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Analytical Biochemistry 335 (2004) 30–41
ANALYTICAL BIOCHEMISTRY
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Reference genes identified in SH-SY5Y cells using custom-made gene arrays with validation by quantitative polymerase chain reaction

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Received 1 June 2004
Available online 1 October 2004

Abstract

Transcriptomic methods are widely used as an initial approach to gain a mechanistic insight into physiological and pathological processes. Because differences in gene regulation to be assessed by RNA screening methods (e.g., SAGE, Affymetrix GeneChips) can be very subtle, these techniques require stable reference genes for accurate normalization. It is widely known that housekeeping genes, which are routinely used for normalization, can vary significantly depending on the tissue, and experimental test. In this study, we aimed at identifying stable reference genes for a fibrillar $A\beta_{42}$ peptide-treated, human tau-expressing SH-SY5Y neuroblastoma cell line derived to model aspects of Alzheimer's disease in tissue culture. We selected genes exhibiting potential normalization characteristics from public databases to create a custom-made microarray allowing the identification of reference genes for low, intermediate, and abundant mRNAs. A subset of these candidates was subjected to quantitative real-time polymerase chain reaction and was analyzed with geNorm software. By doing so, we were able to identify GAPD, M-RIP, and POLR2F as stable and usable reference genes irrespective of differentiation status and $A\beta_{42}$ treatment.
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Keywords: Quantitative RT-PCR; Reference genes; Housekeeping genes; Transcriptional analysis

Gene expression analysis is increasingly used to dissect dynamic molecular signaling and complex physiological mechanisms in species ranging from several bacteria to higher organisms such as *Homo sapiens.* The visualization of gene regulation patterns helps to decipher overall signaling mechanisms and global effects of individual genes that are otherwise not discernible through analysis of a single gene [1,2]. The simultaneous analysis of thousands of genes by microarray analysis provides a powerful means to identify novel genes involved in pathophysiological signaling cascades [3,4]. This has particular application for complex multifactorial

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[2] *Abbreviations used:* AD, Alzheimer's disease; qRT-PCR, quantitative real-time polymerase chain reaction; NFT, neurofibrillary tangle; PHF, paired helical filaments; FTDP-17, frontotemporal dementia with Parkinsonism linked to chromosome 17; APP, amyloid precursor protein; NT, nontransfected SH-SY5Y human neuroblastoma cells; M, mock-transfected SH-SY5Y cells; P, cells with stable expression of the longest human tau isoform with the pathogenic mutation P301L; W, cells with stable expression of the longest human tau isoform without the pathogenic mutation P301L; siRNA, short interfering RNA; GFP, green fluorescent protein; LP, laser power; PMT, photomultiplier; ASP, automated slide processor; BHQ, Black Hole Quencher; HEX, hexachloro-6-carboxyfluorescein; RA, retinoic acid; BDNF, brain-derived nerve growth factor; PP2A, protein phosphatase 2A.

0003-2697/$ - see front matter © 2004 Elsevier Inc. All rights reserved.
doi:10.1016/j.ab.2004.08.028

disorders such as Alzheimer's disease (AD)[2] [5–7]. Dissecting the contribution made by differentially expressed genes in the brains of individuals with AD depends on meaningful characterization and validation. Quantitative real-time polymerase chain reaction (qRT-PCR) is well suited because it provides a wide quantitative dynamic range and allows an accurate analysis, albeit for a limited number of genes in parallel [8,9]. However, both microarray analysis and qRT-PCR depend greatly on a precise normalization procedure for the extraction of subtle, yet reproducible, differences in gene expression.

The current most universally applied normalization methods for both technologies rely on the relative comparison of constitutively expressed endogenous reference genes that are often termed housekeeping or maintenance genes [10]. Reference gene transcripts necessarily undergo the analogous experimental processing as the mRNAs within the cells of interest and provide a better control than does the inclusion of exogenous defined amounts of synthetic DNA, RNA, or rRNAs given that their levels do not reflect total cellular mRNA levels [11]. Many previous studies have assumed that the expression levels of constitutively expressed endogenous reference genes remain constant between treatments and/or cell lines or tissues. This oversimplification has manifested despite the wide recognition that typically all genes experience independent regulation; no single gene transcript can be granted a universal role as calibrator for all quantitative mRNA analytical measurements [12]. Moreover, the definition of constant or stable expression also varies [8–10]. One of the most commonly used human cell lines in neuroscience research (>1400 citations in *PubMed*), and more specifically in neuropathology, is the SH-SY5Y neuroblastoma cell line (205 citations vs. 47 citations for N2a cells or 42 citations for HEK cells as publised to this point). Therefore, it is astonishing to find that no reference gene cohort has yet been established for the study of gene expression in cultured SH-SY5Y cells.

In this study, we aimed at identifying constitutively expressed endogenous reference genes in a tissue culture system of human tau-expressing SH-SY5Y cells that were treated with fibrillar preparations of $A\beta_{42}$ to model aspects of AD [13].

Tau is a microtubule-associated protein that is the major proteinaceous component of neurofibrillary tangles (NFTs). In these tangles, tau forms filaments that are described mainly as paired helical filaments (PHFs) and as narrow-twisted ribbons. These are abundant in many neurodegenerative diseases, including AD and frontotemporal dementia with Parkinsonism linked to chromosome 17 (FTDP-17) [14–16]. Transgenic mice that express pathogenic mutations of human tau such as P301L develop NFTs [6,17]. Crossing P301L mutant tau with $A\beta_{42}$-producing amyloid precursor protein (APP) transgenic mice caused a sevenfold increase of NFT formation compared with P301L single transgenic mice [17]. Intracerebral microinjection of $A\beta_{42}$ into P301L mutant tau transgenic mice also greatly accelerated the rate of NFT formation [18]. Furthermore, we have shown that formation of PHFs can be reproduced in a human tissue culture system [19]. When human tau was expressed with and without pathogenic mutations in SH-SY5Y cells, followed by exposure of the cells to aggregated synthetic $A\beta_{42}$ peptide for 5 days, this caused a decreased solubility of tau along with the generation of PHF-like tau-containing filaments. Together, these data established a mechanistic relationship of $A\beta_{42}$ toxicity with NFT formation.

