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PRESENILIN-1 DETECTION IN BRAIN NEURONS AND FOXp3 IN PERIPHERAL BLOOD MONONUCLEAR CELLS: NORMALIZER GENE SELECTION FOR REAL TIME REVERSE TRANSCRIPTASE PCR USING THE ΔΔCt METHOD

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1. ABSTRACT

Quantification of gene expression is important to confirm changes in levels of gene expression in disease. Prior quantification methods include standard curves, absolute quantification, and relative quantification. This paper describes an analytic method for the relative quantification of Presenilin-1 (PS-1) in neurons and Forkhead-box (FOX) p3 in PBMNCs using real-time PCR analytic techniques. A comparative Ct method (ΔΔCt) is described in which the quantity of target normalized to a normalizer gene reference is given by 2-ΔΔCt where ΔΔCt = [Ct of the gene of interest in the unknown specimen - Ct normalizer gene in the unknown specimen] - [Ct of the gene of interest in the calibrator specimen - Ct normalizer gene in the calibrator specimen]. The calibrator specimen is ideally from a non-treated control specimen and is analyzed on every assay plate with the unknown specimens of interest. The use of the ΔΔCt methodology allows for a higher throughput and a more economical approach to investigate gene expression. We applied this methodology to the quantification of PS-1 and FOXp3 genes and compare the levels of expression by normalizing to different normalizer genes using the ΔΔCt methodology. We find that use of GAPDH is the optimum normalizer gene for the genes analyzed in neurons from human brain and in PBMNCs

2. INTRODUCTION

2.1. Gene expression quantitation

Gene expression analysis is increasingly important in biological research and with real time reverse transcription PCR (RT-PCR) it is the method of choice for high-throughput, sensitive, and accurate expression profiling and gene identification (1-4). Quantitative estimates of specific mRNAs provide new insights into health and disease mechanisms with a high potential for clinical applications including monitoring the response to chemotherapies and the early detection of bacterial or viral pathogens (5). Gene expression analysis also offers the advantage of profiling individual genes to assess their possible roles in the context of specific pathways and networks (6,7).

Given the increased sensitivity, reproducibility, and wide dynamic range, real time PCR is the method of choice for the accurate quantification of mRNA molecules. However, the accuracy of real time PCR depends on the fidelity of the cDNA amplicons generated from highly heterogeneous mRNA pools, their quality, and the amplification efficiencies during PCR. Furthermore, without an absolute standard for quantification, the amplification of an normalizer control should be performed to standardize the target RNA under study. Including a reference gene as a normalizer ensures that changes in the level of expression of the gene of interest are precisely measured with respect to the entire RNA molecular population in the specimen (3). The reference gene choices in molecular biology classically have been what are termed housekeeping genes. These genes are generally categorized as such since they are expressed in virtually all cells and are fundamental to the any cell's functions (8).

Prior methods to determine relative quantification of gene expression included comparison of the experimental signal to that of the reference gene signal generated through standard curves and absolute quantification included using a standard curve based on the identical nucleic acid as the PCR target (3,9,10,11). Normalizer reference genes provide a useful constant reference to normalize the expression of test genes in different cells, tissues, and conditions. Moreover, this is important when estimates of gene expression must be provided in relative rather than absolute units of measurement. The normalizer reference genes are thus used as common denominator in gene expression studies where the expression of a test gene is described as the relative ratio with respect to an arbitrarily selected control presumed to be stably expressed in all circumstances relevant to the experiment. Most frequently, glyceraldehydes-3-phosphate dehydrogenase (GAPDH), albumin (for hepatocytes), β-, γ-actins, cyclophilin, α-, β-tubulins, hypoxantine phosphoribosyl-transferase (HRPT), L32 and 18S, 28S ribosomal RNA (rRNA) have been used as normalizer reference genes. However, the levels of expression of genes commonly used as normalizer reference like GAPDH, cyclophilin and β-actin fluctuate in different tissues, disease stage and are affected by cell behavior including proliferation (12). Recent approaches to more rapidly and efficiently quantify the expression of a particular gene include the comparative Ct method in which the amount of target normalized to an normalizer gene reference is given by $2^{-\Delta\Delta Ct}$ (13). In an effort to validate the importance of normalizer gene selection in gene expression analysis we selected Presenilin-1 (PS-1) and FOXp3 genes and compared the levels of expression by normalizing to different normalizer genes via the ΔΔCt methodology.

