Supplementary Data.

Optimized nickase- and nuclease-based prime editing in human and mouse cells

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Supplementary Figure S1. Sequence view of hU6-pegRNA and hU6-gRNA (second-nick) cassettes and the golden gate cloning sites in PEA1 construct.

**Benchling sequence view of hU6-pegRNA cassette of PEA1**

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gaggctatctttcctcatattgtcgatattctgagagagaattttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttt```
Supplementary Figure S2. Example of Bbs1 check digest of plasmids resulting from one-step digestion-ligation cloning to generate PEA1-Puro VEGFA +4 C ins. All plasmids except for plasmid 2 and plasmid 6 had complete integration of the oligo pairs. Plasmid 6 seemed to lack one pair of oligo duplex integration and therefore could be digested and produced linear plasmid.
Supplementary Figure S3. Prime Editing Target Locator (PETAL) – an online tool for designing prime editing applications. As input, PETAL takes the target sequence to be edited (top) as well as the final edited sequence (bottom, new inserted sequence highlighted in blue). PETAL identifies all valid guide sequences within the target sequence (top, edited sequence highlighted in dark grey). Users are then able to select the desired pegRNA (blue) and Second Nick (yellow) sequences. PETAL then provides the necessary oligo sequences for both gRNA’s as well as those required for the template strand (bottom). The density plot enables users to zoom into the regions of interest.

**Target Selection and Oligos:**

| Class Type | Name   | Sequence                      |
|------------|--------|-------------------------------|
| pegRNA     | Guide  | GCTCTGCGACGCCGAGAT5A          |
| pegRNA     | Oligo1 | CAGGAACGGAAGCAAGAGAATG       |
| pegRNA     | Oligo2 | CAGCGGAAGCAAGAGAATG          |
| Second Nick| Guide  | AGAGAGCGGGCGGCGGCGGCGG       |
| Second Nick| Oligo1 | GGTGTAGACGTTGACGTTGACGTTGACG |
| Second Nick| Oligo2 | CAGCTGCTGCTGCTGCTGCTGCTGCTG |
| Template   | Guide  | CAAAACCGGCTCGCTGCGGCGG       |
| Template   | Oligo1 | CAGCTGCTGCTGCTGCTGCTGCTGCTG |
| Template   | Oligo2 | CAGCTGCTGCTGCTGCTGCTGCTGCTG |
Supplementary Figure S4. Mechanism of partial template duplication (PTD) events.

DSB by nuclease prime editor + pegRNA

RT-template of pegRNA (PBS) anneals with the edited strand.

Reverse transcription, genomic DNA copies the RT-template. Edited strand contains ssDNA containing the edits (red) and the homology sequences.

Instead of annealing with the homology counterpart, this ssDNA undergoes end-joining with the DNA break junction.

Extension of the non-edited strand (blue) and ligation to join the DNA, generating PTD end product.
Supplementary Figure S5. Partial template duplications were also observed as unintended editing outcomes of PE3 in HEK293T cells.
Supplementary Figure S6. Examples of edits found in mouse ES cells targeted with PEA1-Nuc Col12a1 +2 A to C that indicate re-cutting events after prime editing events.

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| TGCCCTGATTAAACCTATTGGAAAGAGCTTTCCTCCATGTGTCCTTGCTCCATGATTGGCTGGGCCCTGTGTTTTAT | 14.37% (n=3) |
| TGGCCCTGATTAAACCTATTGGAAAGAGCTTTCCTCCATGTGTCCTTGCTCCATGATTGGCTGGGCCCTGTGTTTTAT | 2.77% (n=3)   |
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Supplementary Figure S7. Frequency of PE, indels, PTD and WT alleles in individual mice generated by nuclease prime editor.

# indicates 3 or more different alleles were detected in this individual mouse. Alleles with 2 or more bp non-correct insertions were classified as PTDs.

