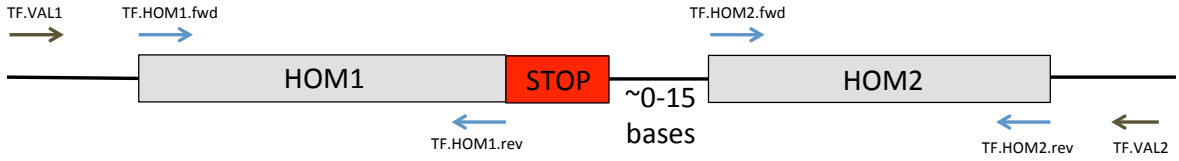
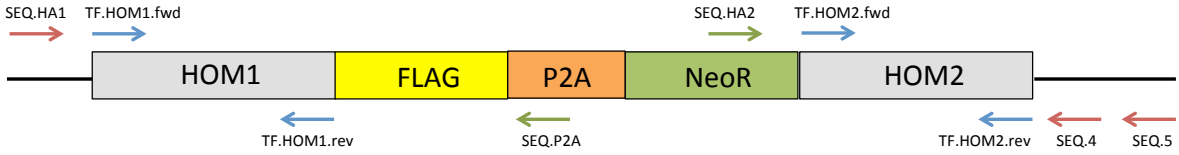
Mendenhall and Myers Lab Groups
HudsonAlpha Institute for Biotechnology
FETCh-seq Protocols

Scheme of Design of Vectors and Establishment of Mammalian Cell Lines With Flag Tagged Endogenous Proteins For ChIP-Seq.

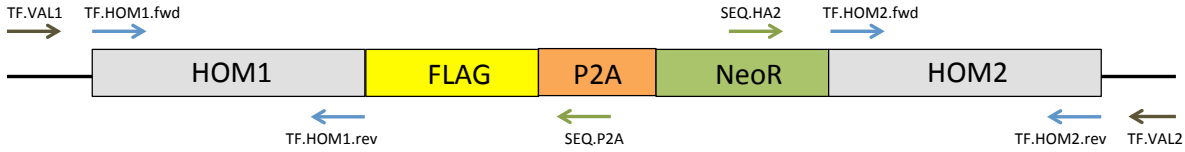
Wild type genomic sequence:



Donor vector sequence with homology arms:



Tag-integrated genomic sequence:



I. Design of Homology Arms

1. Identify the target protein's stop codon, and design gRNAs to target and cut within -5 to +15 basepairs from stop codon. Any CRISPR/Cas9 system should work. We use PX458 or PX462 from Feng Zhang's lab.

II. Option A: Design of 700-800 basepair Homology arms (HOM) primers for Gibson Assembly

1. Add Gibson Assembly tails (noted in blue) to 5' ends of fwd and rev primers for HOM1 and HOM2. Be sure to design the red, highlighted NNN's in the HOM1 rev primer to the codon immediately upstream of the stop codon. The other HOM Arm1 Reverse and HOM Arm 2 Forward should ideally be within 15-20 basepairs of the Cas9 cut site.

Note: In cases where the gRNA will recognize, bind, and direct Cas9 to cut HOM1, Option B outlined below (gBlock design) MUST be used. In these cases, modify the gBlock sequence as necessary, using the redundancy of the genetic code to maintain the correct amino acid codons but modifying the sequence to disrupt gRNA recognition.

1. Gibson Assembly Primer Design
HOM Arm1 Forward, TCCCCGACCTGCAGCCCAGCTNNNNNNNNNNNNNNNNNN
HOM Arm1 Reverse, CCGGAACCTCCTCCGCTCCCNNNNNNNNNNNNNNNNNN
HOM Arm 2 Forward, AGTTCTTCTGATTCGAACATCNNNNNNNNNNNNNNNNNN
HOM Arm 2 Reverse, TGGAGAGGACTTTCCAAGNNNNNNNNNNNNNNNNNN

Note: We recommend you arrange the fragments in correct order: upstream vector, HOM Arm1, downstream vector *in silico* to confirm the open reading frame is correct.
PCR amplification of homology arms for Gibson Assembly:

Forward primer (10uM)	2.5 ul
Reverse primer (10uM)	2.5 ul
gDNA from target cell line (~20ng)	1.0 ul
Phusion High-Fidelity PCR Master Mix	25.0 ul
Nuclease Free Water	17.0 ul
	50 ul

PCR thermocycler conditions:
1.) 98°C for 3 minutes
2.) 98°C for 15 seconds
3.) 60°C for 20 seconds
4.) 72°C for 30 seconds (Repeat 2-4 for 30 cycles)
5.) 72°C for 2 minutes
6.) 4°C hold

Note: Annealing temperature and cycle number may be optimized for each primer pair. Run 10 ul of PCR reaction on an agarose gel to verify amplification of a single band of the expected size for each homology arm. If there are multiple bands present in addition to the expected one, the correct band may be isolated by gel extraction. Clean up reactions with Qiagen PCR purification kit and elute in 30 ul dH2O. Determine concentration with a Qubit or other fluorometer and proceed with Gibson Assembly.

