**scCRISPR protocol for NHEJ**

Ordering sgRNA oligos:

For 20 bp sgRNA

TGGAAAGGACGAAACACCGN19GTTTAAGAGCTATGCTGGAAAC

For 21 bp sgRNA

GGAAAGGACGAAACACCGN20GTTTAAGAGCTATGCTGGAAAC

For 19 bp sgRNA

TGGAAAGGACGAAACACCGN18GTTTAAGAGCTATGCTGGAAACA

Amplifying oligos

Note that we use Onetaq since it is the cheapest polymerase, but other polymerases should work as well. Also note that this protocol includes three successive PCR steps (with no purification in between). scCRISPR also works with product of the first or second PCR (make sure to perform 35 cycles of the final PCR step whichever it is), but for maximal efficiency we recommend the protocol below.

PCR1 for 60 bp oligos (Onetaq, Ta=60 degrees, 10 cycles with both primer sets, typically 20 uL reaction)—293 bp product

sgRNA\_HDRstep1\_fw TGTTTTAAAATGGACTATCATATGCTTACCGTAACTTGAAAGTATTTCGATTTCTTGGCTTTATATATCTTGTGGAAAGGACGAAACACC

sgRNA\_HDRstep1\_rv GTTGATAACGGACTAGCCTTATTTAAACTTGCTATGCTGTTTCCAGCATAGCTCTTAAAC

sgRNA\_HDRstep2\_fw GTACAAAATACGTGACGTAGAAAGTAATAATTTCTTGGGTAGTTTGCAGTTTTAAAATTATGTTTTAAAATGGACTATCATATGCTTACC

sgRNA\_HDRstep2\_rv ATTTTAACTTGCTATTTCTAGCTCTAAAACAAAAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTAAAC

2X Onetaq master mix with standard buffer: 50% of reaction volume

20 uM 60 bp oligo: 2.5% of reaction volume

10 uM mix of sgRNA\_HDRstep1\_fw and sgRNA\_HDRstep1\_rv: 5% of reaction volume

10 uM mix of sgRNA\_HDRstep2\_fw and sgRNA\_HDRstep2\_rv: 5% of reaction volume

dH2O: 37.5% of reaction volume

PCR2 for 60 bp oligos (Onetaq, Ta=60 degrees, 10 cycles, typically 20 uL reaction)—379 bp product

sgRNA\_HDRstep3\_fw CGATACAAGGCTGTTAGAGAGATAATTAGAATTAATTTGACTGTAAACACAAAGATATTAGTACAAAATACGTGACGTAGAAAGTAATAA

sgRNA\_HDRstep3\_rv TCAATGTATCTTATCATGTCTGCTCGATTTTAACTTGCTATTTCTAGCTCTAAAACAAAA

2X Onetaq master mix with standard buffer: 50% of reaction volume

Unpurified product of PCR1: 5% of reaction volume

10 uM mix of sgRNA\_HDRstep3\_fw and sgRNA\_HDRstep3\_rv: 5% of reaction volume

dH2O: 40% of reaction volume

PCR3 for 60 bp oligos (Onetaq, Ta=60 degrees, 35 cycles, typically >100 uL reaction volume)—415 bp product

sgRNA\_HDRstep4\_fw GGCCTATTTCCCATGATTCCTTCATATTTGCATATACGATACAAGGCTGTTAGAGAGATA

sgRNA\_HDRstep4\_rv TCAATGTATCTTATCATGTCTGCTCGA

2X Onetaq master mix with standard buffer: 50% of reaction volume

Unpurified product of PCR1: 5% of reaction volume

10 uM mix of sgRNA\_HDRstep4\_fw and sgRNA\_HDRstep4\_rv: 5% of reaction volume

dH2O: 40% of reaction volume

Note that this PCR is 35 cycles!

Final amplicon:

Test 2 uL of PCR on 2% gel, expecting 415 bp. Then PCR purify/minElute purify

GGCCTATTTCCCATGATTCCTTCATATTTGCATATACGATACAAGGCTGTTAGAGAGATAATTGGAATTAATTTGACTGTAAACACAAAGATATTAGTACAAAATACGTGACGTAGAAAGTAATAATTTCTTGGGTAGTTTGCAGTTTTAAAATTATGTTTTAAAATGGACTATCATATGCTTACCGTAACTTGAAAGTATTTCGATTTCTTGGCTTTATATATCTTGTGGAAAGGACGAAACACCGAGGCGTCTGGGTGGCTCTTGGTTTAAGAGCTATGCTGGAAACAGCATAGCAAGTTTAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGCTTTTTTGTTTTAGAGCTAGAAATAGCAAGTTAAAATCGAGCAGACATGATAAGATACATTGA

Transfection/electroporation (can be scaled up or down):

For transfection into 12-well, transfect PCR-purified product from 25 uL of PCR with 500 ng CBh-Cas9-BlastR + 500 ng sgPal7-HygR. Select with 10 ug/mL Blasticidin from hours 24-72 after transfection. Expect ~50% cutting efficiency.