**PE-Nuc mice Chd2 +1 CTC ins**

**PE-Nuc mice Chd2 +5 G to C**

**PE-Nuc mice Col12a1 +1 GTG ins**
| PEA1 targeting construct | Oligo pair 1 (gRNA) | Oligo pair 2 (repair template) | Oligo pair 3 (second nick gRNA) |
|-------------------------|---------------------|-------------------------------|-------------------------------|
| **HEK3** +1 A ins       | caccGGCCAGACTGAGCAGCTGAG | gttgTCCTGCAACTACTTCGTCATG    | accgTCAACCATACCTTCCTCCTGGT  |
|                         | aaaaTCAGTGCTACCTGCTCTGGCC | aaaaCGACGGATGCAGATGGGCAGA   | taaaAGCCGCGGTATCTGTTGTA    |
| **HEK3** +1 CTT ins     | caccGGCCAGACTGAGCAGCTGAG | gttgTCCTGCAACTACTTCGTCATG    | accgTCAACCATACCTTCCTCCTGGT  |
|                         | aaaaTCAGTGCTACCTGCTCTGGCC | aaaaCGACGGATGCAGATGGGCAGA   | taaaAGCCGCGGTATCTGTTGTA    |
| **HEK3** +1 T del       | caccGGCCAGACTGAGCAGCTGAG | gttgTCCTGCAACTACTTCGTCATG    | accgTCAACCATACCTTCCTCCTGGT  |
|                         | aaaaTCAGTGCTACCTGCTCTGGCC | aaaaCGACGGATGCAGATGGGCAGA   | taaaAGCCGCGGTATCTGTTGTA    |
| **HEK3** +1-3 TGA del   | caccGGCCAGACTGAGCAGCTGAG | gttgTCCTGCAACTACTTCGTCATG    | accgTCAACCATACCTTCCTCCTGGT  |
|                         | aaaaTCAGTGCTACCTGCTCTGGCC | aaaaCGACGGATGCAGATGGGCAGA   | taaaAGCCGCGGTATCTGTTGTA    |
| **HEK3** +1 T to G PM   | caccGGCCAGACTGAGCAGCTGAG | gttgTCCTGCAACTACTTCGTCATG    | accgTCAACCATACCTTCCTCCTGGT  |
|                         | aaaaTCAGTGCTACCTGCTCTGGCC | aaaaCGACGGATGCAGATGGGCAGA   | taaaAGCCGCGGTATCTGTTGTA    |
| **RNF2** +1 T ins       | caccGTACATTGATCTTACCTG    | gttgACGAAACACCTCAGTAGAATG    | accgTCACCATTTAAGCAGAAAAACAT |
|                         | aaaaCACGTTATGACTAAGATGAC | aaaaCATCTTATGCACTTACTTCCTAGTT | taaaAGTTTTGCTTAAATGTTGTA  |
| **RNF2** +1 GTA ins     | caccGTACATTGATCTTACCTG    | gttgACGAAACACCTCAGTAGAATG    | accgTCACCATTTAAGCAGAAAAACAT |
|                         | aaaaCACGTTATGACTAAGATGAC | aaaaCATCTTATGCACTTACTTCCTAGTT | taaaAGTTTTGCTTAAATGTTGTA  |
| **RNF2** +4 A del       | caccGTACATTGATCTTACCTG    | gttgACGAAACACCTCAGTAGAATG    | accgTCACCATTTAAGCAGAAAAACAT |
|                         | aaaaCACGTTATGACTAAGATGAC | aaaaCATCTTATGCACTTACTTCCTAGTT | taaaAGTTTTGCTTAAATGTTGTA  |
| **RNF2** +3-5 GAG del   | caccGTACATTGATCTTACCTG    | gttgACGAAACACCTCAGTAGAATG    | accgTCACCATTTAAGCAGAAAAACAT |
|                         | aaaaCACGTTATGACTAAGATGAC | aaaaCATCTTATGCACTTACTTCCTAGTT | taaaAGTTTTGCTTAAATGTTGTA  |
| **RUNX1** +1 C ins      | caccGCATTTCAGAGGAGAGCAGA  | gttgTCCTGCAACTACTTCGTCATG    | accgTAAGAGACTTGTTGAGGACTGAGA |
|                         | aaaaTCAGTTCTCTCTGAAAAATGC | aaaaATTTTCAGAGGAGAGCAGAAGTTCAGAAGA | taaaACGTACCCACAGTCAGAGCAT |
| **RUNX1** +1 ATG ins    | caccGCATTTCAGAGGAGAGCAGA  | gttgTCCTGCAACTACTTCGTCATG    | accgTAAGAGACTTGTTGAGGACTGAGA |
|                         | aaaaTCAGTTCTCTCTGAAAAATGC | aaaaATTTTCAGAGGAGAGCAGAAGTTCAGAAGA | taaaACGTACCCACAGTCAGAGCAT |
| **RUNX1** +2 G del      | caccGCATTTCAGAGGAGAGCAGA  | gttgTCCTGCAACTACTTCGTCATG    | accgTAAGAGACTTGTTGAGGACTGAGA |
|                         | aaaaTCAGTTCTCTCTGAAAAATGC | aaaaATTTTCAGAGGAGAGCAGAAGTTCAGAAGA | taaaACGTACCCACAGTCAGAGCAT |
| **RUNX1** +2-4 GAT del  | caccGCATTTCAGAGGAGAGCAGA  | gttgTCCTGCAACTACTTCGTCATG    | accgTAAGAGACTTGTTGAGGACTGAGA |
|                         | aaaaTCAGTTCTCTCTGAAAAATGC | aaaaATTTTCAGAGGAGAGCAGAAGTTCAGAAGA | taaaACGTACCCACAGTCAGAGCAT |
| **RUNX1** +1 C to G     | caccGCATTTCAGAGGAGAGCAGA  | gttgTCCTGCAACTACTTCGTCATG    | accgTAAGAGACTTGTTGAGGACTGAGA |
|                         | aaaaTCAGTTCTCTCTGAAAAATGC | aaaaATTTTCAGAGGAGAGCAGAAGTTCAGAAGA | taaaACGTACCCACAGTCAGAGCAT |
RUNX1
+3 A to T
caccG CATT TTCAG GAGGAA GACGCA G
   gttg TGTCTGTAAGCAACGCGTCTCT C CTTGAAAAT
       accgATGAACACTGTTGGTGACGTA
   aaacT CGCCTTTCTCTGAAAAGTCC
   aaaaATTTTCTGGAAGAGCGCTG T GCTCTGACAGA

