Supplemental Figure 1. Related to Figure 1. A. Diagram of PE2 expression plasmid *pAct-PE2*. **B.** Schematic of experiments to detect and quantify insertion events in transfected S2R+ cells. **C.** DNA gel images of targeted PCR amplification of the insertion site. **D**. Sequence structure of 23bpBC insertion in *ebony* with sanger sequence chromatogram of PCR amplified insert. Features include the binding sites of ebony_F and BC_R (see B and C), pegRNA spacer, 23bpBC insertion site, and PAM. Asterisk indicates possible PCR or sequencing error. **E.** Approximate quantification of precise $ebony^{23bpBC}$ insertion and indel percentage from S2R+ transfection experiments by amplicon sequencing. **F.** *white* and *forked* genomic region showing target site and edits ($white^{A134X}$ and $forked^{D111X}$).

Supplemental Figure 2. Related to Figure 2. Images of adult flies with somatic editing using *tub>PE2*. Views of the dorsal side of whole adults (top), scutellum (middle), and eye (bottom). Negative control is *attP40* and classical loss of function allele shown on right. Females shown for editing of *ebony* and *forked*, males shown for *white* editing.

Supplemental Figure 3. Related to Figure 3. A. Diagram of PE2 expression transgene *nos-PE2*. *nos, nanos*; NLS, Nuclear localization sequence; 3' UTR, nanos 3' UTR; *v+*, *vermillion+* rescue transgene; *attB*, phiC31 recombination site. **B-E.** Quantification of *ebony* transmission and edit type using transgenic crossing. pegRNA only = $pCFD3\text{-}PE\text{-}ebony^{G111X}$, pegRNA + sgRNA = $pCFD5\text{-}PE3\text{-}ebony^{G111X}$. Sex of G1 parents and sample size indicated on graph unless otherwise noted. **B-C.** Quantification G2 *ebony* progeny transmitted from single G1 crosses. Results from all single G1 crosses shown. Sample size of progeny counted for each cross is 76 or higher. **D-E.** Quantification of sequenced edit types in individual G2 flies from single G1 crosses.

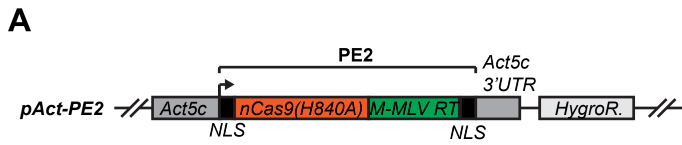
Supplemental Figure 4. Related to Figure 3. A-B. Quantification of *ebony* transmission and edit type using embryo injection of plasmid DNA or synthetic pegRNA. **A.** Quantification G2 *ebony* progeny transmitted from single G1 crosses. Results from all single G1 crosses shown. Sample size of progeny counted for each cross is 41 or higher. **B.** Quantification of sequenced edit types in individual G2 flies from single G1 crosses.

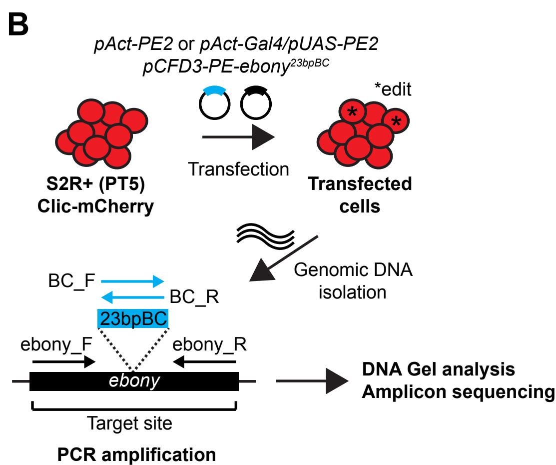
Supplemental Figure 5. Related to Figure 3. A. Quantification of single G1 flies that transmit at least one *ebony* progeny (designated a founder) using *nos>PE2* II or *nos>PE2* III. **B-C.** Quantification G2 *ebony* progeny transmitted from single G1 crosses in (**A**). **B.** Summed data from single G1 crosses. **C.** Data from all single G1 crosses. Sample size of progeny counted for each cross is 54 or higher.

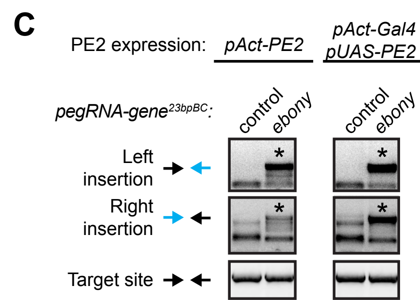
Supplemental File 1. pegRNA and sgRNA sequences

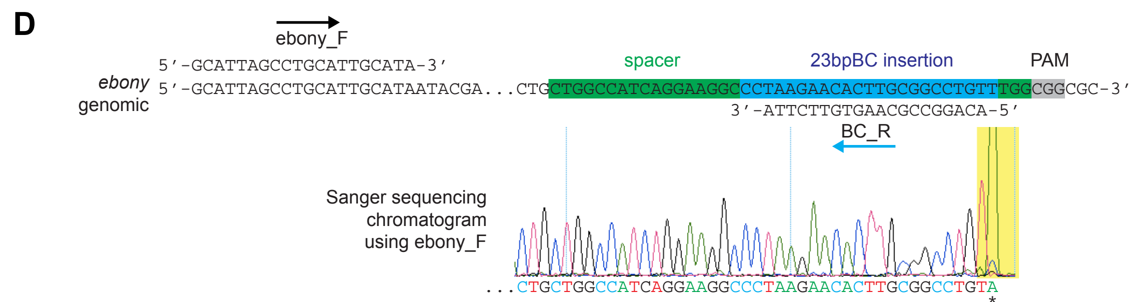
Supplemental File 2. Oligo and dsDNA sequences