For electroporation into 12-well, electroporate minElute-purified product from 100 uL of PCR with 4 ug CBh-Cas9-BlastR + 4 ug sgPal7-HygR. Select with 10 ug/mL Blasticidin + 100 ug/mL Hygromycin from hours 24-72 after electroporation. Expect ~99% cutting efficiency.

**scCRISPR protocol for Homologous recombination**

**Identifying genomic region for knock-in**

Use UCSC genome browser (<http://genome.ucsc.edu/>) to identify the genomic sequence surrounding the gene of interest. If you are interested in making a C-terminal GFP fusion construct, identify the stop codon of the transcript you would like to tag (typically the primary RefSeq transcript). Zoom into this genomic region and copy ~500 bp of genomic DNA sequence centered on the stop codon. Make sure to copy the sequence in the orientation of the coding sequence which may require reverse complementing the entire sequence. Annotate the stop codon as shown below for the example mouse Pou5f1 gene:

Pou5f1 3’ end

CGAGTATGGTTCTGTAACCGGCGCCAGAAGGGCAAAAGATCAAGTATTGAGTATTCCCAACGAGAAGAGTATGAGGCTACAGGGACACCTTTCCCAGGGGGGGCTGTATCCTTTCCTCTGCCCCCAGGTCCCCACTTTGGCACCCCAGGCTATGGAAGCCCCCACTTCACCACACTCTACTCAGTCCCTTTTCCTGAGGGCGAGGCCTTTCCCTCTGTTCCCGTCACTGCTCTGGGCTCTCCCATGCATTCAAACTGAGGCACCAGCCCTCCCTGGGGATGCTGTGAGCCAAGGCAAGGGAGGTAGACAAGAGAACCTGGAGCTTTGGGGTTAAATTCTTTTACTGAGGAGGGATTAAAAGCACAACAGGGGTGGGGGGTGGGATGGGGAAAGAAGCTCAGTGATGCTGTTGATCAGGAGCCTGGCCTGTCTGTCACTCATCATTTTGTTCTTAAATAAAGACTGGGACACACAGTAGATAGCTGAATTTTGTTTTCCTTCAG

**Designing CrispR guide RNA template**

Guide RNA target genomic regions should have the form **NNNNNNNNNNNNNNNNNNNNNGG** or **(N20)NGG** where red is the guide RNA protospacer and blue the required PAM in the genome which is not part of the protospacer. The NGG can be on either strand, so the sequence **CCN(N20)** is also an acceptable target. In order to make a C-terminal fusion protein, you want to find the NGG on either strand that is closest to the stop codon, as homologous recombination works best when the homology arms are as close as possible to the double-strand break. The only limitation is that you need to make sure that the protospacer that you use will NOT continue to cut after homologous recombination has been performed. In practice, this means that NGG sequences prior to or containing the stop codon should not be used, but NGG sequences after the stop codon and CCN sequences immediately prior to, including, or after the stop codon can be used.

Using the example sequence from above as well as a second example sequence, I have labeled NGG and CCN sequences adjacent to the stop codons of the Pou5f1 and Hoxa1 genes and have annotated why I chose particular NGG/CCN sequences to build the GFP fusion lines:

Pou5f1

CGAGTATGGTTCTGTAACCGGCGCCAGAAGGGCAAAAGATCAAGTATTGAGTATTCCCAACGAGAAGAGTATGAGGCTACAGGGACACCTTTCCCAGGGGGGGCTGTATCCTTTCCTCTGCCCCCAGGTCCCCACTTTGGCACCCCAGGCTATGGAAGCCCCCACTTCACCACACTCTACTCAGTCCCTTTTCCTGAGGGCGAGGCCTTTCCCTCTGTTCCCGTCACTGCTCTGGGCTCTCCCATGCATTCAAACTGAGGCACCAGCCCTCCCTGGGGATGCTGTGAGCCAAGGCAAGGGAGGTAGACAAGAGAACCTGGAGCTTTGGGGTTAAATTCTTTTACTGAGGAGGGATTAAAAGCACAACAGGGGTGGGGGGTGGGATGGGGAAAGAAGCTCAGTGATGCTGTTGATCAGGAGCCTGGCCTGTCTGTCACTCATCATTTTGTTCTTAAATAAAGACTGGGACACACAGTAGATAGCTGAATTTTGTTTTCCTTCAG

Hoxa1

CAGCGATGAGAAAACGGAAGCATGAAGCAGAAGAAGCGTGAGAAGGAGGGGCTCCTGCCCATCTCCCCTGCCACTCCTCCTGGCAGCGATGAGAAAACGGAAGAATCATCTGAGAAATCTAGCCCCTCGCCCAGTGCCCCTTCTCCGGCATCGTCTACCTCAGACACTCTGACTACCTCCCACTGAGGCTACTCCAGCCCAACTCTGCAGCCCAGGCTTCTCCCTGGGCTGGGATTTCTTACCCAAAGCACATTCTTAGCTTATCTTCCTTTCTTTACAGACTCTCTCTTCCTTTCTCGTCCCATCTGGGGAGCTCCTGGCCAAGATAAGGTATTTCCAGAGAATCCTTTCTCGTCCCATCTGG

