GENOTYPING PROTOCOL

MUTANT MOUSE RESOURCE & RESEARCH CENTER: UC DAVIS

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530-754-MMRRC

Protocol Name: **CR10596 Ube4a C425Ffs HDR** Stock #: 66878

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:

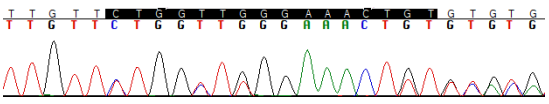
	Name	Nucleotide Sequence (5' - 3')
1.	TM_Ube4a_WT-F	GATGCTGAAGAACTTGCTCCA
2.	TM_Ube4a_WT-R	GCCTGCATTTGCATGCAAAC
3.	Ube4a-WT Orange 560 BHQ-1 Probe	Orange 560-pdU-G-pdU-pdU-pdC-pdU-GG-pdC-pdU-pdU-GGAAA-pdC-pdU-G-pdU-pdU-BHQ-1
4.	TM_Ube4a_KI-F	GATGCTGAAGAACTTGCTCCA
5.	TM_Ube4a_KI-R	GCCTGCATTTGCATGCAAAG
6.	Ube4a-KI Fam BHQ-1 Probe	Fam-pdU-pdC-pdU-pdU-G-pdU-pdU-pdU-pdU-GG-pdU-pdU-GGGAAA-pdC-pdU-pdU-BHQ-1

Allele Description: The mouse C425F frameshift (fs) and early termination signal 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 deletion and fs. Zygotes were electroporated and subsequent progeny were screened for the presence of the correctly targeted allele via homology directed repair (HDR). The GT deletion causing the fs is 11 bp from the cleavage site, and 3 silent protospacer mutations were engineered into ssODN to prevent cleavage of the variant allele by Cas9. Key progeny were sequence confirmed.

gatgctgaagaacttgctccagctctctccagaaaccaagcactgtatcttgtt**T**tgg**T**t**G**ggaaac**T**ttgcatgcaaatgcaggccgcaccaagatctgggccaatc

WT TGT > T (GT deletion with frameshift)

Sample	ΔCt	Genotype
Ube4a-ntc		No Rxn
Ube4a-WT	16.7	WT
CR10596-74	-0.87	Het
CR10541-65	-8.09	Hom



Het

tG**T**ttg
WT
tttg
KO and fs

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:

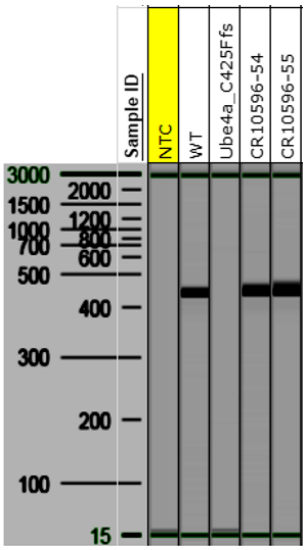
Name	Nucleotide Sequence (5' - 3')
1. CR_Ube4a_C425Ffs-IVF	GACCAGAAAAGGTCTCCTAAGTATCCAG
2. CR_Ube4a_C425Ffs-IVR	GTCCTTGAGGACACAGTACGTGGG

Electrophoresis Protocol:

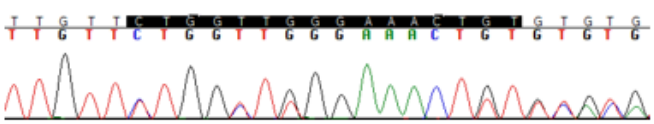
Agarose: **1.5%** : **90**		
Estimated **90** min.		
Primer	**Band** (bp)	**Seq Primer**
1 & 2	513	CR_Ube4a_C425Ffs-IVF

Sequencing across Exon 9 to verify the HDR using standard PCR and PCR purification is used to determine the presence of HDR.

Note: Pup #54 was not sequenced as previous TM data indicated that this pup did not have HDR. It is necessary to sequence all WT-sized bands to verify HDR when using only standard PCR.



Sample	Genotype
CR10596-55	Het



Het

tGTttg
WT

tttg
KO and fs