Supplementary Information

Double nicking by RNA-guided CRISPR Cas9 for Enhanced Genome Editing Specificity

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PRIMER SEQUENCES

SURVEYOR assay

primer name	genomic target	primer sequence (5' to 3')
SUV901	*EMX1*	CCATCCCCTTCTGTGAATGT
SUV902	*EMX1*	GGAGATTGGAGACACGGAGA
DYRK1A-F	*DYRK1A*	GGAGCTGGTCTGTTGGAGAA
DYRK1A-R	*DYRK1A*	TCCCAATCCATAATCCCACGTT
GRIN2B-F	*GRIN2B*	CAGGAGGGCCAGGAGATTTG
GRIN2B-R	*GRIN2B*	TGAAATCGAGGATCTGGGCG
F1	*VEGFA*	CAAAGGACCCCAGTCACTCC
R1	*VEGFA*	GAGGAGGGAGCAGGAAAGTG
F2	*VEGFA*	GACACTTCCCAAAGGACCCC
R2	*VEGFA*	TGAGAGCCGTTCCCTCTTTG
F3	*VEGFA*	GACAGGGGCAAAGTGAGTGA
R3	*VEGFA*	TTCATGGTTTCGGAGGCCC
F4	*VEGFA*	TGAGTGACCTGCTTTTGGGG
R4	*VEGFA*	GTTCATGGTTTCGGAGGCCC

Next-generation deep sequencing and HR

primer name	primer sequence (5' to 3')
EMX1-F	GGAGGACAAAGTACAAACGGC
EMX1-R	ATCGATGTCCTCCCCATTGG
EMX1-HR-F	CCATCCCCTTCTGTGAATGT
EMX1-HR-R	GGAGATTGGAGACACGGAGA
EMX1-OT1-F	TGGGAGAGAGACCCCTTCTT
EMX1-OT1-R	TCCTGCTCTCACTTAGACTTTCTC
EMX1-OT2-F	GACATTCCTCCTGAGGGAAAA
EMX1-OT2-R	GATAAAATGTATTCCTTCTCACCATTC

EMX1-OT3-F	CCAGACTCAGTAAAGCCTGGA
EMX1-OT3-R	TGGCCCCAGTCTCTCTTCTA
EMX1-OT4-F	CACGGCCTTTGCAAATAGAG
EMX1-OT4-R	CATGACTTGGCCTTTGTAGGA
EMX1-OT5-F	TGGGGTTACAGAAAGAATAGGG
EMX1-OT5-R	TTCTGAGGGCTGCTACCTGT
VEGFA-F	TGAAGCAACTCCAGTCCCAA
VEGFA-R	CCCGGCTCTGGCTAAAGAG
VEGFA-OT1.1-F	TGGGTGTGCACATCTAAGGA
VEGFA-OT1.1-R	CCACTGAGTCAACTGTAAGCA
VEGFA-OT2.1-F	GCAGAGGAATATGTGACATGAGG
VEGFA-OT2.1-R	TACTCCCTGCTGTCCTCTCC
VEGFA-OT3.1-F	TTCCTGGCCAAGTCGATTCC
VEGFA-OT3.1-R	GGATACCAGCATGGGCTACC
VEGFA-OT4.1-F	ACTCTTGAGATTGGAACGGGA
VEGFA-OT4.1-R	CCACACTTATCTACGCCCCA
VEGFA-OT5.1-F	AGCCAGAAGAGAACATCCACG
VEGFA-OT5.1-R	AGTTGCTCTTTGTTGAGAGGGA
MECP2-F	TGACCGGGGACCTATGTATGA
MECP2-R	ACAAGACTTGCTCTTACTTACTTGA

qRT-PCR for Cas9 and sgRNA expression

primer name	**primer sequence (5’ to 3’)**
sgRNA reverse-strand synthesis	AAGCACCGACTCGGTGCCAC
EMX1.3 sgRNA qPCR F	AGTCCGAGCAGAAGAAGAAGTTT
EMX1.3 sgRNA qPCR R	TTTCAAGTTGATAACGGACTAGCCT
Cas9 qPCR F	AAACAGCAGATTCGCCTGGA

