Designing Oligos for Universal Knock-in Vectors using Type 2 Restriction Enzymes:

Note In the following document when orientation words are used, they are used in the context of the reading frame of your genetic loci of interest. e.g. A 5' Reverse CRISPR means that the target site for the CRISPR is on the reverse strand at your locus and is toward the 5' end of the gene. Upstream Homology Domains are 5' of the CRISPR cut and Downstream Homology Domains are 3' of the cut. Also note: Upper case and lower case bases are the same; they are typed the way they are as a visual marker of the different parts of the homology domains.

For the 5' Homology Domain:

1) Open the sequence file for the gene of interest and identify the CRISPR site. (In this example it is a Reverse CRISPR target in Yellow, the PAM is in Orange)

Copy the 48 bp 5' of the CRISPR cut into a new sequence file; this is the 5' homology.

2) Observe the next three bases immediately upstream of the 48 bp of 5' homology, and pick a base not present to be the 3 bp spacer between the homology and the Universal PAM in the vector. (Here the three bases are "GGA" so "ccc" was chosen for the spacer)

Add the spacer to the new file 5' (in front) of the homology.
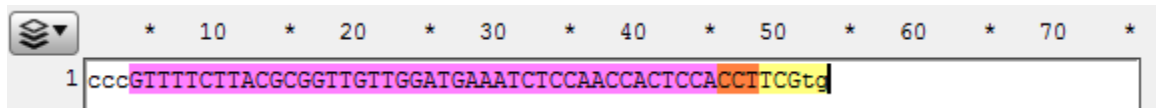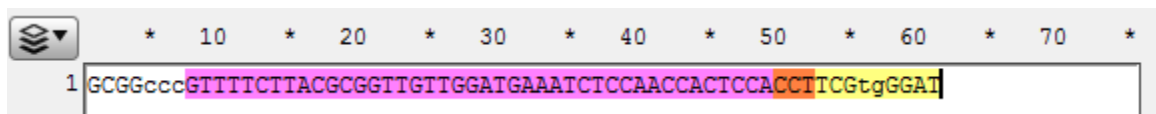
3) Determine where the last codon is in the 5' homology. Here the 3' G in the homology domain is the first base in the last codon upstream of the CRISPR cut.

Complete the codon by adding the remaining bases for that codon from the gene sequence to ensure the integration event will be in frame.

```
          *    10    *    20    *    30    *    40    *    50    *    60    *    70    *
1 cccGTTTTCTTACGCGGTTGTTGGATGAAATCTCCAACCACTCCACCTTCGtg
```

4) Add the BfuAI overhang sequences for cloning, to the ends of the 5' homology domain. 5'GCGG and 3'GGAT (or 3'CTTC for the pPRISM series). (Here both overhangs are set to prevent errors in copying sequence for the oligos in the next two steps.)

```
          *    10    *    20    *    30    *    40    *    50    *    60    *    70    *
1 GCGGcccGTTTTCTTACGCGGTTGTTGGATGAAATCTCCAACCACTCCACCTTCGtgGGAT
```

5) The 5' Homology Oligo A will be this sequence from the beginning to the end of the last codon. Copy and paste this into a new file and save it. (In this example, this oligo sequence is 5'GCGGcccGTTTTCTTACGCGGTTGTTGGATGAAATCTCCAACCACTCCACCTTCGtg3')
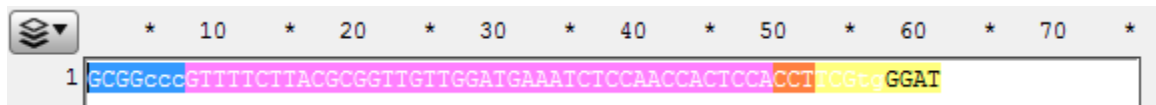
```
          *    10    *    20    *    30    *    40    *    50    *    60    *    70    *
1 GCGGcccGTTTTCTTACGCGGTTGTTGGATGAAATCTCCAACCACTCCACCTTCGtgGGAT
```

6) The 5' Homology Oligo B will be the reverse compliment of this sequence from beginning of the spacer to the end of the sequence. Copy the reverse compliment, paste it into a new file, and save it. (In this example, this oligo sequence is 5'ATCCcaCGAAGGTGGAGTGGTTGGAGATTTCATCCAACAACCGCGTAAGAAAACggg3')

```
          *    10    *    20    *    30    *    40    *    50    *    60    *    70    *
1 GCGGcccGTTTTCTTACGCGGTTGTTGGATGAAATCTCCAACCACTCCACCTTCGtgGGAT
```

For the 3' Homology Domain:

7) Open the sequence file for the gene of interest and identify the CRISPR site. (Reverse CRISPR target in Yellow, PAM in Orange)

Copy the 48 bp 3' of the CRISPR cut into a new sequence file; this is the 3' homology.