Option B: Design of Homology arms with IDT gBlocks (preferred method)

1. Insert tail sequences for Gibson Assembly at 5' and 3' ends of gBlocks.
Upstream tail sequence of HOM Arm1: TCCCCGACCTGCAGCCCAGCT
Downstream tail sequence of HOM Arm1: NNNGGGAGCGGAGGAGGTTCCGG
Upstream tail sequence of HOM Arm2: AGTTCTTCTGATTCGAACATC
Downstream tail sequence of HOM Arm1: CTTGGAAAGTCCTCTCCA

1) We recommend PCR amplification of gBlocks to ensure the correct length and concentration for Gibson Assembly. This can be done using the tail sequences as universal primers.

Forward primer (10uM)	2.5 ul
Reverse primer (10uM)	2.5 ul
gBLOCK DNA (~10ng/ul)	1.0 ul
Phusion High-Fidelity PCR Master Mix	25.0 ul
Nuclease Free Water	17.0 ul
	50 ul

PCR thermocycler conditions:

1.) 98°C for 3 minutes
2.) 98°C for 15 seconds
3.) 60°C for 20 seconds
4.) 72°C for 30 seconds (Repeat 2-4 for 15 cycles)
5.) 72°C for 2 minutes
6.) 4°C hold

Clean up reactions with Qiagen PCR purification kit and elute in 20 ul dH2O. Determine concentration with a Qubit or other fluorometer and proceed with Gibson Assembly.

2) Digestion of EMM0021 Flag Tag Backbone vector for single step Gibson assembly

pFETCh Donor Backbone vector (1 ug)	X ul
BsaI	1.0 ul
BbsI	1.0 ul
NEBuffer 2.1	2.5 ul
ddH20	X ul
	25 ul

Incubate at 37C for >1 hour. Clean up with Qiagen PCR purification kit and elute in 50 ul dH2O. Determine concentration and proceed with single step Gibson Assembly.

3) Single step Gibson Assembly Reaction

2x Gibson Assembly Master Mix (NEB)	10 ul
BsaI + BbsI digested vector from step 1 (50 ng)	X ul
HOM A1 (PCR product, 3:1 molar ratio)	2.1 ul
HOM A2 (PCR product. 3:1 molar ratio)	2.1 ul
ddH20	X ul
	20 ul

Incubate at 50C for 1 hour. Transform into high efficiency E. coli (we use MegaX DH10B cells, C6400-03: Life Technologies) and screen colonies for correct ligation.
Note: Using a 3:1 molar ratio of insert fragments to vector has worked well for us, but if no colonies are seen upon transformation, higher insert:vector ratios may be used.

IV. Verification of correct donor plasmid assembly:

1. Initial verification can be achieved by restriction enzyme digestion/gel visualization. With PvuI + ClaI double digestion, the following bands should be visualized:

Empty vector: 300b, 500b, 1.3kb, 3.4kb
Single homology arm: 300b, 500b, 1.3kb+HOM, 3.4kb
Two homology arms: 300b, 500b, 1.3kb+HOM1+HOM2, 3.4kb

Note: Screen *in silico* for presence of PvuI (CGATCG) or ClaI (ATCGAT) recognition sites in homology arm sequences that might alter expected band sizes.

2. Sanger sequence from both ends of each homology arm, using SEQ.HA1, SEQ.P2A, SEQ.HA2, and SEQ.4 primers (see Appendix 2 for primer sequences and Appendix 3 and Figure 1 for overview of primer locations).

Creation of Flag Tagged Mammalian Cell Lines

1. Use optimized protocol for your particular cell line being transfected. We use either Lonza Nucleofection or Lipid based methods. High transfection efficiency is crucial for simultaneous delivery of multiple plasmids into the same cell.

2. Transfect simultaneously the appropriate amount of donor plasmid and gRNA plasmid(s). We find a ratio of 2:1 of pFETCh_Donor and gRNA/Cas9 plasmid works well for most gRNAs designed.

