

Name of Mouse model or mutation:**SQSTM1-G331D-EM1-B6N****SQSTM1-G331D-EM2-B6N****Description:**

Point mutation model made using CRISPR/Cas9.

Type of mutation:

SNP: G331D

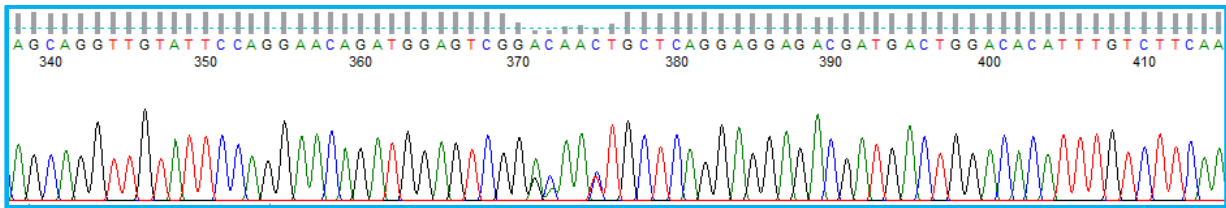
Sequence details**WT**

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CCCACTACCCCAGAAAGTTCCAGCACAGGCACAGAAGACAAGAGTAACACTCAGCCAAGCAGCTGC
TCTTCGGAAGTCAGCAAACCTGACGGGGCTGGGGAGGGCCCTGCTCAGTCTCTGACAGAGCAAATG
AAAAAGATAGCCTTGGAGTCGGTGGGACAGCCAGAGGTAGGTCTACTAGCTTCAGCCTGAGGAATC
CTGTCCTTCTACTGTTACCCTGAGCCTCCTGATAGAACTCTGTAGAGAAAATTTACTTAGCTCTGGAG
CTGTAACATCATACAGCTGGCGCCCATGAGGCCCCAGGTTTCAGTCCCTGGCTGGGAACAGATAGT
GGGGTTAGACTTGTCATTGCTTAGGCCTGATGCTCACAGCAGGTTGTATTCCAGGAACAGATGGAGT
CGGGAAACTGCTCAGGAGGAGACGATGACTGGACACATTTGTCTTCAAAGAAGTGGACCCATCTA
CAGGTGAACTCCAGTCTCTACAGATGCCAGAATCGGAAGGGCCAAGCTCTCTAGACCCCTCACAGG
AAGGACCCACAGGGCTGAAGGAAGCTGCCCTATACCACATCTCCCACCAGGCATGTGGTCACCAG
AAGTTTCATTTTCCTTCTTTGCTTTCAGAGGATCCTGTCCTGTCTAGTCTTGCCAACACAAAAAGGGTT
G
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SQSTM1-G331D-EM1-B6N & SQSTM1-G331D-EM2-B6N

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CCCACTACCCCAGAAAGTTCCAGCACAGGCACAGAAGACAAGAGTAACACTCAGCCAAGCAGCTGC
TCTTCGGAAGTCAGCAAACCTGACGGGGCTGGGGAGGGCCCTGCTCAGTCTCTGACAGAGCAAATG
AAAAAGATAGCCTTGGAGTCGGTGGGACAGCCAGAGGTAGGTCTACTAGCTTCAGCCTGAGGAATC
CTGTCCTTCTACTGTTACCCTGAGCCTCCTGATAGAACTCTGTAGAGAAAATTTACTTAGCTCTGGAG
CTGTAACATCATACAGCTGGCGCCCATGAGGCCCCAGGTTTCAGTCCCTGGCTGGGAACAGATAGT
GGGGTTAGACTTGTCATTGCTTAGGCCTGATGCTCACAGCAGGTTGTATTCCAGGAACAGATGGAGT
CGGACAATTGCTCAGGAGGAGACGATGACTGGACACATTTGTCTTCAAAGAAGTGGACCCATCTA
CAGGTGAACTCCAGTCTCTACAGATGCCAGAATCGGAAGGGCCAAGCTCTCTAGACCCCTCACAGG
AAGGACCCACAGGGCTGAAGGAAGCTGCCCTATACCACATCTCCCACCAGGCATGTGGTCACCAG
AAGTTTCATTTTCCTTCTTTGCTTTCAGAGGATCCTGTCCTGTCTAGTCTTGCCAACACAAAAAGGGTT
G
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SQSTM1-G331D-EM1-B6N & SQSTM1-G331D-EM2-B6N Heterozygous F1 animal sequence trace:



Please note that the SQSTM1-G331D-EM1-B6N & SQSTM1-G331D-EM2-B6N alleles are the same but derived from different founder animals.