3. MATERIALS AND METHODS

3.1. PRESENILIN-1

3.1.1 Brain tissue

Autopsied cryopreserved brain tissue was obtain from the NIH sponsored National NeuroAIDS Tissue Consortium (14). *Globus pallidus* from an HIV positive individual was embedded in OCT, 10-µm sections were cut in a cryostat at - 23°C and mounted on slides specially design for laser capture microdissection (LCM) (Microoptics of Florida, Palm Beach, FL.). The slides were placed immediately in dry ice and cryopreserved at -80°C until microdissection.

3.1.2. LCM and RNA extraction

Immediately, prior to LCM, slides were fixed in 75% ethanol and stained singly with NISSL (Arcturus Inc, Mountain View, CA) for 20 seconds. After rinsing once in DNAse-RNAse free water for 5 seconds, the slides were step-wise dehydrated: 75% ethanol for 30 seconds, 95% ethanol for 30 seconds, and 100% ethanol for 2 minutes. The slides were then placed in Xylenes for 5 minutes and air-dried for 5 minutes. LCM was performed within an hour after staining and dehydration. The entire staining procedure was done with all reagents maintained on ice to avoid RNA degradation. Reagents were prepared freshly in 50 mL centrifuge tubes daily. LCM was performed using a LEICA Laser Microdissection Microscope (Leica Corp, Bannockburn, IL) following the manufacturer's recommendations.

For the neuronal RNA, neurons were specifically microdissected, collected in approximately 200 cell batches, and immediately resuspended in 20 µL extraction buffer as part of the Picopure RNA extraction kit (Arcturus Inc). Tubes were placed onto the CapSure-ExtractureSure assembly incubation block with the cover (Arcturus Inc.) and incubated at 42˚C for 30 minutes. The tubes were then cryopreserved at –80˚C and the RNA was extracted within 5 days according to the kit procedure. Five aliquots of 200 cells in 20 µl of extraction buffer each were pooled immediately prior to extraction. RNA was directly amplified using the RiboAmp HS kit (Arcturus Inc), and analyzed after the first round of amplification using isotachophoresis. RNA was then cryopreserved at -80°C or PCR was immediately performed. For the normal tissue RNA, we used commercially available total RNA extracted from normal complete brain and from *Globus pallidus* (Ambion Corp., Austin, TX).

3.1.3. Real Time PCR

ABI-Prism Primer Express was used to design primers and probes for real time RT-PCR assay specific for

PS-1. Sense primer 5'-ccctgtgactctctgcatggt-3' (nt.508-528) and antisense primer 5'-gcccatccttccgggtataa-3' (nt. # 562-581), probe 5' VIC-agctgactgacttaatggtagccacgacca- 3' (nt. 531-560) from mRNA sequence (GenBank ID U40380) corresponding to the transcript for presenilin I-374 (15). Primer final concentrations were 900 nM, probe final concentration was 250 nM, and reaction tubes were subjected to the following cycling conditions: 30 minutes at 48^0C for reverse transcription, 10 minutes at 95^0C, followed by 40 cycles of steps at 94^0C for 15 seconds and at 60^0C for one minute (Shapshak P, Haliko S, Thinakaron G, Duncan R, Real Time Reverse Transcriptase PCR Method for Quantification of Presenilin (PS)-1 mRNA in cultured cells, *in progress*, 2005.) TaqMan real time PCR was performed using the ABI PRISM 5700HT Sequence Detection System using the manufacturer's instructions (Applied Biosystems, Foster City, CA).

3.2. FOXp3

3.2.1. Cells

Peripheral Blood Mononuclear Cells from a previously *M. tuberculosis*-infected donor were isolated from heparin anti-coagulated blood samples by Ficoll density gradient centrifugation (Amersham Biosciences). Cells were resuspended in R10 medium at a concentration of 2.6 x 10^6 cells/mL. 1mL of cells was then cultured at 37^oC under 5% CO_2 with and without stimulation by *Mycobacterium bovis*, strain Bacillus Calmette Guerin (obtained from Statens Seruminstitut, Copenhagen) at a multiplicity of infection of 0.1 bacillus/PBMNC. Cells were harvested at 0, 2, 6, 12, 18 and 24 hours. 2.6 x 10^6 cells were harvested at each time point for each condition.