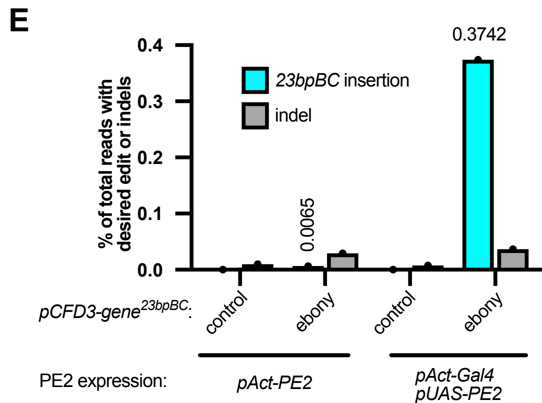
Supplemental File 3. pegRNA design and cloning protocols

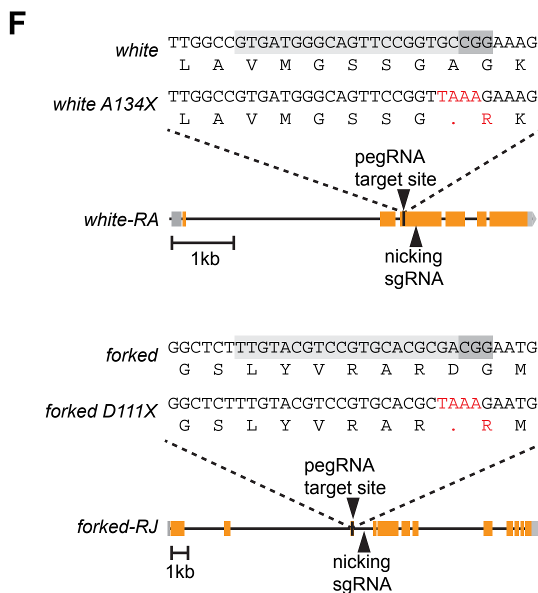




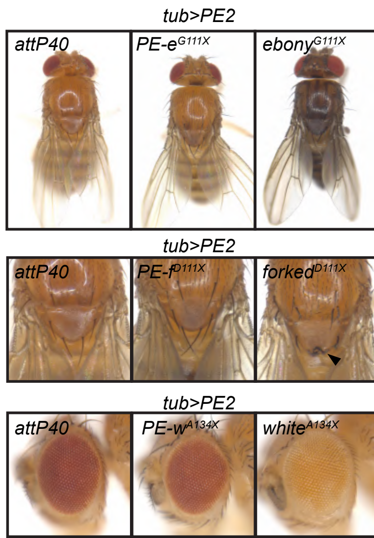






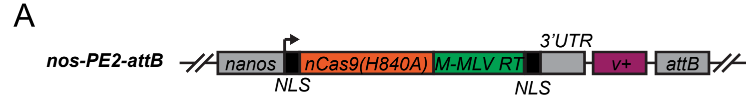


Supplemental Figure 1



Supplemental Figure 2

A



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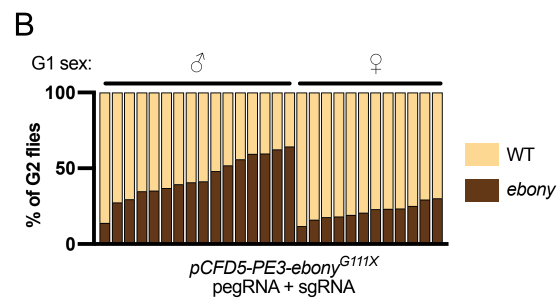

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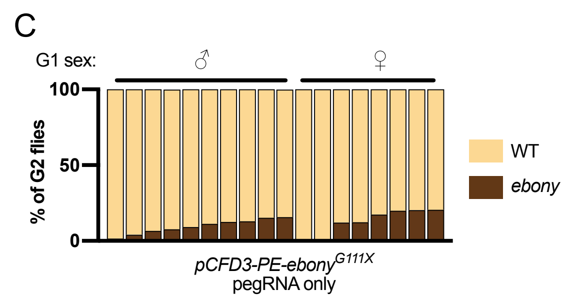
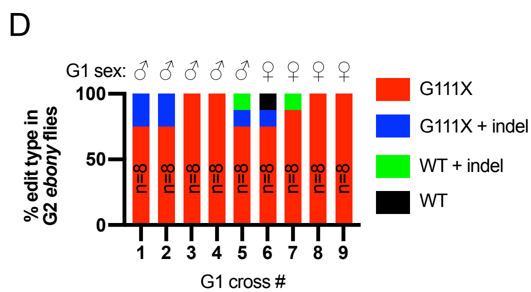
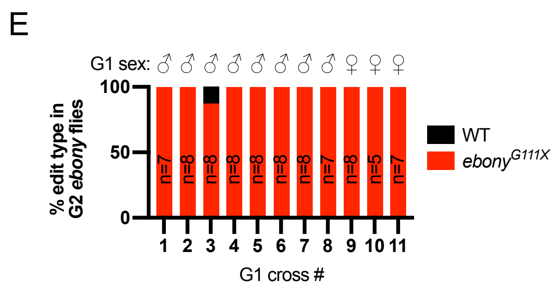


