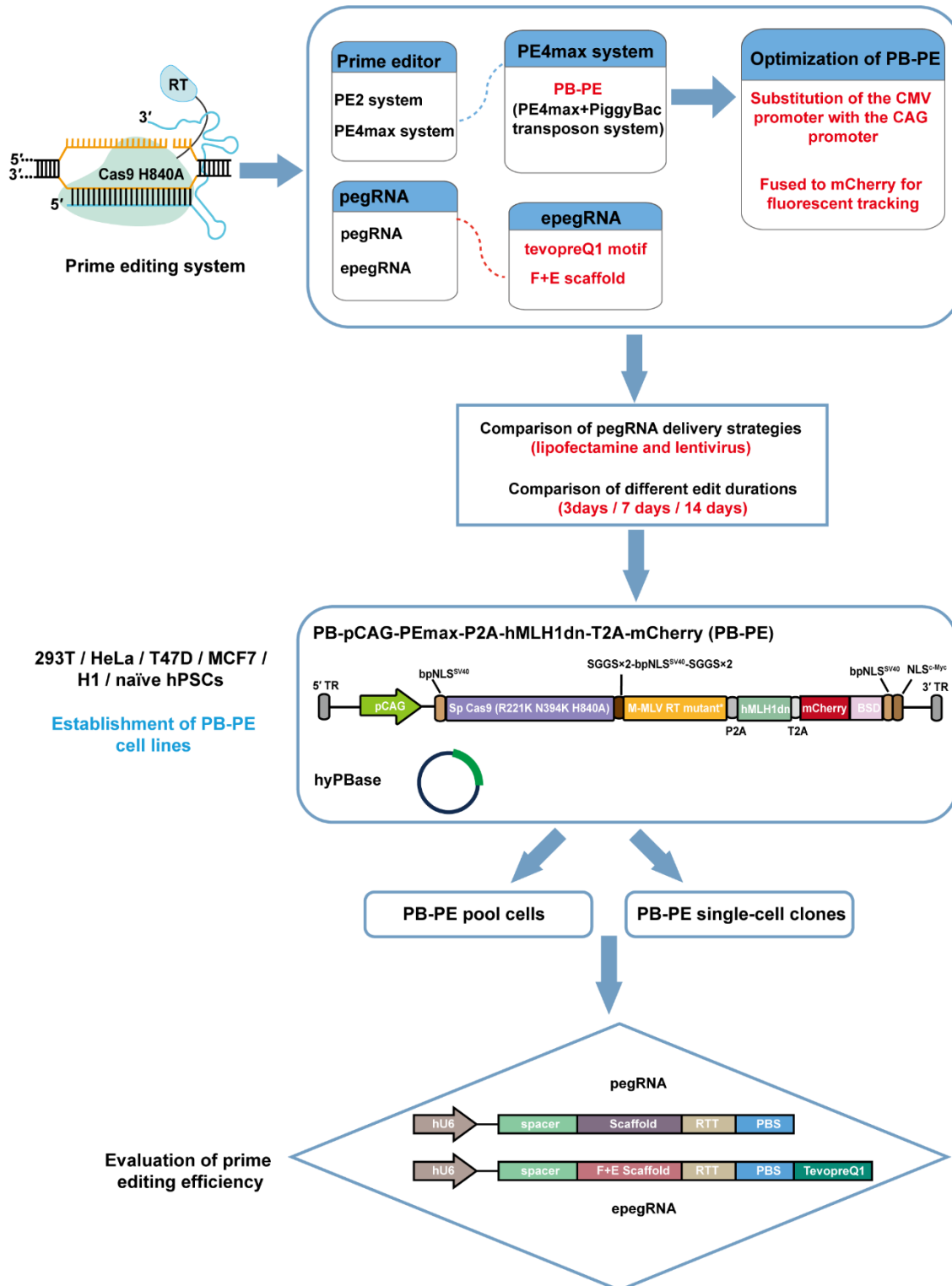
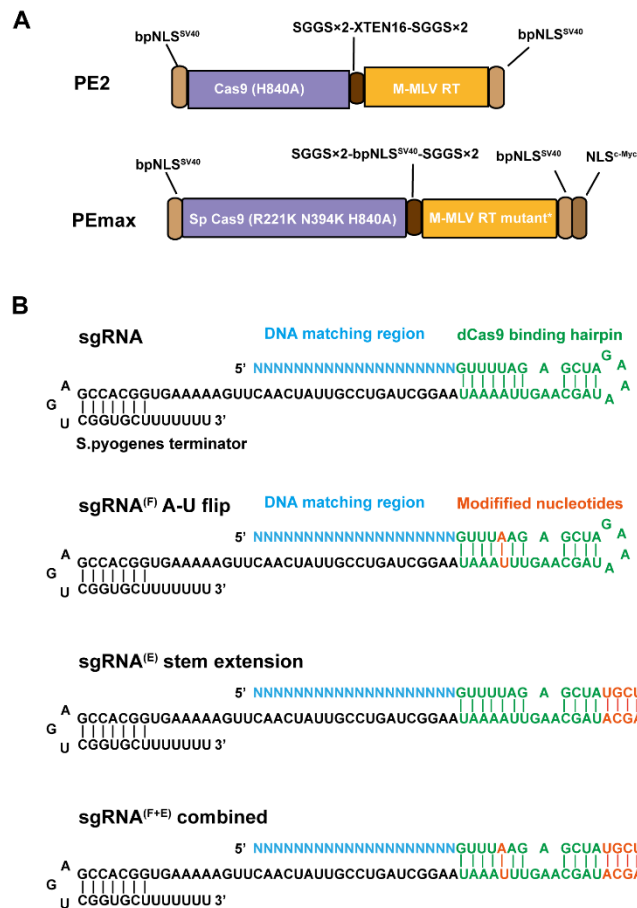


