Mary Lyon Centre at MRC Harwell

Name of Mouse model or mutation:
Lrrk2-Bromotag-EM1-B6J

Description:
Knock-in of EGFP-IRES-Bromotag into exon ENSMUSE00000503724 of the *Lrrk2* gene made by CRISPR/Cas gene editing

Type of mutation:
Knock-in: EGFP-IRES-Bromotag

Delivery method:
Pronuclear injection into 1-cell embryos.

Genetic Background:
C57BL/6J

Nuclease:
Cas9 mRNA

sgRNAs:

Protospacer sequence	PAM sequence
CTGACAGGCGCCACTGGCCA	TGG
GCCGGCTGCACCATGGCCAG	TGG

Donor Sequence (5' to 3'):

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DEFINITION     Lrrk2_Bromotag_pUC57
SOURCE
  ORGANISM
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similar
                     variants"
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similar
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     protein_bind    complement(3508..3530)
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                     relieved by adding lactose or
                     isopropyl-beta-D-thiogalactopyranoside (IPTG)."
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carbenicillin,
                     and related antibiotics"
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 601 GCTCCCAGAT CCTGCCTGCA AACTCAAAAC AGAGAGAGGC CTTTTGGCAA CCACAACCAG
 661 AGCTCAGGAT AACCAACAAG CAAACAACTC CTAAAGCCCA TGTAGCTGGC CGATTTTGAT
 721 TGTTAAACGC TGCATTCTTT GGGGAGCAAA TTACTCCTCT CCCTATAGGG GGAACTCTTA
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 841 TTGCTATTCT TAGTGGCGGG GTGGCGGCTT TCGCTTTTCC CCGGCGGAGT TCCCAGCGGC
 901 GCGATGCGGC TGGGCAGGCC GGGCTGAGAC TGGGCAGGGG CGGTCGCCGT CACCGTCTGG
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Pronuclear Microinjection Mixes:

Microinjection buffer (MIB; 10 mM Tris–HCl, 0.1 mM EDTA, 100 mM NaCl, pH7.5) was prepared and filtered through a 2 nm filter and autoclaved. Cas9 mRNA, sgRNAs and plasmid were diluted and mixed in MIB to the working concentrations of 100 ng/µl, 50 ng/µl each and 50 ng/µl, respectively. Injected embryos were re-implanted in CD1 pseudo-pregnant females. Host females were allowed to litter and rear F0 progeny.

Sequence details

Lrrk2 WT sequence

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Lrrk2-Bromotag-EM1-B6J sequence