In the current study, we selected genes whose expression levels could be considered stable irrespective of the transfection status (mock, wildtype, or mutant tau) or the state of differentiation (neuronal or nonneuronal). To this end, we created a "custom-made" microarray composed of 43 housekeeping genes derived from a microarray screen of 7000 human genes in adult and fetal tissues [12]. Once stable genes were selected, a subset of these was chosen for validation by qRT-PCR and analyzed with geNorm software [20]. Thus, we were able to identify genes with constant expression at intermediate and high expression levels.

This enabling study is essential for our study program targeting the identification of genes involved in the regulation of molecular and cellular processes of tau aggregation.

Materials and methods

Cell culture conditions

Mainly, four cell lines were used: nontransfected SH-SY5Y human neuroblastoma cells (NT), mock-transfected SH-SY5Y cells (M), and cells with stable expression of the longest human tau isoform either with (P) or without (W) the pathogenic mutation P301L. These cells were cultivated, neuronally differentiated, and treated with fibrillar preparations of $A\beta_{42}$ as described previously [13]. The conditions used in the current study are described in Fig. 1. For a subset of data, the cells were either kept undifferentiated or neuronally differentiated for 5, 7, or 10 days. In addition, short interfering RNAs (siRNAs) were generated with the Silencer siRNA construction kit (Ambion, Austin, TX, USA). These were transfected transiently, followed by differentiation of the cells. Furthermore, pSUPER expression vectors [21] were generated targeting the PR55γ subunit of protein phosphatase 2A (PP2A/PR55γ), and stable clones were obtained based on selection for green fluorescent protein (GFP) expression. All transfections were done in DMEM using OptiMEM and Lipofectamine (Invitrogen, Basel, Switzerland).

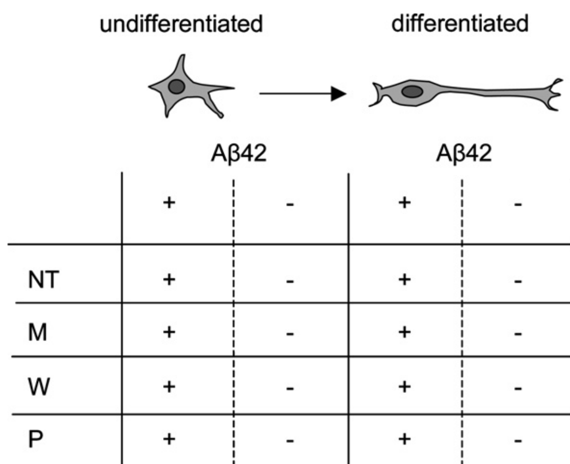


Fig. 1. Cell lines and conditions tested for stable reference gene expression. All four SH-SY5Y neuroblastoma cell lines were cultivated according to Ferrari et al. [13]. NT corresponds to untransfected SH-SY5Y cells, M to mock-transfected SH-SY5Y cells, W to stably transfected wild-type tau-expressing cells, and P to stably transfected P301L mutant tau-expressing cells. Cells were neuronally differentiated (arrow) by incubating for 5 days with 10 μM RA, followed by 5 days with 50 nM BDNF. In addition, both undifferentiated and differentiated cells were treated with (+) or without (−) 10 μM fibrillar preparations of $A\beta_{42}$ for 5 days.

Plasmid preparation and DNA sequencing

After selection on the basis of the presumed function as maintenance genes [12], 53 cDNA clones were obtained from the MRC HGMP Resource Centre (Cambridge, UK) as agar plugs. The plasmid DNAs were isolated using QIAprep (Qiagen, Basel, Switzerland). To verify plasmid inserts, cycle sequencing reactions were carried out on an ABI GeneAmp PCR system 9700 (Applied Biosystems, Rotkreuz, Switzerland) using the ABI Prism Dye Terminator Cycle Sequencing Ready Reaction Kit together with AmpliTaq DNA polymerase (Applied Biosystems) and M13 universal forward and reverse primers (Microsynth, St. Gallen, Switzerland). Automated fluorescent DNA sequencing was performed on an ABI 370 DNA Sequencer (Applied Biosystems). Sequences were verified with the NCBI BLASTN 2.2.1 engine and were considered to be identical to the gene of interest if their derived E values were less than 10^{-36}.

Spotting and array design of the maintenance gene chips

Plasmid DNAs (12.5 μl) at a concentration of 2 μg/μl were mixed with an equal volume of 2× NoAb Printing Buffer (NoAb Diagnostics, Mississauga, Ontario, Canada). Using a custom-built piezo arrayer (kind gift from Remo Hochstrasser, Hofmann-LaRoche, Basel, Switzerland), the plasmids were spotted in triplicate onto 75 × 25-mm epoxy-coated covalent binding glass slides (NoAb Diagnostics). After arraying, the quality of the first slide was controlled by a 5-min incubation with SYBR Green I solution (Molecular Probes, Leiden, Netherlands). The slides were dried by centrifugation for 2 min at 1200*g* and were scanned with a Scan Array 5000 Scanner (Perkin–Elmer/Packard Biosciences, Zürich, Switzerland) using a laser power (LP) of 70% and a photomultiplier (PMT) gain set at 65%.

RNA preparation and labeling

Total RNA was isolated from 10-cm culture dishes using the RNeasy mini-kit according to the manufacturer's instructions (Qiagen). For the undifferentiated condition, RNA was extracted from three M, one W, and one P dishes. Similarly, for the condition of neuronal differentiation, RNA was extracted from one M, one W, and one P culture dishes. Finally, RNA was extracted from one plate of $A\beta_{42}$-treated and neuronally differentiated P cells (Fig. 1). Total RNA (50 μg) was dissolved in RNAse-free water, reverse transcribed with Superscript II (Invitrogen), and labeled with 50 nM Cy3-dCTP using 0.2 mM dCTP and 0.5 mM dATP, dTTP, and dGTP (Amersham–Pharmacia Biotech, Dübendorf, Switzerland). After reverse transcription, 40 μl of this mixture was hydrolyzed for 20 min by adding 5 μl 0.5 M EDTA and 10 μl 1 M NaOH at 65 °C to destroy all traces of RNA. The samples were then neutralized with 6 μl 1 M HCl and 2 μl 1 M Tris–HCl, pH 7.5, and cleaned with the Qiagen PCR cleanup kit. The labeled cDNA was stored at −20 °C until use.