D



E



Supplemental Figure 3

A

% of G2 flies
100
50
0

WT
ebony

Conc. (ng/ul)
1000 800 600 400 200 1000 800 600 400 200

pCFD3-PE-ebony^{G111X}
pegRNA only

pCFD5-PE3-ebony^{G111X}
pegRNA + sgRNA

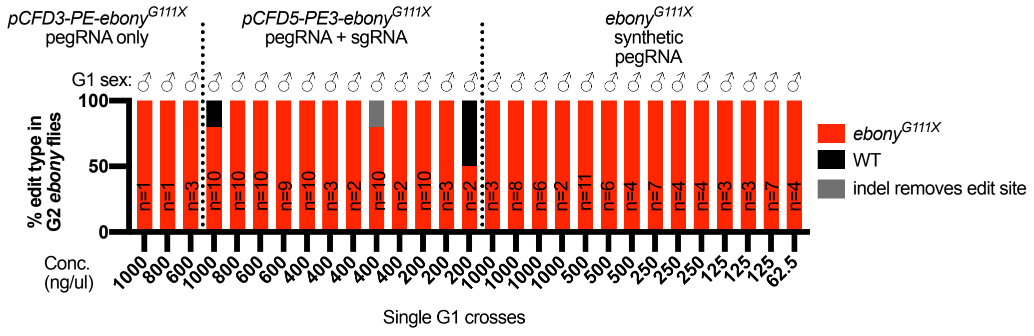
% of G2 flies
100
50
0

WT
ebony

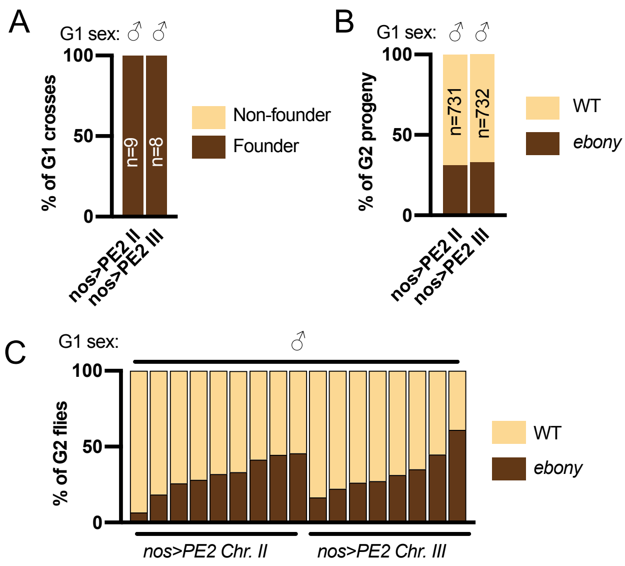
Conc. (ng/ul)
1000 500 250 125 62.5 31.25

ebony^{G111X}
synthetic pegRNA

B



Supplemental Figure 4



Supplemental Figure 5

Supplemental File 1 - pegRNA and sgRNA sequences						
pegRNA	**pegRNA spacer sequence**	**3' extension**	**PBS length**	**RT template length**	**nicking sgRNA**	**nicking sgRNA spacer sequence**
ebony_pFP545_23bpBC	CTGGCCATCTGGAAGGCTGG	GAAGCTGGGATCGATGGGCAAATACGCGCCGCCAaacaggccgcaagtgttcttaggGCCTTCCAGATGG	13	34		
ebony_pFP545_G111X	CTGGCCATCTGGAAGGCTGG	GGCAAATACGCGCtttaAGCCTTCCAGATGG	13	18	ebony_+57	GCTTCGCCTCCAGCAGTATG
white_ex3-3_A134X	GTGATGGGCAGTTCCGGTGC	AGGGTCGTCTTTCtttaACCGGAACTGCCCA	13	18	white_+70	TTGAGCAGTCGCATCCCGGA
forked_pFP801_D111X	TTGTACGTCCGTGCACGCGA	ATCGGTGCCATTCtttaGCGTGCACGGACGT	13	18	forked_+57	ATCTACTCACCATCCATTTG