Supplementary Material

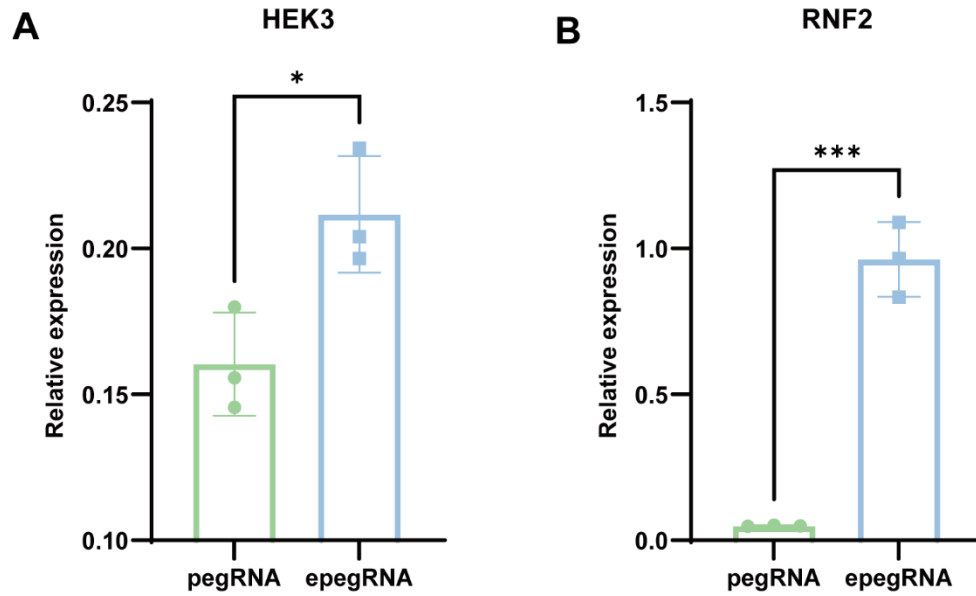
Optimization of prime editing systems



Supplementary Figure 1. Flowchart of the prime editing optimization strategy. The optimization of the prime editing system is categorized into prime editor and pegRNA element optimization. After systematic evaluation of both PE2 and PE4max architectures, the PE4max system was engineered for enhanced performance by associating with the piggyBac transposon, resulting in the development of the PB-PE system. The optimized PB-PE system was subsequently engineered through promoter replacement and mCherry reporter fusion. Engineered pegRNAs were created through incorporation of tevopreQ1 motif and scaffold replacement. We compared the effects of different pegRNA delivery methods and the impact of varying editing durations. Next, the PB-PE system was successfully established across multiple cell lines, with its editing efficiency was verified at multiple target loci.



Supplementary Figure 2. A schematic representation of the prime editing system components. **(A)** Schematic representation of PE2 and PEmax. **(B)** Schematic representation of sgRNA scaffold and 'flip and extension' (F+E) sgRNA scaffold.



Supplementary Figure 3. Quantitative RT-qPCR analysis of pegRNA and epegRNA expression levels in the PE4max system. The expression levels of pegRNA and epegRNA were quantified separately at 7 days post-lentiviral transduction. **(A)** Quantitative RT-qPCR analysis of HEK3 locus. **(B)** Quantitative RT-qPCR analysis of RNF2 locus. Data are presented as mean $\pm$ SD from three independent experiments (n=3). Statistical significance was determined by Student's t-test (*p < 0.05, **p < 0.01). The relative expression levels ($2^{-\Delta Cq}$) of pegRNA and epegRNA were compared between groups using an unpaired two-tailed Student's t-test. * p < 0.05; ** p < 0.01; *** p < 0.001; **** p < 0.0001; ns, p > 0.05.