3.2.2. RNA extraction

RNA extraction was performed using the RNeasy Mini Kit (Qiagen) according to the manufacturer's protocol.

3.2.3. Real Time PCR

Real time RT-PCR was performed using Taqman One Step RT-PCR Kit (Applied Biosystems) using FOXp3 Gene Expression Assay (Applied Biosystems assay ID# Hs00203958 m1). TaqMan real time PCR was also performed using the ABI PRISM 5700HT Sequence Detection System as described above and using the manufacturer's instructions (Applied Biosystems).

3.3. ΔΔCt Methodology

The ΔΔCt method utilizes a formula, 'fold'-gene expression change = $2^{-\Delta\Delta Ct}$ where ΔΔCt = [*Ct* gene of interest in the unknown specimen - *Ct* normalizer in the unknown specimen] - [*Ct* gene of interest in the calibrator specimen - *Ct* normalizer in the calibrator specimen]. The calibrator specimen can be any specimen selected to represent the expression of the gene of interest, should be ideally from an untreated control and is analyzed on every assay plate with the unknown specimens of interest (16).

The inverse linear relationship between C_t and the log of the input RNA concentration needs to be established for each of the genes under study (17). Briefly, the C_t (threshold cycle) indicates the fractional cycle number at which the amount of amplified target reaches a 'set' threshold. For amplicons designed and optimized according to Applied Biosystems guidelines (amplicon size < 150 bp) the efficiency of amplification is close to one. The ΔCt value is determined by subtracting the average Ct normalizer value from the average Ct of the gene of interest and the ΔΔCt involves subtraction by the ΔCt calibrator value.

The ΔΔCt method is validated by demonstrating that the efficiency of the target amplification (gene of interest) and the efficiency of the reference amplification (housekeeping gene) are comparable or equal. A sensitive method for assessing if two amplicons have the same efficiency is to examine the relationship between ΔCT variations with template dilution. For the -ΔΔCt calculation to be valid, the efficiency of the target amplification and the efficiency of the reference amplification must be approximately equal with the difference in $r^2 < 0.1$.

For each of the different gene assays, reactions for each point of the curve were run in 96-well plates, and reagent reactions were prepared by mixing 20XMMPCR (TAQMAN "human endogenous controls") with ONE STEP RT-PCR kit (Applied Biosystems, CA) in a total of 20 ul. Serial 1:10 dilutions from a stock of 200 ng/ul of total RNA from each different source was prepared in RNAse-DNAse free water and 5 ul were added to each reaction for a total volume of 25 microliter. The linear range established was approx 10pg - 1 microgram.

3.4. Statistics

Statistical tests are required to evaluate whether any observed fold differences in nucleic acid concentrations are significant. The efficiencies of PCR for each of the standard curves is based on the equation ($10^{-1/\text{SLOPE}}$) -1 (ABI 1999). The ΔΔCt methodology requires that the r^2 linear regression analysis for each standard curve must be greater than 0.9. The C_t values are used to calculate the mean, standard error, and standard deviation for each housekeeping gene and across all samples under study. We also combined C_t from experimental replicas in different runs in an effort to encompass biological variation with identical thresholds applied to all experiments. In order to avoid any possible contribution from interassay variability to the statistical analysis, the ΔΔCt values for the FOXp3 gene expression were calculated from experiments where both FOXp3 and the respective normalizer gene were run in the same plate. The normalizer gene should not be affected under the control, experimental, or study conditions of the experiment. The relative quantitative objective of the assay is often described as a fold difference. It is important to determine the variance of the system as one wishes to minimize the number of replicas. The student's t- test was applied with a Confidence Interval of 99% (p value = 0.01).

4. RESULTS

Neuropathological aspects of the AIDS case we studied are shown in stained cryosections in Figure 1. Figure 1a shows a photomicrograph of an H&E stained cryosection and Figure1b a NISSL stained cryosection. Nissl stained cryosections were used for neuron LCM.