Nucleotide Alignment:

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                *      20      *      40      *      60      *      80      *      100     *      120
Sqstm1_WT      : CCCACTACCCAGAAAGTTCAGCACAGGCACAGAAGACAAGAGTAACACTCAGCCAAGCAGCTGCTCTTCGGAAGTCAGCAAACCTGACGGGGCTGGGGAGGGCCCTGCTCAGTCTCTGACAGA
Sqstm1_G331D   : CCCACTACCCAGAAAGTTCAGCACAGGCACAGAAGACAAGAGTAACACTCAGCCAAGCAGCTGCTCTTCGGAAGTCAGCAAACCTGACGGGGCTGGGGAGGGCCCTGCTCAGTCTCTGACAGA
                *      140     *      160     *      180     *      200     *      220     *      240     *
Sqstm1_WT      : GCAAATGAAAAAGATAGCCTTGAGTCGGTGGGACAGCCAGAGGTAGGTCTACTAGCTTCAGCCTGAGGAATCCTGTCCTTCTACTGTTACCCTGAGCCTCCTGATAGAACTCTGTAGAGAAAAT
Sqstm1_G331D   : GCAAATGAAAAAGATAGCCTTGAGTCGGTGGGACAGCCAGAGGTAGGTCTACTAGCTTCAGCCTGAGGAATCCTGTCCTTCTACTGTTACCCTGAGCCTCCTGATAGAACTCTGTAGAGAAAAT
                *      260     *      280     *      300     *      320     *      340     *      360     *
Sqstm1_WT      : TTACTTAGCTCTGGAGCTGTAACATCATACAGCTGGCGCCCATGAGGCCAGGTTTCAGTCCCTGGCTGGGAACAGATAGTGGGGTTAGACTTGTTCATTGCTTAGGCCTGATGCTCACAGCAG
Sqstm1_G331D   : TTACTTAGCTCTGGAGCTGTAACATCATACAGCTGGCGCCCATGAGGCCAGGTTTCAGTCCCTGGCTGGGAACAGATAGTGGGGTTAGACTTGTTCATTGCTTAGGCCTGATGCTCACAGCAG
                *      380     *      400     *      420     *      440     *      460     *      480     *      500
Sqstm1_WT      : GTTGTATTCCAGGAACAGATGGAGTCGGGAAATGCTCAGGAGGAGACGATGACTGGACACATTTGTCTTCAAAGAAGTGGACCCATCTACAGGTGAACTCCAGTCTCTACAGATGCCAGAATC
Sqstm1_G331D   : GTTGTATTCCAGGAACAGATGGAGTCGGGAAATGCTCAGGAGGAGACGATGACTGGACACATTTGTCTTCAAAGAAGTGGACCCATCTACAGGTGAACTCCAGTCTCTACAGATGCCAGAATC
                *      520     *      540     *      560     *      580     *      600     *      620
Sqstm1_WT      : GGAAGGGCCAAGCTCTCTAGACCCCTCACAGGAAGGACCCACAGGGCTGAAGGAAGCTGCCCTATACCCACATCTCCACCAGGCATGTGGTCACCAGAAGTTTCATTTTCCTTCTTTGCTTTCA
Sqstm1_G331D   : GGAAGGGCCAAGCTCTCTAGACCCCTCACAGGAAGGACCCACAGGGCTGAAGGAAGCTGCCCTATACCCACATCTCCACCAGGCATGTGGTCACCAGAAGTTTCATTTTCCTTCTTTGCTTTCA
                *      640     *      660
Sqstm1_WT      : GAGGATCCTGTCCTGTCTAGTCTTGGCAACACAAAAGGGTTG
Sqstm1_G331D   : GAGGATCCTGTCCTGTCTAGTCTTGGCAACACAAAAGGGTTG

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Predicted Protein Alignment:

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                *      20      *      40      *      60      *      80      *      100
Sqstm1_WT      : IEVDIDVEHGGKRSRLPTTPESSSTGTEDKSNTPSSCSSEVSKPDGAGEGPAQSLTEQMKKIALESVGPPEEQMESNCSGGDDDWTHLSSKEVDPST-
Sqstm1_G331D   : IEVDIDVEHGGKRSRLPTTPESSSTGTEDKSNTPSSCSSEVSKPDGAGEGPAQSLTEQMKKIALESVGPPEEQMESNCSGGDDDWTHLSSKEVDPST-
                *      100
Sqstm1_WT      : IEVDIDVEHGGKRSRLPTTPESSSTGTEDKSNTPSSCSSEVSKPDGAGEGPAQSLTEQMKKIALESVGPPEEQMESNCSGGDDDWTHLSSKEVDPST
Sqstm1_G331D   : IEVDIDVEHGGKRSRLPTTPESSSTGTEDKSNTPSSCSSEVSKPDGAGEGPAQSLTEQMKKIALESVGPPEEQMESNCSGGDDDWTHLSSKEVDPST