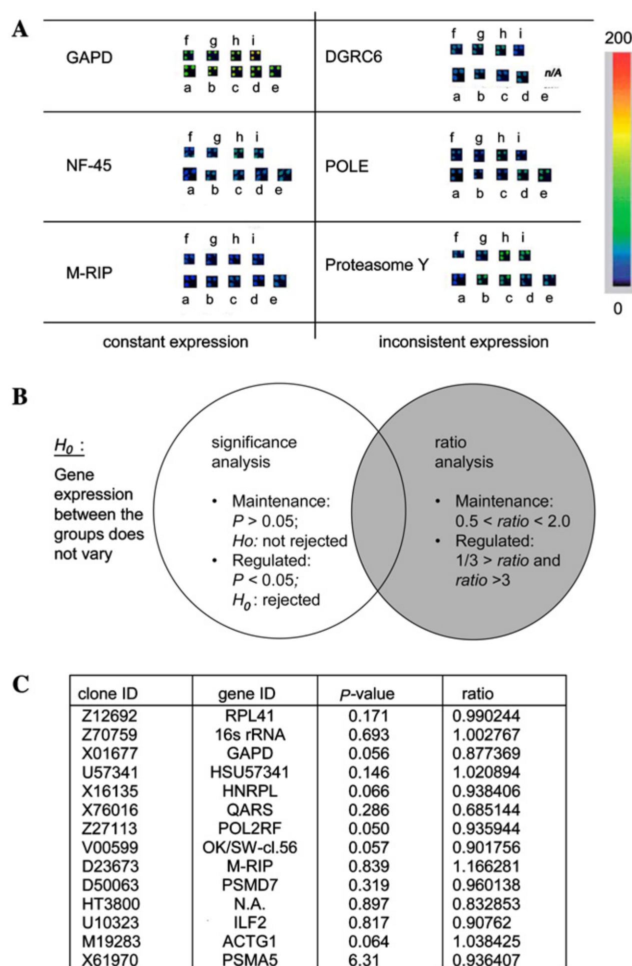
Hybridization of the custom-made arrays

The arrays on the epoxy slides were incubated with blocking buffer (NoAb Diagnostics) by gentle agitation at room temperature for 2 h and denatured by boiling for 2 min in water. Then the slides were treated with 20 μl of 50% formamide/3× SSC for 5 min at 76 °C. After this prehybrization, the slides were placed for 2 min in H_2O, then for 2 min in 70% EtOH, and finally for another 2 min in 100% EtOH, followed by a centrifugation of the slides for 2 min at 1000 rpm.

The labeled cDNA samples were denatured at 95 °C for 5 min and chilled on ice. Then 220 μl of SlideHyb No. 1 Hybridization Buffer (Ambion, Huntingdon, Cambs, UK) was preheated to 65 °C, mixed with 40 μl of the denatured cDNA samples, and incubated at 65 °C for 10 min. Before adding it to the hybridization chamber of the automated slide processor (ASP, Amersham–Pharmacia Biotech, Little Chalfont, Buckinghamshire, UK), each loaded slide chamber was primed with 1× SSC/0.2% SDS for 5 min at 42 °C. The hybridization mixture was injected into the hybridization chamber of the ASP and hybridized for 16 h at 42 °C with continuous agitation. Two washes were performed with 1× SSC/0.2% SDS and 0.1× SSC/0.2% SDS for 10 min each at 30 °C. The slides were then automatically flushed with isopropanol and dried in an airstream.

Scanning of the arrays and image analysis

The hybridized slides were scanned using the Scan Array 5000 Scanner (Perkin–Elmer/Packard Biosciences). The LP and PMT were initially set at 70 and 65%, respectively. To conserve lower intensity hybridization signals, the slides were finally scanned with the PMT and LP set such that spots previously identified as representing the full intensity range showed an intensity distribution between the 50th and 100th percentiles. Image analysis and quantification were performed using ImaGene 3.0 software (BioDiscovery, Los Angeles, CA, USA). Data transformation was achieved with GeneSight 3.0 software (BioDiscovery). The microarray analysis was composed of a total of nine slides. From these slides, we created nine data sets divided into two groups: "controls" comprised the three chips that were hybridized with cDNAs from the untreated undifferentiated M cells, whereas "treated" comprised the six other samples (undifferentiated W and P cells, differentiated W, M, and P cells, and differentiated $A\beta_{42}$-treated P cells). Furthermore, the control data set was duplicated to obtain two groups of similar size. The data were modified first by adjusting for local background, then by combining replicates and omitting empty or poor spots (defined as equivalent to or less than 0.4 times the standard deviation of background signal intensity), and finally by normalizing using the globalization approach [22]. In selecting reference genes from the microarray data set, two methods were combined to enhance the stringency of the selection. First, we used the Student's two-tailed t test with H_0 stating that gene expression does not vary between the two groups, and we selected those genes for which H_0 was not rejected (Fig. 2). Then we performed a pairwise ratio analysis, using the ratio of gene expression (corresponding to the average spot intensity) from each treated sample divided by that of each control sample, to select candidates for which the ratio (r) was between 0.5 and 2.0. Reference gene candidates were selected when they satisfied both criteria.



clone ID	gene ID	*P*-value	ratio
Z12692	RPL41	0.171	0.990244
Z70759	16s rRNA	0.693	1.002767
X01677	GAPD	0.056	0.877369
U57341	HSU57341	0.146	1.020894
X16135	HNRPL	0.066	0.938406
X76016	QARS	0.286	0.685144
Z27113	POL2RF	0.050	0.935944
V00599	OK/SW-cl.56	0.057	0.901756
D23673	M-RIP	0.839	1.166281
D50063	PSMD7	0.319	0.960138
HT3800	N.A.	0.897	0.832853
U10323	ILF2	0.817	0.90762
M19283	ACTG1	0.064	1.038425
X61970	PSMA5	6.31	0.936407

Fig. 2. Reference gene selection strategy using custom-made microarrays. (A) Nine custom-made microarrays were hybridized with Cy3-labeled cDNA derived from three M cells (a,b,c) and one each from undifferentiated W (d) and P cells (e), differentiated M, W, and P cells (f,g,h), and differentiated $A\beta_{42}$-treated P cells (i). The spot triplicate images result from glow-over/glow-under intensity-coded images after the initial scanning. Genes were selected if they showed a consistent stable expression throughout all nine arrays (shown for GAPD, M-RIP, and NF-45) but were rejected when gene expression varied between samples (DGCR6) or when inconsistencies were observed in the three array replicates (Proteasome Y, a,b,c). (B) Reference genes were selected when they satisfied both maintenance conditions for the significance analysis and the ratio analysis. (C) Of the 43 spotted genes, 14 were chosen as stable reference genes for further validation by qRT-PCR. clone ID, image consortium clone number; gene ID, gene identification code; ratio, average ratio (r) of the pairwise ratios of all samples. P values were obtained with the Student's two-tailed t test comparing controls (the three duplicated M data sets) with the treated group (the remaining six data sets). N.A., not available.