Cas9 qPCR R	TCATCCGCTCGATGAAGCTC
GAPDH qPCR F	TCCAAAATCAAGTGGGGCGA
GAPDH qPCR R	TGATGACCCTTTTGGCTCCC

Multiplex nicking screening primers

Deletion size (kb)	Forward primer (5' to 3')	Reverse primer (5' to 3')
0.5	AGGTTGTTGCTGTTGCTTTACA	AGCACGGTTAATTTGCATACATCT
1	AGGTTGTTGCTGTTGCTTTACA	TCCAGGCAGTTTTCTTCTGGT
2	AGGTTGTTGCTGTTGCTTTACA	CCAGAGAGCCAGCATTCCAA
6	AGGTTTCACCTGGTTTGGGG	AGGGCTCCCACTAGAAGAGG

***VEGFA* off-target sites**

sgRNA	Off-target site	Off-target sequence (5’ to 3’)
96	OT-1.1	GGATGGAGGGAGTTTGCTCCTGG
98	OT-2.1	CGCCCTCCCCACCCCGCCTCCGG
98	OT-3.1	TACCCCCCACACCCCGCCTCTGG
98	OT-4.1	GGGCCCCTCCACCCCGCCTCTGG
98	OT-5.1	CTACCCCTCCACCCCGCCTCCGG

SUPPLEMENTARY SEQUENCES

All sequences are in the 5' to 3' direction. For U6 transcription, the string of underlined Ts serve as the transcriptional terminator.

> sgRNA containing +85 tracrRNA (*Streptococcus pyogenes* SF370)
gagggcctatttcccatgattccttcatatttgcatatacgatacaaggctgttagagagataattggaattaatttg
actgtaaacacaaagatattagtacaaaatacgtgacgtagaaagtaataatttcttgggtagtttgcagttttaaaa
ttatgttttaaaatggactatcatatgcttaccgtaacttgaaagtatttcgatttcttggctttatatatcttgtgg
aaaggacgaaacaccNNNNNNNNNNNNNNNNNNNNgttttagagctagaaatagcaagttaaaataaggctagtccgt
tatcaacttgaaaaagtggcaccgagtcggtgcTTTTTTT
(guide sequence is highlighted in blue and the tracrRNA fragment is highlighted in red)

> 3xFLAG-NLS-SpCas9-NLS
ATGGACTATAAGGACCACGACGGAGACTACAAGGATCATGATATTGATTACAAAGACGATGACGATAAGATGGCCCCA
AAGAAGAAGCGGAAGGTCGGTATCCACGGAGTCCCAGCAGCCGACAAGAAGTACAGCATCGGCCTGGACATCGGCACC
AACTCTGTGGGCTGGGCCGTGATCACCGACGAGTACAAGGTGCCCAGCAAGAAATTCAAGGTGCTGGGCAACACCGAC
CGGCACAGCATCAAGAAGAACCTGATCGGAGCCCTGCTGTTCGACAGCGGCGAAACAGCCGAGGCCACCCGGCTGAAG
AGAACCGCCAGAAGAAGATACACCAGACGGAAGAACCGGATCTGCTATCTGCAAGAGATCTTCAGCAACGAGATGGCC
AAGGTGGACGACAGCTTCTTCCACAGACTGGAAGAGTCCTTCCTGGTGGAAGAGGATAAGAAGCACGAGCGGCACCCC
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CTGACCCCCAACTTCAAGAGCAACTTCGACCTGGCCGAGGATGCCAAACTGCAGCTGAGCAAGGACACCTACGACGAC
GACCTGGACAACCTGCTGGCCCAGATCGGCGACCAGTACGCCGACCTGTTTCTGGCCGCCAAGAACCTGTCCGACGCC
ATCCTGCTGAGCGACATCCTGAGAGTGAACACCGAGATCACCAAGGCCCCCCTGAGCGCCTCTATGATCAAGAGATAC
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TACCCATTCCTGAAGGACAACCGGGAAAAGATCGAGAAGATCCTGACCTTCCGCATCCCCTACTACGTGGGCCCTCTG
GCCAGGGGAAACAGCAGATTCGCCTGGATGACCAGAAAGAGCGAGGAAACCATCACCCCCTGGAACTTCGAGGAAGTG
GTGGACAAGGGCGCTTCCGCCCAGAGCTTCATCGAGCGGATGACCAACTTCGATAAGAACCTGCCCAACGAGAAGGTG
CTGCCCAAGCACAGCCTGCTGTACGAGTACTTCACCGTGTATAACGAGCTGACCAAAGTGAAATACGTGACCGAGGGA
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CGGTTCAACGCCTCCCTGGGCACATACCACGATCTGCTGAAAATTATCAAGGACAAGGACTTCCTGGACAATGAGGAA
AACGAGGACATTCTGGAAGATATCGTGCTGACCCTGACACTGTTTGAGGACAGAGAGATGATCGAGGAACGGCTGAAA
ACCTATGCCCACCTGTTCGACGACAAAGTGATGAAGCAGCTGAAGCGGCGGAGATACACCGGCTGGGGCAGGCTGAGC
CGGAAGCTGATCAACGGCATCCGGGACAAGCAGTCCGGCAAGACAATCCTGGATTTCCTGAAGTCCGACGGCTTCGCC