Once you have selected the appropriate protospacer, you will design an oligonucleotide containing the protospacer to be ordered. Because the U6 promoter which will be used to transcribe the gRNA is most efficient when the first transcribed base is a ‘G’, we use the following scheme to design the protospacer and the oligonucleotide allowing its amplification:

Protospacers should have 19-20 bp of homology to the genome immediately preceding the NGG “PAM” sequence:

a. If the genome sequence is GNNNNNNNNNNNNNNNNNNN NGG (GN19NGG), the protospacer sequence should be GNNNNNNNNNNNNNNNNNNN (GN19)

b. If a is not satisfied but GNNNNNNNNNNNNNNNNNN NGG (GN18NGG) is satisfied, the protospacer sequence should be GNNNNNNNNNNNNNNNNNN (GN18)

c. If a and b are not satisfied, the protospacer sequence should be GNNNNNNNNNNNNNNNNNNNN (GN20) where the genomic sequence is NNNNNNNNNNNNNNNNNNNN NGG (N20NGG) – it does not matter if the first G is in the genome.

If the genomic sequence targeted is NCC(N20), then reverse complement that entire stretch and apply these rules to the reverse complemented sequence, which should then have the form (N20)NGG.

After applying these rules, you should have a protospacer sequence that is 19-21 bp. The oligonucleotide you should order contains this 19-21 bp sequence flanked by primer sequences that will be used to PCR amplify the protospacer into a functional amplicon for the scCRISPR system. The oligonucleotide should obey the following format:

For 20 bp protospacer:

TGGAAAGGACGAAACACCGN19GTTTAAGAGCTATGCTGGAAAC

For 21 bp protospacer:

GGAAAGGACGAAACACCGN20GTTTAAGAGCTATGCTGGAAAC

For 19 bp protospacer:

TGGAAAGGACGAAACACCGN18GTTTAAGAGCTATGCTGGAAACA

As examples, here are the oligonucleotides for the Pou5f1 and Hoxa1 examples shown above:

Pou5f1\_gRNA\_oligo GGAAAGGACGAAACACC GACAGCATCCCCAGGGAGGGC GTTTAAGAGCTATGCTGGAAAC

Hoxa1\_gRNA\_oligo GGAAAGGACGAAACACC GTTGGGCTGGAGTAGCCTCAG GTTTAAGAGCTATGCTGGAAAC

**Designing primers for GFP knock-in**

The goal of this strategy is to use CrispR homologous recombination to insert a C-terminal GFP in-frame before the stop codon of the gene of interest. We have found that introducing GFP with 75-80 bp homology arms is sufficient to induce knock-in at appreciable frequency. Because of cost and PCR efficiency, we add these homology arms using two rounds of PCR, each of which adds 35-40 bp of homology arm to GFP—each primer will be 60 bp, which is the maximum allowed by IDT before a price hike. Please note that this protocol in principle allows knock-in of any construct. We have knocked in construct up to 2 kb. HR replacement of short regions such as SNP repair occurs in 20-50% of cells. Longer constructs are possible but would likely undergo less frequent homologous recombination and be trickier to PCR. Both would use roughly the same protocol.

Homology arms are designed to encode GFP in-frame immediately upstream of the stop codon of the gene and to include a stop codon after the GFP ORF. Homology arms were designed so as not to overlap with the gRNA sequence by more than 5 bp on either side. For the forward arm, the homology arm should always include the entire last codon before the stop codon to allow in-frame C-terminal GFP fusion. For the reverse arm, the rule of not overlapping with the gRNA sequence by more than 5 bp means that a portion of the 3’ UTR will be removed. We have not found this to be a problem for gene expression, but we limit the extent of this removal by designing gRNAs as close to the stop codon as possible (see above section) and designing homology arm primers with the maximum allowable 5 bp of overlap with the gRNA. The GFP ORF to be amplified is shown below:

GFP ORF lacking ATG for C-terminal GFP fusion

GTGAGCAAGGGCGAGGAGCTGTTCACCGGGGTGGTGCCCATCCTGGTCGAGCTGGACGGCGACGTAAACGGCCACAAGTTCAGCGTGTCCGGCGAGGGCGAGGGCGATGCCACCTACGGCAAGCTGACCCTGAAGTTCATCTGCACCACCGGCAAGCTGCCCGTGCCCTGGCCCACCCTCGTGACCACCCTGACCTACGGCGTGCAGTGCTTCAGCCGCTACCCCGACCACATGAAGCAGCACGACTTCTTCAAGTCCGCCATGCCCGAAGGCTACGTCCAGGAGCGCACCATCTTCTTCAAGGACGACGGCAACTACAAGACCCGCGCCGAGGTGAAGTTCGAGGGCGACACCCTGGTGAACCGCATCGAGCTGAAGGGCATCGACTTCAAGGAGGACGGCAACATCCTGGGGCACAAGCTGGAGTACAACTACAACAGCCACAACGTCTATATCATGGCCGACAAGCAGAAGAACGGCATCAAGGTGAACTTCAAGATCCGCCACAACATCGAGGACGGCAGCGTGCAGCTCGCCGACCACTACCAGCAGAACACCCCCATCGGCGACGGCCCCGTGCTGCTGCCCGACAACCACTACCTGAGCACCCAGTCCGCCCTGAGCAAAGACCCCAACGAGAAGCGCGATCACATGGTCCTGCTGGAGTTCGTGACCGCCGCCGGGATCACTCTCGGCATGGACGAGCTGTACAAGTAAAGCGGCCGCAATTCACTCCTCA

The first homology primer pair is of the following format:

LocusX\_GFPhomologyarm\_fw [LocusX pre-stop40bp] GTGAGCAAGGGCGAGGAGCT

LocusX\_GFPhomologyarm\_rv [LocusX post-stop reverse complement 40bp] TGAGGAGTGAATTGCGGCCG

The second homology arm primer pair overlaps with the first homology arms by 20-30 bp and extends them by 30-40 bp on each end—each primer is designed to be exactly 60 bp. To determine how much overlap to design with the first primer pair, we choose the minimal overlap such that the overlapping region is estimated to have a Tm of >65 degrees using the NEB Tm calculator (<http://tmcalculator.neb.com/#!/)--> use the default Q5 settings. It is OK if the estimated temperature is above 65 with a 20 bp overlap—we never use less than 20 bp of overlap to ensure efficient PCR. Examples of homology arm primers for Pou5f1 and Hoxa1 are shown below:

Pou5f1

TGTATCCTTTCCTCTGCCCCCAGGTCCCCACTTTGGCACCCCAGGCTATGGAAGCCCCCACTTCACCACACTCTACTCAGTCCCTTTTCCTGAGGGCGAGGCCTTTCCCTCTGTTCCCGTCACTGCTCTGGGCTCTCCCATGCATTCAAACTGAGGCACCAGCCCTCCCTGGGGATGCTGTGAGCCAAGGCAAGGGAGGTAGACAAGAGAACCTGGAGCTTTGGGGTTAAATTCTTTTACTGAGGAGGGATTAAAAGCACAACAGGGGTGGGGGGTGGGATGGGGAAAGAAGCTC

061015\_sgPou5f13’\_GFPHDR\_fw TGTTCCCGTCACTGCTCTGGGCTCTCCCATGCATTCAAAC GTGAGCAAGGGCGAGGAGCT

061015\_sgPou5f13’\_GFPHDR\_rv CCAGGTTCTCTTGTCTACCTCCCTTGCCTTGGCTCACAGC TGAGGAGTGAATTGCGGCCG

061015\_sgPou5f13’\_HDRExt\_fw TCTACTCAGTCCCTTTTCCTGAGGGCGAGGCCTTTCCCTCTGTTCCCGTCACTGCTCTGG

061015\_sgPou5f13’\_HDRExt\_rv TAATCCCTCCTCAGTAAAAGAATTTAACCCCAAAGCTCCAGGTTCTCTTGTCTACCTCCC

Hoxa1

CAGCGATGAGAAAACGGAAGCATGAAGCAGAAGAAGCGTGAGAAGGAGGGGCTCCTGCCCATCTCCCCTGCCACTCCTCCTGGCAGCGATGAGAAAACGGAAGAATCATCTGAGAAATCTAGCCCCTCGCCCAGTGCCCCTTCTCCGGCATCGTCTACCTCAGACACTCTGACTACCTCCCACTGAGGCTACTCCAGCCCAACTCTGCAGCCCAGGCTTCTCCCTGGGCTGGGATTTCTTACCCAAAGCACATTCTTAGCTTATCTTCCTTTCTTTACAGACTCTCTCTTCCTTTCTCGTCCCATCTGGGGAGCTCCTGGCCAAGATAAGGTATTTCCAGAGAATCCTTTCTCGTCCCATCTGG