8) Observe the next three bases downstream of the 48 bp of homology, and pick a base not present to be the 3 bp spacer between the homology and the Universal PAM in the vector. (Here the bases are "CTG" so "aaa" was chosen for the spacer.)

Add the spacer to your new file 3' of (after) your homology.

9) Add the BspQI overhang sequences for cloning, to the ends of the 3' homology domain. 5'AAG and 3'CCG. (Here both overhangs are set to prevent errors in copying sequence for the oligos in the next two steps.)


10) The 3' Homology Oligo A will be this sequence from the beginning of the sequence to the end of the spacer. (Here = 5'AAGTGGGCAAGATATGGCTCACGTTATTCATCATCTTCCGCATTGTTTTGAaaa3')

11) The 3' Homology Oligo B will be the reverse compliment of this sequence from the beginning of the homology to the end of the sequence. (Here = 5'CGGtttTCAAAACAATGCGGAAGATGATGAATAACGTGAGCCATATCTTGCCCA3')
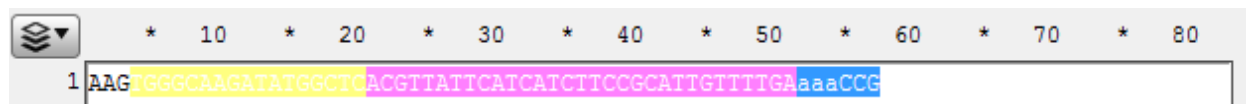
Codon Usage Table:

Reference table for Zebrafish codon usage. The table lists the codon base composition the amino acid result, and the useage number from the codon Usage Database <https://www.kazusa.or.jp/codon/>. Entries are color coded by amino acid.

TTT	F	18.2	TCT	S	16.9	TAT	Y	12.6	TGT	C	11.3
TTC	F	20.8	TCC	S	15.2	TAC	Y	17	TGC	C	11.2
TTA	L	7	TCA	S	13.2	TAA	*	1.1	TGA	*	1.4
TTG	L	12.3	TCG	S	5.6	TAG	*	0.6	TGG	W	11.6
CTT	L	12.7	CCT	P	16.6	CAT	H	10.9	CGT	R	6.9
CTC	L	17	CCC	P	12.7	CAC	H	14.8	CGC	R	9.6
CTA	L	6.2	CCA	P	15.7	CAA	Q	11.8	CGA	R	6.7
CTG	L	37.6	CCG	P	8.2	CAG	Q	33.5	CGG	R	6.6
ATT	I	16.5	ACT	T	14.5	AAT	N	16.3	AGT	S	13.2
ATC	I	23.7	ACC	T	16.2	AAC	N	24.1	AGC	S	18.4
ATA	I	7.7	ACA	T	17	AAA	K	29.3	AGA	R	14.3
ATG	M	25.5	ACG	T	7.4	AAG	K	30.7	AGG	R	10.2
GTT	V	14.1	GCT	A	20.9	GAT	D	24.8	GGT	G	13.7
GTC	V	14.8	GCC	A	19.5	GAC	D	27.8	GGC	G	17.2
GTA	V	6.7	GCA	A	16.6	GAA	E	24.4	GGA	G	21.5
GTG	V	28.3	GCG	A	8.6	GAG	E	42.8	GGG	G	10

For pPRSIM-Fuse vectors:

> Note: pPRISM-Fuse cassettes are for making 3' protein fusions, thus require the addition of any remaining codons 3' of the CRISPR cut. CRISPR targets as close to the stop codon as possible are recommended.

Homology arms are designed as in steps 1-11 above using pPRISM vector overhangs, with the following changes.

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a) To the end of the 5' homology domain in step 3 all remaining bases of the coding sequence besides the stop codon are added, instead of just those bases needed to complete the codon.

b) At least one 'G' in the PAM in the 5'homology oligos should be mutated to result in a silent mutation. (make this change between steps 3 and 4)

c) In all cases where the 3' homology arm would overlap with 20 or more base pairs of the completed coding sequence, the overlapping codons in the 5' homology arm oligos, 3' of the homology, should be shuffled* to prevent unwanted recombination during integration.

