MIQE information for RT-qPCR experiments

Experimental design:

The experiment was designed to examine gene expression in three different age groups in BALB and C57BL/6 mice. The groups consisted of young (2-3 mo), middle-aged (12-13 mo), and aged (20-24 mo) cohorts. Control group is set to young animals; middle-aged and aged groups were defined as experimental.

The number of mice in each group:

BALB: young 10, middle-aged 7, and aged 10.

C57BL/6: young 11, middle-aged 15, and aged 10.

Sample:

Description: Cerebellar and hypothalamic whole tissue samples were dissected from the mouse brain and processed into a single cell suspension.

Processing procedure:

Purchase Papain Dissociation System. (Worthington biochemical Corporation Cat#: LK003150)

Make up solutions:

Reconstitute Vial 2 with 5 ml of EBSS (vial 1). Reconstitute Vial 3 with 0.5ml EBSS (vial 1). Reconstitute Vial 4 (Ablumin ovomucoid inhibitor) with 32 ml EBSS (vial 1) and dissolve. Place vials in 37 °C for 20 min prior to use.

1. Obtain mouse. Decapitated and removed brain. Placed hemicerebellum and the hypothalamus in separate Falcon tube containing 2 ml of 1x PBS .
2. Mince tissue with spatula. Allow tissue to settle for 5 min. Remove PBS with 1 ml pipette careful not to remove tissue. Add 1300 μl vial 2 to tissue. Mix gently.
3. Incubate tissue in +37 °C CO_2 incubator for 40 min. Gently shake contents in tube every 10 min. Remove from incubator.
4. Triturate very slowly with a 1 ml pipette until mixture is homogeneous. Filter the suspension with a 40 μm cell strainer into 50 ml falcon tube. Centrifuge for 3 min 300xg.
5. While centrifuging, make 2700 μl of vial 1 (EBSS), 300 μl vial 4 (albumin ovomucoid inhibitor) and 150 μl vial 3 (DNase).
6. Remove supernant with 1 ml pipette. Add 787.5 μl of solution from step 5 (avoid breaking pellet).

7. Add 1250 μl of vial 4 to each sample. Insert the pipette tip all the way to the bottom of the tube. Add slowly allowing pellet to remain on top. Centrifuge for 10 min 300xg at room temp.
8. Make buffer: 1L PBS (pH 7.2), 0.5% bovine serum albumin, and 2 mM EDTA.
9. Resuspend pellet in 2250 μl buffer.
10. Add 33 μl (young), 50 μl (middle-aged), 75 μl (aged) myelin removal beads (Miltenyi Biotec #130-096-433). Mix well and incubate at 4 °C for 15 min.
11. Wash cells by adding 22.5 ml of buffer. Centrifuge for 10 min 300xg.
12. Discard supernant. Resuspend in 500 μl buffer.
13. Use automax column program "Depl05".
14. Collect negative elutant.
15. Centrifuge 10 min 300xg. Resuspend in 90 μl buffer. Add 10 μl CD11b beads (Miltenyi Biotec #130-093-634).
16. Mix well and incubate in 4 °C for 15min.
17. Add 2 ml of buffer to cells and centrifuge for 10 min 300xg. Discard supernant and resuspend in 500 μl buffer.
18. Use automax column program "Possel".
19. Collect positive elutant. Discard neg. Rinse automax and run possel again with positive elutant.
20. Wash 3x500 μl with buffer.
21. Centrifuge 10 min 300xg. Remove supernant.

Sample Frozen: Not Applicable

Sample Fixed: Not Applicable

Sample storage conditions and duration: Not Applicable.

Nucleic acid extraction:

Procedure and /or instrument: mRNA isolation

Name of kit : uMACS mRNA Isolation kit (Miltenyi Biotec #130-075-201)

1. Place lysis and wash buffer at room temp. Heat waterbath to 70 °C.
2. Add 1 ml of lysis/binding buffer. Vortex for 3-5 min.
3. Place lysate clear column in the centrification tube and apply sheared lysate sample on top of lysate clear column. Centrifuge for 13000xg for 3 min. Lysate now in centrifuge tube.
4. Add 50 μl oligo-T microbeads to tube. Short vortex. Place elution column in magnetic field.
5. Rinse column with 100 μl lysis binding buffer. Discard elutant.