Supplementary Table1. Plasmid construction primer

Description	Sequence (5'-3')	PCR fragment
pCAG-F	GGCCTCCACGGCCGACATTGATTATTGACTAGTTATTAATAGTAATCAATTACGGGGT	CAG promoter 1741 bp
CAG-R	GAATTCTTTGCCAAAATGAT	
T7-cas9-F	GTCTCATCATTTTGGCAAAGAATTCTAATACGACTCACTATAGGGA	T7-SV40 NLS-Cas9 141 bp
SV40NLS-Cas9-R	GTCCAGGCCGATGCTGTACTTCTTGTGACITTTCCGCTTCTCTTTGGT	
T2A-mcherry-F	CCTGCCCCGACCTTTATAAGGTTTTTCGGCAGTGGAGAGGGCAGAGG	T2A-mCherry 828bp
mcherry-pmeI-R	GTGTTTCAGTTAGCCTCCCCGTTTAAACTTACTTGTACAGCTCGTCCA	
MLH1dn-sbfi-F	TCATCAACCTGACCTCCGTGCTGAGCCTGCAGGAGGAGATCAACGAGCAGGGC	hMLH1dn fragment 761 bp
MLH1dn-R	GAAAACCTTATAAAGGTCGG	
pCAG-AgeI-Kozak-hyPBase-F	CGACGGTACCGCGGGCCCGGATCCACCGGTCGCCACCATGGGAA	hyPBase fragment 1857 bp
hyPBase-NotI-pCAG-R	GATAGGCAGCCTGCACCTGAGGAGTGC GGCCGCTTTAGAAAACAGGACTGGCACAATGT	
Align-LGP-NsiI-F	ACATTTTTTAAATGTTTCATCTTCAAATGCAT	region1 fragment 202 bp
LGP-BsmBI- tevopreQ1-R	TTCTAGTTGGTTTAACGCGTAAGTAGATAGAACCGCGAGAGACGTACAAAAAAGAGC AAGAAGC	
tevopreQ1-T6-LGP-align-F	CGCGGTTCTATCTAGTTACGCGTTAAACCACTAGAATTTTTTAAGCTTGGCGTAACTA GATCTTGAG	region2 fragment 293 bp
Align-LGP-XmaI-R	GTTTAAAACTTTATCCATCTTTGCACCCGGG	
F+E-scaffold-F	GTTTAAGAGCTAAGCTGGAAACAGCATAGCAAGTTTAAATAAGGCTAGTCCGTTATCA AC	F+E-scaffold 86 bp
F+E-scaffold-R	AGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTAACTTGC TA	
HEK3-CTTins-gSP_LV_F	TATATATCTTGTGGAAAGGACGAAACACCGGGCCAGACTGAGCACGTGAGTTTAAG AGCTAAGCTGGAA	HEK3 epegRNA 193 bp
HEK3-CTTins_LV_R	TGGTTTAACGCGTAAGTAGATAGAACCGCGCAGACTGAGCACGCTTTGATGGCAGAA GCACCGACTCGGTGCCACTTTTTCA	
RNF2-gSP_LV_F	TATATATCTTGTGGAAAGGACGAAACACCGGTCTCTTAGTCATTACCTGGTTTAAGA GCTAAGCTGGAA	RNF2 epegRNA 197bp
RNF2-Tevopre_LV_R	TGGTTTAACGCGTAAGTAGATAGAACCGCGCATCTTAGTCATTACCTGATGTGTTTCGT AGCACCGACTCGGTGCCACTTTTTCA	
CDK4-gSP_LV_F	TATATATCTTGTGGAAAGGACGAAACACCGACCATCTTTCTACTGACCAC GTTTAAGAGCTAAGCTGGAA	CDK4 epegRNA 199 bp
CDK4-Tevopre_LV_R	TGGTTTAACGCGTAAGTAGATAGAACCGCGCTTTCTACTGACCACGGGTGCAAGTGCC ATCTAGCACCGACTCGGTGCCACTTTTTCA	
AAVS1-gSP_LV_F	TATATATCTTGTGGAAAGGACGAAACACCGAGGGCTCAACATCGGAAGGTTTAAG AGCTAAGCTGGAA	AAVS1 epegRNA 194bp
AAVS1-Tevopre_LV_R	TGGTTTAACGCGTAAGTAGATAGAACCGCGCTCAACATCGGAAGAGGAGAAGTCGA AGCACCGACTCGGTGCCACTTTTTCA	
RB1-gSP_LV_F	TATATATCTTGTGGAAAGGACGAAACACCGACATGAATGTAATATAGATGGTTTAAGA GCTAAGCTGGAA	RB1 epegRNA 193 bp
RB1-Tevopre_LV_R	TGGTTTAACGCGTAAGTAGATAGAACCGCGTGAATGTAATATAGATGAGATAATTTAG CACCGACTCGGTGCCACTTTTTCA	
OCT4-gSP_LV_F	TATATATCTTGTGGAAAGGACGAAACACCGGGACTGGCCTCCCCAGGGAGTTTAAGA GCTAAGCTGGAA	OCT4 epegRNA 190 bp
OCT4-Tevopre_LV_R	TGGTTTAACGCGTAAGTAGATAGAACCGCGCCTCCCCAGGGGCCGTATTCAAGCAC CGACTCGGTGCCACTTTTTCA	