*Codon shuffling: Utilizing a codon usage table for the target organism, for each codon select and change the endogenous codon (in the oligo) to the next best codon with similar (+/- 2 units) or better usage score coding for the same amino acid. Note: leucine, serine, and arginine codons have more alternate codons, and for these choosing the most different base pair composition should take precedence over usage score.

d) The 3' homology arm may be omitted if codons are unable to be satisfactorily shuffled.

e) For 5' homology oligos longer than 150 bp, it may be cheaper to order a gene-block with the following additions to each end; 5'acctgccaca and 3'acacgcaggt. Then the gene-block should either be digested with BfuAI before cloning, or pre-blunt-cloned into an intermediate vector before digesting with BfuAI and purifying the correct size fragment for cloning into the pPRISM vector of choice.

For UFlip vectors:

Note: UFlip vectors contain a revertible cassette, and can be integrated in either an Gene-On/Cassette-Off position (where the cassette does not affect expression of the locus) or a Gene-Off/Cassette-On position (where the locus splices into the cassette for expression of RFP from the locus). In either case, the CRISPR target and homology arms are targeted to intron sequence and it is highly recommended to sequence the intron of interest before beginning, as well as to avoid targeting the natural splicing branch point in the intron (by targeting upstream of the branch point).

For designing homology domains for integration in the gene-OFF orientation:

Homology arms are designed as above using pPRISM vector overhangs, with the following change. The 5' homology domain does not need to be adjusted for frame (step 3). Frame is taken care of by selection of the UFlip vector 0, 1, or 2 depending/matching of the end phase of the preceding exon.
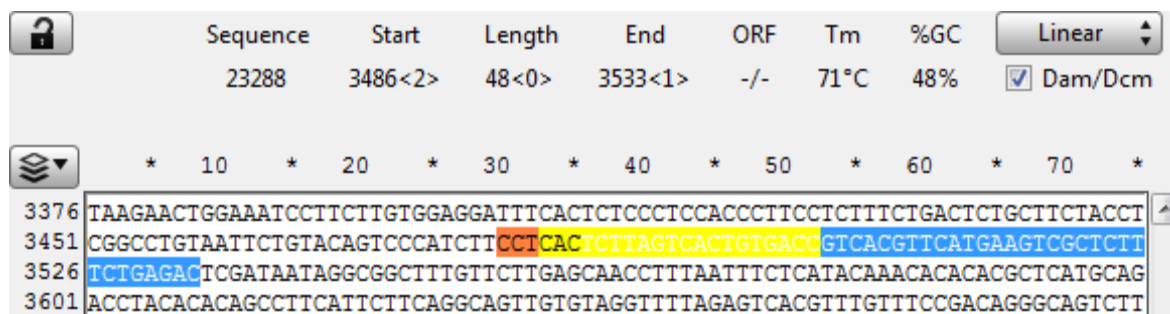
For designing homology arms for integration in the gene-ON orientation:
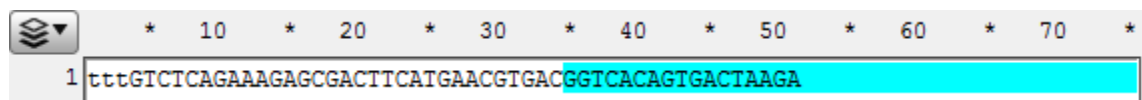
5' gene-On homology domain:

12) Open sequence file for the gene of interest and identify the intron CRISPR site. (Reverse CRISPR target in Yellow, PAM in Orange)

Copy the reverse compliment of the 48 bp 3' of the CRISPR cut into a new sequence file; this is the 5' homology for these vectors.