VEGFA
+4 C ins
caccGAT GTCTA CGAGGCCAGCATGAT
   gttgATGTCGCAATCTTGAGCCCGCT
   CATCATTGGCTGCAGAACACAT
   aaaaTCTGAGGCGGACATGAAACAGG GGCCTACAGGGACACATT

VEGFA
+2 ACA ins
caccGAT GTCTC ACGGACAGCATGAT
   gttgATGTCGCAATCTTGAGCCCGCT
   CATCATTGGCTGCAGAACACAT
   aaaaTCTGAGGCGGACATGAAACAGG GGCCTACAGGGACACATT

VEGFA
+3 A del
caccGAT GTCTC ACGGACAGCATGAT
   gttgATGTCGCAATCTTGAGCCCGCT
   CATCATTGGCTGCAGAACACAT
   aaaaTCTGAGGCGGACATGAAACAGG GGCCTACAGGGACACATT

VEGFA
+2 GAG del
caccGAT GTCTC ACGGACAGCATGAT
   gttgATGTCGCAATCTTGAGCCCGCT
   CATCATTGGCTGCAGAACACAT
   aaaaTCTGAGGCGGACATGAAACAGG GGCCTACAGGGACACATT

VEGFA
+1 T to G
caccGAT GTCTC ACGGACAGCATGAT
   gttgATGTCGCAATCTTGAGCCCGCT
   CATCATTGGCTGCAGAACACAT
   aaaaTCTGAGGCGGACATGAAACAGG GGCCTACAGGGACACATT

VEGFA
+2 G to A
caccGAT GTCTC ACGGACAGCATGAT
   gttgATGTCGCAATCTTGAGCCCGCT
   CATCATTGGCTGCAGAACACAT
   aaaaTCTGAGGCGGACATGAAACAGG GGCCTACAGGGACACATT

Chd2
+1 CTC ins
caccCGT GATGCTCCGAAGACGCTGTA
   gttgATGTCGCAATCTTGAGCCCGCT
   CATCATTGGCTGCAGAACACAT
   aaaaTCTGAGGCGGACATGAAACAGG GGCCTACAGGGACACATT

Chd2
+5 G to C
caccCGT GATGCTCCGAAGACGCTGTA
   gttgATGTCGCAATCTTGAGCCCGCT
   CATCATTGGCTGCAGAACACAT
   aaaaTCTGAGGCGGACATGAAACAGG GGCCTACAGGGACACATT

Col12a1
+1 GTG ins
caccGACTT CCATG GGCTCCACA A
   gttgATGTAACACTTGACGACAGCAT
   aaaaTTCCATGGGCTTCAATGCTGGAA
   aaaaTTTCCATTGGTACATGGACTG T GTC

Col12a1
+2 A to C
caccGACTT CCATG GGCTCCACA A
   gttgATGTAACACTTGACGACAGCAT
   aaaaTTCCATGGGCTTCAATGCTGGAA
   aaaaTTTCCATTGGTACATGGACTG T GTC

Col12a1
+3 CAA to ACC
caccGACTT CCATG GGCTCCACA A
   gttgATGTAACACTTGACGACAGCAT
   aaaaTTCCATGGGCTTCAATGCTGGAA
   aaaaTTTCCATTGGTACATGGACTG T GTC