Supplementary Table2. pegRNA sequence

pegRNA	spacer sequence	3' extension	PBS length (nt)	RTT length (nt)
HEK3 + 1 CTT ins	GGCCCAGACTGAGCACGTGA	TCTGCCATCAAAGCGTGCTCAGTCTG	14	12
RNF2 + 5 G to T	GTCATCTTAGTCATTACCTG	AACGAACACATCAGGTAATGACTAAGATG	15	14
CDK4 T172A	ACCATCTTTCTACTGACCAC	AGATGGCACTTGACCCCGTGGTCAGTAGAAAG	12	20
AAVS1_55116850_1_G_1_A	GAGGGGCTCAACATCGGAAG	TCGACTTCTCCTCTCCGATGTTGAGC	12	15
RB1(c.861+1 G>A)	ACATGAATGTAATATAGATG	AAATTATCTCATCTATATTACATTCA	14	12
OCT4 K177A	GGACTGGCCTCCCCAGGGA	TGAATACGGCCCTGGGGGAGGC	14	9

Construction of pB-pCMV-PEmax-P2A-hMLH1dn vector

Step 1: Digest pCMV-PEmax-P2A-hMLH1dn plasmid

pCMV-PEmax-P2A-hMLH1dn plasmid	1.5 µg
SpeI-HF(NEB)	1.5 µL
PmeI (NEB)	1.5 µL
10× rCutSmart Buffer	5.0 µL
ddH ₂ O	to 50.0 µL
Total reaction volume	50.0 µL

Incubate at 37 °C for 3 hours

Isolate 9,390 bp fragment from cut plasmid

Step 2: Digest pB-CAGGS-dCas9-KRAB-MeCP2 plasmid

pB-CAGGS-dCas9-KRAB-MeCP2 plasmid	1.5 µg
SpeI-HF(NEB)	1.5 µL
PmeI (NEB)	1.5 µL
10× rCutSmart Buffer	5.0 µL
ddH ₂ O	to 50.0 µL
Total reaction volume	50.0 µL

Incubate at 37 °C for 3 hours

Isolate 5,772 bp fragment from cut plasmid

Step 3: Ligate the linearized plasmid

pCMV-PEmax-P2A-hMLH1dn fragment (9,390 bp)	1.0 µL
pB-CAGGS-dCas9-KRAB-MeCP2 fragment (5,772 bp)	1.0 µL
2×T4 DNA Ligase Buffer (NEB)	2.0 µL
T4 DNA Ligase (NEB)	1.0 µL
ddH ₂ O	to 20.0 µL
Total reaction volume	20.0 µL

Incubate at 16 °C overnight

Transform the ligation product into competent cells. Cells transformed with the assembled plasmid were selected using ampicillin resistance.

Construction of pB-pCAG-PEmax-P2A-hMLH1dn vector**Step 1: Digest PB-pCMV-PEmax-P2A-hMLH1dn plasmid**

PB-pCMV-PEmax-P2A-hMLH1dn plasmid	1.0 µg
SpeI-HF (NEB)	1.5 µL
SallI-HF (NEB)	1.5 µL
10× rCutSmart Buffer	5.0 µL
ddH ₂ O	to 50.0 µL
Total reaction volume	50.0 µL

Incubate at 37 °C for 3 hours

Isolate 14,465 bp fragment from cut plasmid

Step 2: PCR amplification of the CAG promoter and T7-SV40 NLS-Cas9 fragments

pCAG-hyPBase plasmid	20.0 ng
pCAG-F (10 µM)	1.0 µL
CAG-R (10 µM)	1.0 µL
2×KapaHIFI	25.0 µL
ddH ₂ O	to 50.0 µL
Total reaction volume	50.0 µL

The PCR program was as follows: 95 °C for 3 minutes, followed by 30 cycles of (98 °C for 20 seconds, 55 °C for 15 seconds, 72 °C for 1 minute), with a final extension at 72 °C for 5 minutes, and hold at 4 °C. The amplified CAG promoter fragment was 1,741 bp.

pCMV-PEmax-P2A-hMLH1dn plasmid	20.0 ng
T7-cas9-F (10 µM)	1.0 µL
SV40NLS-Cas9-R (10 µM)	1.0 µL
2×KapaHIFI	25.0 µL
ddH ₂ O	to 50.0 µL
Total reaction volume	50.0 µL

The PCR program was as follows: 95°C for 3 minutes, followed by 30 cycles of (98 °C for 20 seconds, 65 °C for 15 seconds, 72 °C for 15 seconds), with a final extension at 72 °C for 5 minutes, and hold at 4 °C. The amplified T7-SV40 NLS-Cas9 fragment was 141 bp.