Supplemental File 2 - Oligo and dsDNA sequences		
Name	**Sequence**	**Purpose**
JB1633_pCFD3-NS_gBlock_BbsI-XbaI	agttcgtatatatagacctattttcaatttaacgtcggggtcttcgagaagacctttttttgcctacctggagcctgagagttgttcaataaaataaaaatgtttcgtttttttgctttcgccagtatttattatttttcatcaatatgtattcaatttggtatgtatttagtaattgtaatatatagacaatggttttccgttgacgtacatacatctgacgtgtgtttatttagacataatagttatgttttcacatctttttaatgttcgcttaatgcgtatgcattctagacaattgtgctcggcaacagtatatttgt	gBlock to create pCFD3-NS
JB1690_gBlock_pCFD5-NS	agttcgtatatatagacctattttcaatttaacgtcggggctttgagtgtgtgtagacatcaagcatcggtggttcagtggtagaatgctcgcctgccacgcgggcggcccgggttcgattcccggccgatgcagggtcttcgagaagacctttttttgcctacctggagcctgagagttgttcaataaaataaaaatgtttcgtttttttgctttcgccagtatttattatttttcatcaatatgtattcaatttggtatgtatttagtaattgtaatatatagacaatggttttccgttgacgtacatacatctgacgtgtgtttatttagacataatagttatgttttcacatctttttaatgttcgcttaatgcgtatgcattctagacaattgtgctcggcaacagtatatttgt	gBlock to create pCFD5-NS
JB265_Gibson_pEntr_1F	AAGGGTGGGCGCGCCGAC	Amplifies pEntr backbone for Gibson assembly
JB266_Gibson_pEntr_1R	GGTGAAGGGGGCGGCCGC	Amplifies pEntr backbone for Gibson assembly
JB1615_PE2_pEntr_F	ccgcggccgccccccttcaccatgaaacggacagccgac	Amplifies PE2 coding sequence to assemble with pEntr backbone by Gibson
JB1616_PE2_stop_pEntr_R	gggtcggcgcgcccaccctttagactttcctcttcttcttggg	Amplifies PE2 coding sequence to assemble with pEntr backbone by Gibson
JB1613_PE2-nosbackbone_XbaI-AvrII_F	TCGCCtgAATTgagatctcTCTAGAggtacCGCCACCatgaaacggacagccgac	Amplifies PE2 coding sequence to assemble with pNos backbone by Gibson
JB1614_PE2-nosbackbone_XbaI-AvrII_R	TAAACCTcgagtggatcctcctaggtgctagAttagactttcctcttcttcttggg	Amplifies PE2 coding sequence to assemble with pNos backbone by Gibson
JB157_Gibson_MT-GW_backbone_1F	ACCGAGAGCATCTGGCCA	Amplifies MK33-GW backbone to assemble with Actin5c promoter
JB158_Gibson_MT-GW_backbone_1R	GATCCAGACATGATAAGATACATTGATGAG	Amplifies MK33-GW backbone to assemble with Actin5c promoter
JB159_Gibson_MT-GW_actP_1F	tatcttatcatgtctggatcGCATGCAATTCTATATTCTAAAAACACAAATG	Amplifies Actin5c promoter to insert into MK33-GW by Gibson
JB160_Gibson_MT-GW_actP_1R	attggccagatgctctcggtATCTGGATCCGGGGTCTC	Amplifies Actin5c promoter to insert into MK33-GW by Gibson
ebony_targetsite_F	GAGGGATTGGTCACCACACT	Amplifies indicated target site from genomic DNA, used to amplify in ebony23bpBC experiments
ebony_targetsite_2F	CCGGTTCCTGCAGCCAAACA	Amplifies indicated target site from genomic DNA, used to amplify in ebonyG111X experiments
ebony_targetsite_R	GGGATTTGGCATACAGTTCG	Amplifies indicated target site from genomic DNA
white_targetsite_F	TTCGCAGTCGGCTGATCTGT	Amplifies indicated target site from genomic DNA
white_targetsite_R	CACAGGTTGGCCATTGAGCA	Amplifies indicated target site from genomic DNA
forked_targetsite_F	ACGATGTCACGCCCGTTTAC	Amplifies indicated target site from genomic DNA
forked_targetsite_R	CAACTGCTGCAGTTGGCCAA	Amplifies indicated target site from genomic DNA
JB1647_BC_F	TAAGAACACTTGCGGCCTGT	Binds to inserted 23bp BC for amplification of insertions
JB1647_BC_R	ACAGGCCGCAAGTGTTCTTA	Binds to inserted 23bp BC for amplification of insertions
JB1637_scaffold_top	GTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCG	sgRNA scaffold for annealing and cloning a pegRNA into pCFD3-NS
JB1638_scaffold_bot	GCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTA	sgRNA scaffold for annealing and cloning a pegRNA into pCFD3-NS
JB1639_ebony_pFP545_spacer_top	gtcgCTGGCCATCTGGAAGGCTGG	ebony_23bpBC pegRNA spacer for annealing and cloning into pCFD3-NS
JB1640_ebony_pFP545_spacer_bot	aaacCCAGCCTTCCAGATGGCCAG	ebony_23bpBC pegRNA spacer for annealing and cloning into pCFD3-NS
JB1641_ebony_pFP545_3'ext_top	gtgcGAAGCTGGGATCGATGGGCAAATACGCGCCGCCAAACAGGCCGCAAGTGTTCTTAGGGCCTTCCAGATGG	ebony_23bpBC pegRNA 3'extension for annealing and cloning into pCFD3-NS
JB1642_ebony_pFP545_3'ext_bot	aaaaCCATCTGGAAGGCCCTAAGAACACTTGCGGCCTGTTTGGCGGCGCGTATTTGCCCATCGATCCCAGCTTC	ebony_23bpBC pegRNA 3'extension for annealing and cloning into pCFD3-NS
JB1721_pCFD5-NS_ebony_pFP545_G111X_PE3_gBlock1	cgggttcgattcccggccgatgcagcttcgcctccagcagtatggttttagagctagaaatagcaagttaaaataaggctagtccgttatcaacttgaaaaagtggcaccgagtcggtgctaacaaagcaccagtggtctagtggtagaatagtaccctgccacggtacagacc	ebonyG111X sgRNA-tRNA-pegRNA to clone into pCFD5-NS
JB1724_pCFD5-NS_ebony_pFP545_G111X_PE3_gBlock2	taacaaagcaccagtggtctagtggtagaatagtaccctgccacggtacagacccgggttcgattcccggctggtgcactggccatctggaaggctgggttttagagctagaaatagcaagttaaaataaggctagtccgttatcaacttgaaaaagtggcaccgagtcggtgcGGCAAATACGCGCtttaAGCCTTCCAGATGGtttttttgcctacctggagcctgag	ebonyG111X sgRNA-tRNA-pegRNA to clone into pCFD5-NS
JB1722_pCFD5-NS_white_ex3-3_A134X_PE3_gBlock1	cgggttcgattcccggccgatgcattgagcagtcgcatcccggagttttagagctagaaatagcaagttaaaataaggctagtccgttatcaacttgaaaaagtggcaccgagtcggtgctaacaaagcaccagtggtctagtggtagaatagtaccctgccacggtacagacc	whiteA134X sgRNA-tRNA-pegRNA to clone into pCFD5-NS
JB1725_pCFD5-NS_white_ex3-3_A134X_PE3_gBlock2	taacaaagcaccagtggtctagtggtagaatagtaccctgccacggtacagacccgggttcgattcccggctggtgcagtgatgggcagttccggtgcgttttagagctagaaatagcaagttaaaataaggctagtccgttatcaacttgaaaaagtggcaccgagtcggtgcAGGGTCGTCTTTCtttaACCGGAACTGCCCAtttttttgcctacctggagcctgag	whiteA134X sgRNA-tRNA-pegRNA to clone into pCFD5-NS
JB1723_pCFD5-NS_forked_pFP801_D111X_PE3_gBlock1	cgggttcgattcccggccgatgcaatctactcaccatccatttggttttagagctagaaatagcaagttaaaataaggctagtccgttatcaacttgaaaaagtggcaccgagtcggtgctaacaaagcaccagtggtctagtggtagaatagtaccctgccacggtacagacc	forkedD111X sgRNA-tRNA-pegRNA to clone into pCFD5-NS
JB1726_pCFD5-NS_forked_pFP801_D111X_PE3_gBlock2	taacaaagcaccagtggtctagtggtagaatagtaccctgccacggtacagacccgggttcgattcccggctggtgcattgtacgtccgtgcacgcgagttttagagctagaaatagcaagttaaaataaggctagtccgttatcaacttgaaaaagtggcaccgagtcggtgcATCGGTGCCATTCtttaGCGTGCACGGACGTtttttttgcctacctggagcctgag	forkedD111X sgRNA-tRNA-pegRNA to clone into pCFD5-NS