Tyr
+1 TGT ins
caccGAAA AGAATGTGCACCCACC A
   gttgATCACCACATCCATGGGACCT G
   aaaaAGAATGTCCTGCTTGAAGAT GGTTGAT

Tyr
+6 G to A
caccGAAA AGAATGTGCACCCACC A
   gttgATCACCACATCCATGGGACCT G
   aaaaAGAATGTCCTGCTTGAAGAT GGTTGAT
   aaaaAGGATATCTTCTTGTCACCT

Tyr
HA-Tag

caccGTTTTC ATGAGATGTCACAGA
   gttgTCAGAGCCATCTGACCCCATACAAGCT
   aaaaCTTGGATAGATGCCATTCAACCACCAT G GAT GCTGTCGGA
   aaaaGAGGATACTTCTTGTCACCT

MisII
+1 CTT ins (Nick +48)
caccGCAAGT GGAAGATGTCGGA TAC
   gttgTCACAGCACGACATGAAACGCGGCTACATTC G
   aaaaTGGAATGTCGTCGAATGCTG T CAGT GTCGCTGGA
   aaaaAGCTGAACAGACATGCGCT G
   aaaaAGCTGAACAGACATGCGCT G

MisII
+1 A to G (Nick +48)
caccGCAAGT GGAAGATGTCGGA TAC
   gttgTCACAGCACGACATGAAACGCGGCTACATTC G
   aaaaTGGAATGTCGTCGAATGCTG T CAGT GTCGCTGGA
   aaaaAGCTGAACAGACATGCGCT G
   aaaaAGCTGAACAGACATGCGCT G

MisII
+1 ACA del (Nick +48)
caccGCAAGT GGAAGATGTCGGA TAC
   gttgTCACAGCACGACATGAAACGCGGCTACATTC G
   aaaaTGGAATGTCGTCGAATGCTG T CAGT GTCGCTGGA
   aaaaAGCTGAACAGACATGCGCT G
   aaaaAGCTGAACAGACATGCGCT G
| gRNA | Target Site | Oligo | gRNA Sequences | gRNA Sequences | gRNA Sequences |
|------|-------------|-------|----------------|----------------|----------------|
| **Mixl1** | +1 CTT ins (Nick -60) | caccGCAAGTGGATGTCTGGGTAC | gttgTCCGACAGACCATGTAagACCACGACCCATCAC | taaaGGATCTGGAGTCCGGTAC | acgcCTACCCGAGTCCAGGATCCgt |
| |  | aaaaGTGGATGTCTGGGTcttACATG | GTCGGTAGTCCGGA | | |
| **Mixl1** | +1 A to G (Nick -60) | caccGCAAGTGGATGTCTGGGTAC | gttgTCCGACAGACCATGtcACCCAGACATCCAC | taaaGGATCTGGAGTCCGGTAC | acgcCTACCCGAGTCCAGGATCCgt |
| |  | aaaaGTGGATGTCTGGGTcttACATG | GTCGGTAGTCCGGA | | |
| **Mixl1** | +1-3 ACA del (Nick -60) | caccGCAAGTGGATGTCTGGGTAC | gttgTCCGACAGACCATGtcACCCAGACATCCAC | taaaGGATCTGGAGTCCGGTAC | acgcCTACCCGAGTCCAGGATCCgt |
| |  | aaaaGTGGATGTCTGGGTcttACATG | GTCGGTAGTCCGGA | | |
| **EphB2** | loxP site 1 (R) | caccGCAATGGTCTCAAGGATAC | gttgTTCGAGTCTGGTGATACATCCAC | taaaGGATCTGGAGTCCGGTAC | acgcCTACCCGAGTCCAGGATCCgt |
| |  | | GTCGGTAGTCCGGA | | |
| **EphB2** | loxP site 2 (L2) | caccGCAAGTGGATGTCTGGGTAC | gttgTCCGACAGACCATGtcACCCAGACATCCAC | taaaGGATCTGGAGTCCGGTAC | acgcCTACCCGAGTCCAGGATCCgt |
| |  | aaaaGTGGATGTCTGGGTcttACATG | GTCGGTAGTCCGGA | | |
| **EphB2** | loxP site 3 (L3) | caccGCAATGGTCTCAAGGATAC | gttgTTCGAGTCTGGTGATACATCCAC | taaaGGATCTGGAGTCCGGTAC | acgcCTACCCGAGTCCAGGATCCgt |
| |  | | GTCGGTAGTCCGGA | | |
| **Cftr** | +1-3 CTT del | caccATCAAAGAAAATATCATCTTT | gttgATCATAGGAAAACACCATGATGTTTTTCTTTGAT | taaaACAAAGAAAATATCATGTCGGTAGT | acgcGGCCAGCAGGGCTTACGTCGGAGTGGG |
| |  | | GTCGGTAGTCCGGA | | |