6. Apply lysate on top of the column matrix. Rinse column 2x200 μl lysis buffer. Rinse column with 4x100 μl wash buffer.
7. Apply 70 °C elution buffer 27 μl. Discard flow thru.
7. Place RNAase-free eppendorf below column. Add 75 μl (70 °C) elution buffer.
8. Save isolated mRNA. Freeze sample in -80 °C.
9. Following day thaw and concentrate to final volume 12 μl. Nanodrop and qRT-PCR.

Details of DNase or RNase treatment: not performed.

Contamination assessment: Not performed; insufficient material from microglial RNA preparation for analysis

Nucleic acid quantification: Not performed; insufficient material from microglial RNA preparation for analysis

Instrument and method: Nanodrop (Thermo Scientific Nanodrop 2000 Spectrophotometer) was used.

1. Arm was lifted up and 1 μl DEPC water was placed on pedestal to blank the nanodrop.
2. 1 μl of sample was placed on the pedestal, RNA was selected as sample type and clicked on measure.

RNA integrity : method/instrument: Per bioanalyzer, 28S peak > 18S peak and RIN.

Inhibition testing (C_q dilutions, spike, or other): Not applicable.

Reverse transcription:

Complete reaction conditions: We used kit Verso SYBR- 1-step QRT Fluorescein (Thermo Scientific #AB-4017) to make our reaction mix.

Amount of RNA and reaction volume: the follow reaction mix was made for each sample.

	Volume	Final Concentration
Verso Enzyme Mix	0.125 μl	
1-Step SYBR Fluorescein	6.25 μl	1X
RT Enhancer	0.675 μl	Not applicable
Qiagen Primer	0.5 μl	
Template (RNA)	1 μl	1 ng
Water (DEPC)	3.95 μl	

(Total Volume 12.5 μl)

qPCR target information:

Gene symbol	Sequence accession number	Amplicon length	In silico specificity screen (proprietary Qiagen catalog #)
gfap	NM_010277(nlm)	127 bp	QT00101143
Neun			
Itgam	NM_008401(nlm)	139 bp	QT00156471
CD68	NM_009853(nlm)	67 bp	QT00254051
C1qc	NM_007574(nlm)	87 bp	QT00250789
C3	NM_009778(nlm)	108 bp	QT00109270
Lilrb3	XM_974271(nlm)	76 bp	QT01540147
H2-Q1	NM_010390(nlm)	92 bp	QT00282331
Tlr2	NM_011905(nlm)	62 bp	QT00129752

Location of each primer by exon or intron: Not Applicable.

What splice variants are targeted: Not Applicable.

qPCR oligonucleotides:

Primer sequences: See chart above Qiagen catalog number.

Location and identity of any modifications: No modifications.

qPCR protocol:

Complete reaction conditions :

1-Step qRT-PCR themal cycling program

	Temp	Time	Number of cycle
cDNA Synthesis	50 °C	15 min	1 cycle
Thermo-Start activation	95 °C	15 min	1 cycle

Denaturataion	95 °C	15 sec	40 cycles
Annealing	50-60 °C	30 sec	
Extension	72 °C	30 sec	

Melt curve program at end

Denaturation	95 °C	15 sec	1 cycle
Starting tem	60 °C	15 sec	1 cycle
Melting step	95 °C	15 sec	80 cycles

Exact chemical composition of the buffer: Not Applicable

Additives: SYBR Green

Manufacturer of plates: Fisherbrand (#14230244)
Manufacturer of qPCR instrument: Eppendorf (Mastercycler ep realplex)

qPCR Valication:
Specificity: Melt
Calibration curves with slope and y intercept: Not Applicable
PCR efficiency calculated from the slope: Not performed
I^2 of calibration curve: Not Applicable
Linear dynamic range: Not Applicable
C_q variation of LOD: Not performed
Evidence of LOD: Not Applicable

Data Analysis:

qPCR analysis program : Realplex 2.2 (Eppendorf)

Method of C_q determination: by program

Outlier identification and disposition: C_q more than 3 sd from technical replicate mean (statistical outlier), evidence of multiple product amplification, evidence of no amplification.