061015\_sgHoxa13’\_GFPHDR\_fw TCCGGCATCGTCTACCTCAGACACTCTGACTACCTCCCAC GTGAGCAAGGGCGAGGAGCT

061015\_sgHoxa13’\_GFPHDR\_rv GAAATCCCAGCCCAGGGAGAAGCCTGGGCTGCAGAGTTGG TGAGGAGTGAATTGCGGCCG

061015\_sgHoxa13’\_HDRExt\_fw AATCATCTGAGAAATCTAGCCCCTCGCCCAGTGCCCCTTCTCCGGCATCGTCTACCTCAG

061015\_sgHoxa13’\_HDRExt\_rv GTAAAGAAAGGAAGATAAGCTAAGAATGTGCTTTGGGTAAGAAATCCCAGCCCAGGGAGA

The last set of required primers are genomic DNA PCR primers, which will be used to test whether GFP knock-in cells exist in the population after electroporation and will be used to test clones for GFP knock-in. To order these, use Primer3 (<http://bioinfo.ut.ee/primer3/>) using standard settings. Primers must be outside of the amplified homology arms to avoid background of unintegrated homology arm construct, so paste the 500 bp genomic sequence into Primer 3 placing [ ] at the end of the homology arms. Primer3 should give you one forward primer before the homology arm and one reverse primer after the homology arm. These should be ordered. These primers will typically be paired with GFP primers to look for locus-specific GFP integration. The GFP primers listed below can be used to pair with the locus-specific ones.

032514\_GFPV5\_early\_newrv GTCCAGCTCGACCAGGATG

061714\_GFPV5ORF\_late\_fw GGATCACTCTCGGCATGGAC

Examples of flanking primers are shown below for Pou5f1 and Hoxa1:

Pou5f1 3’ end

TGTATCCTTTCCTCTGCCCCCAGGTCCCCACTTTGGCACCCCAGGCTATGGAAGCCCCCACTTCACCACAC[TCTACTCAGTCCCTTTTCCTGAGGGCGAGGCCTTTCCCTCTGTTCCCGTCACTGCTCTGGGCTCTCCCATGCATTCAAACTGAGGCACCAGCCCTCCCTGGGGATGCTGTGAGCCAAGGCAAGGGAGGTAGACAAGAGAACCTGGAGCTTTGGGGTTAAATTCTTTTACTGAGGAGGGATTA]AAAGCACAACAGGGGTGGGGGGTGGGATGGGGAAAGAAGCTC

061015\_sgPou5f13’\_up\_fw TGTATCCTTTCCTCTGCCCC

061015\_sgPou5f13’\_dwn\_rv GAGCTTCTTTCCCCATCCCA

Hoxa1

CAGCGATGAGAAAACGGAAGCATGAAGCAGAAGAAGCGTGAGAAGGAGGGGCTCCTGCCCATCTCCCCTGCCACTCCTCCTGGCAGCGATGAGAAAACGGAAG[AATCATCTGAGAAATCTAGCCCCTCGCCCAGTGCCCCTTCTCCGGCATCGTCTACCTCAGACACTCTGACTACCTCCCACTGAGGCTACTCCAGCCCAACTCTGCAGCCCAGGCTTCTCCCTGGGCTGGGATTTCTTACCCAAAGCACATTCTTAGCTTATCTTCCTTTCTTTAC]AGACTCTCTCTTCCTTTCTCGTCCCATCTGGGGAGCTCCTGGCCAAGATAAGGTATTTCCAGAGAATCCTTTCTCGTCCCATCTGG

061015\_sgHoxa13’\_up\_fw CAGCGATGAGAAAACGGAAG

061015\_sgHoxa13’\_dwn\_rv CCAGATGGGACGAGAAAGG

**Amplification of gRNAs and homology arm-GFP constructs**

*Amplification of gRNA*

To create gRNA homology fragments for use in scCRISPR, you will perform three successive PCRs, only testing and purifying the product at the end. Note that we use Onetaq since it is the cheapest polymerase, but other polymerases should work as well. You will need to order the 8 stock primers listed below, as these are used for all scCRISPR PCRs.

PCR1 (Onetaq, 94 degrees for 30s, 10 cycles of 94 degrees for 15 seconds followed by 60 degrees for 30 seconds followed by 68 degrees for 30 seconds, then a final hold at 68 degrees for 5 min, typically 20 uL volume)—293 bp product

sgRNA\_HDRstep1\_fw TGTTTTAAAATGGACTATCATATGCTTACCGTAACTTGAAAGTATTTCGATTTCTTGGCTTTATATATCTTGTGGAAAGGACGAAACACC

sgRNA\_HDRstep1\_rv GTTGATAACGGACTAGCCTTATTTAAACTTGCTATGCTGTTTCCAGCATAGCTCTTAAAC

sgRNA\_HDRstep2\_fw GTACAAAATACGTGACGTAGAAAGTAATAATTTCTTGGGTAGTTTGCAGTTTTAAAATTATGTTTTAAAATGGACTATCATATGCTTACC

sgRNA\_HDRstep2\_rv ATTTTAACTTGCTATTTCTAGCTCTAAAACAAAAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTAAAC

