

Novel reference genes for normalization of real-time PCR data in normal human tissues and an application to gene expression profiling

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Answers That Matter.

Objectives:

- Establish a human tissue panel consisting of cDNA from peripheral and central nervous system (CNS) tissues to test the expression profile of novel drug candidates
- Set up and validate PCR assays for reference genes and target genes
- Test a panel of reference gene candidates to select the best ones to normalise real-time RT-PCR data for the tissue panel
- Compare the stability of the expression of housekeeping genes frequently used in the literature with those selected on the basis of public microarray data
- Demonstrate the usefulness of the cDNA panel by establishing the gene expression profile of potential drug target genes

Methods

The human cDNA panel consists of 12 neural (parietal, frontal, temporal lobe, thalamus, striatum, hippocampus, hypothalamus, substantia nigra, nucleus accumbens, cerebellum, spinal cord, dorsal root ganglia) and 12 peripheral tissues (liver, lung, heart, pancreas, adrenal gland, small intestine, spleen, skeletal muscle, kidney, bladder, stomach and thyroid). Pooled RNA samples (obtained commercially from Becton-Dickinson and Ambion) derived from at least 4 donors were used for cDNA synthesis. The TaqMan Archival Reverse Transcription Kit (Applied Biosystems) was used to synthesise first strand cDNA. The final concentration of RNA in the reaction was 1 µg / 100µl. Quantitative real-time RT-PCR was performed using SYBR Green chemistry with a final concentration of 250 ng/ml template in the PCR on an ABI Prism 7900HT real-time PCR system (Applied Biosystems, 384-well plate, 5 µl final reaction volume). The following cycling conditions were used: 50°C for 2min (stage1); 95°C for 10min (stage 2); 95°C for 15sec; 60°C for 1min (stage 3) x 40. Primers were designed to amplify cDNA regions that are present in all known splicing isoforms of the target genes, optimised and used for quantitative real-time PCR. Reference gene candidates were selected on the basis of minimal variation of their expression across human tissues, described in a public microarray database (HUGE database, Haverty et al., NAR, Jan 2002; 30: 214 – 217). We used the best three genes to normalise our data according to the method of Vandesompele et al. (Genome Biol. 2002, 3:RESEARCH0034 (2002) [1].

Quality control of RNA samples

Name of tissue	Source	No. of individuals	Cause of death	Race	Age	Sex
Peripheral tissues						
Heart	BD Biosciences	10	Trauma	Caucasian	21-51	Female/Male
Skeletal muscle	BD Biosciences	7	Sudden death	Caucasian	20-88	Female/Male
Adrenal gland	BD Biosciences	51	Sudden death	Caucasian	15-81	Female/Male
Pancreas	Ambion	5	Head trauma/accident/trauma	Caucasian	1-73	Female/Male
Thyroid	BD Biosciences	65	Head trauma	Caucasian	15-81	Female/Male
Stomach	Ambion	5	Car accident/trauma	Caucasian	18-78	Female/Male
Human small intestine	BD Biosciences	5	Sudden death	Caucasian	20-61	Female/Male
Kidney	Ambion	5	Trauma/traumatic/accident bleed	Caucasian	21-86	Female/Male
Liver	Ambion	5	Sickle/Stroke/traumatic hemorrhage	Caucasian	17-69	Female/Male
Spleen	BD Biosciences	14	Sudden death	Caucasian	20-86	Female/Male
Bladder	BD Biosciences	20	Sudden death	Caucasian	17-90	Female/Male
Uterus	BD Biosciences	4	Sudden death	Caucasian	20-86	Female/Male
CNS tissues						
Brain - frontal lobe	BD Biosciences	4	Sudden death	Caucasian	22-21	Female/Male
Brain - parietal lobe	BD Biosciences	4	Sudden death	Caucasian	20-39	Female/Male
Brain - temporal	BD Biosciences	20	Sudden death	Caucasian	22-21	Female/Male
Hippocampus	BD Biosciences	20	Sudden death	Caucasian	15-24	Female/Male
Cerebellum	BD Biosciences	20	Sudden death	Caucasian	15-70	Female/Male
Substantia nigra	BD Biosciences	19	Sudden death	Caucasian	15-84	Female/Male
Spinal Cord	BD Biosciences	49	Sudden death	Caucasian	15-86	Female/Male
Dorsal root ganglia	BD Biosciences	5	Trauma	Caucasian	21-59	Male
Thalamus	Ambion	5	Heart failure/myocard infarct/trach cancer	Caucasian	45-84	Female/Male
CNS (Caudate nuclei)	BD Biosciences	23	Sudden death	Caucasian	22-55	Female/Male
Nucleus accumbens	BD Biosciences	6	Sudden death	Caucasian	22-56	Female/Male
Hippocampus	Ambion	5	Heart failure/Obd. Alzheim disease	Caucasian	28-80	Female/Male

