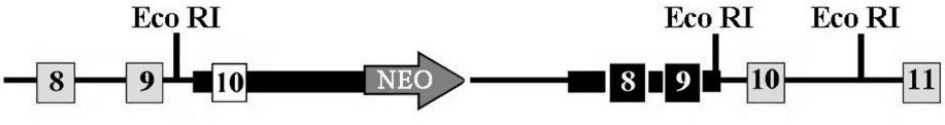
09.10.10 MS
08.07.13 MLS

Genotyping Protocol: **MMRRC 11588**

Strain Characteristics: Acadm (acetyl-Coenzyme A dehydrogenase, medium chain) knockout

Assay Type: PCR to confirm presence of Neomycin gene. Can only distinguish positive from negative animals. Cannot distinguish heterozygous from homozygous mutant animals.

Mice have a knockin of redundant exons 8-10 inverted between exon 9 and 10 of the wild type gene with a neomycin gene in between redundant exons 10 and 8 (see figure below). Details can be found in Tolwani et al (2005) PLoS Genet. 1(2): e23.



Neomycin PCR

DNA Extraction: DNA from tail snips was extracted using Qiagen's DNeasy Blood and Tissue kit (Cat# 69506). Kit directions for animal tissues were performed with a few minor modifications as follows: repeat AW1 and AW2 wash steps one time, elute in 200µl of AE buffer once.

Primer Information:
1) Name: Neo F Sequence: 5'-CATTCGACCACCAAGCGAAACATC-3'
2) Name: Neo R Sequence: 5'-ATATCACGGGTAGCCAACGCTATG-3'

Assay Name: Neomycin PCR

PCR Master Mix Components:

Component	Manufacturer	Concentration	µl/rxn
Buffer with $MgCl_2$ (green cap)	Roche	10x	2
dNTPs	Promega (Cat# U1515)	1.25 mM	3.2
Neo F	Sigma	25 µM	0.3
Neo R	Sigma	25 µM	0.3
FastStart *Taq*	Roche (Cat# 12032953001)	5 U/µl	0.2
Sterile Water			13

PCR Setup:
Final Reaction: 19 µl master mix & 1µl DNA template (10-20ng/µl of genomic DNA)
All reactions were performed in 200µl thin walled PCR tubes and were run in an Applied Biosystems 2700 thermocycler.

Cycle Parameters:
1) 95°C 5 minutes
2) 94°C 30 seconds
3) 68 °C 30 seconds
4) 72°C 1 minute
5) Repeat steps 2-3 34 times for a total of 35 cycles
6) 72°C 10 minutes
7) 4°C hold

Product Analysis:
All products were analyzed on the Qiaxcel (instrument and all supplies from Qiagen) with the Qiaxcel DNA Screening Kit (Cat# 929004).
Alignment Marker: QX Alignment Marker 15bp/3Kb (Cat# 929522)
Size Marker: QX DNA Size Marker 100-3Kb (Cat# 929553)
Method: AM320 Injection: 10s at 5KV
Separation: 320s at 6KV

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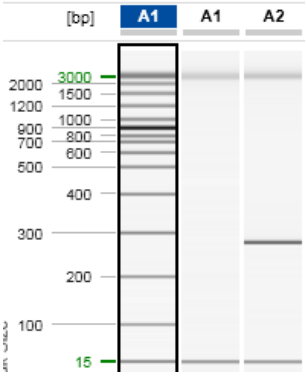
Expected products:
Positive: 289 bp
Negative: no product

Example gel:



Lane A1 (highlighted in blue) displays a 15bp-3kb size marker.
Lane A1 (unhighlighted) displays a sample negative for the neomycin gene (no product).
Lane A2 displays a sample positive for the neomycin gene (289bp product).

Please note: the 15bp and 3kb bands are reference markers specific to the Qiaxcel method and do not represent expected products.

MMRRC 11588 rtPCR: (alternative assay)

Assay Type: RT-PCR to detect wild type transcript. Wild type and heterozygous animals produce a single 550 bp product and therefore this assay cannot distinguish between wild type and heterozygous animals.

Because of the redundant exons, numerous RT-PCR products appear to be formed in homozygous mutant animals giving a specific pattern of products resulting in a ladder effect with product sizes of ~700 bp, 800bp, 1000 bp, and larger. This array of products is not seen in wild type or heterozygous animals.

RNA Extraction: RNA from ear punches or liver was extracted using Qiagen's RNeasy Mini kit (Cat# 74104). Kit directions for animal tissues were performed following kit directions with a single elution of 40µl.

Primer Information:
1) Name: M11588 Forward Sequence: 5'-ATG TGG CGG CCA TTA AGA CCA AAG-3'
2) Name: M11588 Reverse Sequence: 5'-GCT GAT TGG CAA TGT CTC CAG CAA-3'

Primer location: Forward primer binds to exon 7and reverse primer binds to exon 11 to produce 550 bp product

Assay Name: MMRRC 11588 rtPCR

RT-PCR Master Mix Components:
Master Mix for RT-PCR uses Qiagen OneStep RT-PCR Kit (Cat# 210212):

component	manufacturer	concentration	µl/rxn
Rnase free water	Qiagen		21
5X RT-PCR Buffer	Qiagen	5X	10
dNTP mix	Qiagen		2
5X Q-Solution	Qiagen	5X	10
M11588 Forward	IDT	20µM	1.5
M11588 Reverse	IDT	20µM	1.5
One Step Enzyme Mix	Qiagen		2

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PCR Setup:
Final Reaction: 48µl master mix & 2µl RNA template

All reactions were performed in 200µl thin walled PCR tubes and were run in Perkin Elmer 2400 thermocycler or Applied Biosystems 2700 thermocycler.

Cycle Parameters:

1)	50°C	30 minutes
2)	95°C	15 minutes
3)	94°C	30 seconds
4)	64°C	1 minute
5)	72°C	2 minute
6)	Repeat steps 3-5 39 times for a total of 40 cycles	
7)	72°C	10 minutes
8)	4°C	hold until refrigerate product

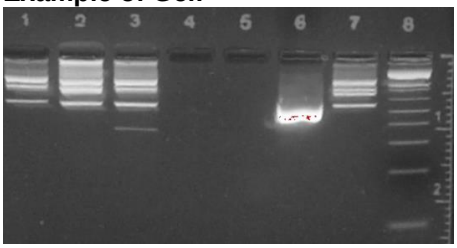
Product Analysis:
All products were analyzed on a 3% agarose gel with ethidium bromide staining.

Wild type mRNA product: 550 bp

Knockout gene product: ladder effect: weak or absent band at 550 bp, strong bands at 700, 800, 1000-bp and larger, easily detected in homozygous animals.

A mixture of 50% wildtype RNA with 50% homozygous RNA results in only amplification of the wild type transcript (550bp product) and this has been confirmed in known heterozygous animals (see Case #13316). Because of this, the assay cannot be used to distinguish between wild type and heterozygous animals.

Example of Gel:



Lanes 1-3 display homozygous samples (ladder of products: 700bp, 800bp, 1000bp bands).
Lanes 4 and 5 are extraction and PCR blanks, respectively.
Lane 6 displays a WT control (550bp band).
Lane 7 displays a homozygous control (ladder of products).
Lane 8 displays 1Kb+ Ladder size marker (Invitrogen Cat# 10787-018).

Interpretation if both assays are used:

Wild type: negative for neo and 550 bp RT-PCR product
Heterozygous: positive for neo and 550 bp RT-PCR product
Homozygous mutant: positive for neo and ladder effect in RT-PCR assay