*G*RNA sequences are underlined. Red highlight indicates extra G was added to the gRNA sequences. Oligo pair 3 for PEA1-Nuc targeting constructs used a sham targeting oligos which are the same oligos highlighted in blue.*
## Supplementary Table S2. Primers to generate IVT template of pegRNAs for mouse zygote injections.

| Target | Forward primer | Reverse primer |
|--------|----------------|----------------|
| Chd2 +1 CTC ins | TTAATACGACCTCACTATAGCGGGTAGCTCCCAGAA | aaaaTAGCTCCCAGAACGACGGTGACATC |
| Chd2 +5 G to C | TTAATACGACCTCACTATAGCGGGTAGCTCCCAGAA | aaaaTAGCTCCCAGAACGACGGTGACATC |
| Col12a1 +1 GTG ins | TTAATACGACCTCACTATAGTGACTTCCATGGTTCCACAA | aaaaTTCCATGGTGTTCAATGGGTCCCATT |
| Col12a1 +2 A to C | TTAATACGACCTCACTATAGTGACTTCCATGGTTCCACAA | aaaaTTCCATGGTGTTCAATGGGTCCCATT |
| Col12a1 +1-3 CAA to ACC | TTAATACGACCTCACTATAGTGACTTCCATGGTTCCACAA | aaaaTTCCATGGTGTTCAATGGGTCCCATT |
| Tyr +1 TGT ins | TTAATACGACCTCACTATAGCAAAAAGAATGCTGCCACCA | aaaaAGAATGCTGCCACATGGGTGAT |
| Tyr +6 G to A | TTAATACGACCTCACTATAGCAAAAAGAATGCTGCCACCA | aaaaAGAATGCTGCCACATGGGTGAT |
| Tyr HA-Tag | TTAATACGACCTCACTATAGTTTCCTAGGATGTTCACATTAAGCGTAATCTGGAACATC | aaaaCTAGGATGTTCAATGCGTAATCTGGGACATC |
| Cfr +1-3 CTT del | TTAATACGACCTCACTATAGATCAAAGAAAATATCATTGGTGTTTCCTATAGTTC | aaaaCAAAGAAAATATCATTGGTGTTTCCTATAGTTC |

T7 promoter sequences are highlighted in green. The reverse primers are the same as the bottom primers used for oligo pair 2 for generating PEA1 targeting constructs.
### Supplementary Table S3. List of PCR primers for sequencing.

| Target sites | Forward | Reverse |
|--------------|---------|---------|
| **HEK3**     | GGGAAAGCCCATGCAATTA | CAGAGATCAACCAGATTACCCCA |
| **RNF2**     | ACGTGGAAATTTTGTGGGACA | ACAGATGTGCAACCAACCATGGA |
| **RUNX1**    | AGAGAGATGTAGGGCTAGAGGG | CACTTGACAAGGTTCTCACCAC |
| **VEGFA**    | CTCCACAGTCGATACGTTGGG | CCCTAGTGACTGGCCGTCTG |
| **Chd2**     | CTGCGAGTCAAGGAGACTGG | CTCTCCATCACCTCAGGCT |
| **Col12a1**  | CAGTGTGAAGTCAAGTGGG | CAAAGAAGACAGAGTGGG |
| **Tyr**      | GTCTGTGACACTCATATACCTATTGCT | TCAACTGCGGAAACTGTAAGTGG |
| **Tyr-HA Tag** | GGAGCTGTATATGCTGAGICT | ACCAGCTCAATTAAGTGAAGAGG |
| **Mixl1**    | CGGTTCATCCCATCTCC | GACTTCCCAGCACCCTCCACT |
| **EphB2 LoxP site 1** | AGGTAGCCACCCACATGATC | AGGCTGGCATGCGTTAGTTC |
| **EphB2 LoxP site 1** | GACCACTCCACCAGTAAAGAAAGG | CAACCGATTGAGGGAACAG |
| **EphB2 LoxP site 1** | GCCAGGTGGATCTCTGAGTTTG | CCACCTCTGTCTATCTACTAGTCA |
| **Cftr**     | TCACAGCAATTAAATGAGGGGC | GGGATGATACCGGTACCCTGAGTGG |

For NGS PCRs, primers contain Nextera adapter sequences at the 5’ end. The adapter sequences for the forward primer are TCGTCGGCCACGGCTACAGTGATGTAGTGTAAGAGACAG. The adapter sequences for the reverse primer are GTCTCGGTGGGCTCGAGATGTGTAAGAGACAG.
Supplementary Note S1. One-step digestion-ligation protocol using PEA1 to generate PE targeting constructs.