Step 3: Ligate the linearized vector and PCR fragments

Digested PB-pCMV-PEmax-P2A-hMLH1dn plasmid (14465 bp)	1.0 µL
CAG promoter fragment	1.0 µL
T7-SV40 NLS-Cas9 fragment	1.0 µL
2×MultiF Seamless Assembly Mix (Abclonal)	5.0 µL
ddH ₂ O	to 10.0 µL
Total reaction volume	10.0 µL

Incubate at 50 °C for 30 minutes

Transform the ligation product into competent cells. Cells transformed with the assembled plasmid were selected

using ampicillin resistance.

Construction of pB-pCAG-PEmax-P2A-hMLH1dn-T2A-mCherry vector

Step 1: Digest pCAG-PEmax-P2A-hMLH1dn plasmid

PB-pCAG-PEmax-P2A-hMLH1dn plasmid	1.0 µg
PmeI (NEB)	1.0 µL
SbfI-HF (NEB)	1.0 µL
10x rCutSmart Buffer	5.0 µL
ddH ₂ O	to 50.0 µL
Total reaction volume	50.0 µL

Incubate at 37 °C for 3 hours

Isolate 15,502 bp fragment from cut plasmid

Step 2: PCR amplification of the T2A-mCherry and hMLH1dn fragments

Lenti-dCas9-Zim3-mCherry plasmid	20.0 ng
T2A-mCherry-F (10 µM)	1.0 µL
mCherry-PmeI-R (10 µM)	1.0 µL
2×KapaHIFI	25.0 µL
ddH ₂ O	to 50.0 µL
Total reaction volume	50.0 µL

The PCR program was as follows: 95°C for 3 minutes, followed by 30 cycles of (98 °C for 20 seconds, 65 °C for 15 seconds, 72 °C for 30 seconds), with a final extension at 72 °C for 5 minutes, and hold at 4 °C. The amplified T2A-mCherry fragment was 828 bp.

pB-pCAG-PEmax-P2A-hMLH1dn plasmid	20.0 ng
MLH1dn-SbfI-F (10 µM)	1.0 µL
MLH1dn-R (10 µM)	1.0 µL
2×KapaHIFI	25.0 µL
ddH ₂ O	to 50.0 µL
Total reaction volume	50.0 µL

The PCR program was as follows: 95°C for 3 minutes, followed by 30 cycles of (98 °C for 20 seconds, 50 °C for 15 seconds, 72 °C for 30 seconds), with a final extension at 72 °C for 5 minutes, and hold at 4 °C. The amplified hMLH1dn fragment was 761 bp.

Step 3: Ligate the linearized vector and PCR fragments

Digested PB-pCAG-PEmax-P2A-hMLH1dn plasmid (9730 bp)	1.0 µL
hMLH1dn fragment	1.0 µL
T2A-mcherry fragment	1.0 µL
2×MultiF Seamless Assembly Mix (Abclonal)	5.0 µL
ddH ₂ O	to 10.0 µL
Total reaction volume	10.0 µL

Incubate at 50 °C for 30 minutes

Transform the ligation product into competent cells. Cells transformed with the assembled plasmid were selected

using ampicillin resistance.

Construction of pCAG-hyPBase vector

Step 1: Digest CAG-CBE4max-SpG-P2A-EGFP plasmid

CAG-CBE4max-SpG-P2A-EGFP plasmid	1.0 µg
AgeI-HF (NEB)	1.0 µL
NotI-HF (NEB)	1.0 µL
10x rCutSmart Buffer	5.0 µL
ddH ₂ O	to 50.0 µL

Total reaction volume	50.0 µL
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Incubate at 37 °C for 3 hours

Isolate 4,826 bp fragment from cut plasmid

Step 2: PCR amplification of the hyPBase fragment

AgeI-KOZAK-hyPBase-NotI synthesis template	20.0 ng
pCAG-AgeI-Kozak-hyPBase-F (10 µM)	1.0 µL
hyPBase-NotI-pCAG-R (10 µM)	1.0 µL
2×KapaHIFI	25.0 µL
ddH ₂ O	to 50.0 µL

Total reaction volume	50.0 µL
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The PCR program was as follows: 95°C for 3 minutes, followed by 30 cycles of (98 °C for 20 seconds, 65 °C for 15 seconds, 72 °C for 1 minute), with a final extension at 72 °C for 5 minutes, and hold at 4 °C. The amplified hyPBase fragment was 1,857 bp.

Step 3: Ligate the linearized vector and PCR fragments

Digested CAG-CBE4max-SpG-P2A-EGFP plasmid (4826 bp)	1.0 µL
hyPBase fragment	1.0 µL
2×MultiF Seamless Assembly Mix (Abclonal)	5.0 µL
ddH ₂ O	to 10.0 µL

Total reaction volume	10.0 µL
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Incubate at 50 °C for 30 minutes

Transform the ligation product into competent cells. Cells transformed with the assembled plasmid were selected using ampicillin resistance.

