GENOTYPING PROTOCOL

MUTANT MOUSE RESOURCE & RESEARCH CENTER: UC DAVIS

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530-754-MMRRC

Protocol Name: **CR10687 Dph5 H260R** Stock #: 66779

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:

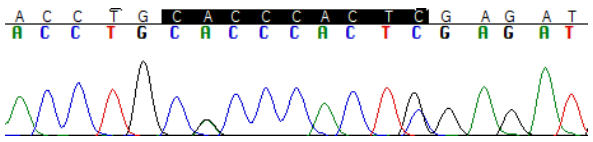
	Name	Nucleotide Sequence (5' - 3')
1.	TCRD Forward Primer	CAGACTGGTTATCTGCAAAGCAA
2.	TCRD Reverse Primer	TCTATGCCAGTTCCAAAAAACATC
3.	TCRD MGB VIC Probe	VIC-ATTATAACGTGCTCCTGG-MGB
4.	TM_Dph5-F	TCCGGTATAGAGAAGAGACTTAGCA
5.	TM_Dph5-R	CAGTGAGCTTGGGAGAACCA
6.	Dph5 MGB FAM Probe	Fam-GCGCCCACTCGAG-MGB

Allele Description: The mouse H260R 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 3 bp from cleavage site, and one silent PAM mutation was engineered into the ssODN to protect the HDR derived locus from auto-cutting by Cas9. Key progeny were sequence confirmed.

tgtgcacagtgagcttgggagaaccactgcattctttggtcattacaggggcaacctgcGccactCgagatggagatgctaagtctcttctctataccgga

WT cAC > cGC KI
H > R

Sample	ΔCt	Genotype
Dph5-ntc		No Rxn
Dph5-wt	16.89	WT
CR10687-76	2.08	Het
CR10685-77	2.05	Het



Het

cAcccactG
WT

cGcccactC
KI

Note: Homozygous animals will have a deltaCt around -6.1 (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:

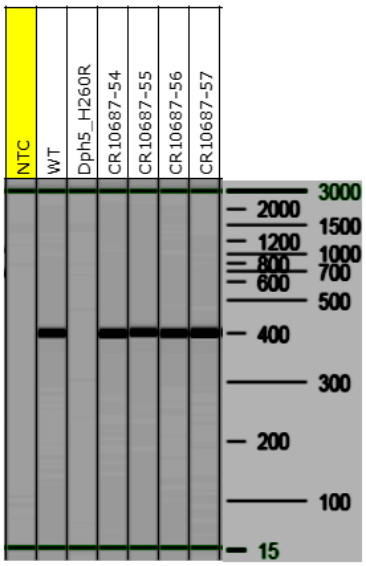
Name	Nucleotide Sequence (5' - 3')
1. CR-Dph5-IVF	GAAAGTTGACTAAGGCGATGGATGC
2. CR-Dph5-IVR	GCTGTCGTTCAACCTGCTTCTTCTT

Electrophoresis Protocol:

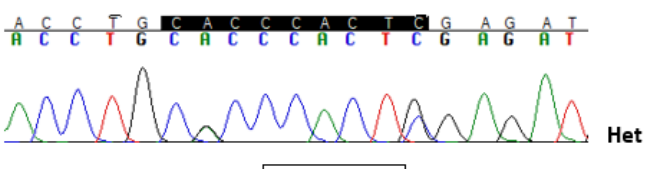
Agarose: **1.5%** : **90**		
Estimated **90** min.		
Primer	**Band** (bp)	**Seq Primer**
1 & 2	425	CR-Dph5-IVF

Sequencing across Exon 8 to verify the HDR using standard PCR and PCR purification is used to determine the presence of HDR.

Note: Pups #54 and #56 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
CR10687-55	Het
CR10687-57	Het



cAcccactG
WT

cGcccactC
KI

PCR protocol developed by MMRRC at University of California, Davis