	Sequence	Start	Length	End	ORF	Tm	%GC	Linear ⬍
🔓	23288	3486<2>	48<0>	3533<1>	-/-	71°C	48%	☑ Dam/Dcm

```
      *    10    *    20    *    30    *    40    *    50    *    60    *    70    *
3376 TAAGAACTGGAAATCCTTCTTGTGGAGGATTTCACTCTCCCTCCACCCTTCCTCTTTCTGACTCTGCTTCTACCT
3451 CGGCCTGTAATTCTGTACAGTCCCATCTTCCTCACTCTTAGTCACTGTGACCGTCACGTTCATGAAGTCGCTCTT
3526 TCTGAGACTCGATAATAGGCGGCTTTGTTCTTGAGCAACCTTTAATTTCTCATACAAACACACACGCTCATGCAG
3601 ACCTACACACAGCCTTCATTCTTCAGGCAGTTGTGTAGGTTTTAGAGTCACGTTTGTTTCCGACAGGGCAGTCTT
```
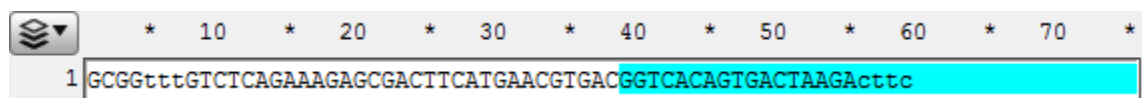
13) Observe the reverse compliment of the next three bases downstream of the 48 bp of 5' homology, and pick a base not present to be the 3 bp spacer between the homology and the Universal PAM in the vector. (Here the bases are "CGA" so "ttt" was chosen for the spacer.)

Add the spacer to a new file 5' of (before) the homology.

```
      *    10    *    20    *    30    *    40    *    50    *    60    *    70    *
   1 tttGTCTCAGAAAGAGCGACTTCATGAACGTGACGGTCACAGTGACTAAGA
```

14) Add the BfuAI overhang sequences for cloning, to the ends of the 5' homology domain. 5'GCGG and 3'CTTC. (Here we are setting both overhangs to prevent errors in copying sequence for the oligos in the next two steps.)

```
      *    10    *    20    *    30    *    40    *    50    *    60    *    70    *
   1 GCGGtttGTCTCAGAAAGAGCGACTTCATGAACGTGACGGTCACAGTGACTAAGActtc
```

15) The 5' Homology Oligo A will be this sequence from the beginning of the sequence to the end of the homology. (Here = 5'GCGGtttGTCTCAGAAAGAGCGACTTCATGAACGTGACGGTCACAGTGACTAAGA3')

```
      *    10    *    20    *    30    *    40    *    50    *    60    *    70    *
   1 GCGGtttGTCTCAGAAAGAGCGACTTCATGAACGTGACGGTCACAGTGACTAAGActtc
```
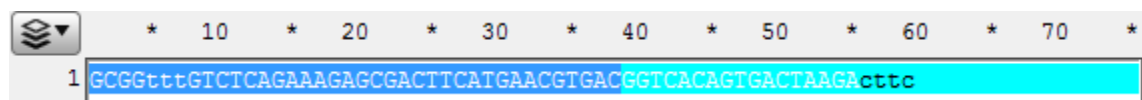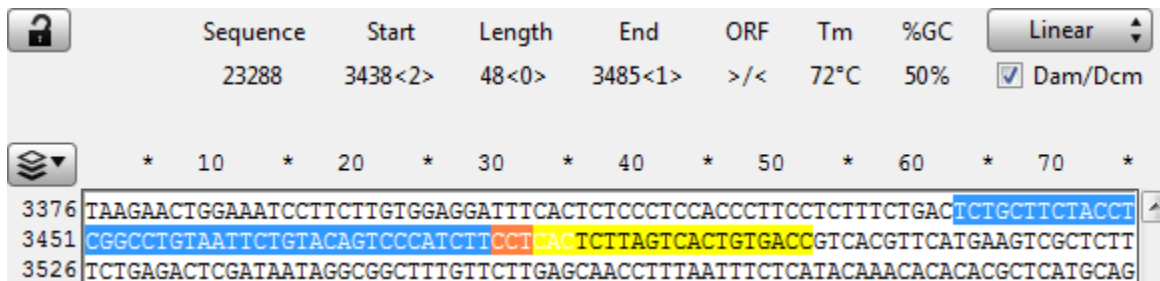
16) The 5' Homology Oligo B will be the reverse compliment of this sequence from the beginning of the spacer to the end of the sequence. (Here = 5' gaagTCTTAGTCACTGTGACCGTCACGTTCATGAAGTCGCTCTTTCTGAGACaaa3')
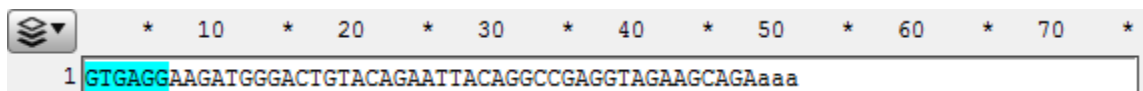