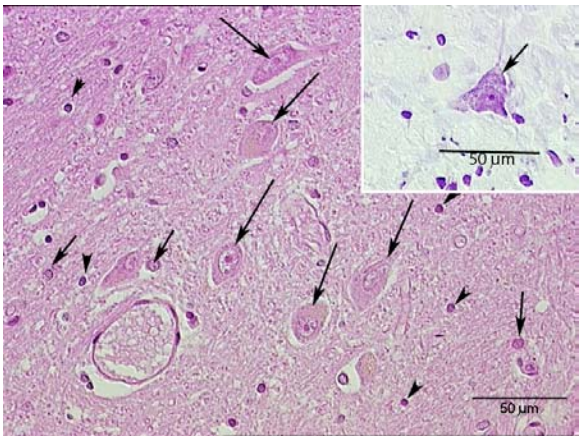

Figure 1. Cells in brain sections. H&E. Formalin fixed paraffin embedded (FFPE) section of *Globus Pallidus* from male Caucasian patient, age 50 years, HIV-1 seropositive, homosexual, without HIV associated dementia (HAD) and without HIV encephalitis (HIVE). Five micron thickness, the bar represents 50 microns, Hematoxylin and Eosin stain. Small arrows: oligodendrocytes; Medium arrows: astrocytes; Large arrows: neurons. Inset: Nissl stain Cryo-section of *Globus Pallidus* from male Caucasian patient, age 62 years, intravenous drug user, HIV-1 seropositive, with HAD and without HIVE. Five micron thickness, the bar represents 50 microns, Nissl stain; Large arrow: neuron of the type used for LCM.

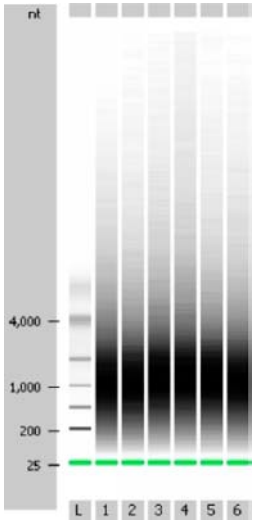

Figure 2. Electrophoretic analysis of amplified PS-1 RNA. This figure shows the amplified RNA electrophoretic profiles (AGILENT, Palo Alto CA) from 3 different specimens in duplicate and illustrates the distribution of amplified aRNA with sizes ranging between 200 and 2,000 nt. As described in the text, RNA was purified and amplified after LCM of neurons from cryosections of basal ganglia brain tissue. Lanes 1 and 2 show the aRNA used for the analysis of PS-1 aRNA also analyzed in other tables and figures. This RNA was from a patient who had HAD and HIVE. Lanes 3 and 4 are from a patient who did not have HAD and had HIVE. Lanes 5 and 6 are from a patient who did not have either HAD or HIVE. All three patients were HIV-positive. L corresponds to the molecular weight ladder (Ambion).

Microdissected neuron-specific RNA was then purified and analyzed using isotachophoresis after the first round of amplification. As shown in Figure 2 the amplified RNA (aRNA) profile (Agilent Inc, Mountain View, CA) has a size distribution with lengths ranging from 200 to 2,000 nt and the absence of accumulation of lower molecular weights that would be indicative of degradation.

Figure 3 shows the inverse linear relationship graphs between C_t and the log of the input RNA concentration (Ct vs. log ng of RNA) for each one of the genes in Total RNA from normal brain tissue. Real time reverse transcriptase PCR was done for PS-1, β-actin, β_2-microglobulin, GADPH, and 18S rRNA. Based on the equation $(10^{-1/\text{SLOPE}})$ -1, the efficiencies of PCR for each of the standard curves were as follows: PS-1 100 % (showing the lowest level of sensitivity – 100 pg) β-actin 85%, β_2-microglobulin 86% , GAPDH 88% , and 18S rRNA 93%. The graphs also show a linear range of approximately 10pg – 1ug RNA. The r^2's were 0.99 for all standard curves except for PS1 for which r^2 was 0.93.

Table 1 shows the Ct raw data for the genes PS-1, β_2-microglobulin, GADPH, and 18S rRNA. Of all candidate genes studied for purposes of normalization, GAPDH shows the lowest SD and SE across all biological and experimental replicas. For the GAPDH gene, the largest difference in Ct/RNA mass is the one observed between neuronal RNA and *Globus pallidus* RNA (3.29 increase in Ct mean value when comparing RNA from the two different sources). However, when averaged, differences in mean Ct's from all three different sources are the lowest (data not shown). Based on the criterion that the best normalizer gene is the one with the ratio of gene to RNA mass that fluctuates least from sample to sample. GAPDH exemplifies this. It should be noted that we were unable to detect β-actin signal in the neuronal amplified RNA.