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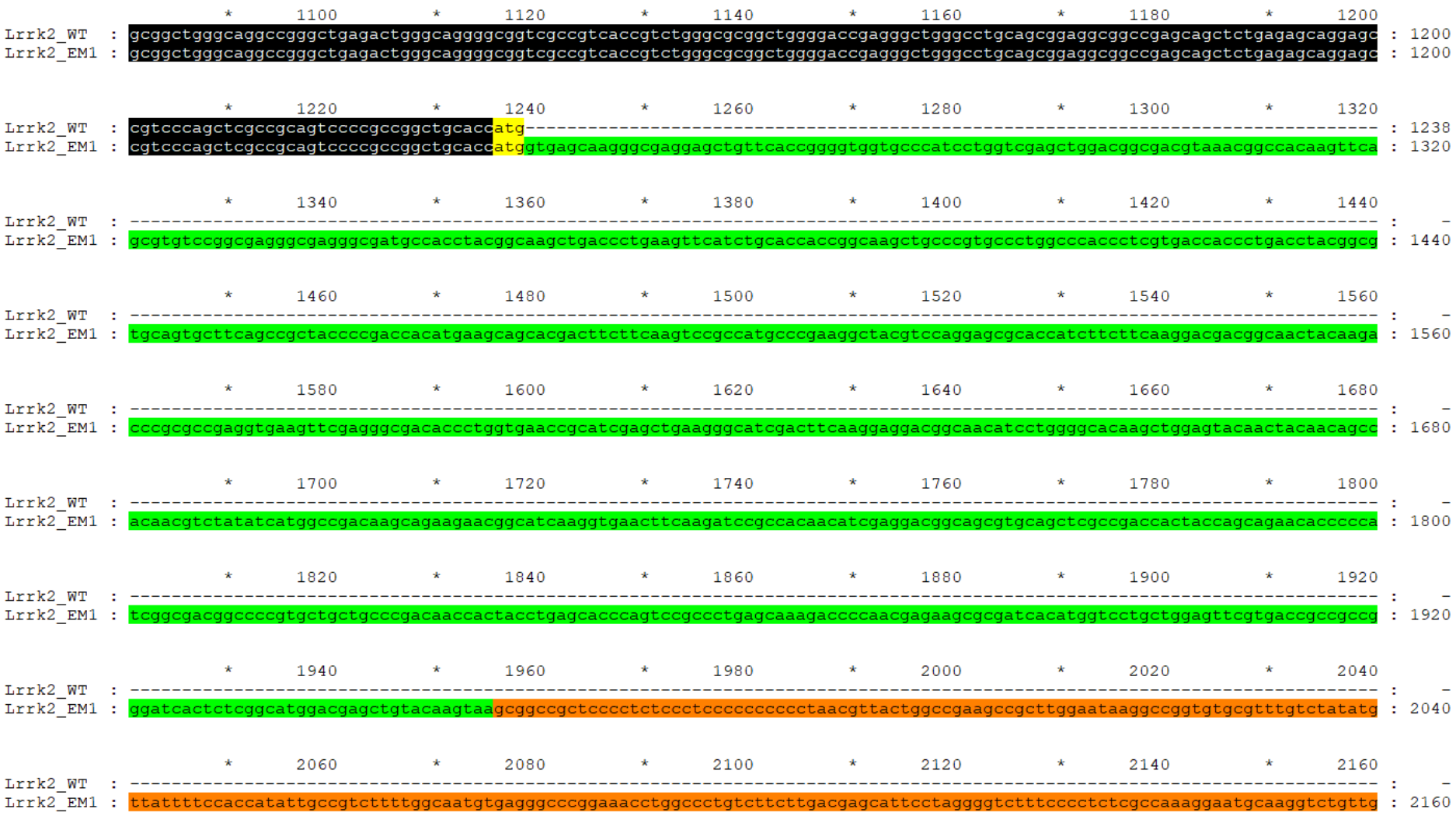
Nucleotides in bold are the **start codon**. Nucleotides highlighted in green = eGFP sequence, nucleotides highlighted in yellow = IRES sequence and nucloetides highlighted in blue = bromotag sequence.