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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_SQSTM1_G331D_F1 primer (5'-3')	CCCACTACCCCAGAAAGTTCC
Geno_SQSTM1_G331D_R1 primer (5'-3')	CAACCCTTTTGTGTTGCCA
Taq Polymerase used	ThermoFisher SuperFi PCR Kit
Annealing Temperature (°C)	55
Elongation time (min)	0.5
WT product size (bp)	668
Mutant product size (bp)	668
Notes	Sequence in forward direction using primer Geno_SQSTM1_G331D_F2 primer (5'-3': GATAGCCTGGAGTCGGTGG)

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 used were checked with the following primers:

Off-target site	Sequence	Type	Primers used
18:21210046-21210068	ATGGAGTCTGGAAACTGCTA AGG	Intronic	Geno_SQSTM1_G331D_OT1F2 primer (5'-3': CTGGAGGAACTATGGGGTGATG) Geno_SQSTM1_G331D_OT1R2 primer (5'-3': CAGCTGGTAAGTAGTTTTGCCA)

All amplicons were sent for Sanger sequencing, no evidence of off-target activity was detected.

Copy counting by ddPCR

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	Sqstm1-G331D-UNI1
Forward Primer (5'-3')	TGCTCACAGCAGGTTGTATT
Reverse Primer (5'-3')	CTGTAGATGGGTCCACTTCTTT
Probe (5'-3')	AGGAGGAGACGATGACTGGACACA
Label	FAM-BHQ1

This ddPCR assay is universal to both the WT allele and the mutant allele of the Sqstm1 gene. WT controls are expected to call at 2 copies and a correct mutation is expected to call at 2 copies for F1 (HET) animals.

Assay name	Sqstm1-G331D-MUT1
Forward Primer (5'-3')	GAACAGATGGAGTCGGacAAt
Reverse Primer (5'-3')	AGACTGGAGTTCACCTGTAGA
Probe (5'-3')	AGGAGGAGACGATGACTGGACACA
Label	FAM-BHQ1

This ddPCR assay is potentially unique to the mutant allele of the Sqstm1 gene as it sits across the mutated region. WT controls are expected to call at 0 copies and 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.



Allele Description

This is a CRISPR/Cas9 induced mutation creating a series of point mutations; G331D, in *SQSTM1*. The stock was generated at MRC Harwell via microinjection of CRISPR/Cas9 reagents into 1-cell stage embryos.

qPCR Copy Counting Genotyping Strategy

The genotyping strategy presented here has been optimized for reagents and conditions used by the Genotyping Core at MRC Harwell. To genotype animals, we recommend researchers validate the assay independently. PCR cycling temperature and times may require additional optimization based on the specific genotyping reagents used.

Samples are genotyped using qPCR copy counting with both a wild type and a mutant assay against a known reference assay (*Dot1l* on chromosome 10; 2 copies present). Samples for this line are genotyped using the following primers and probe:

- Wild type (WT) assay with probe and reverse primer binding to the WT bases mutated in the mutant allele.
- Mutant assay with probe and reverse primer binding to the G601R, F606Y and R609H point mutations.

For autosomal genes that have been targeted, the following results would be expected:

Genotype of the Modified allele	WT Assay	Mutant Assay
Wildtype	2	0
Heterozygous	1	1
Homozygous mutant	0	2



SQSTM1-G331D

SQSTM1-G331D-WT1 assay (FAM labelled)

GGGGTTAGACTTGTCATTGCTTAGGCCTGATGCTCACAGCAGGTTGTATTCCAGGAACAGATGGAGT
CGGgaAAcTGCTCAGGAGGAGACGATGACTGGACACATTTGTCTTCAAAGAAGTGG**ACCCATCTA**
CAGGTGAACTCCAGTCT**CTACAGATGCCAGAATCGGAAG**GGCCAAGCTCTCTAGACCCCTCACAGG

Lower case letters denote bases changed in the mutant allele.
 Probe sequence is in bold and shaded grey.
 Primer sequences are in bold and underlined.

Oligo SQSTM1- G331D	5' label	Sequence 5' → 3'	3' label	Oligo Type
SQSTM1- G331D-WT_F	n/a	<u>GTCGGGAAACTGCTCAGG</u>	n/a	Wild type Forward
SQSTM1- G331D- WT_PROBE	FAM	<u>ACCCATCTACAGGTGA</u> ACTCCAGTCT	ZEN/IBFQ	Wild type Probe
SQSTM1- G331D-WT_R	n/a	<u>CTTCCGATTCTGGCATCTGT</u>	n/a	Wild type Reverse

SQSTM1-G331D-MUT1 assay (FAM labelled)

GGGGTTAGACTTGTCATTGCTTAGGCCTGATGCTCACAGCAGGTTGTATTCCAGGAACAGATGGAGT
CGGacAAcTGCTCAGGAAGGAGACGATGACTGGACACATTTGTCTTCAAAGAAGTGG**ACCCATCTAC**
AGGTGAACTCCAGTCT**CTACAGATGCCAGAATCGGAA**GGCCAAGCTCTCTAGACCCCTCACAGGA

Lower case letters denote bases changed in the mutant allele.
 Probe sequence is in bold and shaded grey.
 Primer sequences are in bold and underlined.