TaqMan real-time quantitative PCR

RNA was extracted from SH-SY5Y cells for all conditions described in Fig. 1 using 250 μg glycogen/ml Trizol and RNase-free DNase I (Roche Diagnostics, Mannheim, Germany) for 15 min at 37 °C, followed by a phenol/chloroform extraction. The cDNAs were generated with the SuperScript First-Strand Synthesis System for RT-PCR using random hexamers as primers. The cDNA was diluted twofold prior to use in the qRT-PCR.

Black Hole Quencher (BHQ) TaqMan probes and primers (Table 1) were designed with Primer Express 1.5 software (PE Biosystems, Foster City, CA, USA). The specificity of the PCR amplification for each primer pair was confirmed on a 3% agarose gel (data not shown). The PCR was carried out using the TaqMan Universal PCR Master Mix (Applied Biosystems), 333 nM oligonucleotide primers (Metabion, Martinsried, Germany), 200 nM fluorogenic probe (labeled at the 5′ end with hexachloro-6-carboxyfluorescein (HEX) and at the 3′ end with BHQ-1) (Biosearch Technologies, Novato, CA, USA) and 1 μl of template cDNA. For signal detection,

Table 1
Genes selected for spotting on the custom-made housekeeping arrays

Number	Gene name	GenBank entry	Image ID	Expression level
1	Lysosomal hyaluronidase	AJ000099	4545669	m
2	KIAAO106	D14662	4654913	lm
3	KIAA0120	D21261	3619603	m
4	HH109	D23673	4840784	m
5	Proteasome subunit Y	D29012	4810620	m
6	HLA class-I (HLA-A26) heavy chain (clone cMIY-1)	D32129	4849824	m
7	Proteasome subunit p40/Mov34 protein	D50063	4640894	m
8	KIAA0142	D63476	3834336	lm
9	MHC classe I	X12432	4703878	m
10	Elongation factor EF-1-α	J04617	4341808	h
11	Ribosomal protein L37a (RPL37A)	L06499	5016324	h
12	RNA-binding protein (clone E 5.1)	L37368	4850931	m
13	clone S20iii 15, 3′ end of CDS	L40395	4763838	lm
14	c-yes-1	M15990	449421	lm
15	Ca 2-activated neutral protease large subunit (CANP)	M23254	3929691	m
16	Chromosome 1q subtelomeric squence D1S553	U06155	4331154	h
17	18-kDa Alu RNA-binding protein	U07857	4328576	m
18	Nuclear factor (NF) 45	U10323	4642434	m
19	Gps 1	U20285	4899251	m
20	Tax1-binding protein TXBP151	U33821	4838204	m
21	E2 ubiquitin conjugation enzyme UbcH5B	U39317	4279322	l
22	Tetratricopeptide repeat protein (tpr1)	U46570	4864394	m
23	Histone deacetylase HD1	U50079	4855663	lm
24	Lysphosphatidic acid acyltransferase-α	U56417	3945415	m
25	Neurofilament triplet L protein	U57341	4815916	mh
26	Myelodysplasia/myeloid leukemia factor 2 (MLF2)	U57342	476569	m
27	Hypotehetical protein 384D8_2	U62317	4135830	m
28	Branched-chain amino acid aminotransferase (ECA 40)	U62739	4861163	m
29	SR31747-binding protein 1	U79528	4859914	m
30	Phosphomannomutase	U86070	4908320	m
31	Novel heterogeneous nuclear RNP protein, L protein	X16135	3939440	m
32	Ezrin	X51521	4620773	m
33	IFN-inducible γ-2 protein	X59892	4299127	m
34	Macropain subunit ζ	X61970	4842426	lm
35	Caltractin	X72964	4454992	m
36	Protein phosphatase 1-γ	X74008	4672352	lm
37	QRSHs glutaminyl-tRNA synthetase	X76013	4864470	m
38	MLN51	X80199	4565568	lm
39	5-HT2c receptor	X80763	4243520	m
40	ATP synthase	X83218	4684987	m
41	Thioredoxin reductase	X96484	4072281	lm
42	DGCR6 protein	Z12962	4346073	m
43	Homologue to yeast ribosomal protein L41	Z27113	4295040	lm
44	RNA polymerase II subunit 14.4 kDa	Z47727	3607380	lm
45	RNA polymerase II subunit	Z70759	4795702	h
46	Mitochondrial 16S rRNA	J03191	4764352	m
47	Profiling	M10277	4899685	lm
48	Cytoplasmic β-actin	M10277	5088253	h
49	Cytoskeletal γ-actin	M19283	5087969	m
50	Liver glyceraldehyde-3-phosphate dehydrogenase	X01677	4906960	h
51	Lactate dehydrogenase-A	H05914	43550	ND
52	Heat shock protein (hsp 70)	M11717	4852172	m
53	β-tubulin (from clone D-β-1)	V00599	4762093	mh

Note. Based on a previous study by Warrington et al. [12] and sequencing information, we spotted 43 of 53 genes onto epoxy slides to create a custom-made housekeeping array. The previously published gene information includes gene name, GenBank entry (GenBank accession number), image ID (clone number of the image consortium agar clone databank), and expression level (h, more than 100 copies/cell; mh, 50–100 copies; m, 50 copies; lm, 10–50 copies; l, less than 10 copies; ND, not determined).

the ABI Prism 7700 Sequence Detector System was programmed with an initial sterilization step of 2 min at 50 °C, followed by 10 min denaturation at 95 °C and 40 temperature cycles for 15 s at 95 °C and 1 min at 60 °C. All measurements were produced in duplicate, and for each qRT-PCR primer/probe set, reaction efficiency estimates were derived from standard curves that were generated using serial dilutions of the corresponding

plasmid. These were then used to transform the cycle threshold (*Ct*) values for analysis with geNorm 1.3 software [20]. The geNorm VBA applet for Microsoft Excel determines the most stable housekeeping genes from a set of genes in a given panel of cDNA samples. It calculates a gene expression normalization factor for each tissue sample based on the geometric mean of a user-defined number of housekeeping genes [20]. The software can be downloaded (http://medgen31.ugent.be/jvdesomp/genorm).