3. Selection and growth. Selection should proceed with the lowest amount of G418 possible as determined by kill-curve with untransfected or mock transfected cells. (This is often roughly half the concentration we normally use for selection with PGK-Neo constructs. In our lab we use 400ug/mL for HepG2, 250 ug/mL for K562 and 50 ug/mL for mouse ES cells).

4. If possible, count number of G418 resistant colonies to estimate number of correctly engineered cells. We find FETCh-seq works best with polyclonal populations derived from >50 colonies. If necessary scale the transfection up or pool multiple transfections.

5. Expand the polyclonal population of cells. Crosslink using standard ChIP-seq protocol; Myers lab protocol is available online (http://research.hudsonalpha.org/Myers/?page_id=142).

6. Standard flash-frozen cell pellets can be used for protein extraction, gDNA isolation, and RNA purification to confirm correct tagging by PCR and/or western blot. We recommend PCR and sequence confirmation of both insert/homology arm junctions and if desired, deep sequencing of PCR product from the endogenous gRNA target for frequency of NHEJ events in the polyclonal population.

Appendix 1: Standard primer tail sequences

Gibson Assembly HOM1 fwd tail: tccccgacctgcagcccagct
Gibson Assembly HOM1 rev tail: ccggaacctcctccgctccc
Gibson Assembly HOM2 fwd tail: agttcttctgattcgaacatc
Gibson Assembly HOM2 rev tail: tggagaggactttccaag

Appendix 2: Standard primer sequences

SEQ.HA1: acgcctgtgaaaccgtacta
SEQ.HA2: ggccgcttttctggattcat
SEQ.P2A: cagcaggctgaagttagtagc
SEQ.4: gacggccagtgaattggag
SEQ.5: aactgttgggaagggcgatc

Appendix 3: Sequence Map and Feature Annotation for pFETCh Homology Arm Cloned Vector

Seq.HA1 Forward Sequencing Primer

Glycine-Serine Linker Sequence

3XFLAG Sequence

P2A Linker Sequence

Seq.P2A Reverse Sequencing Primer

Neo Resisance Gene

Seq.HA2 Forward Sequencing Primer

Seq.5 Reverse Sequencing Primer

Partial Sequence of Donor Cassette in Plasmid After Gibson Assembly

acgcctgtgaaaccgtactaagtctcccgtgtattcttatcaccatcaggtgacatcctcgcccaggctgtcaatcatgccggtatcgattccagtagc
accggccccacgctgacaacccactcttgcagcgttagcagcgcccctcttaacaagccgacccccaccagcgtcgcggttactaacactcctctcc
ccgacctgcagcccagct-[HOM Arm 1-sequence]-
gggagcggaggaggttccggtggaggtggttctggagattacaaggatgacgacgataagggcgattacaaggatgacgacgataagggagatt
acaaggatgacgacgataaggtttcaggaagcggagctactaacttcagcctgctgaagcaggctggagacgtggaggagaaccctggacctgg
atcgtttcgcatgattgaacaagatggattgcacgcaggttctccggccgcttgggtggagaggctattcggctatgactgggcacaacagacaatcg
gctgctctgatgccgccgtgttccggctgtcagcgcaggggcgcccggttctttttgtcaagaccgacctgtccggtgccctgaatgaactgcaggacg
aggcagcgcggctatcgtggctggccacgacgggcgttccttgcgcagctgtgctcgacgttgtcactgaagcgggaagggactggctgctattggg
cgaagtgccggggcaggatctcctgtcatctcaccttgctcctgccgagaaagtatccatcatggctgatgcaatgcggcggctgcatacgcttgatcc
ggctacctgcccattcgaccaccaagcgaaacatcgcatcgagcgagcacgtactcggatggaagccggtcttgtcgatcaggatgatctggacga
agagcatcaggggctcgcgccagccgaactgttcgccaggctcaaggcgcgcatgcccgacggcgaggatctcgtcgtgacccatggcgatgcct
gcttgccgaatatcatggtggaaaatggccgcttttctggattcatcgactgtggccggctgggtgtggccgaccgctatcaggacatagcgttggctac
ccgtgatattgctgaagagcttggcggcgaatgggctgaccgcttcctcgtgctttacggtatcgccgctcccgattcgcagcgcatcgccttctatcgcc
ttcttgacgagttcttctgattcgaacatc-[HOM Arm 2-sequence]-
cttggaaagtcctctccactgactgtagcctccaattcactggccgtcgttcactggccgtcgttttacaacgtcgtgactgggaaaaccctggcgttacc
caacttaatcgccttgcagcacatccccctttcgccagctggcgtaatagcgaagaggcccgcaccgatcgcccttcccaacagtt