Supplemental File 3
pegRNA cloning for Prime Editing in *Drosophila*, Nov. 2020, Version 1.0
Justin Bosch, Perrimon Lab, Harvard Medical School

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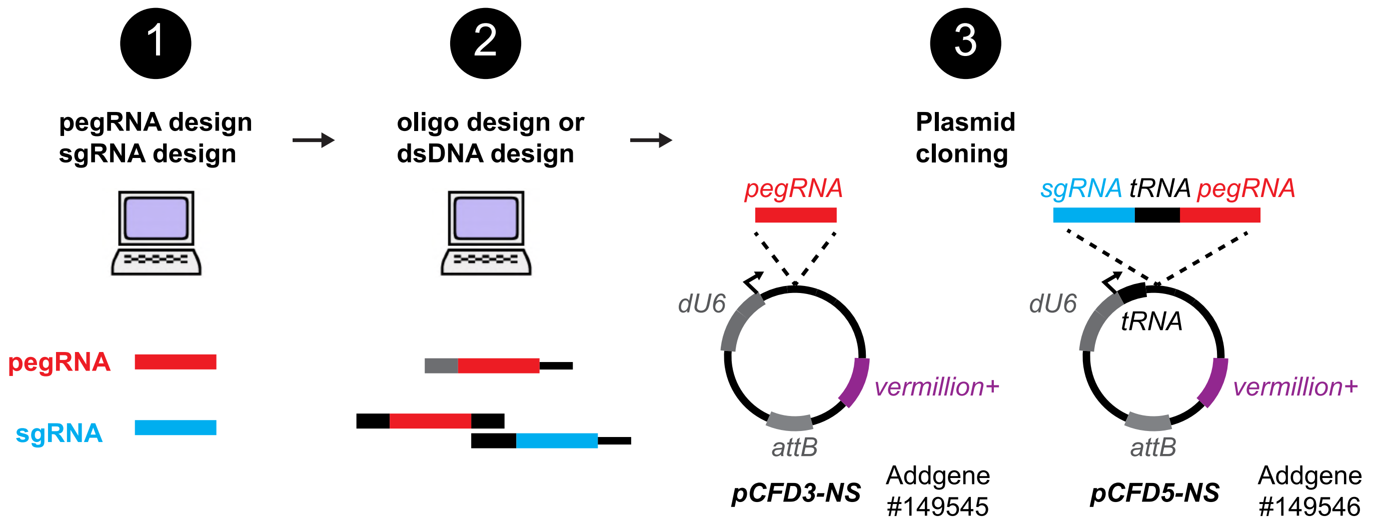
B. pegRNA and nicking sgRNA design
C. Oligo and dsDNA design
D. Cloning protocol for *pCFD3-NS* using annealed oligos
E. Cloning protocol for *pCFD3-NS* using a dsDNA fragment
F. Cloning protocol for *pCFD5-NS* using two dsDNA fragments

A. Introduction: These protocols are used to assemble plasmids to express pegRNAs under the control of the *Drosophila U6-3* promoter. pegRNAs are designed to make a precise edit in the genome, and optional nicking sgRNAs are designed to enhance prime editing efficiency (PE3 system). To express pegRNAs and sgRNAs, they are encoded in annealed oligos or dsDNA fragments, and then cloned into one of two empty expression plasmids. *pCFD3-NS* is used for expression of a single pegRNA. *pCFD5-NS* is used for expression of a pegRNA/sgRNA pair. *pCFD3-NS* and *pCFD5-NS* do not contain a sgRNA scaffold (NS = No Scaffold), and are slight modifications of the sgRNA-expression plasmids *pCFD3* and *pCFD5* (1, 2). *pCFD3-NS* and *pCFD5-NS* contain an *attB* site for phiC31 integration and a *vermillion+* marker to select transgenic flies.