Results

Selection of reference gene cDNAs from custom-made microarrays

We chose 47 genes based on a previous selection [12], added a further six classical employed reference genes (cytoplasmic β-actin, cytoskeletal γ-actin, liver glyceraldehyde-3-phosphate dehydrogenase, lactate dehydrogenase-A, heat shock protein 70, and β-tubulin), and spotted the corresponding cDNA clones onto epoxy slides to create our own custom-made housekeeping array (Table 1). High expression was defined as more than 100 copies/cell, medium expression as a copy number between 10 and 50, and low expression as less than 5 copies/cell, in accordance with previous studies [6]. After sequencing and alignment with the published GenBank sequences, 43 of the 53 cDNA clones were verified and spotted onto the epoxy slide.

To specifically select for candidates that could be used as stable reference genes in our tissue culture system, RNA was isolated for a subset of the conditions described in Fig. 1. These included plates with nondifferentiated mock (M), wild-type tau (W), and P301L tau-overexpressing cells (P). Moreover, the M, W, and P lines were sequentially incubated with retinoic acid (RA) and brain derived nerve growth factor (BDNF) to induce neuronal differentiation. In addition, RNA was extracted from differentiated P cells that were treated for 5 days with $A\beta_{42}$ [13]. The RNA was reverse transcribed using Cy3-dCTP and was hybridized to the housekeeping array (Fig. 2A). After normalization for global pixel intensities and exclusion of empty and poor spots, the nine microarrays were compared and analyzed. The data were divided into those for “controls” and “treated.” The control group comprised the signals derived from the three M samples duplicated to obtain equally sized groups, whereas the treated group contained the data of the other six samples.

Using the Student’s two-tailed *t* test to compare the control with treated groups, genes were considered stable when they showed a $P > 0.05$ and when the ratio of intensities of the compared samples was between 0.5 and 2.0 (Fig. 2B). Furthermore, candidates showing no reproducibility among the three M replicates (e.g., proteasome Y in Fig. 2A) were also excluded from further analysis. As such, we obtained a list of 14 candidates that included GAPD (Fig. 2C). Of these genes, five were predicted to be expressed at a high level (with more than 100 copies/cell), nine at a medium level (with 10–50 copies/cell), and one at a low level (with less than 5 copies/cell). Pixel intensities of spots corresponding to these three expression categories were mostly in accordance with the predicted levels using the glow-over/glow-under scala to interpret pixel intensities. These ranged from 0 (black) to 100 (green) to 200 (white). Genes with high copy numbers ranged from 140 to 200 (yellow–red to white). Medium copy number genes comprised intensity values ranging from 80 to 150 (blue–green). Genes predicted to be lowly expressed showed intensities between 30 and 80 (deep blue to light blue). Of these 14 genes (Fig. 2C), 8 were validated by qRT-PCR (Table 2).

Validation of eight candidate genes by qRT-PCR using the geNorm normalization approach

To validate the candidate genes selected from the housekeeping array, we created nine qRT-PCR primer and probe sets (eight candidates plus ATP50) that were tested in biological triplicates for all conditions described in Fig. 1. Primers and qRT-PCR probes were selected to hybridize specifically to human cDNA. Furthermore, the following criteria were applied for the selection and design of the qRT-PCR primer and probe sets: constitutive expression in the two differentiation states of the SH-SY5Y cells and on $A\beta_{42}$ treatment. Also, when known, the probes were made to span conserved exon–exon boundaries to avoid priming from contaminating DNA. The sequences of the primers and probes, the sizes of the amplicons, and the GenBank accession numbers are listed in Table 2.

The specificity of each qRT-PCR primer and probe set was checked on agarose gels and confirmed when a single band of the predicted size was obtained (data not shown). For each specific primer and probe set, we used 10-fold dilutions of either the plasmid or the oligonucleotide of the corresponding qRT-PCR primer and probe set to create a standard curve that was used for an absolute quantification of the samples. The efficiency and detection range of each specific set was evaluated by creating a pool of all samples with a series of twofold dilutions. The average efficiency was 87.5% (SD = 20%), and the average correlation coefficient of detection was 0.98 (SD = 0.02, range 0.960–0.982).

Of the 43 genes spotted on the custom-made microarray, ATP50 (Tables 1 and 2 and Fig. 3) was not considered as a stable gene but was included for the qRT-PCR as a negative control. For each specific qRT-PCR primer and probe set, a standard curve was generated