Table 2 shows the differences in fold expression and the significance of the fold of expression when different genes are alternately used as normalizers. Of the use of the three normalizer genes, GAPDH shows the highest t-test value for both comparison of PS-1 in LCM neurons vs. brain total RNA and *Globus pallidus* RNA.

Figure 4 demonstrates the flow cytometric gating strategy employed to identify the small proportion of peripheral blood lymphocytes that express the CD4 and CD25 antigens. This population of $CD4^+CD25^+$ T cells contains the regulatory subset that expresses FOXP3. Figure 4A shows the Lymphocyte population identified as small cells with low granularity in a 2 dimensional plot of forward scatter (FSC: proportional to cell size) and side scatter (SSC: proportional to cellular granularity). Figure 4B shows gating on the lymphocyte population from Figure 4A: T cells are identified as expressing CD3. Figure 4C shows gating on T cells from Figure 4 B and CD4 cells are identified that also express CD25. This population contains cells known as the regulatory T cells.

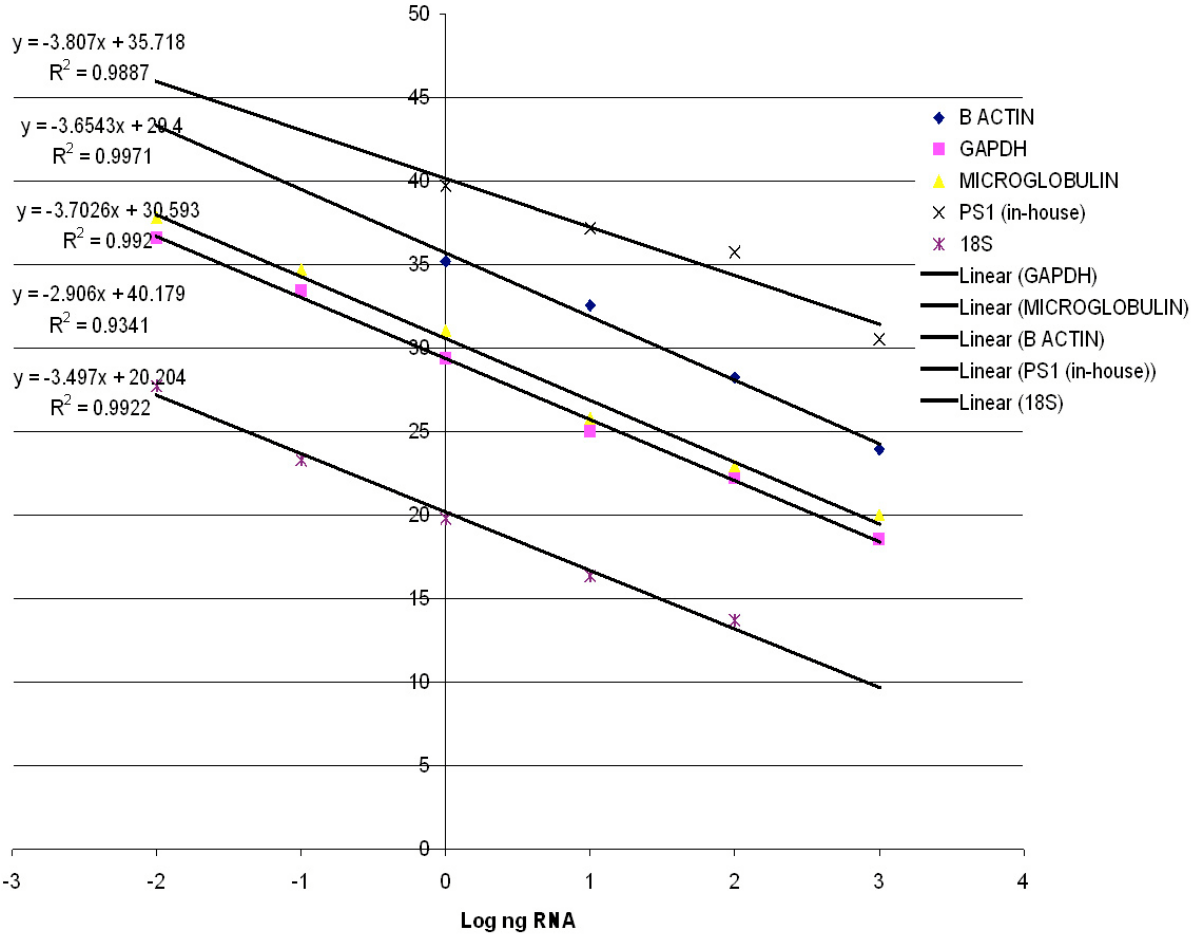

Figure 3. Reverse transcriptase real time PCR for PS-1, GAPDH, β-actin, β2-microglobulin, and 18S ribosomal-RNA This figure shows the linear range, sensitivity, and the slope of the standard curve for each of the genes under study in total RNA from total human brain tissue.

In Figure 5, as in Figure 3, the plots of Ct vs. log ng of RNA are shown. Real time reverse transcriptase PCR was done for FOXp3, β-actin, and GADPH. The efficiencies for each standard curve are: FOXp3 80%, GAPDH 108%, and β-actin 76%. The graph shows a linear range of approximately 100 pg-1 ug for these genes in RNA extracted from PBMCs. The r^{2}'s were approximately 0.99 for all standard curves.