Summary of pegRNA-expression plasmids:

Plasmid	Addgene #	Promoter	Used to express	Cloning methods	Fly marker	Bacterial resistance
pCFD3-NS	149545	*dU6:3*	pegRNA	Annealed oligos/T4 Ligase 1 dsDNA fragment/Gibson	*vermillion+*	Ampicillin
pCFD5-NS	149546	*dU6:3*	sgRNA + pegRNA	2 dsDNA fragments/Gibson	*vermillion+*	Ampicillin

B. pegRNA and nicking sgRNA design

Automatic design (recommended):

PrimeDesign (3): http://primedesign.pinellolab.org/
pegFinder (4): http://pegfinder.sidichenlab.org/

Manual design (optional):

1. Create wild-type (WT) and edited sequence files for annotation
2. WT sequence - select a pegRNA spacer near the desired edit, ensuring the edit is 3' to nick site.
3. Edited sequence - annotate the primer binding site (PBS) by selecting ~13bp 5' to the nick site.
4. Edited sequence - annotate the reverse transcribed (RT) region by selecting ~13-18bp 3' to nick site.
5. Edited sequence – The reverse complement of the PBS-edit-RT sequence is the pegRNA 3' extension.
6. WT sequence - select a sgRNA target on the non-edited strand between +40 and +90 from the pegRNA nick.

Notes:

- Avoid starting pegRNA 3' extension with a "C".
- Edits or silent mutations that affect the PAM or pegRNA spacer sequence increase efficiency.
- Use a shorter RT sequence if region has high G:C content.

Example pegRNA and nicking sgRNA design:

pegRNA spacer
nicking sgRNA spacer
PAM
nick = |
PBS
RT
edit
scaffold

>ebony_WT
CCGGTTCCTGCAGCCAAACAGCGATGGTGACTTCATCGTGGCTGTGTGCATGCAGCCGTCGGAGGGATTGGTCACCACACT
GCTGGCCATCTGGAAGGC|TGGCGGCGCGTATTTGCCCATCGATCCCAGCTTCCCGGCGAACCGCATTCACCACAT|ACTG
CTGGAGGCGAAGCCCACCTTGGTGATTCGCGACGATGACATCGACGCCGGCCGTTTCCAGGGAACTCCCACGTTATCCACC
ACCGAACTGTATGCCAAATCCC

>ebony_GGCG331-334TAAA_G111X
CCGGTTCCTGCAGCCAAACAGCGATGGTGACTTCATCGTGGCTGTGTGCATGCAGCCGTCGGAGGGATTGGTCACCACACT
GCTGGCCATCTGGAAGGCTtaaaGCGCGTATTTGCCCATCGATCCCAGCTTCCCGGCGAACCGCATTCACCACATACTGCT
GGAGGCGAAGCCCACCTTGGTGATTCGCGACGATGACATCGACGCCGGCCGTTTCCAGGGAACTCCCACGTTATCCACCAC
CGAACTGTATGCCAAATCCC

>pegRNA_spacer
CTGGCCATCTGGAAGGCTGG

>pegRNA_extension
GGCAAATACGCGCtttaAGCCTTCCAGATGG

>nicking_sgRNA_spacer
GCTTCGCCTCCAGCAGTATG

>pegRNA
CTGGCCATCTGGAAGGCTGGGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTG
GCACCGAGTCGGTGCGGCAAATACGCGCtttaAGCCTTCCAGATGG

>nicking_sgRNA
GCTTCGCCTCCAGCAGTATGGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTG
GCACCGAGTCGGTGC

C. Oligo and dsDNA design

C1. For cloning into pCFD3-NS by T4 ligation (single pegRNA) (See section D)

Order oligos with overhangs (5’ lowercase sequence)

```
>pegRNA_spacer_top
gtcgCTGGCCATCTGGAAGGCTGG

>pegRNA_spacer_bot
aaacCCAGCCTTCCAGATGGCCAG

>Scaffold_top:
gtttTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCG

>Scaffold_bot:
gcacCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTA

>pegRNA_extension_top
gtgcGGCAAATACGCGCtttaAGCCTTCCAGATGG

>pegRNA_extension_bot
aaaaCCATCTGGAAGGCTtaaaGCGCGTATTTGCC
```

Annealed oligos:

```
>pegRNA_spacer
5’-gtcgCTGGCCATCTGGAAGGCTGG-3’
    3’-GACCGGTAGACCTTCCGACCcaaa-5’

>Scaffold
5’-gtttTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCG-3’
    3’-ATCTCGATCTTTATCGTTCAATTTTATTCCGATCAGGCAATAGTTGAACTTTTTCACCGTGGCTCAGCcacg-5’

>pegRNA_extension
5’-gtgcGGCAAATACGCGCtttaAGCCTTCCAGATGG-3’
    3’-CCGTTTATGCGCGaaatTCGGAAGGTCTACCaaaa-5’
```

Cloning:

```
>pCFD3-NS cut w/ BbsI
5’-agacctattttcaatttaac          tttttgcctacctggagcctgag-3’
3’-tctggataaaagttaaattgcagc          aacggatggacctcggactc-5’

>pCFD3-pegRNA_final
agacctattttcaatttaacgtcgCTGGCCATCTGGAAGGCTGGGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGC
TAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGCGGCAAATACGCGCtttaAGCCTTCCAGATGGtttttg
cctacctggagcctgag
```

C2. dsDNA to clone into pCFD3-NS by Gibson assembly (single pegRNA) (See section E)