Justification of choice of reference gene: Gusb common standard

Description of normalization method: Per reference 7 (methods),

Number and stage (RT or qPCR) of technical replicates: 3

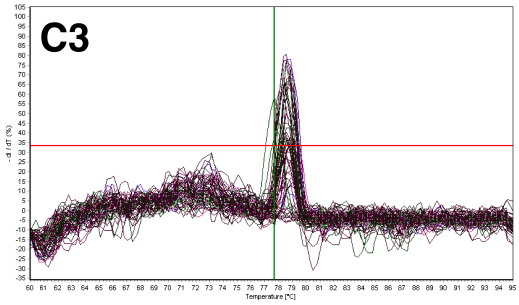
Statistical methods for results significance: means compared by two-tailed t-test (against normalized reference gene) with Bonferonni correction for number of comparisons.

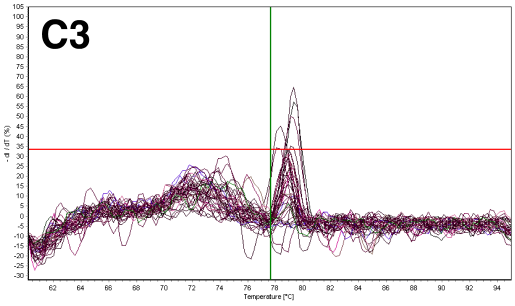
Software (Source, version): MATLAB R6.5, R2011b.

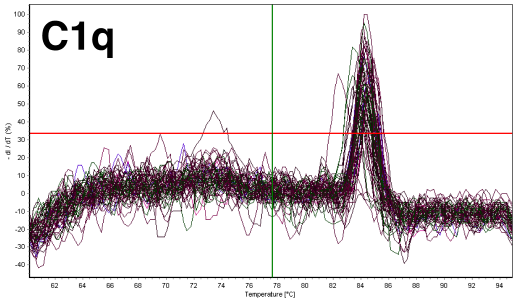
Example melt curves for microglial RT-qPCR (first set), and human cerebellar RT-qPCR (second set):

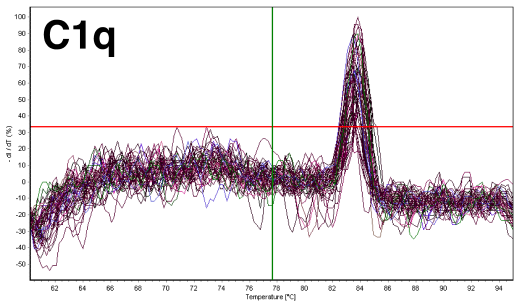
C57BL/6

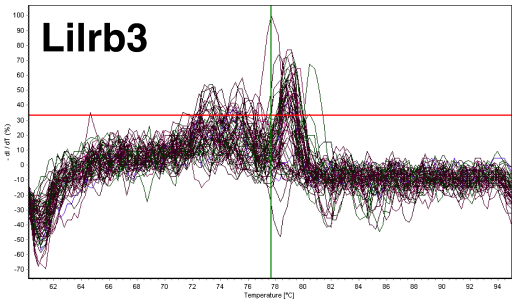
BALB

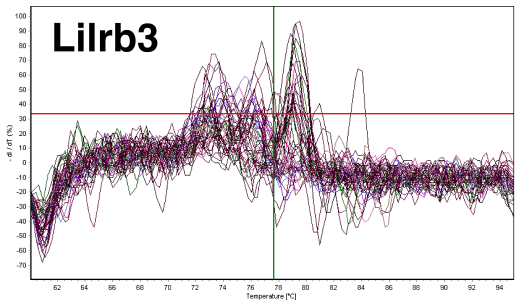

C3
- dI / dT (%)
Temperature [°C]

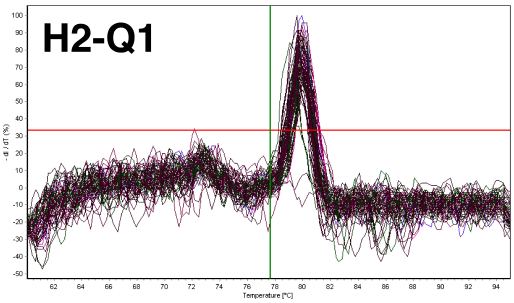

C3
- dI / dT (%)
Temperature [°C]

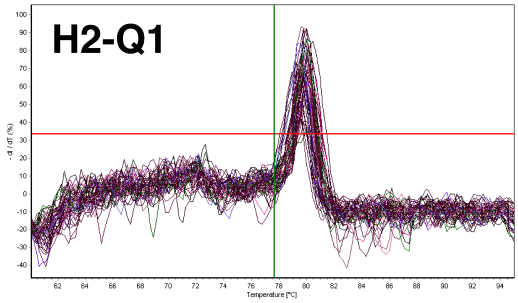

C1q
- dI / dT (%)
Temperature [°C]