Oligo SQSTM1- G331D	5' label	Sequence 5' → 3'	3' label	Oligo Type
SQSTM1- G331D- MUT_F	n/a	<u>GTCGGACAATTGCTCAGGA</u>	n/a	Mutant Forward
SQSTM1- G331D- MUT_PROBE	FAM	<u>ACCCATCTACAGGTGA</u> ACTCCAGTCT	BHQ	Mutant Probe
SQSTM1- G331D- MUT_R	n/a	<u>TTCCGATTCTGGCATCTGTAG</u>	n/a	Mutant Reverse



Dot1l internal control (VIC labelled)

CTGATGGGTGTGGGCAGATCCTACAGAGTCCCATTGGCCACCATGTGTGCTACGCCTGAAATAAAGCCTT**GCC**
CCAGCACGACCATTCAGGG**CCAGCTCTCAAGTCG**ACTGTAAGATGAAGCATAAGGATGCCAACTACTAACA
GAAAACGACTAGAGGGGAAAAGAACAAGGAAACAGAAGACGCAGCACTCCGGCTTCCCTGGGTTGGCCAGT
CACCTATGA

Oligo SQSTM1-G331D	5' label	Sequence 5' → 3'	3' label	Oligo Type
Dot1l_Forward	n/a	<u>GCCCCAGCACGACCATT</u>	n/a	WT Forward
Dot1l_Probe	VIC	CCAGCTCTCAAGTCG	BHQ	WT Probe
Dot1l_Reverse	n/a	<u>TAGTTGGCATCCTTATGCTTCATC</u>	n/a	WT Reverse

Probe sequence is in bold and shaded grey

Primer sequences are in bold and underlined

DNA extraction method

DNA is extracted from ear clips using Applied Biosystems Taqman Sample-to-SNP Kit and qPCR run using 1:10 dilution from the crude preparation.

qPCR master mix

1X

Applied Biosystems GTX Taqman master mix	5 µl
Dot1l_Forward (20 µM)	0.225 µl
Dot1l_Reverse (20 µM)	0.225 µl
Dot1l_Probe (5 µM)	0.2 µl
FAM Assay (probe 5 µM & primers 15 µM each)	0.3 µl
ddH2O	1.55 µl
DNA (1:10 dilution of ABI Sample-to-SNP prep)	2.5 µl

Each sample is ran in technical duplicate. Seven WT and/or mutant controls are also included in duplicate along with non-template controls.

qPCR cycling conditions

qPCR instrument: Applied Biosystems 7500/7900 or ThermoFisher QuantStudio 7

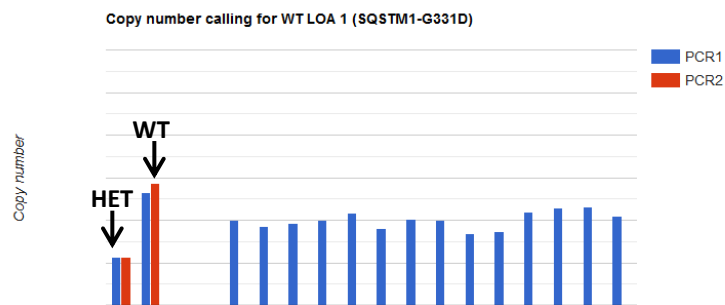
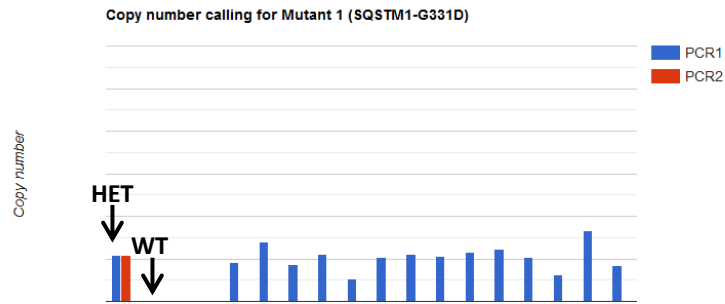
95°C for 20 sec
Then 40 cycles of;
95°C for 3 sec
60°C for 30 sec



Analysis

The results are analysed using CopyCaller software v2.0 from Applied Biosystems or in-house software that is based on CopyCaller v2.0.

SQSTM1-G331D-WT1 and SQSTM1-G331D -MUT1 assays copy called results, image showing copy number chart for WT and Mutant assays (Task 294549 results)



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Approved by: Rumana Zaman