Append homology arms (black, lowercase) to pegRNA that overlap with pCFD3-NS cut w/ BbsI.

```
>dsDNA_fragment_pCFD3-NS
agacctattttcaatttaacgtcgCTGGCCATCTGGAAGGCTGGGTTTTAGAGCTAGAAATAGCAAGTTAAAATAA
GGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGCGGCAAATACGCGCtttaAGCCTTCCAGATGGt
tttttgcctacctggagcctgag
```

<u>Cloning:</u>

```
>pCFD3-NS cut w/ BbsI
5'-agacctattttcaatttaac          tttttgcctacctggagcctgag-3'
3'-tctggataaaagttaaattgcagc          aacggatggacctcggactc-5'
```

>pCFD3-pegRNA_final
agacctattttcaatttaacgtcgCTGGCCATCTGGAAGGCTGGGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGC
TAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGCGGCAAATACGCGCtttaAGCCTTCCAGATGGtttttg
cctacctggagcctgag

Note: If needed, homology arms can be extended longer (~100bp each). This can help decrease complexity scores using IDT gBlocks.

C3. dsDNAs to clone into pCFD5-NS by Gibson assembly (nicking sgRNA and pegRNA) (See section F)

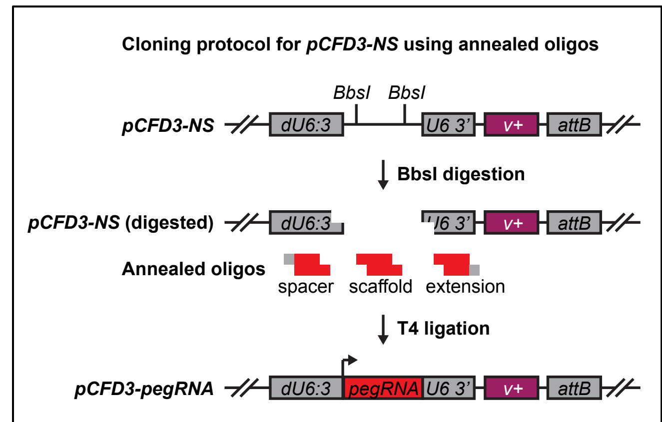
<u>Append homology arms (black, lowercase) to nicking sgRNA and pegRNA that overlap with pCFD3-NS cut w/ BbsI and encode rice Os-tRNA^{Gly} (lowercase, italic)</u>

>dsDNA_fragment1_pCFD5-NS
cgggttcgattcccggccgatgcaGCTTCGCCTCCAGCAGTATGGTTTTAGAGCTAGAAATAGCAAGTTAAAATAA
GGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGC*aacaaagcaccagtggtctagtggtagaatag*
taccctgccacggtacagacc

>dsDNA_fragment2_pCFD5-NS
aacaaagcaccagtggtctagtggtagaatagtaccctgccacggtacagacccgggttcgattcccggctggtgc
*a*CTGGCCATCTGGAAGGCTGGGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAA
AAAGTGGCACCGAGTCGGTGCGGCAAATACGCGCtttaAGCCTTCCAGATGGtttttgcctacctggagcctgag

>pCFD5-sgRNA-tRNA-pegRNA_final
cgggttcgattcccggccgatgcaGCTTCGCCTCCAGCAGTATGGTTTTAGAGCTAGAAATAGCAAGTTAAAATAA
GGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGC*aacaaagcaccagtggtctagtggtagaatag*
*taccctgccacggtacagacccgggttcgattcccggctggtgca*CTGGCCATCTGGAAGGCTGGGTTTTAGAGCT
AGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGCGGCAAATACGC
GCtttaAGCCTTCCAGATGGtttttgcctacctggagcctgag

D. Cloning protocol for *pCFD3-NS* (Addgene # 149545) using annealed oligos



D1. Design pegRNA and order oligos (see Sections B&C).

D2. Digest/dephosphorylate *pCFD3-NS*

5μg pCFD3-NS
3μl BpiI (cuts BbsI) (Fermentas, FD1014)
3μl FastAP (Fermentas, EF0651)
6μl 10x FastDigest Buffer
Xμl H20
60ul total

D3. Gel-purify digested *pCFD3-NS* backbone (~6.2kb).

D4. Phosphorylate and anneal each pair of oligos in PCR tubes

1μl Top oligo (100μM)
1μl Bottom oligo (100μM)
1μl 10x T4 Ligation buffer (NEB, B0202S)
6.5μl H20
.5μl T4 PNK (NEB, M0201)
10μl total

37˚C for 30min, 95˚C for 5min, then ramp down to 25˚C at 5˚C/min

D5. Dilute annealed/phosphorylated oligos 1:200 in H20

D6. Ligate annealed oligos into digested *pCFD3-NS*

Xμl digested pCFD3-NS (50ng)
1μl **spacer** diluted annealed oligo
1μl **scaffold** diluted annealed oligo
1μl **3' extension** diluted annealed oligo
1.5μl 10x T4 Ligation Buffer (NEB, B0202S)
Xμl H20
1μl T4 DNA ligase (NEB, M0202)
15μl total

Incubate reaction at room temperature for 30min.