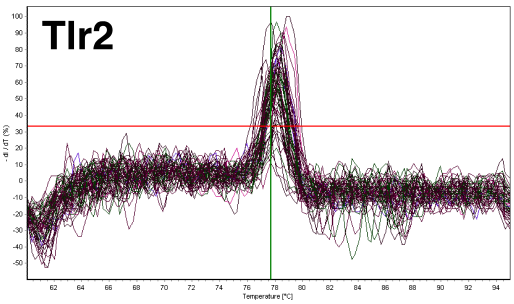

C1q
- dI / dT (%)
Temperature [°C]

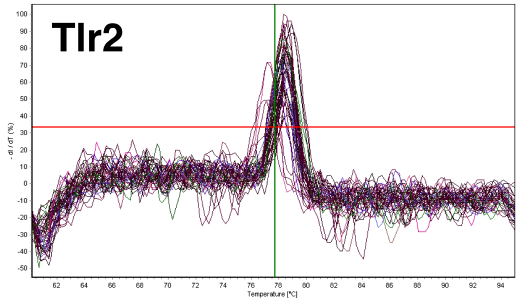

Lilrb3
- dI / dT (%)
Temperature [°C]

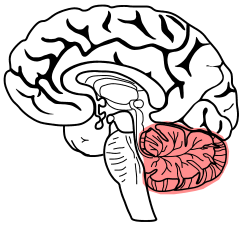

Lilrb3
- dI / dT (%)
Temperature [°C]

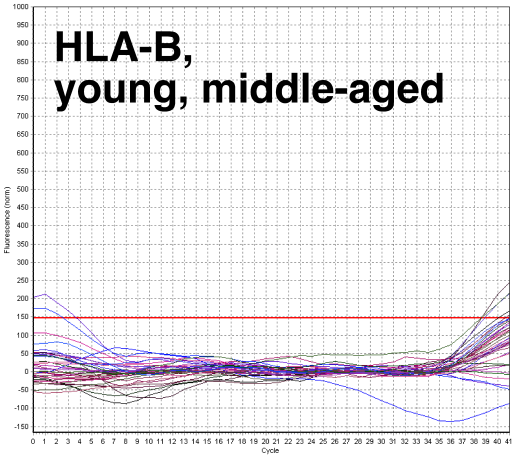

H2-Q1
- dI / dT (%)
Temperature [°C]

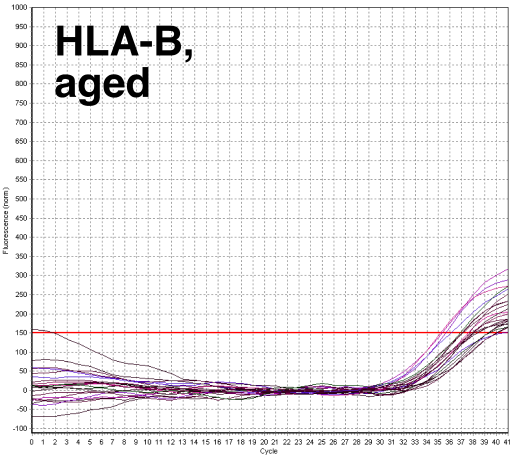

H2-Q1
- dI / dT (%)
Temperature [°C]

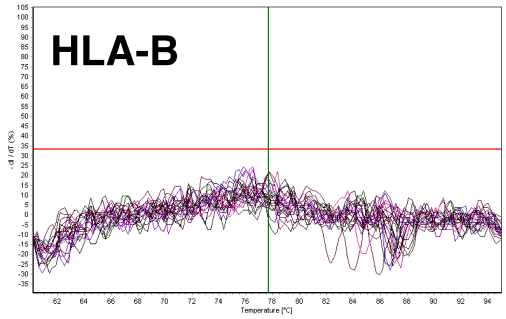

Tlr2
- dI / dT (%)
Temperature [°C]