Nucleotide Alignment: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Lrrk2_EM1 : gcggctgggcaggccgggctgagactgggcaggggcggtcgccgtcaccgtctgggcgcggctggggaccgagggctgggcctgcagcggaggcggccgagcagctctgagagcaggagc : 1200
* 1220 * 1240 * 1260 * 1280 * 1300 * 1320
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Lrrk2_EM1 : cgtcccagctcgccgcagtccccgccggctgaccatggtgagcaagggcgaggagctgttcaccggggtggtgcccatcctggtcgagctggacggcgacgtaaacggccacaagttca : 1320
* 1340 * 1360 * 1380 * 1400 * 1420 * 1440
Lrrk2_WT : ---------- : -
Lrrk2_EM1 : gcgtgtccggcgagggcgagggcgatgccacctacggcaagctgaccctgaagttcatctgcaccaccggcaagctgcccgtgccctggcccaccctcgtgaccaccctgacctacggcg : 1440
* 1460 * 1480 * 1500 * 1520 * 1540 * 1560
Lrrk2_WT : ---------- : -
Lrrk2_EM1 : tgcagtgcttcagccgctaccccgaccacatgaagcagcacgacttcttcaagtccgccatgcccgaaggctacgtccaggagcgcaccatcttcttcaaggacgacggcaactacaaga : 1560
* 1580 * 1600 * 1620 * 1640 * 1660 * 1680
Lrrk2_WT : ---------- : -
Lrrk2_EM1 : cccgcgccgaggtgaagttcgagggcgacaccctggtgaaccgcatcgagctgaagggcatcgacttcaaggaggacggcaacatcctggggcacaagctggagtacaactacaacagcc : 1680
* 1700 * 1720 * 1740 * 1760 * 1780 * 1800
Lrrk2_WT : ---------- : -
Lrrk2_EM1 : acaacgtctatatcatggccgacaagcagaagaacggcatcaaggtgaacttcaagatccgccacaacatcgaggacggcagcgtgcagctcgccgaccactaccagcagaacaccccca : 1800
* 1820 * 1840 * 1860 * 1880 * 1900 * 1920
Lrrk2_WT : ---------- : -
Lrrk2_EM1 : tcggcgacggccccgtgctgctgcccgacaaccactacctgagcacccagtccgccctgagcaaagaccccaacgagaagcgcgatcacatggtcctgctggagttcgtgaccgccgccg : 1920
* 1940 * 1960 * 1980 * 2000 * 2020 * 2040
Lrrk2_WT : ---------- : -
Lrrk2_EM1 : ggatcactctcggcatggacgagctgtacaagtaagcggccgctcccctctccctccccccccccctaacgttactggccgaagccgcttggaataaggccggtgtgcgtttgtctatatg : 2040
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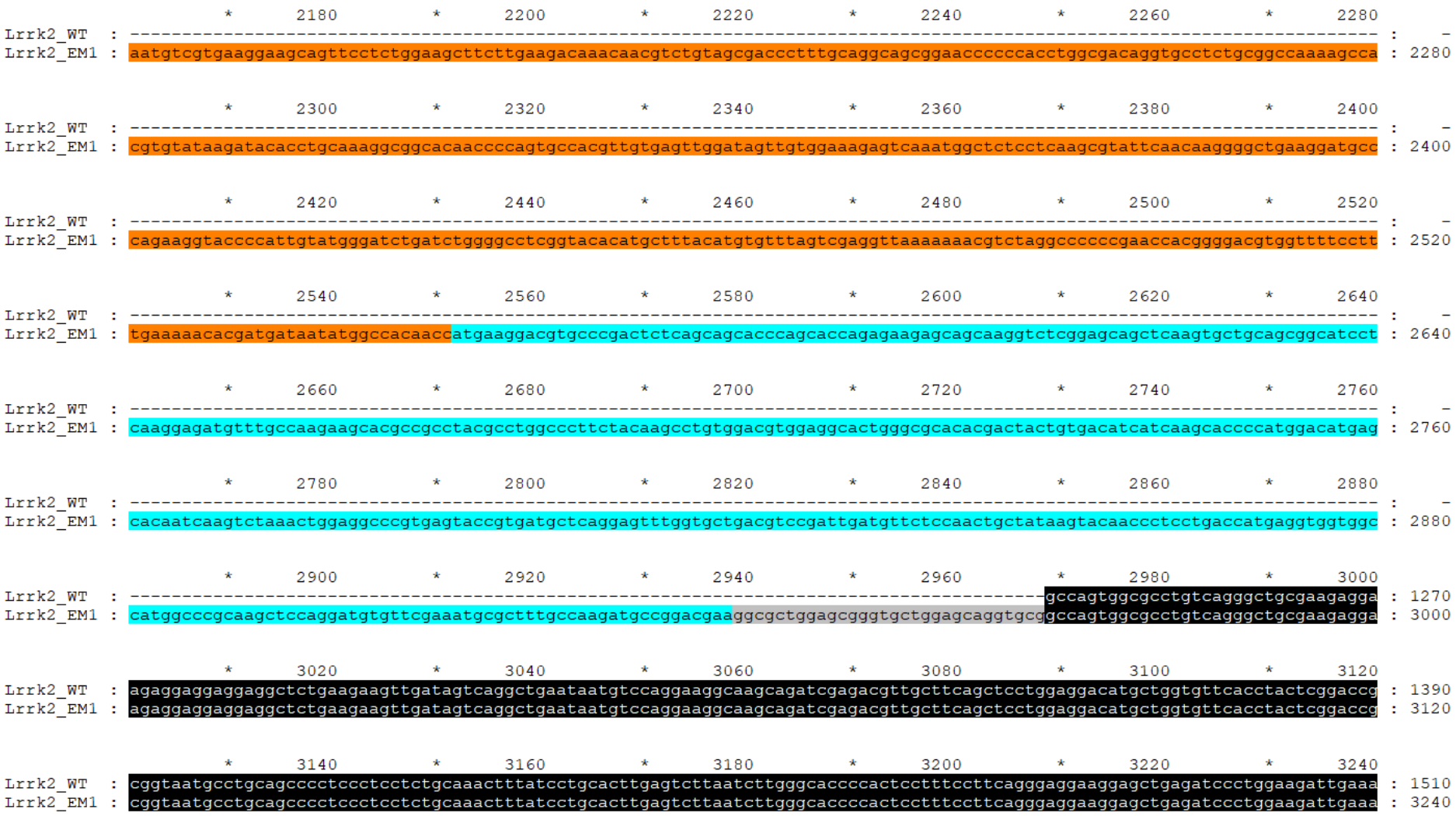
Lrrk2_WT : ---------- : -
Lrrk2_EM1 : ttattttccaccatattgccgtcttttggcaatgtgagggcccggaaacctggccctgtcttcttgacgagcattcctaggggtctttcccctctcgccaaaggaatgcaaggtctgttg : 2160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Lrrk2_EM1 : aatgtcgtgaaggaagcagttcctctggaagcttcttgaagacaaacaacgtctgtagcgacccttgcaggcagcggaacccccacctggcgacaggtgcctctgcggccaaaagcca : 2280
