GENOTYPING PROTOCOL

MUTANT MOUSE RESOURCE & RESEARCH CENTER: UC DAVIS

mmrrc@ucdavis.edu
530-754-MMRRC

Protocol Name: **VR538_612 Alg13 N107S and Glt28d2_N107S HDR** Stock #: 65341

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. **When crossing Alg13 and Glt28d2, the Taqman picks up the "other" mutation, and sequencing will be necessary if mixed.** 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			

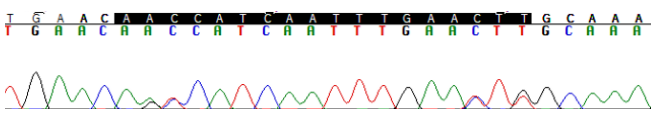
Primers:

	Name	Nucleotide Sequence (5' - 3')
1.	TM_Alg13_WT-F	TCTGGAGAAAGGCAAACCAC
2.	TM_Alg13_WT-R	TCTTTGTGCAACTGCTTTGCC
3.	Alg13-WT Orange 560 BHQ-1 Probe	Orange 560-pdU-GAA-pdC-AA-pdU-pdC-A-pdU-pdC-AA-pdU-pdU-pdU-GAA-pdU-pdU-GG-pdC-BHQ-1
4.	TM_Alg13_KI-F	TCTGGAGAAAGGCAAACCAC
5.	TM_Alg13_KI-R	CTTTGTGCAACTGCTTTGCA
6.	Alg13-KI Fam BHQ-1 Probe	Fam-AA-pdC-AG-pdC-pdC-A-pdU-pdC-AA-pdU-pdU-pdU-GAA-pdC-pdU-pdU-G-pdC-BHQ-1

Allele Description: The mouse N107S 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 nucleotides are 11 and 12 bp from the cleavage site, and silent PAM mutations were engineered into the PAM site and ssODN to prevent cleavage of the KI allele by Cas9. Key progeny were sequence confirmed.

ctgtttggagagtctggagaaaggcaaaccacttgtggtagttgtaaatgaaaagttaatgaac**aGC**catcaatttgaa**CtT**gcaaagcagttgcacaaagaaggccatctctttttattgtac

WT AAT > AGC KI



Het

a**AT**catcagtttgaa**T**t**G**
WT

a**GC**catcagtttgaa**C**t**T**
KI

PCR protocol developed by MMRRC at University of California, Davis

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WT = 99 and 101; Het = 92-94, 96-98, 100; Hom = 95

Protocol Name: VR538_612 Alg13 N107S and Glt28d2_N107S HDR

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. **When crossing Alg13 and Glt28d2, the Taqman picks up the "other" mutation, and sequencing will be necessary if mixed.** 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			

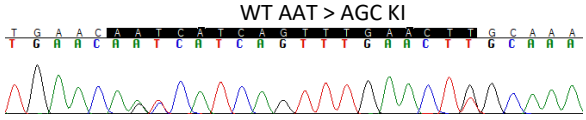
Primers:

	Name	Nucleotide Sequence (5' - 3')
1.	TM_Glt28d2_WT-F	CTGGAGAAAGGCAAACCACTTG
2.	TM_Glt28d2_WT-R	TCTTTGTGCAACTGCTTTGCC
3.	Glt28d2-WT Orange 560 BHQ-1 Probe	Orange 560- AA-pdC-AA-pdU-pdC-A-pdU-pdC-AG-pdU-pdU-pdU-GAA-pdC-pdU-GG-pdC-BHQ-1
4.	TM_Glt28d2_KI-F	CTGGAGAAAGGCAAACCACTTG
5.	TM_Glt28d2_KI-R	CTTTGTGCAACTGCTTTGCA
6.	Glt28d2-KI Fam BHQ-1 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 mutation was engineered into ssODN to prevent cleavage of the KI allele by Cas9. Key progeny were sequence confirmed.

ctgtttggagagtctggagaaaggcaaaccacttgtggtagttgtgaatgaaaagttaatgaacaGCcatcagtttgaactTgcaaagcagttgcacaaagaaggccatctcttctactgtac

WT AAT > AGC KI



Het

aATcatcagtttgaactG
WT

aGCcatcagtttgaactT
KI

PCR protocol developed by MMRRC at University of California, Davis

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Alternative Genotyping Protocol
Standard PCR and Sequencing

When crossing Alg13 and Glt28d2, the Taqman picks up the "other" mutation, and sequencing will be necessary if mixed.

Sample	ΔCt	Genotype
Alg13-ntc		No Rxn
Alg13-WT	15.88	WT
VR538_612-102	1.64	Het
VR538_612-104	16.60	WT
VR538_612-106	-2.99	Hom

Sample	ΔCt	Genotype
Glt28d2-ntc		No Rxn
Glt28d2-WT	16.51	WT
VR538_612-102	0.66	Het
VR538_612-104	17.12	WT
VR538_612-106	-0.14	Hom

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_Alg13-IVF	ATAGACATTACTCCTGGGTGGTGGTG
2. CR_Agl13-IVR	ACCAGTGAAAACCCGAGTAGTACTAGTGA
3. CR_Glt28d2-IVF	TGCGATGAAGAGAGTGTTTGTGACC
4. CR_Glt28d2-IVR	TCCACGAACTGGACTTAGGGGTATT

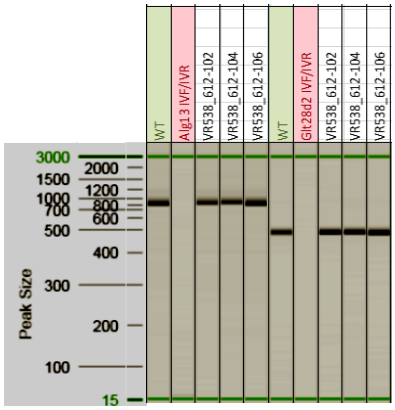
Electrophoresis Protocol:

Agarose: **1.5%** : **90**

Estimated Running **90** min.

Primer Combination	Band (bp)	Seq Primer
1 & 2	922	Alg13-IVF
3 & 4	543	Glt28d2-IVF

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



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Sample ID	Alg13_N107S	Glt28d2_N107S
VR538_612-102	WT	Het
VR538_612-104	WT	WT
VR538_612-106	Hom	Het

Alg13_N107S

WT ATCATCAATTTGAATTG

MUT GCCATCAATTTGAACTT

Glt28d2_N107S

WT ATCATCAGTTTGAACTG

MUT GCCATCAGTTTGAACTT