Construction of *pegRNAs*

Step 1: Construction of Lenti-TevopreQ1-Puro backbone vector

Digest Lenti-guide-puro vector plasmid

Lenti-guide-puro vector plasmid	1.5 µg
NsiI-HF (NEB)	1.0 µL
XmaI (NEB)	1.0 µL
10x rCutSmart Buffer	5.0 µL
ddH ₂ O	to 50.0 µL

Total reaction volume	50.0 µL
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Incubate at 37 °C for 3 hours

Isolate 9,746 bp fragment from cut plasmid

PCR amplification of the region1 and region2 fragments

Lenti-guide-puro vector plasmid	20.0 ng
Align-LGP-NsiI-F (10 µM)	1.0 µL
LGP-BsmBI- tevopreQ1-R (10 µM)	1.0 µL
2×KapaHIFI	25.0 µL
ddH ₂ O	to 50.0 µL

Total reaction volume	50.0 µL
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The PCR program was as follows: 95°C for 3 minutes, followed by 30 cycles of (98 °C for 20 seconds, 55 °C for 15 seconds, 72 °C for 15 seconds), with a final extension at 72 °C for 5 minutes, and hold at 4 °C. The amplified region1 fragment was 202 bp.

Lenti-guide-puro vector plasmid	20.0 ng
tevopreQ1-T6-LGP-align-F (10 µM)	1.0 µL
Align-LGP-XmaI-R (10 µM)	1.0 µL
2×KapaHIFI	25.0 µL
ddH ₂ O	to 50.0 µL

Total reaction volume	50.0 µL
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The PCR program was as follows: 95°C for 3 minutes, followed by 30 cycles of (98 °C for 20 seconds, 60 °C for 15 seconds, 72 °C for 15 seconds), with a final extension at 72 °C for 5 minutes, and hold at 4 °C. The amplified region2 fragment was 293 bp.

Ligate the linearized vector and PCR fragments

Digested Lenti-guide-puro vector plasmid (9746 bp)	1.0 µL
region1	1.0 µL
region2	1.0 µL
2×MultiF Seamless Assembly Mix (Abclonal)	5.0 µL
ddH ₂ O	to 10.0 µL

Total reaction volume	10.0 µL
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Incubate at 50 °C for 30 minutes

Transform the ligation product into competent cells. Cells transformed with the assembled plasmid were selected

using ampicillin resistance.

Step 2: Digest Lenti-TevopreQ1-Puro plasmid

Lenti-TevopreQ1-Puro plasmid	1.5 µg
BsmBI-v2 (NEB)	2.0 µL
r3.1 buffer (NEB)	5.0 µL
ddH ₂ O	to 50.0 µL

Total reaction volume	50.0 µL
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Incubate at 55 °C for 3 hours

Isolate 8,259 bp fragment from cut plasmid

Step 3: Generate the F+E scaffold fragment

F+E-scaffold-F (10 µM)	1.0 µL
F+E-scaffold-R (10 µM)	1.0 µL
2×KapaHIFI	25.0 µL
ddH ₂ O	to 50.0 µL

Total reaction volume	50.0 µL
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The PCR program was as follows: 95°C for 3 minutes, followed by 30 cycles of (98 °C for 20 seconds, 65 °C for 15 seconds, 72 °C for 15 seconds), with a final extension at 72 °C for 5 minutes, and hold at 4 °C. The amplified F+E scaffold fragment was 86 bp.

Step 4: Generate the epeg guide cloning insert using the F+E scaffold fragment as the PCR template

F+E scaffold fragment	20.0 ng
gSP-LV-F (10 µM)	1.0 µL
Tevopre-LV-R (10 µM)	1.0 µL
2×KapaHIFI	25.0 µL
ddH ₂ O	to 50.0 µL

Total reaction volume	50.0 µL
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The PCR program was as follows: 95°C for 3 minutes, followed by 30 cycles of (98 °C for 20 seconds, 65 °C for 15 seconds, 72 °C for 15 seconds), with a final extension at 72 °C for 5 minutes, and hold at 4 °C. The amplified HEK3 fragment was 193 bp (RNF2 fragment was 197 bp, AAVS1 fragment was 194 bp, CDK4 fragment was 199 bp, RB1 fragment was 193 bp, OCT4 fragment was 190 bp).

