GENOTYPING PROTOCOL

MUTANT MOUSE RESOURCE & RESEARCH CENTER: UC DAVIS

mmrrc@ucdavis.edu
530-754-MMRRC

Protocol Name: **CR10582 Actn2 V809I HDR** Stock #: 67174

Reagent/Constituent	Volume (µL)
QuantiTect Multiplex PCR Master Mix **Cat No./ID 204541**	5.0
Water	3.4
Target Probe mix	0.3
-21 µM Mutant Forward Primer	
-21 µM Mutant Reverse Primer	
-7 µM Mutant probe	
TCRD (endogenous control) mix	0.3
-21 µM WT Forward primer	
-21 µM WT Reverse Primer	
-7 µM WT probe	
Sample	1.0
TOTAL VOLUME OF REACTION:	**10.00 µL**

Comments on protocol:

Protocol may work with other DNA extraction methods. WT Vic probe may be substituted for WT Orange 540 probe. Reference: ABI User Bulletin #2 and #5 (updated 10/2001) for multiplex in same tube and validation of each assay to match relative efficiencies of reference and target primer/probe combinations. Also reference: Rapid and accurate determination of zygosity in transgenic animals by real-time quantitative PCR. TransRes (2002).

Strategy:

Steps	Temp (ºC)	Time (m:ss)	# of Cycles
1. Initiation/Melting	95	15:00	**1**
2. Denaturation	95	0:30	**40x**
3. Annealing/Elongation	60	1:00	**40x**
4. To step 2 for 40 cycles			

Primers:

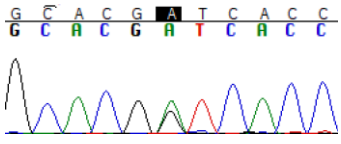
	Name	Nucleotide Sequence (5' - 3')
1.	TM_Actn2_WT-F	CCCGCATTATGACTTTGGTTGAC
2.	TM_Actn2_WT-R	CTGCCGTGTCGGTGTCT
3.	Actn2-WT Orange 560 BHQ-1 Probe	Orange 560-AGG-pdC-A-pdC-GG-pdU-pdC-A-pdC-pdC-pdU-pdU-pdC-
4.	TM_ Actn2_KI-F	CCCGCATTATGACTTTGGTTGAC
5.	TM_ Actn2_KI-R	CTGCCGTGTCGGTGTCT
6.	Actn2-KI Fam BHQ-1 Probe	Fam-pdC-AAGG-pdC-A-pdC-GA-pdU-pdC-A-pdC-pdC-pdU-pdU-BHQ-1

Allele Description: The mouse V809I model was created using optimized CRISPR Cas9 KI technology utilizing Ribonucleoprotein (RNP) in the presence of a synthetic single strand DNA repair template harboring the desired KI. Zygotes were electroporated and subsequent progeny were screened for the presence of the correctly targeted allele via homology directed repair (HDR). The KI nucleotide is 6bp from the cleavage site; the engineered A is within the PAM and will protect the HDR derived locus from auto-cutting by Cas9. Key progeny were sequence confirmed.

cagggtgaagctgaatttgcccgcattatgactttggttgaccccaacggacaaggcacg**ATC**accttccagtccttcattgacttcatgactagagagac

WT GTC > ATC KI

Sample	ΔCt	Genotype
Actn2-ntc		No Rxn
WT	2.85	WT
CR10582-29	-1.35	Het
CR10582-30	2.64	WT



Het

Note: Homozygous animals will have a deltaCt around -5.2 (1 Ct less than heterozygous animals).

Gtc
WT
Atc
KI

PCR protocol developed by MMRRC at University of California, Davis

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Alternative Genotyping Protocol
Standard PCR and Sequencing

Protocol: ***GoTaq® G2 Colorless Master Mix(Promega)***

Reagent/Constituent	Volume (µL)
Water	5.0
GoTaq® G2 Colorless Master Mix,2X	7.5
Primer 1. (stock concentration is 20µM) IVF	0.5
Primer 2. (stock concentration is 20µM) IVR	0.5
DNA (example) extracted w/ "Qiagen DNeasy columns or other similar silica based kits"	1.5
TOTAL VOLUME OF REACTION:	**15.0 µL**

Comments on protocol:

- Protocol may work with other DNA extraction methods.
- When crossing Alg13 and Glt28d2 the Taqman picks up the "other" mutation. Sequencing will be needed if mixed.

Strategy:

Steps		Temp (ºC)	Time (m:ss)	# of Cycles
1. Initiation/Melting	HOT START? ☐	94	2:00	**1x**
2. Denaturation		94	0:10	
3. Annealing	steps 2-3-4 cycle in sequence	65 (↓1ºC/cycle)	0:30	**10x**
4. Elongation		68	2:00	
5. Denaturation		94	0:15	
6. Annealing	steps 5-6-7 cycle in sequence	55	0:30	**25x**
7. Elongation		68	2:00 (↑20sec/cycle)	
8. Finish		4	∞	n/a

Primers:

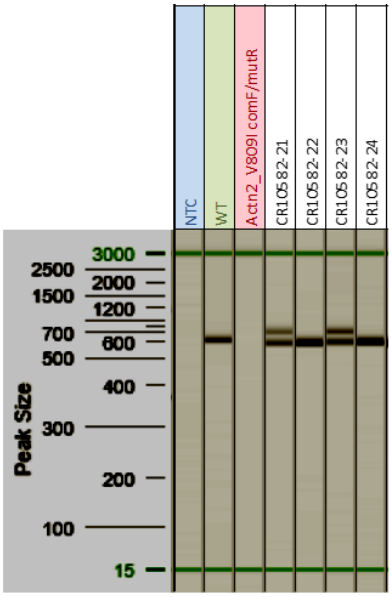
Name	Nucleotide Sequence (5' - 3')
1. CR-Actn2_V809I-IVF	AAATACAACTGCGTTGATTCCTCTGG
2. CR-Actn2_V809I-IVR	CGAGATGAGGGCAAGACTGATGTC

Electrophoresis Protocol:

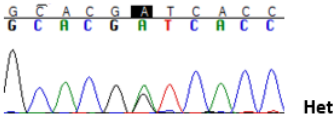
Agarose: **1.5%** : **90**		
Estimated **90** min.		
Primer	**Band** (bp)	**Seq Primer**
1 & 2	610	CR-Actn2_V809I-IVF

Sequencing across Exon 20 to verify the HDR using standard PCR and PCR purification is used to determine the presence of HDR.

Note: Pups #21 and #23 were not sequenced as previous TM data indicated that these pups did not have HDR. It is necessary to sequence all WT-sized bands to verify HDR when using only standard PCR.



Sample	Genotype
CR10582-22	Het
CR10528-24	Het



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