Table 1. Information about the human total RNA samples used in this study. Samples were pooled from at least 4 individuals, and the cause of death was chosen not to interfere with the quality of the tissues.

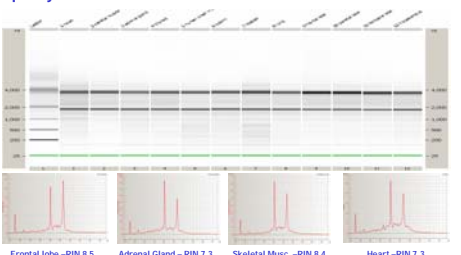


Fig. 1. Integrity of human total RNA samples as measured by the Agilent Bioanalyzer. All 24 samples were above the RNA Integrity Number (RIN) 7. Virtual gel examples of electropherograms are shown above.

Primer sequences for reference gene candidates

Name	Accession Number	Sequence annotation	Product Length
PSMB2PrlHum-1.0	D26589	Homo sapiens mRNA for proteasome subunit HCS74, complete cds.	123
CANXpHum-1.0	L10284	Homo sapiens integral membrane protein, calnexin, (P30) mRNA, complete cds.	137
RPOL2PrlHum-1.0	U37690	Human RNA polymerase II subunit (hRPB10) mRNA, complete cds.	75
CYCpHum-1.0	X82851	Human cyclophilin gene for cyclophilin (EC 5.2.1.8).	75
NACAP1Hum-1.0	X83939	Hsapiens alpha NAC mRNA.	86
TAX1PrlHum-1.0	U33821	Homo sapiens tax-binding protein TBP151 mRNA, complete cds.	148
GPS1PrlHum-1.0	U20265	Human G-protein pathway suppressor 1 (GPS1) mRNA, complete cds.	90
PSMD2PrlHum-1.0	D78151	Human mRNA for 26S proteasome subunit p97, complete cds.	146
ACTB1PrlHum-1.0	NM_001101	Homo sapiens actin, beta (ACTB), mRNA.	100
GAPDP1Hum-1.0	NM_002046	Homo sapiens glyceraldehyde-3-phosphate dehydrogenase (GAPD), mRNA.	126
GAPDP2Hum-2.0	NM_002046	Homo sapiens glyceraldehyde-3-phosphate dehydrogenase (GAPD), mRNA.	94
ACTBP1Hum-2.0	NM_001101	Homo sapiens actin, beta (ACTB), mRNA.	130

Table 2. GenBank accession numbers and names of primers

PCR assay optimisation

Name	Length Calculated	Length estimated with BioAnalyzer	Difference	ΔSS difference
PSMB2PrlHum-1.0 FWD/RS	123	121	-2	2
CANXpHum-1.0 FWD/RS	137	132	-5	5
RPOL2PrlHum-1.0 FWD/RS	75	77	2	2
CYCpHum-1.0 FWD/RS	75	73	-2	2
NACAP1Hum-1.0 FWD/RS	86	88	2	2
TAX1PrlHum-1.0 FWD/RS	148	145	-3	3
GPS1PrlHum-1.0 FWD/RS	90	98	8	8
PSMD2PrlHum-1.0 FWD/RS	146	142	-4	4
ACTB1PrlHum-1.0 FWD/RS	100	100	0	0
GAPDP1Hum-1.0 FWD/RS	126	123	-3	3
GAPDP2Hum-2.0 FWD/RS	94	96	2	2
ACTBP1Hum-2.0 FWD/RS	130	132	2	2