Table 3 shows the raw Ct data for FOXp3, GAPDH and β-actin genes. This table shows a decrease in the Ct mean of β-actin from 21.85 to 18.3 after 6 hour induction of FOXp3 while GAPDH maintains a mean Ct with and standard deviation of 0.18 cycles throughout the curse of treatment. The mean Ct decrease in β-actin of approximately 3.55 cycles (above 10 fold change if PCR efficiency was 100%) before and after treatment is suggestive of an increase in β-actin expression signal after treatment and specifically over time.

Table 4 shows that changes in expression of FOXp3 after BCG treatment are detected consistently only after 6 hours if β-actin is used as the normalizer gene while the induction of expression of FOXp3 is detected after 2 hours if GAPDH is used as the normalizer gene. GAPDH provides a more sensitive assay with statistically significant changes registered only after 2 hours.

Table 5 shows a simulation analysis demonstrating that gene expression of FOXp3 levels would be relatively quantifiable by both β-actin as well as GADPH if the number of replicas were higher (20 replicas at hour 2 and 18 replicas at hour 4). However, in this simulation it is assumed that the SD of the system would be maintained which would be unlikely if the β-actin signal was "induced" by the treatment. It is established that as the number of replicates increases, individual variations are reduced and the standard deviation decreases but in this methodology this is possible only if the ratio Ct/RNA mass for the normalizer gene is consistent throughout experimental and control samples. GAPDH ratios are maintained more consistently across the specimens analyzed.