* 2300 * 2320 * 2340 * 2360 * 2380 * 2400
Lrrk2_WT : ---------- : -
Lrrk2_EM1 : cgtgtataagatacacctgcaaaggcggcacaaccccagtgccacgttgtgagttggatagttgtggaaagagtcaaatggctctcctcaagcgtattcaacaaggggctgaaggatgcc : 2400
* 2420 * 2440 * 2460 * 2480 * 2500 * 2520
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Lrrk2_EM1 : cagaaggtaccccattgtatgggatctgatctggggcctcggtacacatgctttacatgtgtttagtcgaggttaaaaaaacgtctaggccccccgaaccacggggacgtggttttcctt : 2520
* 2540 * 2560 * 2580 * 2600 * 2620 * 2640
Lrrk2_WT : ---------- : -
Lrrk2_EM1 : tgaaaaacacgatgataatatggccacaaccatgaaggacgtgcccgactctcagcagcacccagcaccagagaagagcagcaaggtctcggagcagctcaagtgctgcagcggcatcct : 2640
* 2660 * 2680 * 2700 * 2720 * 2740 * 2760
Lrrk2_WT : ---------- : -
Lrrk2_EM1 : caaggagatgtttgccaagaagcacgccgcctacgcctggcccttctacaagcctgtggacgtggaggcactgggcgcacacgactactgtgacatcatcaagcaccccatggacatgag : 2760
* 2780 * 2800 * 2820 * 2840 * 2860 * 2880
Lrrk2_WT : ---------- : -
Lrrk2_EM1 : cacaatcaagtctaaactggaggcccgtgagtaccgtgatgctcaggagtttggtgctgacgtccgattgatgttctccaactgctataagtacaaccctcctgaccatgaggtggtggc : 2880
* 2900 * 2920 * 2940 * 2960 * 2980 * 3000
Lrrk2_WT : ----------gccagtggcgcctgtcagggctgcgaagagga : 1270
Lrrk2_EM1 : catggcccgcaagctccaggatgtgttcgaaatgcgctttgccaagatgccggacgaaggcgctggagcgggtgctggagcaggtgcggccagtggcgcctgtcagggctgcgaagagga : 3000
* 3020 * 3040 * 3060 * 3080 * 3100 * 3120
Lrrk2_WT : agaggaggaggaggctctgaagaagttgatagtcaggctgaataatgtccaggaaggcaagcagatcgagacgttgcttcagctcctggaggacatgctggtgttcacctactcggaccg : 1390
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* 3140 * 3160 * 3180 * 3200 * 3220 * 3240
Lrrk2_WT : cggtaatgcctgcagcccctccctcctctgcaaactttatcctgcacttgagtcttaatcttgggcaccccactcctttccttcagggaggaaggagctgagatccctggaagattgaaa : 1510
Lrrk2_EM1 : cggtaatgcctgcagcccctccctcctctgcaaactttatcctgcacttgagtcttaatcttgggcaccccactcctttccttcagggaggaaggagctgagatccctggaagattgaaa : 3240