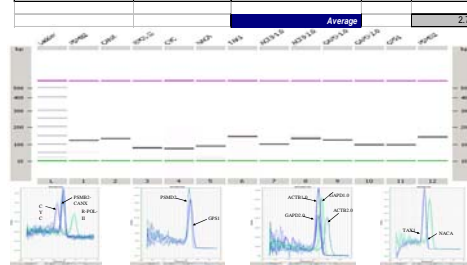


Fig. 2 The PCR reactions resulted in a single product on a virtual gel (Agilent), with the expected molecular weight (average accuracy of +/-3 base pair). Dissociation curves showed one peak with no primer dimer formation detected.

Name	Efficacy I	Efficacy II	Efficacy III	Average	STD
CANX	1.98	1.98	1.98	1.97	0.01
CYC	2.04	2.01	2.01	2.02	0.02
GPS	2.00	2.00	2.00	2.00	0.00
PSMB2	1.95	2.00	2.01	1.99	0.03
PSMD2	2.02	1.98	2.02	2.01	0.02
RPOL-2	1.90	2.02	1.98	2.00	0.02
TAX1	2.01	1.92	1.94	1.92	0.01
NACA	2.01	2.05	1.92	1.99	0.07
ACTB1.0	1.95	2.02	1.98	1.98	0.03
ACTB2.0	1.99	2.00	2.06	2.02	0.04
GAPD1.0	1.98	2.01	1.99	1.99	0.01
GAPD2.0	1.95	1.98	1.98	1.97	0.02

Fig. 3. PCR efficiencies of the primer sets for reference gene candidates. The columns represent 3 repeated determinations of the efficiencies. All PCR reactions had near 100% efficiency.

Stability of expression for reference gene candidates in 24 human tissues

Ref. Gene	Sorted by average of ranges			Mean Ct range	Fold range
	Range-Run1	Range-Run2	Range-Run3		
GPS1	2.74	2.43	2.87	2.68	6.41
PSMB2-1.0	2.79	2.70	2.78	2.76	6.76
RPOL-1.0	2.88	2.87	3.06	2.94	7.67
CANX-1.0	3.17	3.58	3.38	3.38	10.39
PSMD2	3.51	3.27	3.46	3.41	10.66
NACA-1.0	3.17	3.53	3.68	3.46	10.99
ACTB-2.0	3.50	3.32	3.63	3.48	11.17
ACTB-1.0	3.02	4.15	4.04	3.74	13.32
GAPD-2.0	5.76	5.97	5.97	5.87	150.40
GAPD-1.0	5.83	5.96	5.86	5.88	150.94
TAX1-1.0	5.94	6.16	5.95	6.02	64.85
CYC-1.0	5.53	5.59	7.30	6.14	70.91

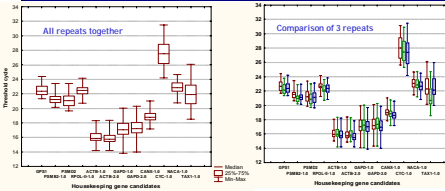


Fig. 4. The range of Ct (max - Ct_min) of threshold cycle values across 24 human cDNA samples (total RNA samples were adjusted to have the same concentration). The three best HK genes are shown in yellow in the table (same results obtained by pairwise comparisons according to Vandesompele et al.)

