GENOTYPING BY PCR PROTOCOL
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NAME OF PCR: C57BL/6-*Tnf*^{M1Btlr}/Mmcd, (*PanR1*) **MMRRC #** 010462-UCD

Protocol:

Reagent/ Constituent	Volume (µL)
Water	12.675
10x Buffer (contains 15mM $MgCl_2$)	2.5
Betaine (stock concentration is 5M) *Optional*	6.5
dNTPs (stock concentration is 25mM)	0.5
DMSO *Optional*	0.325
Primer 1 (stock concentration is 20µM) 10462 PCR F1	0.5
Primer 2 (stock concentration is 20µM) 10462 PCR R1	0.5
Taq Polymerase	0.5
DNA sample extracted with ☐ NaOH ☐ Proteinase K ☒ Other: Any	1.0
TOTAL VOLUME OF REACTION:	**25µL**

Comments on protocol:

- PCR products are verified to contain the correct amplicon size by running ~10µl of the reaction on a gel and the remaining 15µl purified via column based PCR purification method for sequencing.
- Use Touch-Down cycling protocol-first 10 cycles anneal at 65° C decreasing in temperature by 1.0° C; next 30 cycles anneal at 55° C.
- Betaine and DMSO have been standardized due to high GC content. Protocol may be tested without. Also, may adjust $MgCl_2$ to increase reaction or decrease non specific amplifications.

Strategy:

Steps		Temp (°C)	Time (m:ss)	# of Cycles
1. Initiation/Melting HOT START? ☐		94	5:00	**1**
2. Denaturation		94	0:15	
3. Annealing	steps 2-3-4 will cycle in sequence	65 to 55 (↓1°C/cycle)	0:30	**10x**
4. Elongation		72	0:40	
5. Denaturation		94	0:15	
6. Annealing	steps 5-6-7 will cycle in sequence	55	0:30	**30x**
7. Elongation		72	0:40	
8. Amplification		72	5:00	**1**
9. Finish		15	∞	n/a

Primers:

Name	Nucleotide Sequence (5' - 3')
1: 10462 PCR F1	ATT TGA GTC CTT GAT GGT GGT GCA
2: 10462 PCR R1	CAA ACC CTG GTA TGA GCC CAT ATA CCT
3: 10462 _seq (F)	GTC ACT GTC CCA GCA TCT TGT GTT T

Electrophoresis Protocol:

Agarose: 2% **mV:** 80 **Estimated Running Time:** 90 **min**

Primer Combination	Band	Genotype
1 and 2	378 bp	*PanR1*
SNP found at position ~ 225 of sequencing		

Mutation site (red) and flanking sequence:

WT cttgg**G**cag

PanR1 cttgg**T**cag