Step 5: pegRNA assembly

Digested Lenti-TevopreQ1-Puro plasmid (8,259 bp)	1.0 µL
epeg guide cloning insert	1.0 µL
2×MultiF Seamless Assembly Mix (Abclonal)	5.0 µL
ddH ₂ O	to 10.0 µL

Total reaction volume	10.0 µL
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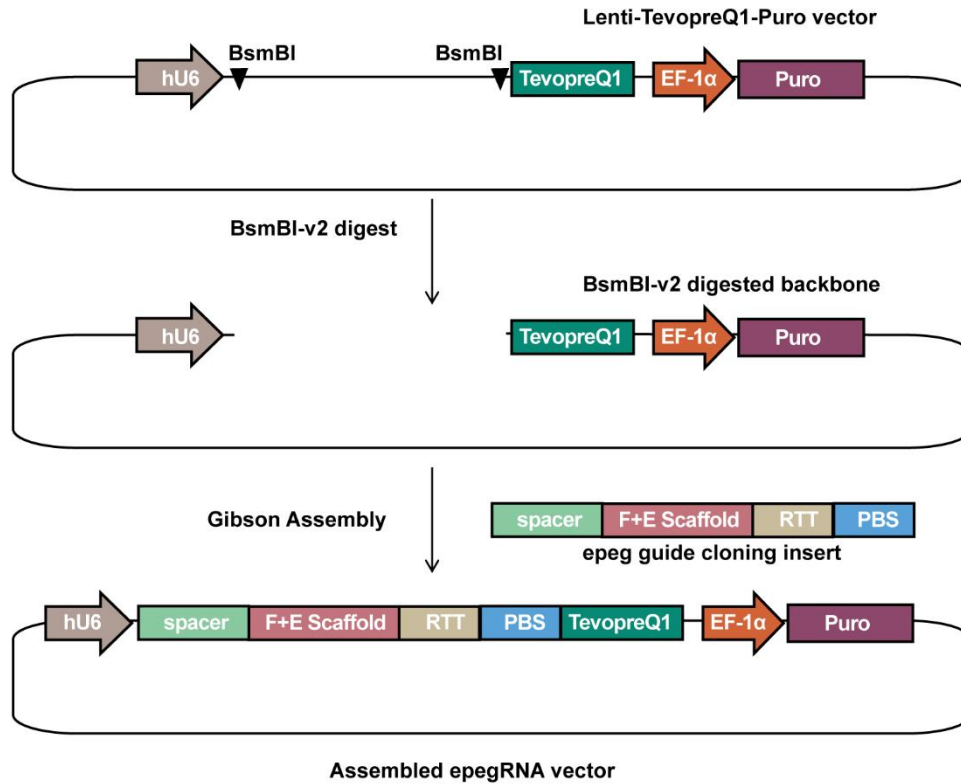
Incubate at 50 °C for 30 minutes

Transform the ligation product into competent cells. Cells transformed with the assembled plasmid were selected using ampicillin resistance.

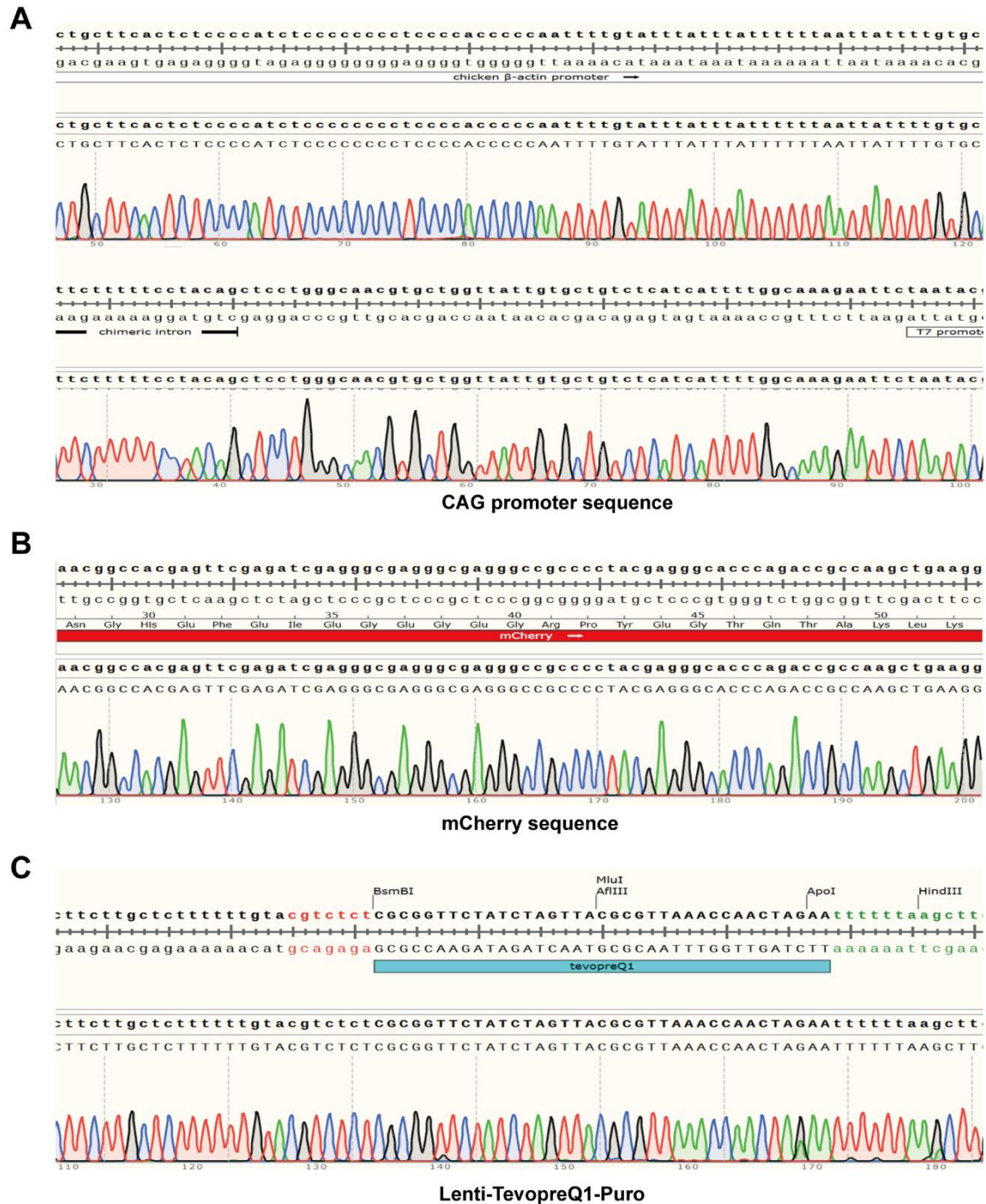
`tatatatctgtggaaggacgaaacaccg`+[20 bp spacer] +[F+E scaffold] +[extension sequence]+`cgcggttctatctagttacgcgttaaacca`