Oligos for guide and RT template insertion into plasmid need to be of the following form:

**pegRNA guide:**

\[
\begin{align*}
5' & -\text{CACCGNNNNNNNNNNNNNNNNNNNN} - 3' \\
3' & -\text{CNNNNNNNNNNNNNNNNNNNNCAAA} - 5'
\end{align*}
\]

**pegRNA RT template**

\[
\begin{align*}
5' & -\text{GTGCNNNNNNNNNNNNNNNNNNNNNNNNNNNNN} - 3' \\
3' & -NNNNNNNNNNNNNNNNNNNNNNNNNNNNAAAT - 5'
\end{align*}
\]

**Second-nick guide**

\[
\begin{align*}
5' & -\text{ACCGNNNNNNNNNNNNNNNNNNNGT} - 3' \\
3' & -NNNNNNNNNNNNNNNNNNNNCAAAAT - 5'
\end{align*}
\]

If the first N on the top strand for each guide is a G, it should be excluded.

1. Mix the following reagents in a [PCR tube](#) for each of the two inserts:

| Reagent                                           | Amount |
|---------------------------------------------------|--------|
| MQ H₂O                                           | 6.5 µL |
| NEB T4 DNA Ligase Buffer with 10 mM ATP (10x)     | 1 µL   |
| top oligo (100 µM)                               | 1 µL   |
| bottom oligo (100 µM)                            | 1 µL   |
| NEB T4 PNK (10 U/µL)                             | 0.5 µL |
| **Total**                                        | **10 µL** |

2. Place each mixtures in thermocycler with the following parameters:

| Step | Temperature | Time |
|------|-------------|------|
| 1    | 37 °C       | 30 min |
| 2    | 95 °C       | 5 min  |
| 3    | Ramp to 25 °C @ 0.1 °C/s | ∞ |

3. Dilute the 3 sets of phospho-annealed oligos 1:250 with [MQ H₂O](#) in a [1.5 mL tube](#):

| Reagent                        | Amount |
|--------------------------------|--------|
| MQ H₂O                         | 249 µL |
| phospho-annealed oligo         | 1 µL   |
| **Total**                      | **250 µL** |

- For the guides, the N’s (typically 20 bases) in the two top strands comprise the guide sequence, which target the identical gRNA binding sequences followed by PAMs in the genomic DNA.
- The overhangs allow the oligos to bind the complementary overhanging DNA at the cut sites in the plasmid created by BbsI digestion.
- The U6 promoter is more efficient if it starts transcription with a G, this is the reason for the extra G/C in the first pair of oligos, this doubles as the first base in the guide which is why it should be excluded if the guide starts with a G. The G is also present as part of the overhang in the second pair of oligos.
- The extra GT/CA in the second pair of oligos completes the gRNA scaffold.
4. Mix the following reagents in a PCR tube:

| Reagent                      | Amount         |
|------------------------------|----------------|
| MQ H₂O                       | 10 µL          |
| PEA1 empty plasmid (100 ng/µL) | 1 µL          |
| phospho-annealed oligo pair 1 (1:250) | 1 µL |
| phospho-annealed oligo pair 2 (1:250) | 1 µL |
| phospho-annealed oligo pair 3 (1:250) | 1 µL |
| NEBuffer 2.1 (10x)           | 2 µL          |
| DTT (10 mM)                  | 1 µL          |
| ATP (10 mM)                  | 1 µL          |
| NEB BbsI (5 U/µL)            | 1 µL          |
| T4 DNA Ligase (400 U/µL)     | 1 µL          |
| Total                        | 20 µL         |

Note: We showed that removing NEBuffer 2.1, DTT, ATP components and replacing it with 2 ul of 10x T4 Ligase Buffer (add extra MQ to reach total volume of 20 ul) could lead to successful reaction.

5. Place in thermocycler with the following parameters:

| Step | Temperature °C | Time min |
|------|----------------|----------|
| 1    | 37             | 5        |
| 2    | 16             | 5        |
| 3    | Go to step 1   | 5 times  |

6. Transform to competent cells.
   Recommended: Incubating the reaction overnight at 4 C before transformation could lead to higher number of colonies.

7. Check digest using BbsI. Plasmids with complete integrations remain circular.

8. Sequence verify using primers GGTTCGCCACCTCTGACTTG and CACTCCCACTGCTTTCCTAATA.
Supplementary Note S2. Generation of pegRNA protocol.