2X Onetaq master mix with standard buffer: 50% of reaction volume

20 uM 60 bp gRNA protospacer oligo: 2.5% of reaction volume

10 uM mix of sgRNA\_HDRstep1\_fw and sgRNA\_HDRstep1\_rv: 5% of reaction volume

10 uM mix of sgRNA\_HDRstep2\_fw and sgRNA\_HDRstep2\_rv: 5% of reaction volume

dH2O: 37.5% of reaction volume

PCR2 (Onetaq, 94 degrees for 30s, 10 cycles of 94 degrees for 15 seconds followed by 60 degrees for 30 seconds followed by 68 degrees for 30 seconds, then a final hold at 68 degrees for 5 min, typically 20 uL volume)—379 bp product

sgRNA\_HDRstep3\_fw CGATACAAGGCTGTTAGAGAGATAATTAGAATTAATTTGACTGTAAACACAAAGATATTAGTACAAAATACGTGACGTAGAAAGTAATAA

sgRNA\_HDRstep3\_rv TCAATGTATCTTATCATGTCTGCTCGATTTTAACTTGCTATTTCTAGCTCTAAAACAAAA

2X Onetaq master mix with standard buffer: 50% of reaction volume

Unpurified product of PCR1: 5% of reaction volume

10 uM mix of sgRNA\_HDRstep3\_fw and sgRNA\_HDRstep3\_rv: 5% of reaction volume

dH2O: 40% of reaction volume

PCR3: Note that this PCR is 30 cycles!(Onetaq, 94 degrees for 30s, **30** cycles of 94 degrees for 15 seconds followed by 60 degrees for 30 seconds followed by 68 degrees for 30 seconds, then a final hold at 68 degrees for 5 min, typically 200 uL reaction volume)—415 bp product

sgRNA\_HDRstep4\_fw GGCCTATTTCCCATGATTCCTTCATATTTGCATATACGATACAAGGCTGTTAGAGAGATA

sgRNA\_HDRstep4\_rv TCAATGTATCTTATCATGTCTGCTCGA

2X Onetaq master mix with standard buffer: 50% of reaction volume

Unpurified product of PCR2: 5% of reaction volume

10 uM mix of sgRNA\_HDRstep4\_fw and sgRNA\_HDRstep4\_rv: 5% of reaction volume

dH2O: 40% of reaction volume

Final amplicon:

Test 2 uL of PCR on 2% gel, expecting 415 bp. Then minElute purify using one column, eluting in 10 uL EB into a sterile tube.

GGCCTATTTCCCATGATTCCTTCATATTTGCATATACGATACAAGGCTGTTAGAGAGATAATTGGAATTAATTTGACTGTAAACACAAAGATATTAGTACAAAATACGTGACGTAGAAAGTAATAATTTCTTGGGTAGTTTGCAGTTTTAAAATTATGTTTTAAAATGGACTATCATATGCTTACCGTAACTTGAAAGTATTTCGATTTCTTGGCTTTATATATCTTGTGGAAAGGACGAAACACCGAGGCGTCTGGGTGGCTCTTGGTTTAAGAGCTATGCTGGAAACAGCATAGCAAGTTTAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGCTTTTTTGTTTTAGAGCTAGAAATAGCAAGTTAAAATCGAGCAGACATGATAAGATACATTGA

*Amplification of GFP-homology arm construct*

We use two successive PCRs with no purification in between to amplify the GFP-homology arm cassette. We use 2x NEBNext Mastermix (M0541L), which is a proofreading polymerase, as we want to avoid errors in PCR of the GFP ORF. In rare cases, these PCRs fail because the homology arm primers are insufficient. In this case you should troubleshoot the PCRs by trying a range of annealing temps (55-72 degrees) for each PCR and test with and without DMSO. If all else fails, you would have to redesign the homology arm primers, but this is rare. We use a p2Lox-GFP-V5 plasmid as our GFP template, but any GFP-containing plasmid will do.

PCR1: (NEBNext, 98 degrees for 30s, 15 cycles of 98 degrees for 10 seconds followed by 72 degrees for 45 seconds, then a final hold at 72 degrees for 5 min, typically 20 uL volume)—~820 bp product

2X NEBNext master mix: 50% of reaction volume

GFP plasmid at 100 ng/uL: 0.5% of reaction volume

20 uM LocusX\_GFPhomologyarm\_fw: 2.5% of reaction volume

20 uM LocusX\_GFPhomologyarm\_rv: 2.5% of reaction volume

DMSO: 3% of reaction volume

dH2O: 41.5% of reaction volume

We then perform a second PCR using the first PCR reaction as the template without purification.

PCR2: note that this PCR is 30 cycles! (NEBNext, 98 degrees for 30s, 30 cycles of 98 degrees for 10 seconds followed by 72 degrees for 45 seconds, then a final hold at 72 degrees for 5 min, typically 200 uL volume)—~900 bp product

2X NEBNext master mix: 50% of reaction volume

Unpurified product of PCR1: 5% of reaction volume

20 uM LocusX\_homologyarmextension\_fw: 2.5% of reaction volume

20 uM LocusX\_homologyarmextension\_rv: 2.5% of reaction volume

DMSO: 3% of reaction volume

dH2O: 37% of reaction volume

Final amplicon using Pou5f1-GFP as an example:

Test 2 uL of PCR on 2% gel, expecting ~900 bp. There may be a weak lower band but the 900 bp band should be dominant. There is no need to gel purify unless there is an abundance of smaller band. MinElute purify using one column, eluting in 10 uL EB into a sterile tube.