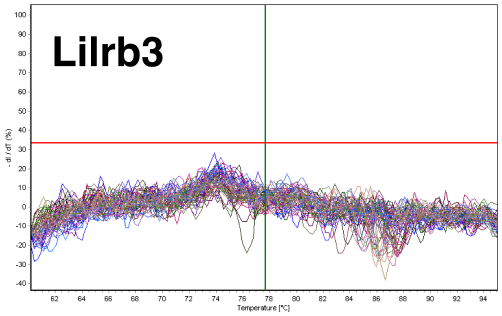

Tlr2
- dI / dT (%)
Temperature [°C]

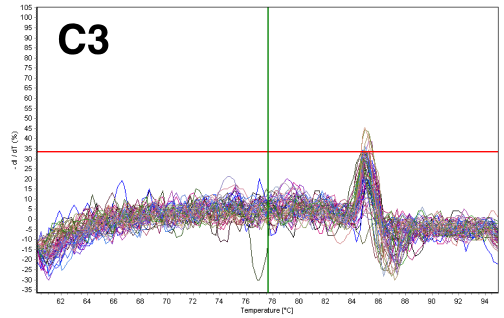


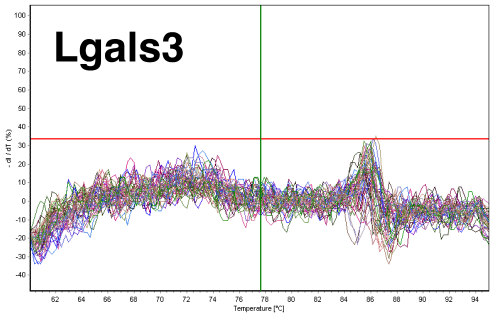

HLA-B,
young, middle-aged
Fluorescence (norm)
Cycle

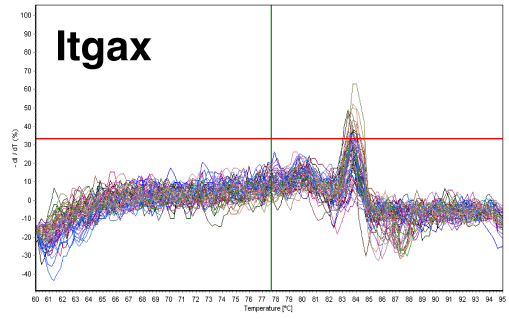

HLA-B,
aged
Fluorescence (norm)
Cycle

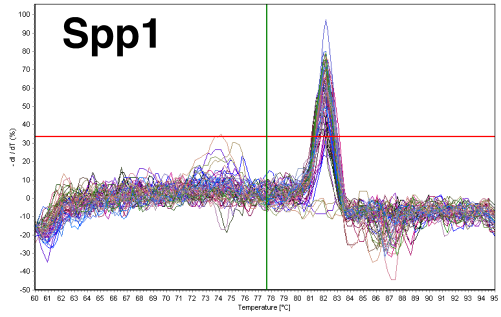

HLA-B
- d / dT (%)
Temperature [°C]

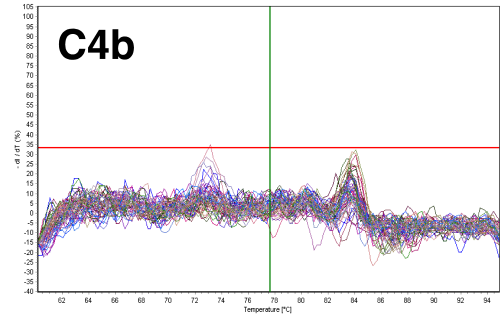

Lilrb3
- d / dT (%)
Temperature [°C]

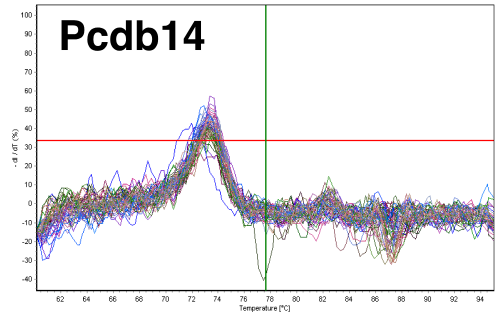

C3
- d / dT (%)
Temperature [°C]


Lgals3
- d / dT (%)
Temperature [°C]


Itgax
- d / dT (%)
Temperature [°C]


Spp1
- d / dT (%)
Temperature [°C]


C4b
- d / dT (%)
Temperature [°C]


Pcdb14
- d / dT (%)
Temperature [°C]