Table 2
Selected qRT-PCR primer and probe sets

Gene ID	Accession number	Oligonucleotides	Amplicon size (bp)
TAU	A001X18	(1) 5′-CAAGACCAAGAGGGTGACACG-3′ (2) 5′-CTGGCCTGAAAGAATCTCCCCTGCA-3′ (3) 5′-TCAGAGCCCGGTTCCTCAG-3′	89
GAPD	X01677	(1) 5′-ATTCCACCCATGGCAAATTC-3′ (2) 5′-GGGATTTCCATTGATGACAAGC-3′ (3) 5′-CACCGTCAAGGCTGAGAACGGGA-3′	71
QARS	X76013	(1) 5′-GGG CAA GTT TTC AGA GGG C-3′ (2) 5′-TCC ATC TTG CCA TCC TCC A-3′ (3) 5′-AGGCCACACTACGGATGAAGCTGGTG-3′	66
POLR2F	Z27113	(1) 5′-CCCGAAAGATCCCCATCAT-3′ (2) 5′-CACCCCCCAGTCTTCATAGC-3′ (3) 5′-TCGCCGTTACCTGCCAGATGGG-3′	65
M-RIP	D23673	(1) 5′-ATCTCAGCCATCGAAGCCAT-3′ (2) 5′-TGGCTCTTCTCCAGCTCCC-3′ (3) 5′-AAGAACGCCCACCGGGAGGAAAT-3′	68
PSMD7	D50063	(1) 5′-AGGAAGCTGAGGAAGTTGGAGTT-3′ (2) 5′-TGTGATCCGCTGGGACAGA-3′ (3) 5′-ACACTTGTTACGAGATATCAAAGACACGACGGTG-3′	84
ILF-2	U10323	(1) 5′-ACAACCCCACCAGACAGCC-3′ (2) 5′-GCAGCCAGAATCTGCAAGC-3′ (3) 5′-TGGCCCTAAACGTTGCATACAGGCG-3′	67
ACTG1	M19283	(1) 5′-GACAGTGAGGCCAGGATGGA-3′ (2) 5′-AGATCATCGCACCCCCAGA-3′ (3) 5′-CCACCGATCCACACCGAGTACTTGC-3′	76
PSMA5	X61970	(1) 5′-AAACAAGTAATGGAGGAGAAGCTGA-3′ (2) 5′-ACATGTGGAAATTCTGGCCAG-3′ (3) 5′-CAACAAACATTGAGCTAGCCACAGTGCAG-3′	78
ATP5O	X83218	(1) 5′-GCCAAGTATTGAAATTGGAGGC-3′ (2) 5′-TCGCCAATGCGCACAA-3′ (3) 5′-AGACTGATCCGTCAATCTTGGGTGGAATG-3′	70

Note. Eight reference genes, tau, and ATP50 are listed together with the GenBank accession numbers, the forward primer (1), the reverse primer (2), the fluorogenic probe (3), and the amplicon size.

to account for the variability of PCR efficiency and to allow the quantification of our samples. Using this quantitative approach, we found that the relative rankings based on copy numbers were conserved but that the absolute copy numbers corresponding to these categories were not. For example, GAPD was predicted to be a highly expressed transcript but showed an average expression level of only 42.6 copies/cell, M-RIP was suggested to be a medium-expressed gene but showed an expression level of only 1.6 copies/cell, and PSMA5 was suggested to be a low- to medium-expressed gene but was expressed at only 0.8 copies/cell.

To finally select the most stable reference genes, we used geNorm software [20] that measures the variability of the ratio of putative reference genes. Specifically, geNorm determines the average stability variable M of a pool of genes as well as the individual M for each gene within this pool. In the first round, the gene with the highest M (i.e., the least stable gene) is excluded. The software repeats this procedure in a stepwise fashion. When applied to our pool of nine genes, GAPD, M-RIP, and POLR2F were identified as the most stable reference genes (Fig. 3A). When we restricted the analysis to samples from differentiated cells (Fig. 1), the most stable reference genes were POLR2F, PSMD7, and GAPD (data not shown). To normalize gene expression, we used a normalization factor based on the geometric mean of the expression levels of three or more selected reference genes calculated by geNorm [20]. The software evaluates the variation of this normalization factor in the different conditions depending on the number of reference genes used [20]. We found that three reference genes were needed to calculate an adequate normalization factor, whereas a fourth reference gene did not improve the normalization (Fig. 3B). However, by using seven reference genes instead of six, the variation of the normalization factor was again decreased, although the use of six genes is not practical. Therefore, the geometric mean based on the best three reference genes (i.e., those with the lowest M values) can be used adequately for future normalizations using SH-SY5Y cells.

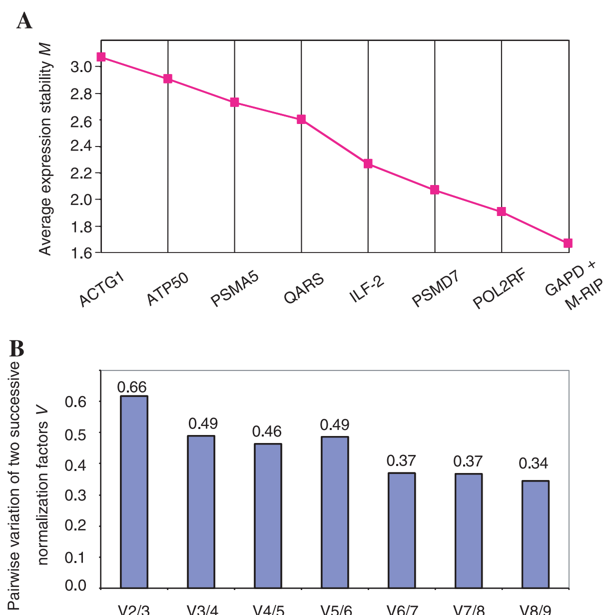


Fig. 3. Identification of the most stable reference genes in our tissue culture system by geNorm analysis of nine specific qRT-PCR primer and probe sets. RNA was isolated from the four cell lines for all conditions described in Fig. 1 using biological triplicates for each condition tested. Furthermore, each qRT-PCR measurement was done in duplicate, and serial dilutions of the corresponding plasmid or oligonucleotide were included to account for plate-to-plate variation and to allow for a quantification of the samples. (A) Algorithmic step-by-step selection of the most stable reference genes according to the average stability of expression measure *M* [20]. geNorm calculates the ratio of two putative reference genes within a pool of genes in a pairwise comparison and determines the standard deviation of the logarithmically transformed expression ratios, thereby defining *M*. For each iteration round, the average and gene-specific *M* is calculated and the gene with the highest *M* value is excluded. The abscissa shows the gene with the highest *M* value within a pool of genes. This gene is excluded from the next iteration round. (B) Determination of the optimal number of reference genes for normalization of all samples. *V* is the pairwise variation between two sequential normalization factors. *V*2/3, for example, is the variation of the normalization with two genes compared with the normalization with three genes. Because *V* does not change when a fourth or fifth reference gene is included, and because using six or more genes is not practical, the adequate normalization factor is calculated with three reference genes.

To further confirm the validity of this approach, we obtained *Ct* values for GAPD and POLR2F and determined their ratios for additional experimental conditions (Table 3). SH-SY5Y cells were either kept undifferentiated or neuronally differentiated for 5, 7, or 10 days. The cells were transfected with pSUPER (siRNA) vectors to target the mRNA of the protein phosphatase 2A (PP2A) regulatory subunit PR55γ. In addition, siRNA oligonucleotides targeting PR55γ were transfected transiently. All experiments were done in duplicate. We found that both reference genes varied little for all conditions tested, as shown by the mean values, the standard deviations and coefficients of variance (CV), which were $Ct_{GAPD} = 14.9 \pm 1.5$ (CV = 10.7%) and $Ct_{POLR2F} = 19.1 \pm 1.6$ (CV = 8.7%). Most important, the Ct_{POLR2F}/Ct_{GAPD} ratio was two to three times more stable than the individual reference genes, as shown by a $CV_{POLR2F/GAPD}$ of 2.8%.