3' gene-On homology domain:

17) Open the sequence file for the gene of interest and identify the CRISPR site. (In this example it is a Reverse CRISPR target in Yellow, the PAM is in Orange)

Copy the reverse compliment of the 48 bp 5' of the CRISPR cut into a new sequence file; this is the 3' homology.
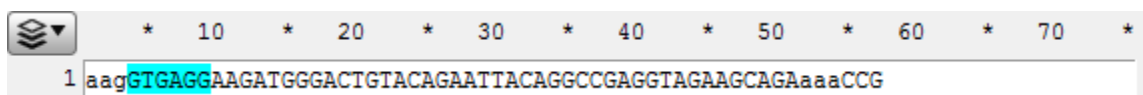

18) Observe the reverse compliment of next three bases immediately upstream of the 48 bp of 3' homology, and pick a base not present to be the 3 bp spacer between the homology and the Universal PAM in the vector. (Here the three bases are "GTC" so "aaa" was chosen for the spacer)

Add the spacer to the new file 3' (behind) of the homology.
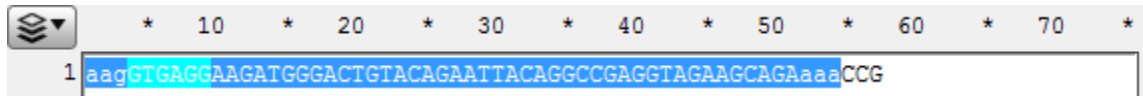

19) Add the BspQI overhang sequences for cloning, to the ends of the 3' homology domain. 5'AAG and 3'CCG. (Here both overhangs are set to prevent errors in copying sequence for the oligos in the next two steps.)
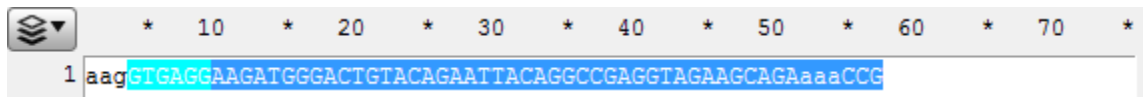
20) The 3' Homology Oligo A will be this sequence from the beginning to the end of the spacer. Copy and paste this into a new file and save it. (In this example, this oligo sequence is 5'aagGTGAGGAAGATGGGACTGTACAGAATTACAGGCCGAGGTAGAAGCAGAaaa 3')

21) The 3' Homology Oligo B will be the reverse compliment of this sequence from beginning of the homology to the end of the sequence. Copy the reverse compliment, paste it into a new file, and save it. (In this example, this oligo sequence is 5' CGGtttTCTGCTTCTACCTCGGCCTGTAATTCTGTACAGTCCCATCTTCCTCAC 3')

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For pPRISM-Splicer vectors:

> Note: pPRISM-Splicer vectors are intended for adding specific SNPs to exons of interest without interfering with natural splicing patterns, while also adding a secondary marker to follow the new allele.

Homology arms are designed as in steps 1-11 above using pPRISM vector overhangs, with the following changes.

a) To the end of the 5' homology domain in step 3 the remaining bases of the targeted exon sequence including the first two bases of the next intron (GT) are added instead of just those bases needed to complete the codon.

b) At least one 'G' in the PAM in the 5'homology oligos should be mutated. (make this change between steps 3 and 4)

c) The desired SNP should be made. (make this change between steps 3 and 4)

d) In all cases where the 3' homology arm would overlap with 20 or more base pairs of the completed coding sequence, the codons in the 5' homology arm oligos 3' of the homology should be shuffled to prevent unwanted recombination.

e) The 3' homology arm may be omitted if codons are unable to be satisfactorily shuffled.

f) For 5' homology oligos longer than 150 bp, it may be cheaper to order a gene-block with the following additions to each end; 5'acctgccaca and 3'acacgcaggt. Then the gene-block should either be digested with BfuAI before cloning, or pre-blunt-cloned into an intermediate vector before digesting with BfuAI and purifying the correct size fragment for cloning into the pPRISM vector of choice.

For instances where a second CRISPR is used to delete part of the target exon, it is recommended to target the intron 5' of the branch point. Also, when using two CRISPR targets the 5' homology should be designed from the 5' CRISPR cut, and the 3' homology should be designed from the 3' CRISPR cut.