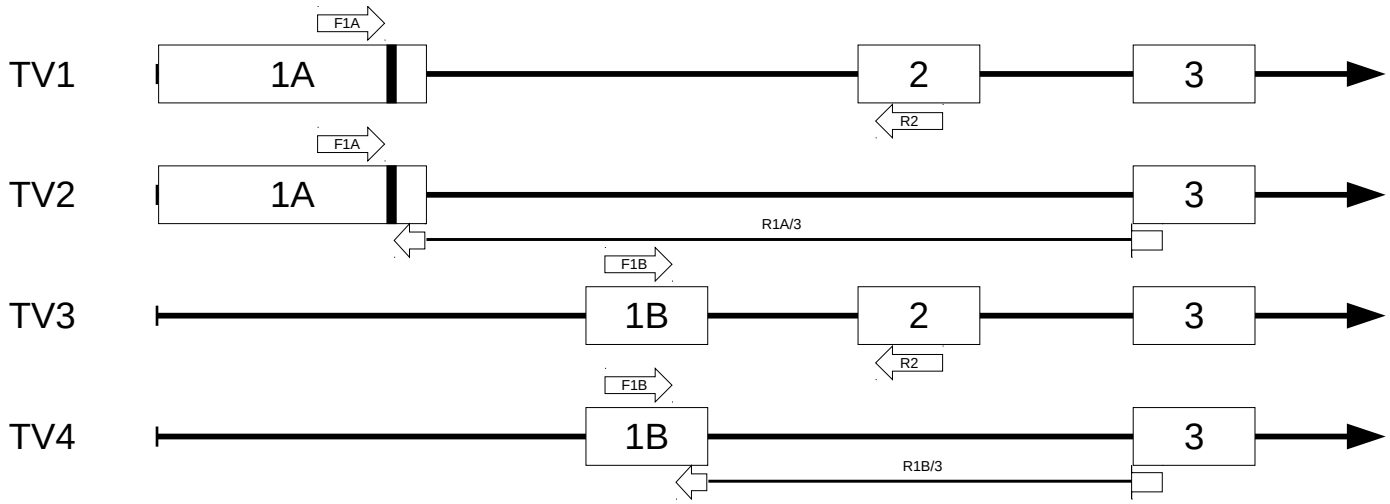
Supplementary Information File 5. Description sheet of the *SLC12A6* RT-qPCR assays and results.

Description:

Taking into account only the first 3 *SLC12A6* exons (1A/1B, 2, 3; numbered as in Garneau *et al*, 2017), 4 different transcript variants can be formed (TV1-4). Four RT-qPCR assays were developed to specifically amplify each of the 4 TVs. A schematic representation is shown below. Blocks are exons, the black bar is the location of the NC_006612.3(XM_014109414.2):c.178_181delinsCATCTCACTCAT variant and arrows are primers. *RPS5* was included as reference gene for normalization.



Primer sequences (IDT, Leuven, Belgium):

Cfam*SLC12A6*_F1A:	5‘-GCCTGAAACAAGCCGCAGTGA-3’
Cfam*SLC12A6*_F1B:	5’-ACCGTGACTAAGGTAGAGGACCCA-3’
Cfam*SLC12A6*_R2:	5’-TGGCTGTGTTCCCCTGTGATGG-3’
Cfam*SLC12A6*_R1A/3:	5’-TATGCCCATCATCCTCGGTAACGTC-3’
Cfam*SLC12A6*_R1B/3:	5’-GCTTTCTTATTATGCCCATCATCTGGTTCA-3’
Cfam*RPS5*_F:	5’-TGCAGTGAAGGAGAAGTATGCCAAGT-3’
Cfam*RPS5*_R:	5’-TGAGTTGGTCAGGCGCTCCAC-3’

RT-qPCR mix (Sigma-Aldrich, Overijse, Belgium):

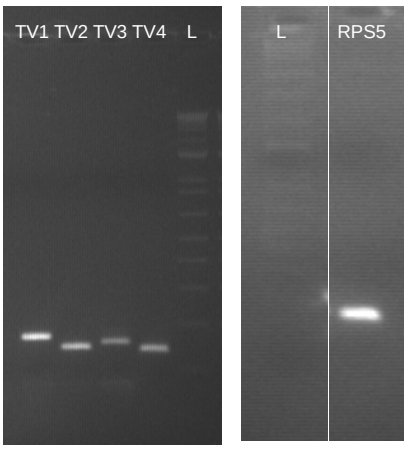
2.0 µl H_2O		[TV1 (156 bp): F1A + R2
5.0 µl 2x KAPA SYBR FAST Universal		[TV2 (131 bp): F1A + R1A/3
1.0 µl Primer mix (5 µM each primer)	→	[TV3 (149 bp): F1B + R2
2.0 µl cDNA (1/10)		[TV4 (134 bp): F1B + R1B/3
10.0 µl Total volume		[RPS5 (109 bp): F + R

RT-qPCR program (CFX96 Real-Time PCR Detection System, Bio-Rad Laboratories, Nazareth, Belgium)

02’40” - 95°C
00’20” - 95°C]
00’40” - 64°C*] x40
Melt curve analysis
00’05” + 0.5°C*] 70°C → 95°C
* signal detection

Gel picture (2% agarose, Gentaur, Kampenhout, Belgium)