Effect of the normalization factor on relative tau mRNA levels

To determine how the newly calculated procedure for normalization affected tau mRNA levels, we compared the relative expression levels of tau for all conditions described in Fig. 1 by qRT-PCR, with the single reference gene GAPD and with the normalization factor calculated as the geometric mean of the three reference genes GAPD, M-RIP, and POLR2F. When a qRT-PCR was performed using a primer/probe set for tau with GAPD as reference (Table 2), P cells showed 4- to 64-fold higher, and W cells showed 16- to 128-fold higher, tau mRNA levels compared with NT or M cells. Differentiation did not affect the expression level of tau in NT, W, or P cells (Fig. 4A). When the new normalization was applied, differentiation caused increased tau levels in NT, M, and W cells (Fig. 4B). Because neuronal differentiation of SH-SY5Y cells is known to cause both increased tau mRNA and increased protein levels [23–25], the results obtained with the new normalization factor are likely more relevant. Interestingly, with the new normalization, the effect of fibrillar $A\beta_{42}$ on tau mRNA levels was observed only in NT cells and not in W cells (Fig. 4).

Discussion

The recent availability of high-density microarray technology is significantly enabling the transcriptomic analysis of different species. RNA can be derived from entire organisms or selected tissues, specialized cell types, or established cell lines analyzed under different controlled experimental conditions. Because transcriptional changes are often subtle, differential expression analysis depends heavily on both a robust baseline and stable reference genes [8]. However, in roughly 90% of the studies published to this point, GAPD, ACTB, 18S, and 28S rRNA have been used as single reference genes without prior verification [26], despite the finding that expression of these housekeeping genes can vary considerably [10,12,27].

Based on a previously published study in which 47 housekeeping genes were selected from a total of 7000 genes [12] and with the inclusion of 6 classical reference genes (cytoplasmic β-actin, cytoskeletal γ-actin, liver glyceraldehyde-3-phosphate dehydrogenase, lactate dehydrogenase-A, heat shock protein 70, and β-tubulin). We created our own custom-made microarray and identified 14 genes that were further selected for qRT-PCR validation. In the first selection step, RNA was obtained

Table 3
Stability of the ratio of POLR2F and GAPD *Ct* values

U/D	Time point	Treatment	*Ct* (GAPD)	*Ct* (POL2RF)	*Ct* (GAPD)/ *Ct* (POL2RF)
U	0	—	14.3	18.4	1.29
U	0	—	14.1	19.2	1.35
U	5	siRNA PP2A/PR55γ	14.5	18.4	1.27
D	5	siRNA PP2A/PR55γ	15.7	20.8	1.32
D	7	siRNA PP2A/PR55γ	18.7	22.9	1.22
D	10	siRNA PP2A/PR55γ	17.7	21.9	1.24
U	0	pSUPER-PP2A/PR55γ	14.1	18.3	1.30
U	10	pSUPER-PP2A/PR55γ	13.6	17.4	1.28
D	10	pSUPER-PP2A/PR55γ	14.8	18.9	1.28
U	5	Mock	13.8	17.7	1.28
D	5	Mock	14.5	18.9	1.30
D	10	Mock	15.5	19.5	1.25
U	0	pSUPER (empty)	13.7	17.6	1.28
D	10	pSUPER (empty)	14.1	18.1	1.29
		Mean	14.9	19.1	1.28
		Standard deviation	1.5	1.6	0.03
		CV (percentage)	10.7	8.7	2.8

Note. We obtained *Ct* values for GAPD and POLR2F and determined their ratio for different experimental conditions. SH-SY5Y cells were either kept undifferentiated (U) or kept neuronally differentiated (D) for 5, 7, or 10 days. The cells were transfected with pSUPER (siRNA) vectors to target the mRNA of the protein phosphatase 2A (PP2A) regulatory subunit PR55γ. Empty vectors were included as controls. In addition, siRNA oligonucleotides targeting PR55γ were transfected transiently. We found that for both reference genes, the mean values, the standard deviations, and the coefficients of variance (CV) showed little variation for all conditions tested: $Ct_{GAPD} = 14.9 \pm 1.5$ (CV = 10.7%) and $Ct_{POLR2F} = 19.1 \pm 1.6$ (CV = 8.7%). Most important, the ratio Ct_{POLR2F}/Ct_{GAPD} was two to three times more stable than were the individual reference genes, as shown by a $CV_{POLR2F/GAPD}$ of 2.8%.

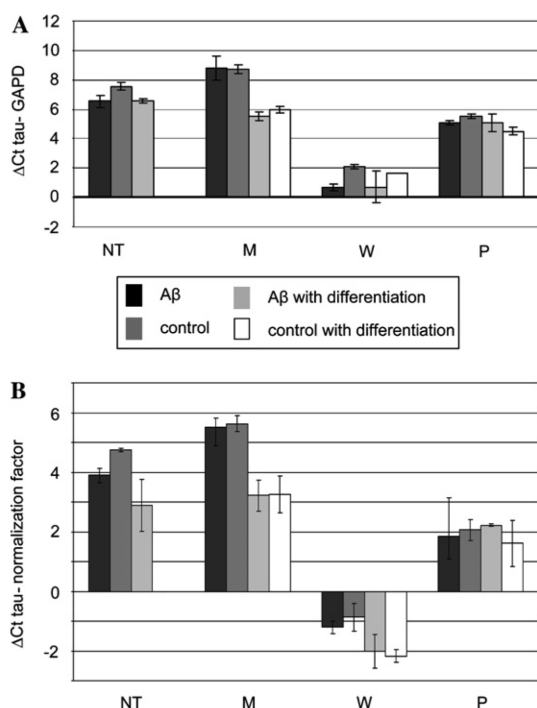


Fig. 4. Comparison of tau expression using either GAPD alone or the geometric mean of GAPD, POLR2F, and M-RIP as a new normalization factor. (A) Relative tau expression levels of all four cell lines for all conditions described in Fig. 1 using GAPD for normalization. (B) Tau expression levels using the geometric mean of GAPD, M-RIP, and POLR2F for normalization. For example, when the new normalization is applied, differentiation reveals increased tau mRNA levels not only in M cells but also in NT and W cells.

for a subset of the conditions described in Fig. 1, whereas in the second step, SH-SY5Y neuroblastoma RNA was derived from a total of 48 samples representing 16 different experimental conditions. In the first selection step, candidates were considered to be stably expressed when they satisfied two criteria: a *P* value greater than 0.05 (when comparing treated and control samples using a Student's two-tailed *t* test) and an average expression ratio between 0.5 and 2.0.