AACAGAAACTTCATGCAGCTGATCCACGACGACAGCCTGACCTTTAAAGAGGACATCCAGAAAGCCCAGGTGTCCGGC
CAGGGCGATAGCCTGCACGAGCACATTGCCAATCTGGCCGGCAGCCCCGCCATTAAGAAGGGCATCCTGCAGACAGTG
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CAGATCCTGAAAGAACACCCCGTGGAAAACACCCAGCTGCAGAACGAGAAGCTGTACCTGTACTACCTGCAGAATGGG
CGGGATATGTACGTGGACCAGGAACTGGACATCAACCGGCTGTCCGACTACGATGTGGACCATATCGTGCCTCAGAGC
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GACAATCTGACCAAGGCCGAGAGAGGCGGCCTGAGCGAACTGGATAAGGCCGGCTTCATCAAGAGACAGCTGGTGGAA
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CCTAAGCTGGAAAGCGAGTTCGTGTACGGCGACTACAAGGTGTACGACGTGCGGAAGATGATCGCCAAGAGCGAGCAG
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CTGTACCTGGCCAGCCACTATGAGAAGCTGAAGGGCTCCCCCGAGGATAATGAGCAGAAACAGCTGTTTGTGGAACAG
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GACAAAGTGCTGTCCGCCTACAACAAGCACCGGGATAAGCCCATCAGAGAGCAGGCCGAGAATATCATCCACCTGTTT
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AAAGAGGTGCTGGACGCCACCCTGATCCACCAGAGCATCACCGGCCTGTACGAGACACGGATCGACCTGTCTCAGCTG
GGAGGCGACAAGCGTCCTGCTGCTACTAAGAAAGCTGGTCAAGCTAAGAAAAAGAAA