D7. Transform ligation into competent cells and grow colonies on LB-agar Ampicillin plates

D8. (Optional) Colony PCR to identify candidate pegRNA plasmids

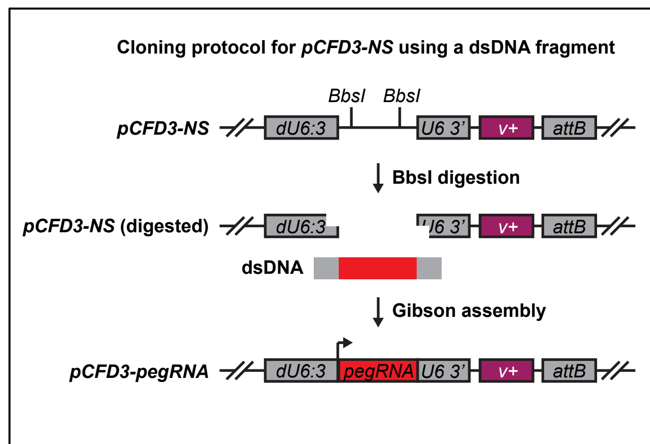
pCFD3genoF ACGTTTTATAACTTATGCCCCTAAG
pCFD3genoR GCCGAGCACAATTGTCTAGAATGC

Uncut backbone = 490bp
Correct insert = 638bp (depends on pegRNA length)

D9. Culture colonies with LB + Ampicillin and sequence confirm plasmids

pCFD3seqF ACCTACTCAGCCAAGAGGC

E. Cloning protocol for *pCFD3-NS* (Addgene # 149545) using a dsDNA fragment



E1. Design pegRNA and order dsDNA fragment (see Sections B&C).

E2. Digest/dephosphorylate plasmid

5µg *pCFD3-NS*
3µl BpiI (cuts BbsI) (Fermentas, FD1014)
3µl FastAP (Fermentas, EF0651)
6µl 10x FastDigest Buffer
Xµl H20
60ul total

E3. Gel-purify digested *pCFD3-NS* backbone (~6.2kb).

E4. Gibson assembly

Xµl digested pCFD3-NS (50ng)
Xµl dsDNA fragment (5ng)
2.5µl Gibson master mix (NEB, E2611)
Xµl H20
5µl total

Incubate reaction at 50˚C for 30min.

E5. Transform ligation into competent cells and grow colonies on LB-agar Ampicillin plates

E6. (Optional) Colony PCR to identify candidate pegRNA plasmids

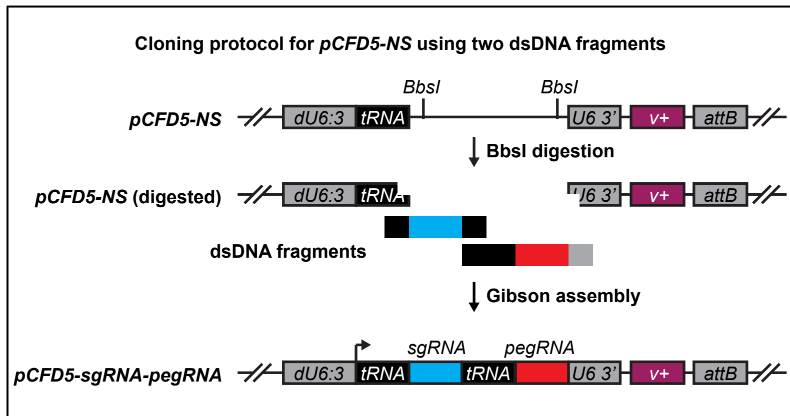
pCFD3genoF ACGTTTTATAACTTATGCCCCTAAG
pCFD3genoR GCCGAGCACAATTGTCTAGAATGC

Uncut backbone = 490bp
Correct insert = 638bp (depends on pegRNA length)

E7. Culture colonies with LB + Ampicillin and sequence confirm plasmids

pCFD3seqF ACCTACTCAGCCAAGAGGC

F. Cloning protocol for *pCFD5-NS* (Addgene # 149546) using two dsDNA fragments



F1. Design pegRNA and nicking sgRNA, and order dsDNA fragments (see Sections B&C).

F2. Digest/dephosphorylate plasmid

5µg *pCFD5-NS*
3µl BpiI (cuts BbsI) (Fermentas, FD1014)
3µl FastAP (Fermentas, EF0651)
6µl 10x FastDigest Buffer
Xµl H20
60ul total

F3. Gel-purify digested *pCFD5-NS* backbone (~6.3kb).

F4. Gibson assembly

Xµl digested pCFD5-NS (50ng)
Xul dsDNA fragment 1 (5ng)
Xul dsDNA fragment 2 (5ng)
2.5µl Gibson master mix (NEB, E2611)
Xµl H20
5ul total

Incubate reaction at 50˚C for 30min.

F5. Transform ligation into competent cells and grow colonies on LB-agar Ampicillin plates

F6. (Optional) Colony PCR to identify candidate pegRNA plasmids

pCFD3genoF ACGTTTTATAACTTATGCCCCTAAG
pCFD3genoR GCCGAGCACAATTGTCTAGAATGC

Uncut backbone = 587bp
Correct insert = ~846bp (depends on pegRNA length)

F7. Culture colonies with LB + Ampicillin and sequence confirm plasmids

pCFD3seqF ACCTACTCAGCCAAGAGGC

References:

1. F. Port, H. M. Chen, T. Lee, S. L. Bullock, Optimized CRISPR/Cas tools for efficient germline and somatic genome engineering in Drosophila. *Proc Natl Acad Sci U S A* **111**, E2967-2976 (2014).
2. F. Port, S. L. Bullock, Augmenting CRISPR applications in Drosophila with tRNA-flanked sgRNAs. *Nat Methods* **13**, 852-854 (2016).
3. J. Y. Hsu *et al.*, PrimeDesign software for rapid and simplified design of prime editing guide RNAs. *bioRxiv* 10.1101/2020.05.04.077750 (2020).
4. R. D. Chow, J. S. Chen, J. Shen, S. Chen, pegFinder: A pegRNA designer for CRISPR prime editing. *bioRxiv* 10.1101/2020.05.06.081612 (2020).