Kit: NEB HiScribe™ T7 Quick High Yield RNA Synthesis.

1. T7 guide primer for PCR of gRNA oligo needs to be of the following form:
   \[5' - \text{TATAACGACTCACTATAGNNNNNNNNNNNNNNNNN} - 3'\]
   Where the N's are identical to your specific guide.

2. Reverse primer for PCR of gRNA oligo needs to be:
   \[5' - \text{AAAA} \text{NNNNNNNNNNNNNNNNNNN} - 3'\]
   Where the N's are the RT template sequence.

   This oligos are the same oligos used for RT template bottom oligos in the digestion-ligation protocol.

1. Generate the relevant PEA1 targeting plasmid (miniprep).
2. Mix the following master mix reagents in a 1.5 mL tube and aliquot into 6 PCR tubes:

| Reagent                                      | Amount | 6x MM |
|----------------------------------------------|--------|-------|
| MQ H2O                                       | 12.3 µL| 73.8 µL|
| NEB Phusion HF Reaction Buffer (5x)           | 4 µL   | 24 µL |
| T7 guide primer (10 µM)                       | 1 µL   | 6 µL  |
| Reverse primer (10 µM)                        | 1 µL   | 6 µL  |
| NTP Mix (10 mM)                               | 0.5 µL | 3 µL  |
| NEB Phusion HF DNA Polymerase (2 U/µL)        | 0.2 µL | 1.2 µL|
| 1 µL PEA1 targeting plasmid (~1-3 ng/µL)      | 1      | 6     |

   **Total** 20 µL 120 µL

3. Place the tubes in a thermocycler with the following parameters:

| 1    | 98 °C | 3 min |
| 2    | 98 °C | 15 s  |
| 3    | 60 °C | 20 s  |
| 4    | 72 °C | 15 s  |
| 5    | Go to step 2 | 32 times |
| 6    | 72 °C | 5 min |
| 7    | 4 °C  | ∞     |

4. Make a 1% agarose gel and run 5 µL of the PCR products:
   - Testing the plasmid has the correct insert.
   - Band should be present at ~100 bp.

5. Combine all PCR reactions and perform Qiagen PCR Purification in a single column.
6. Use NanoDrop to measure concentration of DNA.
   - Confirms the DNA is still present.

7. Perform IVT by mixing the following reagents in a PCR tube:

| Reagent                                      | Amount     |
|----------------------------------------------|------------|
| Nuclease-free MQ H2O                         | up to 40 µL|
| NEB NTP Buffer Mix (20 mM)                   | 20 µL      |
| Purified PCR product                         | ~1000 ng   |
| NEB T7 RNA Polymerase Mix                    | 4 µL       |

   **Total** ~40 µL

   Note: half reaction (total 20 ul) is also possible.

8. Incubate O/N @ 37 °C in thermocycler.
Note: 3 hours is also possible.

9. Transfer 2 µL to PCR tube for testing later.
10. Mix the following reagents in a PCR tube:

| Reagent                                         | Amount |
|-------------------------------------------------|--------|
| Nuclease-free MQ H₂O                            | 60 µL  |
| IVT gRNA product                                 | 40 µL  |
| NEB DNase I (RNase-free) (2 U/µL)                | 4 µL   |
| **Total**                                       | **104 µL** |

(Degrades DNA.)

11. Incubate 15 min @ 37 °C.
12. Transfer 2 µL to PCR tube for testing later.
13. Perform Qiagen RNEasy Mini Kit RNA Cleanup, eluting in 30 µL.
14. Check RNA on gel (RNase free technique should be applied).
Supplementary Note S3. Generation of nuclease prime editor mRNA protocol.

- **Linearize plasmid PE2-Nuc using Pme1**
  - MQ = X ul
  - Cut smart buffer = 6 ul
  - Plasmid = Y ul (10 ug)
  - Pme1 = 3 ul
  - Total 60 ul
  - Incubate 37 C for 2 hours

- **Purify the linearized plasmid using Zymo DNA clean and concentrator 5**
  - Add 200 ul binding buffer
  - Spin
  - Add 200 ul wash buffer spin
  - Repeat wash
  - Add 12 ul RNase-free water
  - Spin

- **Setup IVT using Mm message ultra kit**
  - T7 Arca = 10 ul
  - Buffer = 2 ul
  - Linearized plasmid = X ul (1.5-2 ug)
  - T7 enzyme = 2 ul
  - RNase-free water = Y ul (total 20 ul)
  - Incubate 37 C for 3 hours
  - Add 1 ul of DNAse, incubate 30 min 37 C
  - Add 36 ul water + 20 ul EPAP + 10 ul MnCl2 + 10 ul ATP (all included in the kit)
  - Take 2.5 ul for gel checking
  - Add 4 ul of EPAP enzyme, incubate 37 C for 20 min
  - Keep the reaction on ice
  - Take 2.5 ul for gel checking
  - Proceed to RNA clean up using RNAeasy kit (elute in 35 ul of water).