Overhang complementary to the
BsmBI-v2 digested
Lenti-TevopreQ1-Puro vector

Overhang complementary to the
BsmBI-v2 digested
Lenti-TevopreQ1-Puro vector



Supplementary Figure 4. Schematic representation of epegRNA construction. (1) BsmBI-v2 digested the Lenti-TevopreQ1-Puro vector. (2) PCR amplification of epeg guide cloning insert fragment with homology arms. (3) The BsmBI-digested Lenti-TevopreQ1-Puro vector was assembled with the PCR-amplified epegRNA fragment via homologous recombination.



Supplementary Figure 5. Sequencing results of plasmid construction. **(A)** Results of Sanger sequencing for the pB-pCAG-PEmax-P2A-hMLH1dn vector. **(B)** Results of Sanger sequencing for the pB-pCAG-PEmax-P2A-hMLH1dn-T2A-mCherry vector. **(C)** Results of Sanger sequencing for the Lenti-TevopreQ1-Puro vector.

AgeI-KOZAK-hyPBase-NotI synthesis template

ACCGGTCCGCCACCATGGGAAGCTCACTCGATGACGAACATATCTTGTCCGCTTTGCTCCAAAGCGATGATGAACCTGGTGGGAGAAGATT
 CTGATAGTAGGTTGAGCGACCAACGCTGTGAGAAGATGACGTACAGTCTGACACAGAGGAGGCGCTTCATAGATAGGTTCCATGAGGTTCCA
 ACTACATCTAGCGGACTGTGAGATCCTGGACGACAGAACGTAATTTGAACAACCTGGAAGCAGTTTGGCCAGCACAGGACTCCTTACTC
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 CTTTCTATGTTCTACGTGACGCTTATGAGCCGGGATCGATTGATTCTTCTGATCCGGTGCTGAGGATGGATGACAAGTCCATCGAGCC
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 GGACAACATCTCCAACATCTCCCCAAGGAAGTTCAGGTAATCTGACGACTCAACCGAAGAGCCAGTGTGAAGAAACGCACATACT
 GCACCTATTGCCCGTCAAGATTAGACGAAGGCATCCGCTAGCTGCAAGAAATGCAAGAAGGTCATCTGTCGGGAGCATAACATCGA
 CATGTGCCAGTCTCTGTTCTAGAGCGGCGCG

pU6-epegRNA vector

U6 promoter-Spacer-F+E scaffold-3' extension (RTT+PBS)-TevpreQ1-Plasmid backbone

[illegible]

CATTTTTTTCCTGCTGCTAGTTGTGGTTTGTCCAACTCATCAATGTATCTTATCATGTCTGGCTCTAGCTATCCCGCCCTAACTCCGC
CCATCCCGCCCTAACTCCGCCAGTTCCGCCCATTTCCGCCCATGGCTGACTAATTTTTTTATGTCAGAGGCCGAGGCCGCTC
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pB-pCMV-PEmax-P2A-hMLH1dn vector

Plasmid backbone

CMV enhancer and promoter

SV40 NLS

SpCas9 (R221K N394K H840A)

c-myc NLS

EF-1-alpha core promoter

BSD

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[illegible]

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PB-pCAG-PEmax-P2A-hMLH1dn-T2A-mcherry-BSD vector

Plasmid backbone

CAG promoter

SV40 NLS

SpCas9 (R221K N394K H840A)

c-myc NLS

T2A-mCherry

EF-1-alpha core promoter

BSD

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