> 3xFLAG-NLS-SpCas9n-NLS (the D10A nickase mutation is labeled in red)
ATGGACTATAAGGACCACGACGGAGACTACAAGGATCATGATATTGATTACAAAGACGATGACGATAAGATGGCCCCA
AAGAAGAAGCGGAAGGTCGGTATCCACGGAGTCCCAGCAGCCGACAAGAAGTACAGCATCGGCCTGGCCATCGGCACC
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CGGCACAGCATCAAGAAGAACCTGATCGGAGCCCTGCTGTTCGACAGCGGCGAAACAGCCGAGGCCACCCGGCTGAAG
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GACAGCACCGACAAGGCCGACCTGCGGCTGATCTATCTGGCCCTGGCCCACATGATCAAGTTCCGGGGCCACTTCCTG
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TTCGAGGAAAACCCCATCAACGCCAGCGGCGTGGACGCCAAGGCCATCCTGTCTGCCAGACTGAGCAAGAGCAGACGG
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GACCTGGACAACCTGCTGGCCCAGATCGGCGACCAGTACGCCGACCTGTTTCTGGCCGCCAAGAACCTGTCCGACGCC
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CGGTTCAACGCCTCCCTGGGCACATACCACGATCTGCTGAAAATTATCAAGGACAAGGACTTCCTGGACAATGAGGAA
AACGAGGACATTCTGGAAGATATCGTGCTGACCCTGACACTGTTTGAGGACAGAGAGATGATCGAGGAACGGCTGAAA
ACCTATGCCCACCTGTTCGACGACAAAGTGATGAAGCAGCTGAAGCGGCGGAGATACACCGGCTGGGGCAGGCTGAGC
CGGAAGCTGATCAACGGCATCCGGGACAAGCAGTCCGGCAAGACAATCCTGGATTTCCTGAAGTCCGACGGCTTCGCC
AACAGAAACTTCATGCAGCTGATCCACGACGACAGCCTGACCTTTAAAGAGGACATCCAGAAAGCCCAGGTGTCCGGC
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TCCGAAGAGGTCGTGAAGAAGATGAAGAACTACTGGCGGCAGCTGCTGAACGCCAAGCTGATTACCCAGAGAAAGTTC
GACAATCTGACCAAGGCCGAGAGAGGCGGCCTGAGCGAACTGGATAAGGCCGGCTTCATCAAGAGACAGCTGGTGGAA
ACCCGGCAGATCACAAAGCACGTGGCACAGATCCTGGACTCCCGGATGAACACTAAGTACGACGAGAATGACAAGCTG
ATCCGGGAAGTGAAAGTGATCACCCTGAAGTCCAAGCTGGTGTCCGATTTCCGGAAGGATTTCCAGTTTTACAAAGTG
CGCGAGATCAACAACTACCACCACGCCCACGACGCCTACCTGAACGCCGTCGTGGGAACCGCCCTGATCAAAAAGTAC
CCTAAGCTGGAAAGCGAGTTCGTGTACGGCGACTACAAGGTGTACGACGTGCGGAAGATGATCGCCAAGAGCGAGCAG
GAAATCGGCAAGGCTACCGCCAAGTACTTCTTCTACAGCAACATCATGAACTTTTTCAAGACCGAGATTACCCTGGCC
AACGGCGAGATCCGGAAGCGGCCTCTGATCGAGACAAACGGCGAAACCGGGGAGATCGTGTGGGATAAGGGCCGGGAT
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AGCAAAGAGTCTATCCTGCCCAAGAGGAACAGCGATAAGCTGATCGCCAGAAAGAAGGACTGGGACCCTAAGAAGTAC
GGCGGCTTCGACAGCCCCACCGTGGCCTATTCTGTGCTGGTGGTGGCCAAAGTGGAAAAGGGCAAGTCCAAGAAACTG
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CACAAGCACTACCTGGACGAGATCATCGAGCAGATCAGCGAGTTCTCCAAGAGAGTGATCCTGGCCGACGCTAATCTG
GACAAAGTGCTGTCCGCCTACAACAAGCACCGGGATAAGCCCATCAGAGAGCAGGCCGAGAATATCATCCACCTGTTT

ACCCTGACCAATCTGGGAGCCCCTGCCGCCTTCAAGTACTTTGACACCACCATCGACCGGAAGAGGTACACCAGCACC
AAAGAGGTGCTGGACGCCACCCTGATCCACCAGAGCATCACCGGCCTGTACGAGACACGGATCGACCTGTCTCAGCTG
GGAGGCGACAAGCGTCCTGCTGCTACTAAGAAAGCTGGTCAAGCTAAGAAAAAGAAA

> dsODN ligation insert fwd
AGCAGGCCAATGGGGAGGACATCGATGTCACCTCCAATGACTAcTACCCATACGATGTTCCAGATTACGCTATGGACT
ATAAGGACCACGACGGAGACTACAAGGATCATGATATTGATTACAAAGACGATGACGATAAGaagcttgaaATGGCAT
CAATGCAGAAGCTGATCTCAGAGGAGGACCTGtaa

> dsODN ligation insert rev
TAGTCATTGGAGGTGACATCGATGTCCTCCCCATTGGCCTGCTTTACAGGTCCTCCTCTGAGATCAGCTTCTGCATTG
ATGCCATTTCAAGCTTCTTATCGTCATCGTCTTTGTAATCAATATCATGATCCTTGTAGTCTCCGTCGTGGTCCTTAT
AGTCCATAGCGTAATCTGGAACATCGTATGGGTAG

The RNA-guided Cas9 nuclease can tolerate certain mismatches to the DNA target and thereby promote undesired off-target mutagenesis. Cas9 nickase mutants can be combined with guide RNA pairs to introduce targeted double-strand breaks. Paired nicking reduces off-target activity by 50-1,000 fold in cell lines and facilitates gene knockout in mouse zygotes without sacrificing on-target cleavage efficiency. This versatile strategy enables a wide variety of genome editing applications with higher levels of specificity.