08.04.10 MS
03.15.13 MH

Genotyping Protocol: **MMRRC 27**

Strain Characteristics: Phospholamban knockout

Assay Type: PCR- can distinguish heterozygous animals from homozygous animals

DNA Extraction: DNA from tail snips was extracted using Sigma's Extract-N-Amp Tissue PCR Kit (Cat#XNAT2R). Kit directions for animal tissues were performed with a few minor modifications as follows: Use only 50 µl of Extraction Solution, 12.5 µl Tissue Preparation Solution and 50 µl of Neutralization Solution B.

Primer Information*:
1) Name: HLT7 R4 Sequence: 5'-ACA ACC ACT TCC TCT CTG GGA GAT CA-3'
2) Name: L27 NEO 3' (F) Sequence: 5'-TCC TCG TGC TTT ACG GTA TC-3'
3) Name: 27 WT F Sequence: 5'-CAC GTC AGA ATC TCC AGA ACC-3'
4) Name: 27 WT R Sequence: 5'-TCC CCC TTT AAC TCT CATAAG C-3'

Primer location: WT allele: 27 WT F binds & 27 WT R bind to exon (deleted region in KO)

Knockout (KO) allele: NEO 3' binds to the neomycin cassette and HLT7 R4 binds to Chromosome 10

Assay Name: Phospholamban KO PCR

PCR Master Mix Components:
Run separate reaction to detect KO allele and WT allele:

Master Mix for WT allele:

component	manufacturer	concentration	µl/rxn
Extract-N-Amp PCR Reaction Mix	Sigma (Cat# XNAT2R)	2X	10
27 WT F	IDT	25µM	0.3
27 WT R	IDT	25µM	0.3
sterile water			5.4

Master Mix for KO allele:

component	manufacturer	concentration	µl/rxn
Extract-N-Amp PCR Reaction Mix	Sigma	2X	10
L27 NEO 3'	IDT	25µm	0.3
HLT7 R4	IDT	25µm	0.3
sterile water			5.4

PCR Setup:
WT Final Reaction: 16µl master mix & 4µl DNA template
KO Final Reaction: 16µl master mix & 4µl DNA template

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

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Cycle Parameters:

1)	94°C	3 minutes
2)	94°C	30 sec
3)	59°C KO or 60°C WT	30 sec
4)	72°C	1 minute
5)	Repeat steps 2-4 34 times for a total of 35 cycles	
6)	72°C	10 minutes
7)	4°C	hold until refrigerate product

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

Wild type Phospholamban allele product: 700 bp
Knockout allele product: 400 bp

Wild type: 700 bp band only on WT gel
Heterozygous: 700 bp band on WT gel and 400 bp band on KO gel
Homozygous mutant: 400 bp band only on KO gel

Example Gels:

WT PCR Gel

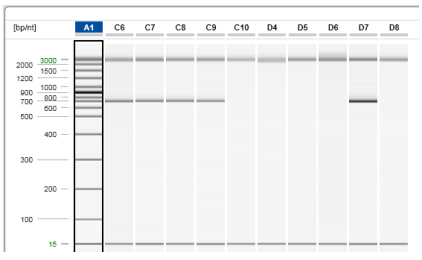
Lane	Sample
A1	15 bp-3 kb Marker
C6	Sample 1
C7	Sample 2
C8	Sample 3
C9	Sample 4
C10	Sample 5
D4	Blank
D5	No DNA
D6	C57BL/6 (WT)
D7	12977-10-1 (het)
D8	11220-09-3 (hom)

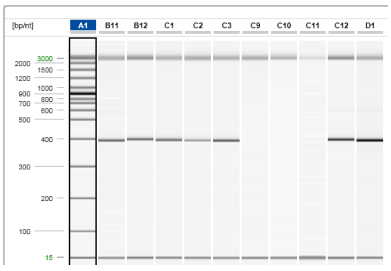
KO PCR Gel

Lane	Sample
A1	15 bp-3 kb Marker
B11	Sample 1
B12	Sample 2
C1	Sample 3
C2	Sample 4
C3	Sample 5
C9	Blank
C10	No DNA
C11	C57BL/6 (WT)
C12	12977-10-1 (het)
D1	11220-09-3 (hom)

Interpretation of Results

Samples 1-4: heterozygous
Sample 5: homozygous mutant





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

Alternative Assay

***** As a backup assay, the primer set MMRRC line 27 HLT7 and L27 NEO 3' which spans the junction between the insertion site and the neomycin cassette used to knockout the gene, can be used to test for the presence of the knockout allele.

Primer Information:
1) Name: MMRRC line 27 HLT7 Sequence: 5'-TGT GGG TTG CAA AGT TAG GC-3'
2) Name: L27 NEO 3' (F) Sequence: 5'-TCC TCG TGC TTT ACG GTA TC-3'

PCR parameters remain the same as those for detecting the KO allele as described earlier. This primer set produces an expected product of 450 bp.