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                 *      3260        *      3280        *      3300        *      3320        *      3340        *      3360
Lrrk2_WT  : ggatgccaggatgccgtggtttgtgactttgttttcatcccccatttgccccctttctttccagcctccaagttatttgaagataaaaatttccacgtgcctctgttgattgtcctggac : 1630
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                 *      3380        *      3400        *      3420        *      3440        *      3460        *      3480
Lrrk2_WT  : tcctacatgagagttgccagtgtacagcaggtaagcgggccttcccatctgttcctgaagactgttctgtgataggccttatgtcccggggtcttgagatttgctttaagaggggaaatc : 1750
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                 *      3500        *      3520        *      3540        *      3560        *      3580        *      3600
Lrrk2_WT  : ttgtcaaatgttggcagaggagaagcacctttgacattatctgggtggtggtagcagcaaaatgaaaacagggctcttattggcaaggtgagtccgttaacctttcaaactattctaaga : 1870
Lrrk2_EM1 : ttgtcaaatgttggcagaggagaagcacctttgacattatctgggtggtggtagcagcaaaatgaaaacagggctcttattggcaaggtgagtccgttaacctttcaaactattctaaga : 3600

                 *      3620        *      3640        *      3660        *      3680        *      3700        *      3720
Lrrk2_WT  : atgctccttaatatacagcgagtttctggtagagcagtgcagtgaatatttccagcaagggagggtaattaaaaaaacagtaataaaacagtcaagtctagggtcagtttgcttttatac : 1990
Lrrk2_EM1 : atgctccttaatatacagcgagtttctggtagagcagtgcagtgaatatttccagcaagggagggtaattaaaaaaacagtaataaaacagtcaagtctagggtcagtttgcttttatac : 3720

                 *      3740        *      3760        *      3780        *      3800        *      3820        *      3840
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Lrrk2_EM1 : ttttaaattagaagacacatttcaaggattcttcctgcagtgtatatcacccagtatctaaagaaagtgatgtttcccgaatgccccctcgcctgccgtgtttacatttctactttgcgg : 3840

                 *      3860        *      3880        *      3900        *      3920        *      3940        *      3960
Lrrk2_WT  : ctgttatcatacaaagtggatacaaaagtagaacttctgtctggatctgtgtcacaggaagtccagaagtaggctatgtaattaactgtgattttaaagcagactcggataagtaagaat : 2230
Lrrk2_EM1 : ctgttatcatacaaagtggatacaaaagtagaacttctgtctggatctgtgtcacaggaagtccagaagtaggctatgtaattaactgtgattttaaagcagactcggataagtaagaat : 3960

                 *      3980        *      4000        *      4020        *
Lrrk2_WT  : ggctttcagtcaggacagaattacttctgaaggtgctaacttcctttagaggtttgcgatctggggaactgaaa : 2304
Lrrk2_EM1 : ggctttcagtcaggacagaattacttctgaaggtgctaacttcctttagaggtttgcgatctggggaactgaaa : 4034
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QC strategy employed at Harwell to check the edited allele:

Genomic DNA was extracted from ear clip biopsies and amplified in a PCR reaction using the following conditions/primer sequences:

Geno_Lrrk2_BTag_F1 (5’ to 3’)	GTGTCTTGGCTCTCAACCCG
Geno_Lrrk2_BTag_R1 (5’ to 3’)	TTTCAGTTCCCCAGATCGCAA
Buffer Used	ThermoFisher SuperFi II PCR kit
Annealing Temperature	60
Elongation Time	2
WT Size	2304
Mutant Size	4034
Notes	PCR external to Knock-in. Primers bind to engineered and WT allele.

eGFP_F2 (5’ to 3’)	cacatgaagcagcacgactt
eGFP_R2 (5’ to 3’)	ttctcgttggggtctttgct
Buffer Used	ThermoFisher SuperFi II PCR kit
Annealing Temperature	60
Elongation Time	0.5
WT Size	0
Mutant Size	413
Notes	Knock-in specific PCR.

Geno_Lrrk2_Btag_F1 (5' to 3')	GTGTCTTGGCTCTCAACCCG
eGFP_R2 (5’ to 3’)	ttctcgttggggtctttgct
Buffer Used	ThermoFisher SuperFi II PCR kit
Annealing Temperature	60
Elongation Time	1

WT Size	0
Mutant Size	1879
Notes	Knock-in specific PCR. Sequenced with the following primers (5'-3'): Seq_Lrrk2_Btag_F1: ATCCTGCCTGCAAACTCAAA

eGFP_F2 (5' to 3')	cacatgaagcagcacgactt
Geno_Lrrk2_BTag_R1 (5' to 3')	TTTCAGTTCCCCAGATCGCAA
Buffer Used	ThermoFisher SuperFi II PCR kit
Annealing Temperature	60
Elongation Time	1.25
WT Size	0
Mutant Size	2568
Notes	Knock-in specific PCR. Sequenced with the following primers (5'-3'): Geno_Lrrk2_Btag_F3: ggatcactctcggcatggac Geno_Lrrk2_Btag_R5: aagcgcatttcgaacacatcc Seq_Lrrk2_Btag_R1: TGCTTCTCCTCTGCCAACAT

All amplicons were sent for Sanger sequencing to check for integration of the donor oligo sequence at the target site. F1 sequences should be heterozygous unless on sex chromosome.