- **Zygote microinjection mix**
  - MQ = X ul
  - 10x injection buffer = 1.5 ul
  - Nuclease prime editor mRNA = Y ul (final 150 ng/ul)
  - pegRNA = Z ul (final 75 ng/ul)
  - Total = 15 ul

### 10X injection buffer

| Reagent                  | Amount     |
|--------------------------|------------|
| pH 8.0 EDTA (0.5 M)      | 10 µL      |
| pH 7.5 Tris (1 M)        | 500 µL     |
| Nuclease-free MQ H2O     | 4.49 mL    |
| **Total**                | **5 mL**   |

(Filter into aliquots in 1.5 mL tubes and stored @ - 20 °C.)
Supplementary Note S4. Data analysis using Rgenome PE-Analyzer.

% Correct PE
Correct PE can be directly gathered from the generated analysis.

| Prime editing |
|---------------|
| 64 (1.4%)     |

% Unmodified (WT)
WT frequency can be gathered directly by clicking the WT column. Ensure you don’t filter any sequence here.

% Unintended edits = 100 - % Correct PE - % WT

% Any intended edits: any alleles containing prime edited sequences (modified and/or unmodified)
To get total PE, filter sequences starting from PBS to 2nt of the edit.

The sequence list to filter to get total PE:
**HEK3:**
+1 A ins: CAGACTGAGCAGaT
+1 CTT ins: CAGACTGAGCAGcT
+1 T del: CAGACTGAGCACGGA
+1-3 TGA del: CAGACTGAGCACGTGG
+1 T to G PM: CAGACTGAGCAGGgG
+2 G to C PM: CAGACTGAGCACGTcA

**RNF2:**
+1 T ins: GaGTAATGACTAAGATG
+1 GTA ins: acGTAATGACTAAGATG
+4 A del: CCCAGGTAATGACTAAGATG
+3-5 GAG del: ACAGGTAATGACTAAGATG
+1 C to G PM: AcGTAATGACTAAGATG
+2 T to A PM: CtGGTAATGACTAAGATG

**RUNX1:**
+1 C ins: ATTTTCAGGAGGAAGCcC
+1 ATG ins: ATTTTCAGGAGGAAGCat
+2 G del: ATTTTCAGGAGGAAGCAT
+2-4 GAT del: ATTTTCAGGAGGAAGCGG
+1 C to G PM: ATTTTCAGGAGGAAGgG
+3 A to T PM: ATTTTCAGGAGGAAGCGT

**VEGFA:**
+4 C ins: TCTGCAGGCCAGATGAGcG
+2 ACA ins: TCTGCAGGCCAGATGacG
+3 A del: TCTGCAGGCCAGATGG
+2 G to A PM: TCTGCAGGCCAGATGacG

Mouse ES cells
Mixl1 +1 CTT ins: agACCCAGACATCCAC
Mixl1 +1-3 ACA del: CAACCCAGACATCCAC
Mixl1 +1 A to G: GcACCCAGACATCCAC

**Tyr** +1 TGT ins: AGAATGCTGCCCAtg
Tyr +6 G to A: AGAATGCTGCCCACCATGaA

Chd2 +1 CTC ins: agGTTCAGGAGCTA
Chd2 +5 G to C: GcCACCAGTCTGGGAGCTA

Col12a1 +1 GTG ins: TTCCATGGTTCACagt
Col12a1 +2 A to C: TTCCATGGTTCACCaA

% Any loxP: any alleles containing loxP sequences (modified and/or unmodified)
LoxP site 1 ATAACTTCGTATAATGTATGCTATACGAAGTTATcaattg
LoxP site 2 ATAACTTCGTATAATGTATGCTATACGAAGTTATgatatc
LoxP site 3 ATAACTTCGTATAATGTATGCTATACGAAGTTATgatatc

% Partial template duplications (PTDs)
To get the frequency of PTDs, filter the same sequences above into the “insertions” column.

If the edit is substitution or deletion, collect the frequency of PTD straight away from the count.

If the edit is insertion, the PTD = the count – the correct PE.

% Indels = % unintended edits - %PTDs