GENOTYPING PROTOCOL

MUTANT MOUSE RESOURCE & RESEARCH CENTER: UC DAVIS

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530-754-MMRRC

Protocol Name: **CR10612 Glt28d2 N107S HDR** Stock #: 66784

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 TCRD Forward primer	
-21 µM TCRD Reverse Primer	
-7 µM TCRD probe	
Sample	1.0
TOTAL VOLUME OF REACTION:	**10.00 µL**

Comments on protocol:

Protocol may work with other DNA extraction methods. 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:

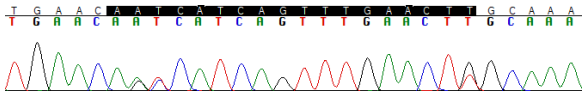
	Name	Nucleotide Sequence (5' - 3')
1.	TCRD Forward Primer	CAGACTGGTTATCTGCAAAGCAA
2.	TCRD Reverse Primer	TCTATGCCAGTTCCAAAAAACATC
3.	TCRD MGB VIC Probe	VIC-ATTATAACGTGCTCCTGG-MGB
4.	TM_Glt28d2-F	CTGGAGAAAGGCAAACCACTTG
5.	TM_Glt28d2-R	CTTTGTGCAACTGCTTTGCA
6.	Glt28d2 BHQ-1 FAM Probe	Fam-A-pdC-AG-pdC-pdC-A-pdU-pdC-AG-pdU-pdU-pdU-GAA-pdC-pdU-pdU-G-pdC-BHQ-1

Allele Description: The mouse N107S model was created to account for the mouse orthologous paralogs to hALG13 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 nucleotides are 10 and 11 bp from the cleavage site, and one silent PAM mutations was engineered into ssODN to prevent cleavage of the KI allele by Cas9. Key progeny were sequence confirmed.

ctgtttggagagtctggagaaaggcaaaccacttgtggtagttgtgaatgaaaagttaatgaac**aGC**catcagtttgaact**T**gcaaagcagttgcacaaagaaggccatctcttctactgtac

WT AAT > AGC KI

Sample	ΔCt	Genotype
Glt28d2-ntc		No Rxn
Glt28d2-WT	16.51	WT
CR10612-87	0.44	Het
CR10685-88	0.50	Het



Het

aATcatcagtttgaactG
WT

aGCcatcagtttgaactT
KI

Note: Homozygous animals will have a deltaCt around -1.1 (1 Ct less than heterozygous animals).

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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:

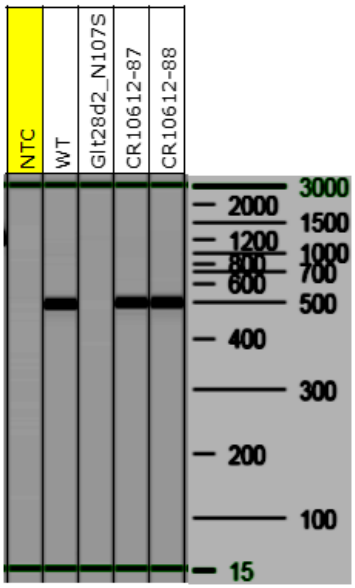
Name	Nucleotide Sequence (5' - 3')
1. CR_Glt28d2-IVF	TGCGATGAAGAGAGTGTTTGTGACC
2. CR_Glt28d2-IVR	TCCACGAACTGGACTTAGGGGTATT

Electrophoresis Protocol:

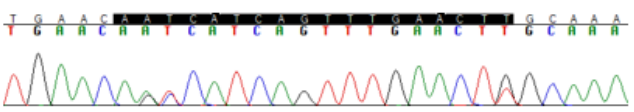
Agarose: **1.5%** : **90**		
Estimated **90** min.		
Primer	**Band** (bp)	**Seq Primer**
1 & 2	543	CR_Glt28d2-IVF

Sequencing across Exon 3 to verify the HDR using standard PCR and PCR purification is used to determine the presence of HDR.

Note: It is necessary to sequence all WT-sized bands to verify HDR when using only standard PCR.



Sample	Genotype
CR10612-87	Het
CR10612-88	Het



Het

aATcatcagtttgaactG
WT

aGCcatcagtttgaactT
KI