Off-target site with ≤2 mismatches for guide(s) used were checked with the following primers:

Off-target site	**Sequence**	**Type**	**Primers used (5'-3')**
15:79720608-79720630	CT**A**A**G**AGG**G**GCCA**T**TGGCCA GGG	Exonic	Lrrk2_Btag_OT1_F1: AGGGAGAGAGGGAAGAGCAG Lrrk2_Btag_OT1_R1: CCAAGCCTCCTTCCAGTGAG
15:25625627-25625649	CTG**TT**AGG**G**GCCA**G**TGGCCA GGG	Intronic	Lrrk2_Btag_OT2_F1: GGGTAGAACTCTGCCCCCTA

			Lrrk2_Btag_OT2_R1: AACTTACACCTGCAGCCCTC Please sequence with the following primer (5'-3'): Lrrk2_Btag_OT2_F2: ACTGGAAAGCGCTGGAATCA
15:87734402-87734424	CTTATAGGTGCCACTGGCAA AGG	Intronic	Lrrk2_Btag_OT4_F1: GCTCAGAACCAGGTACCCAC Lrrk2_Btag_OT4_R1: ACAAGCAAGATGCTACCCCC
15:59348308-59348330	ATGCCAGGAGCCACTGGCCG GGG	Intronic	Lrrk2_Btag_OT5_F2: ACGTGAGGCTATATGGGGGA Lrrk2_Btag_OT5_R2: ACTGACATCTTGGGCAGTGG Please sequence with the following primer (5'-3'): Lrrk2_Btag_OT5_R1: TGGCCTCAGAGTTGCAGAAG
7:63444938-63444960	CTGGCCACGGTGCTGCCGGC GGG	Intronic	Lrrk2_Btag_OT6_F1: ACCCGTTGGCAACCTTGAAT Lrrk2_Btag_OT6_R1: CGTGACCCACGTAGCCATAG Please sequence with the following primer (5'-3'): Lrrk2_Btag_OT6_F2: TACACCCTAAGGGGGCAAAC
18:77487603-77487625	CTGGGCATGGTGCAGCCAGC AGG	Intronic	Lrrk2_Btag_OT7_F1: GTACCCTGAAGCCCACTACTT Lrrk2_Btag_OT7_R1: CTCCATGACATAGGGCCGAG
15:99408335-99408357	CTGTCCTGGGTGCAGCCGGG AGG	Intronic	Lrrk2_Btag_OT10_F1: GTCTTTCAGGCACTGCGTTG Lrrk2_Btag_OT10_R1: ACCTTGCCACACATACTCGG Please sequence with the following primer (5'-3'):

			Lrrk2_Btag_OT10_R2: ACAAAAGCTTTCCTGATGCTTTCA

All amplicons were sent for Sanger sequencing. No off-target activity was detected in the animals selected to establish the colony.

Additional integrations of the donor sequence

Copy counting of the donor sequence was carried out by ddPCR at the F1 stage to confirm donor oligos were inserted once on target into the genome. The following Taqman assay was used to copy count the donor sequence compared against a VIC-labelled reference assay for Dot1l:

Assay Name	Bromotag-MUT1
Forward Primer (5'-3')	CATGGACATGAGCACAATCAAG
Reverse Primer (5'-3')	GTCAGGAGGGTTGTACTTATAGC
Probe Sequence	TGATGCTCAGGAGTTTGGTGCTGA
Label	FAM

This ddPCR assay is specific to the donor used to create the engineered mutation and only mutant alleles are expected to be recognised by this assay. Therefore, WT controls are expected to call at 0 copies and a single integration for a correct mutation is expected to call at 1 copy for F1 (HET) animals.

Reference Assay Name	Dot1l
Forward primer (5'-3')	GCCCCAGCACGACCATT
Reverse primer (5'-3')	TAGTTGGCATCCTTATGCTTCATC
Probe (5'-3')	CCCAACAGGCCTGGATTCTCAATGC
Label	VIC

VIC-labelled reference assay for Dot1l gene.

No additional donor integrations were detected in the animals taken forward to establish the colony.