TCTACTCAGTCCCTTTTCCTGAGGGCGAGGCCTTTCCCTCTGTTCCCGTCACTGCTCTGGGCTCTCCCATGCATTCAAACGTGAGCAAGGGCGAGGAGCTGTTCACCGGGGTGGTGCCCATCCTGGTCGAGCTGGACGGCGACGTAAACGGCCACAAGTTCAGCGTGTCCGGCGAGGGCGAGGGCGATGCCACCTACGGCAAGCTGACCCTGAAGTTCATCTGCACCACCGGCAAGCTGCCCGTGCCCTGGCCCACCCTCGTGACCACCCTGACCTACGGCGTGCAGTGCTTCAGCCGCTACCCCGACCACATGAAGCAGCACGACTTCTTCAAGTCCGCCATGCCCGAAGGCTACGTCCAGGAGCGCACCATCTTCTTCAAGGACGACGGCAACTACAAGACCCGCGCCGAGGTGAAGTTCGAGGGCGACACCCTGGTGAACCGCATCGAGCTGAAGGGCATCGACTTCAAGGAGGACGGCAACATCCTGGGGCACAAGCTGGAGTACAACTACAACAGCCACAACGTCTATATCATGGCCGACAAGCAGAAGAACGGCATCAAGGTGAACTTCAAGATCCGCCACAACATCGAGGACGGCAGCGTGCAGCTCGCCGACCACTACCAGCAGAACACCCCCATCGGCGACGGCCCCGTGCTGCTGCCCGACAACCACTACCTGAGCACCCAGTCCGCCCTGAGCAAAGACCCCAACGAGAAGCGCGATCACATGGTCCTGCTGGAGTTCGTGACCGCCGCCGGGATCACTCTCGGCATGGACGAGCTGTACAAGTAAAGCGGCCGCAATTCACTCCTCAGCTGTGAGCCAAGGCAAGGGAGGTAGACAAGAGAACCTGGAGCTTTGGGGTTAAATTCTTTTACTGAGGAGGGATTA

By the end of these PCRs, you should have 200 uL of gRNA PCR product and 200 uL of GFP homology construct minElute-purified in 10 uL EB. You are now ready for electroporation.

**Electroporation and transient drug selection**

You will need midi/maxipreps of CBh Cas9-BlastR plasmid and sgPal7-HygR plasmid.

For targeting 1 well of a 6-well/12-well (~106) mouse ESCs: we electroporate a mixture of 5 ug of CBh Cas9-BlastR plasmid, 10 uL minElute purified product of 200 uL GFP LocusX homology arm fragment, 5 ug sgPal7-HygR, and 10 uL minElute purified product of 200 uL gRNA PCR product.

Electroporated cells are plated onto a single well of a 6-well/12-well tissue culture plate (BD Falcon) in >2 mL 2i mESC media supplemented with 7.5 uM Y-27632 (Tocris). From 24-72 hours after electroporation, media was refreshed daily with mESC media supplemented with 10 ug/mL (1:1,000) Blasticidin and 100 ug/mL (1:500) Hygromycin. After selection, media was refreshed every day with 2i mESC media and cells were trypsinized and replated when confluent. Testing of homologous recombination efficiency was performed 5-7 days after electroporation.

For targeting 1 well of a 6-well/12-well (~106) human ESCs: we electroporate a mixture of 5 ug of CBh Cas9-BlastR plasmid, 10 uL minElute purified product of 200 uL GFP LocusX homology arm fragment, 5 ug sgPal7-HygR, and 10 uL minElute purified product of 200 uL gRNA PCR product into feeder-free or feeder-depleted hESCs. Electroporated cells were plated onto a single well of a 6-well tissue culture plate (BD Falcon) in >2 mL complete human ESC media supplemented with 10 uM Y-27632 (Tocris). From 24-72 hours after electroporation, media was refreshed daily with complete human ESC media supplemented with 2 ug/mL Blasticidin and 66 ug/mL (1:666) Hygromycin. After selection, media was refreshed every day with standard hESC media and cells were trypsinized and replated when confluent. Testing of CRISPR mutation or homologous recombination efficiency was performed at the first and second passages, circa 7-14 days after electroporation.

For fastest procedure, once selection is complete and cells have grown up enough to split, split cells 3 ways:

Count the cells and plate 96 wells of a 96-well plate at ~10 cells/well.

With the remaining cells, freeze ~1/2 of cells into 1-2 vials.

Collect genomic DNA on ~1/2 of cells using the Purelink Genomic DNA Mini Kit

The more cautious approach would be to split the bulk cells, freezing and collecting genomic DNA to test for integration and keeping some cells in culture, waiting to plate the 96 well limiting dilution until after you have confirmed integration in the bulk culture by PCR.