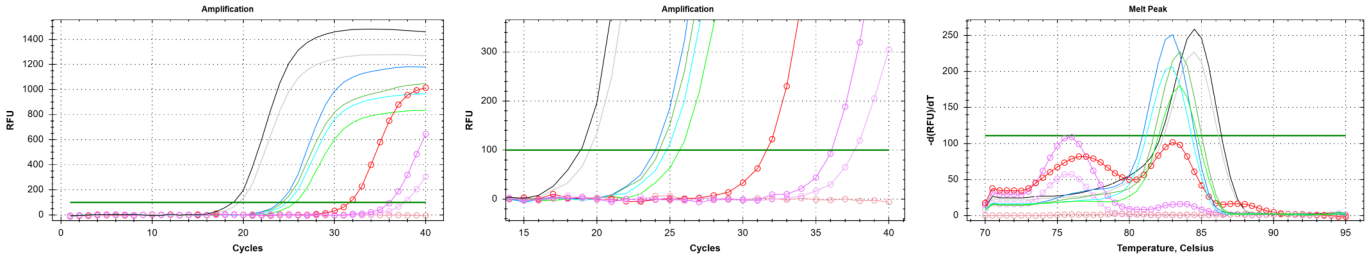
L: 1 Kb+ DNA ladder (Fisher Scientific, Erembodegem, Belgium)
(amplicons were checked on gel and by sequencing)



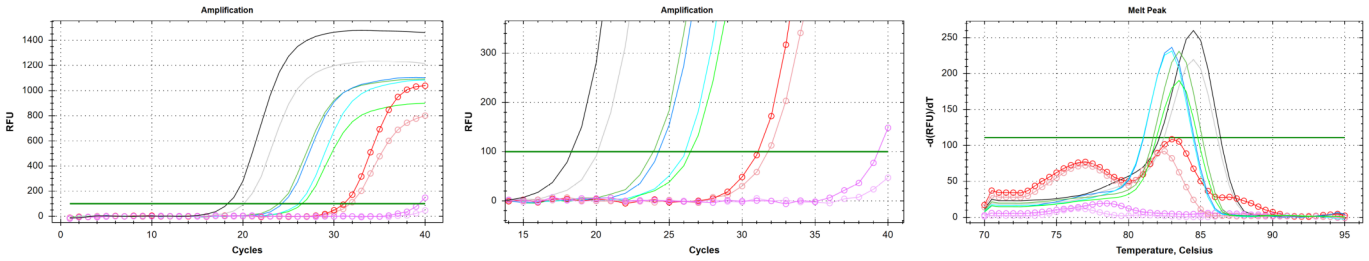
RT-qPCR results

All assays were performed in duplicate. Standard curves (5-point 1/10 dilution series of eluted amplicons) gave PCR efficiencies of 95-105%, correlation coefficients of 0.998-1.000 and dynamic ranges of 18-32 Cq. Melt curve analysis was performed to check for amplicon specificity. No template controls were negative. Assays were performed on cDNA transcribed from DNA-free high-quality RNA isolated from unaffected (cerebrum) and affected (cerebellum and cervical spinal cord) tissues from 2 affected Malinois dogs (for details on method see Van Poucke *et al*, 2016). RT-qPCR analysis showed that TV1 and TV2 are highly expressed in all three tissues from both animals, while TV3 and TV4 are very weakly (at least 50-fold less compared to TV1 and TV2) or not expressed. To illustrate these conclusions, amplification plots (complete and zoomed in) and melt peaks are shown of (A) cerebrum, (B) cerebellum and (C) cervical spinal cord. Biological replicates (2 affected Malinois dogs) are shown in light and dark colors. RPS5 is shown in gray, TV1 in green, TV2 in blue, TV3 in red and TV4 in purple. Amplification plots that are (partially) based on primer dimer signals are marked with circles.

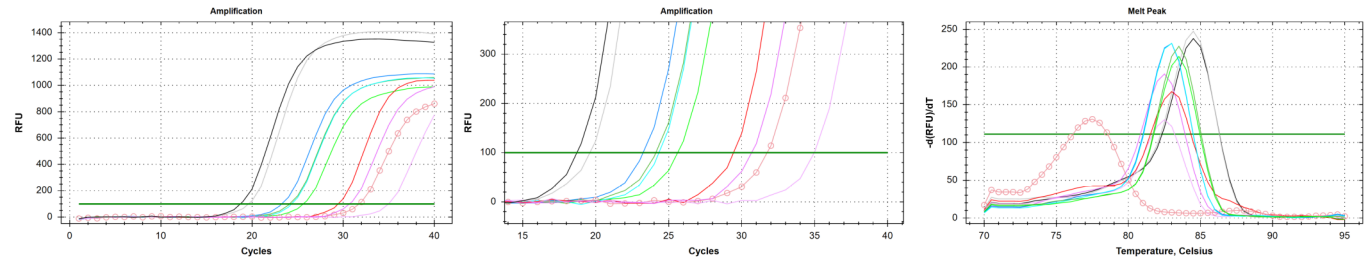
A



B



C



References

Garneau AP, Marcoux AA, Frenette-Cotton R, Mac-Way F, Lavoie JL, Isenring P. Molecular insights into the normal operation, regulation, and multisystemic roles of K+-Cl- cotransporter 3 (KCC3). *Am J Physiol Cell Physiol* 2017; **313:** C516-C532.

Van Poucke M, Martlé V, Van Brantegem L, Ducatelle R, Van Ham L, Bhatti S *et al*. A canine orthologue of the human GFAP c.716G4A (p.Arg239His) variant causes Alexander disease in a Labrador retriever. *Eur J Hum Genet* 2016; **24:** 852–856.