Name of primer	Sequence of primer	Working conc. in PCR (nM)
PSMB2PrlHum-1.0-FWD	AACGGTTCATCCGTAATGTGTGCC	300
PSMB2PrlHum-1.0-RVS	AAGTGGGAGGAGGAGGATGATG	300
CANXpHum-1.0-FWD	AGCAGTGGCTCCCTTCATCATCTC	300
CANXpHum-1.0-RVS	TGGTATCGTGTCTTGTGGCTTTC	300
RPOL2PrlHum-1.0-FWD	CCTGGGAAGTACACCGGCT	300
RPOL2PrlHum-1.0-RVS	AATTCGGGTAGGACGGCTGAG	300
CYCpHum-1.0-FWD	TTCGGGAAGTACAGCATTTTGAAGCC	300
CYCpHum-1.0-RVS	CTACAGAGTGGGACAGGAGC	300
NACAP1Hum-1.0-FWD	ACAGCTGTATGATTAAGGCAATTGA	300
NACAP1Hum-1.0-RVS	TGTGTTCTTAGGGCTCGGACT	300
TAX1PrlHum-1.0-FWD	AAGGCAACACCTAAGAGGACACAA	300
TAX1PrlHum-1.0-RVS	TTAAGTCTGGGACCTGTGGCATAGCC	300
GPS1PrlHum-1.0-FWD	AAGATCTGCGACGAGATGAAGGA	300
GPS1PrlHum-1.0-RVS	ACGGTTCGGAATCTGGGCTTA	300
PSMD2PrlHum-1.0-FWD	AAGGCAAGTCTCTCTTCTACAACTT	300
PSMD2PrlHum-1.0-RVS	CGGAGATGCTGACAGCAAAA	300
ACTBP1Hum-1.0-FWD	CGCCTGAGGAGCAGCTTTCGA	300
ACTBP1Hum-1.0-RVS	CGGATGTCAGCAGTACACTTC	300
ACTBP1Hum-2.0-FWD	GTGGACATCCGCAAGGACCTG	300
ACTBP1Hum-2.0-RVS	TGATCTTGAATCTTGAATGCTGGG	300
GAPDP1Hum-1.0-FWD	TCTCCTCTGACTCAAGAGCGAC	300
GAPDP1Hum-1.0-RVS	CGCTGTCTCTGACCAAGTTC	300
GAPDP2Hum-2.0-FWD	ACAGCTCAAGATCATGAGGATG	300
GAPDP2Hum-2.0-RVS	CCTTCCACGATACCAAGTTCAT	300

Table 3. Sequences and working concentrations of primers

Gene expression profiling examples

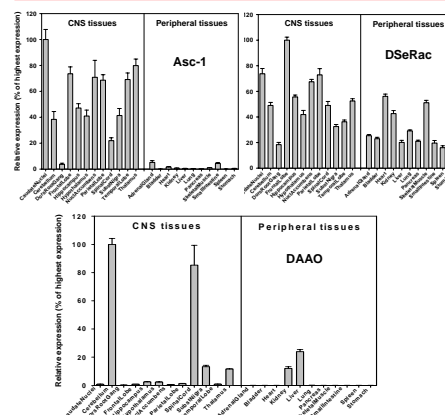


Fig. 5. Gene expression profiles of D-amino-acid oxidase (DAAO), D-serine transporter (Asc-1) and D-serine racemase (DSeRac) in 24 human tissues.

Summary / Conclusions

- We have established a cDNA collection from various human tissues, which can be used to check the distribution of gene expression for novel drug target candidates.
- QPCR assays were designed and validated for the reference gene candidates and target genes.
- We have tested 12 candidate reference genes and found the three best ones to be used for normalisation in human tissues: GPS1, PSMB2 and RPOL2 (showing a variation of 6, 6.3 and 7-fold, respectively). We have described two novel housekeeping gene candidates for human tissues: GPS1 and PSMB2.
- HK genes, which are frequently used in the literature (β-actin, cyclophilin and GAPD) showed a much higher variation (13, 58 and 70-fold respectively) across the 24 human tissues compared to our selected candidates (see above).
- We have established the gene expression profiles of three genes, DAAO, DSeRac and Asc-1, and found that Asc-1 was highly expressed in the CNS with very little expression in the peripheral tissues, whereas DSeRac was expressed widely, including the periphery in most tissues, showing between 20 and 80% expression of the highest signal, which was found in the frontal lobe. DAAO mRNA had the most striking differential distribution, with very high levels in caudate nucleus and spinal cord and relatively little expression in the peripheral tissues with the exception of kidney and liver where there was a significant level of DAAO mRNA.
- The above data helps us select genes with restricted expression in the CNS, as opposed to the peripheral tissues, representing an advantageous distribution for a potential CNS drug.