The below steps are for lines in which GFP is not expressed in ESCs themselves. If GFP is expressed in ESCs, flow cytometric sorting is the method of choice to purify knock-in cells.

**Bulk genomic DNA PCR/qPCR**

Bulk genomic DNA isolation can be done using the Purelink Genomic DNA mini kit (Life Technologies) (see below for 96-well gDNA isolation which is different).

Genomic DNA should be used to check the integration frequency using the primers ordered for this purpose during homology construct generation. There are three possible primer pairs to use:

Locus upstream fw + GFP rv

GFP fw + locus downstream rv

Locus upstream fw + locus downstream rv

You should try all of these combinations, although the third primer pair will most likely only give you the wildtype band and not the band indicating GFP insertion because small bands dominate in PCR and the GFP integration is going to occur in only a small subset of cells. We typically use Onetaq with 60 degree Ta (the standard conditions), and ideally we use ~200 ng genomic DNA per 20 uL reaction although this amount can be varied, and I have gotten useful data even when there is negligible detectable genomic DNA. If the bands are absent, weak, or have competing bands at incorrect sizes, optimize the Ta of this PCR and try with DMSO. This step is important not only to ensure that GFP has integrated in the population but also to optimize primer combinations, as the 96-well genomic DNA PCRs are messier so require robust primers. If PCR is successful, qPCR can optionally be used to estimate integration frequency. To do so, primer pairs that only give a product after correct integration should be used along with genomic DNA control primers that occur twice in every cell. By comparing amplication cycle number, approximate integration frequency can be established. I do not do this routinely but it is a possibility if desired.

Once you have verified integration, you can move on to isolating subclones with the integration.

**96-well genomic DNA PCR (made for mESCs but can be adapted to hESCs)**

Once 96-wells are at least 1/3 confluent, prepare to split. To do so, gelatin-coat and add 100 uL 2i media to an equal number of wells of a 96-well (must be a separate 96-well plate) and prepare 25 uL trypsin/well. Using multichannel, discard media in colony-containing wells, add 50 uL PBS/well, discard, add 25 uL trypsin/well. Incubate for ~5-10 min in 37 degree incubator, pipetting up and down every few minutes. Once colonies are broken up, pipet ~1/2 (10-15 uL, depends how confluent wells were to begin with) to the 2i wells. The remainder (~1/2) will be used for genomic DNA isolation. It is OK that the trypsin hasn’t been quenched since the next step is cell lysis.

Moving to benchtop (sterility not required), to the colonies in the trypsin-containing 96-well plate, add 50 uL genomic DNA lysis buffer (make sure Proteinase K has been added to buffer at 1 mg/mL final concentration). Stocks of genomic DNA lysis buffer can be made but proteinase K must be added fresh—final concentration of lysis buffer is 10 mM TrisHCl (pH7.5 or pH 8.0), 10 mM EDTA, 10 mM NaCl, 0.5% SDS. After transferring appropriate amount of lysis buffer to a boat, add 1:20 of 20 mg/mL Proteinase K then aliquot 50 uL per well using multichannel. Seal plate using parafilm and place plate in humidified staining chamber to avoid evaporation, then place chamber at 60 degrees. This step is ideally done overnight but can be shortened to >3 hrs.

Carefully add 100 uL/well of pre-chilled 100% EtOH + 75 mM NaCl (add NaCl fresh as it doesn’t really go into solution—150 uL 5 M NaCl per 10 mL EtOH) to each well and let sit on benchtop at room temp for 30 min.

After 30 min, carefully invert plate to remove liquid then add 150 uL/well 70% EtOH. Invert and repeat for 2 total 70% EtOH washes. After second wash, invert and then shake plate vigorously to remove all EtOH and blot upside down on paper towels to remove all EtOH. Let plate dry for 10-15 min at room temp then add 30 uL/well TE Buffer/Elution Buffer from miniprep or PCR purification kit. Optionally place back at 60 degrees for several minutes to dissolve DNA or just keep at room temp long enough for DNA to dissolve.

Prepare PCR mixture for 15 uL Onetaq PCR reactions of each colony (small volume to save $$). Aliquot 8.25 uL/well PCR mixture (7.5 uL 2X Onetaq mix + 0.75 uL primer mix) then add 6.75 uL 96-well genomic DNA (pipet up and down to mix genomic DNA before adding to PCR).

If necessary, prepare a larger PCR reaction (~25 uL) to confirm correct clones, gel purify and set up sequencing reactions. Onetaq is still fine for this. Sometimes sequencing isn’t great from 96-well clones so you may have to perform column genomic DNA isolation after splitting and growing up a larger number of cells from correct clones.

Note which wells give positive PCR reactions. These wells have a positive colony, but because they were subcloned at ~10 cells/well, you need to further subclone these by limiting dilution or by colony picking.