A qRT-PCR validation was included to accurately measure gene expression because understanding gene regulation depends not only on stable reference genes but also on an accurate means of detection. Large-scale microarray studies have shown that the amplitude of the vast majority of changes at the transcriptional level is small (<two- to fourfold) [28–32]. Therefore, qRT-PCR that typically exhibits a detection range of five to six orders of magnitude, high accuracy, and high reproducibility is a method of choice to detect subtle changes in gene expression and to validate candidates selected through large screening microarray experiments [8,33–36].

Hence, we developed nine qRT-PCR primer and probe sets for reference gene detection. The average efficiency of these was 87.5% with a correlation coefficient of 0.98. In a related study, oligo-dT was used instead of random hexamers, resulting in an average efficiency of 110% and a correlation efficient of 0.989 [10]. Random hexamers may result in the amplification of nucleic acids coextracted with mRNA, thereby

reducing the overall specific priming efficiency. Also, because the oligo-dT is specifically priming from mRNA, any non-template-related probe hydrolysis may take the efficiency above 100%, although this is only speculative given that the nontemplate controls did not reveal any release of fluorochrome (which might reflect the limitations of optical detection).

Furthermore, our quantification of the copy number of genes per cell showed much lower numbers for all three expression level categories compared with what has been published previously [12]. A possible explanation is that the plasmid DNA used for the standard curves and the cDNAs obtained by reverse transcription employing the random hexamer approach differ in their transcriptional efficiencies. If the plasmid transcription efficiency was higher than that for the sample, this would lead to an underestimation of the absolute copy numbers.

Our second and final selection step relied on the data obtained from the qRT-PCR analysis and on the stability measure M [20] to select the most stable reference genes for all conditions tested (Fig. 1). M is based on the concept that the ratio between two stable reference genes should always be constant irrespective of the experimental conditions used. Other methods simply consider the relative ΔCt values of single reference genes in the control and treated samples for selection [10,37]. However, this approach still necessitates quantification and normalization with total mRNA levels. Indeed, small variations in total mRNA quantities between control and treatment would lead to a nonsystematic difference for exogenous reporters. The advantage of the method used here is that it employs the ratio of reference genes in each sample, thereby eliminating this additional variability factor and the discrepancy observed with the absolute values. As shown in Table 3, the variation of two individual reference genes (GAPD and POLR2F) is significantly higher than the variability of the ratio of the Ct values. This measure is not absolute and cannot define a single "best reference gene," but it allows the definition of reference genes as genes whose relative expression levels are constant over the conditions tested, as measured by the variation of the ratio of their expression. So long as the reference genes have independent functions, this is the best possible evaluation of constant gene expression.

Furthermore, Vandesompele et al. [20] showed that conventional normalizing strategies using only one reference gene lead to erroneous normalization with up to 3-fold differences for 25% of all genes and more than 20-fold differences for some genes. Here we show, for a human neuroblastoma tissue culture system, that the normalization for several experimental conditions is best achieved not by using one gene but rather by using three genes (GAPD, M-RIP, and POLR2F).

Therefore, we analyzed the effect of the new normalization factor on tau mRNA levels for all conditions described in Fig. 1. Our tau-specific qRT-PCR primer and probe set was designed to detect transfected and endogenous tau mRNA. Consistent with our observation that tau levels were increased after differentiation for three conditions instead of one when the new normalization factor, rather than the old one, was applied, Heicklen-Klein et al. [24] showed increased tau promoter activity in stably transfected P19 cells after 4 days of treatment with RA. Similar results have been reported for PC12 cells [38]. Heicklen-Klein et al. [24] used luciferase activity to quantify tau promoter activity and quantified endogenous tau mRNA using GAPD for normalization. Furthermore, in two studies using SH-SY5Y cells differentiated with RA and BDNF, Western blot analysis and immunohistochemistry were employed to demonstrate increased endogenous tau protein levels following differentiation [22,25].

The three genes that define the new normalization factor are GAPD, M-RIP, and POLR2F. M-RIP interacts with the myosin ρ-phosphatase complex, whereas POLR2F is the F constituent of RNA polymerase II. GAPD and POLR2F were selected not only when all samples were considered together but also when the differentiated samples were taken separately as a group (data not shown). This would suggest that at least these two reference genes can be used for further transcriptomic analyses using SH-SY5Y cells in both undifferentiated and differentiated states. The two genes might also be valid for experiments with stably transfected SH-SY5Y cells using a cytomegalovirus-driven expression vector provided that the expressed proteins do not affect reference gene transcription.

In conclusion, the current study has described a method for the identification of stable reference genes with different expression levels in a human tissue culture system using a custom-made microarray analysis combined with a step-by-step selection of specific qRT-PCR primer and probe sets. The three reference genes M-RIP, POLR2F, and GAPD can be used as reference genes in SH-SY5Y cells under both undifferentiated and neuronally differentiated conditions. The method presented here emphasizes the use of multiple reference genes. Although our results do not offer direct extrapolation to other cell types, we believe that they are applicable for additional studies using SH-SY5Y cells and possibly other neuroblastoma cell lines, in particular because these are widely used tools in AD-related research [39–42].

Acknowledgments

We thank Dr. Beat Schäfer for providing cDNA clones and Dr. Oliver Greiner for providing help. This work was supported in part by grants from the ZNZ Zürich, the A + D Fonds of the Swiss Academy of Medical Sciences (SAMS), the Olga Mayenfisch Foundation, the EMDO Stiftung Zürich, the Kurt und Senta

Herrmann Foundation, the Hartmann Müller Stiftung, and the Swiss National Science Foundation.

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