

GENOTYPING PROTOCOL

MUTANT MOUSE RESOURCE & RESEARCH CENTER: UC DAVIS

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530-754-MMRRC

Protocol Name: CR10615/ET16513 Mir208a TtoC Edit/HDR Stock #: 67013

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 (°C)	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:

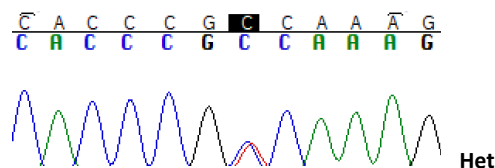
Name	Nucleotide Sequence (5' - 3')
1. TCRD Forward Primer	CAGACTGGTTATCTGCAAAGCAA
2. TCRD Reverse Primer	TCTATGCCAGTTCCAAAAACATC
3. TCRD MGB VIC Probe	VIC-ATTATAACGTGCTCCTGG-MGB
4. TM_Mir208a-F	TGCTCGTCTTATACGTGAGTGTC
5. TM_Mir208a -R	TGGCTGGCCCTTGCTTA
6. Mir208a BHQ-1 FAM Probe	Fam-pdC-pdU-pdC-A-pdC-pdC-G-pdU-pdC-AAAGGA-BHQ-1

Allele Description: The mouse T>C 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 adjacent to the cleavage site and the engineered C at the 17th position of the protospacer will protect HDR derived locus from auto-cutting by Cas9. Key progeny were sequence confirmed.

tttgctcgtcttatacgtgagtgtcagggtataaccggggccaaaagctcaccgcCcaaaggaagtaagcaagggccagccaggcggttacaggttg

WT T > C KI

Sample	ΔCt	Genotype
Mir208a-ntc		No Rxn
Mir208a-wt	12.59	WT
CR10615-3	-0.14	Het
ET16513-1	-0.03	Het



Note: Homozygous animals will have a deltaCt around -1.0 (1 Ct less than heterozygous animals).

cgTca
WT

cgCca
KI

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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 (°C)	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 (↓1°C/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

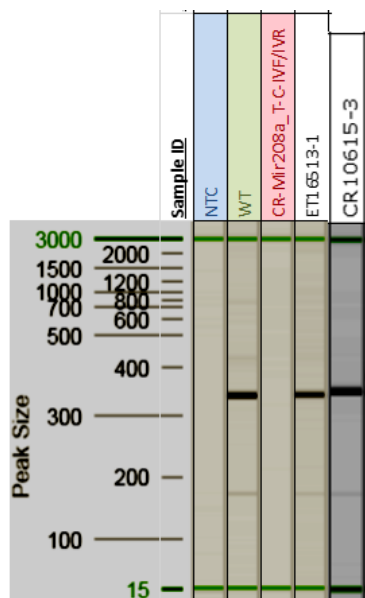
Primers:

Name	Nucleotide Sequence (5' - 3')	Agarose: 1.5% : 90
1. CR-Mir208a T-C-IVF	GCCTAGAGGATGTGAGGGCTACTGG	Estimated 90 min.
2. CR-Mir208a T-C-IVR	CCAAGAGAATGGAGCAGAGCGTCT	
		Primer Band (bp) Seq Primer
		1 & 2 388 CR-Mir208a T-C-IVF

Electrophoresis Protocol:

Sequencing across Exon 1 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
ET16513-1	Het
CR10615-3	Het