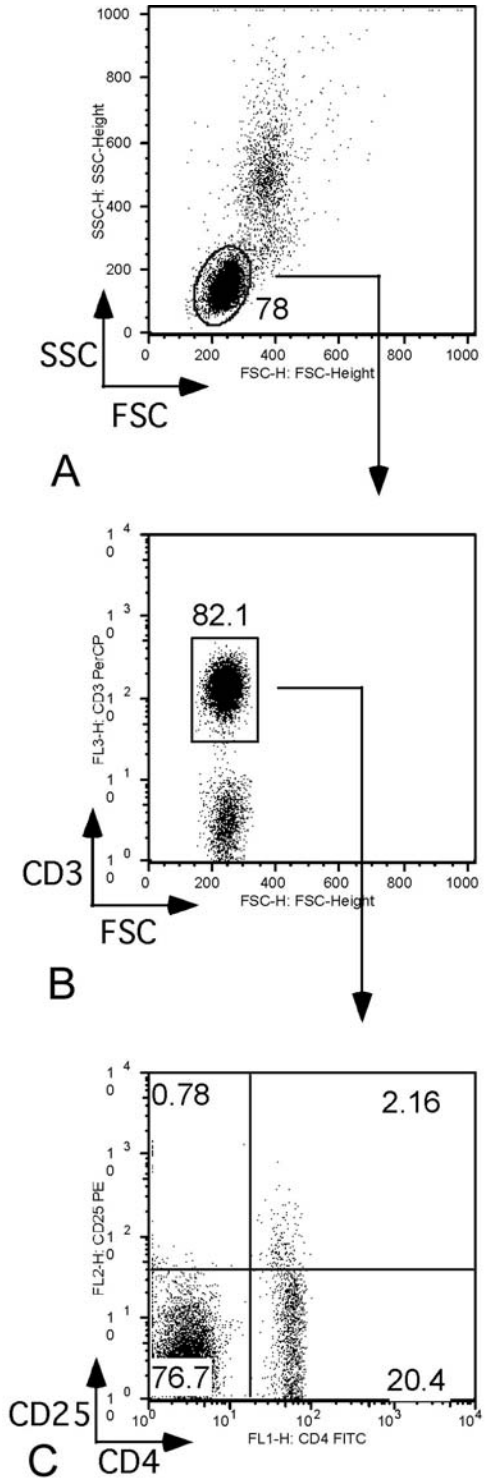

Figure 4. Use of multiparameter flow cytometry to identify $CD3^{-}CD4^{+}CD25^{+}$ regulatory T cells. **A**. Lymphocyte population is identified as small cells with low granularity in a 2 dimension plot of forward scatter (FSC: proportional to cell size) and side scatter (SSC: proportional to cellular granularity); **B.** Gating on the lymphocyte population in **A**., T cells are identified as expressing CD3; **C.** Gating on T cells in **B**, CD4 cells are identified that also express CD25. This population contains the regulatory T cells.

5. DISCUSSION

5.1. PS-1 in neurons from human brain

It has been proposed that the pathogenesis of HIV Associated Dementia (HAD) and minor cognitive motor disorder (MCMD) the altered extracellular milieu produced by activated astrocytes and microglia may induce synaptic and dendritic changes and loss with eventual apoptosis in neurons (18-21). Despite the widespread use of highly active antiretroviral therapy (HAART) in the developed world, MCMD has not been eradicated because it may not progress to HAD and may involve upto 30% of the HIV-infected subjects (22).

There is also a possible connection between aging and the progression of AIDS that is under investigation. In this regard it is of relevance to account for mechanisms for the pathogenesis of Alzheimer's disease (AD) as they may impact on the understanding of the pathogenesis of NeuroAIDS (1). AD results in a progressive degeneration of neurons and appears to be driven by inflammation and oxidative stress resulting from production of amyloid-β (A- β) oligopeptides. The elucidation of the functions of PS-1 may be crucial for the understanding of the pathogenesis of AD. The A-β oligopeptides are derived from the sequential cleavage of an integral trans-membrane β-amyloid precursor protein (βAPP) by a neuron-enriched, membrane associated γ-secretase complex of which PS-1 is an essential component (23). Our work currently involves LCM to microdissect neurons from brain cryosections and this provides a more cell-specific approach in this work. Thus, we selected PS-1 as an important gene to evaluate the application of the ΔΔCt methodology for its rapid quantification and the selection of the optimum normalizer among four different housekeeping genes GAPDH, β-actin, 18S ribosomal RNA and β_2-microglobulin.

5.2. FOXp3 in peripheral blood mononuclear cells

The human Forkhead-box (FOX) gene family consists of at least 43 members, including FOXp3. The 1869 bp long messenger RNA (GenBank ID NM_014009) codes for a 47.2 kDa protein. Recent studies showed that FOXp3 is a master regulatory gene for the maturation and function of $CD4^{+}CD25^{+}$ regulatory T cells. This gene may control activation and repression of gene transcription modulating regulatory T cell development. This is crucial in the maintenance of immunological self-tolerance and down-regulatory control of various components of the immune response (24-28). It thus was also important for us to examine the optimum normalizer gene selection, GADPH, for the rapid relative quantification of FOXp3 mRNA.

5.3. Selecting a gene as normalizer

The method used to derive quantitative values must be taken into account when interpreting gene expression data. Every method requires specific assumptions and it is important to be aware of how these assumptions may impact the resultant data. Previous work demonstrated the linearity of real-time PCR and detailed the use of standard curves for relative quantitation of gene targets (16).

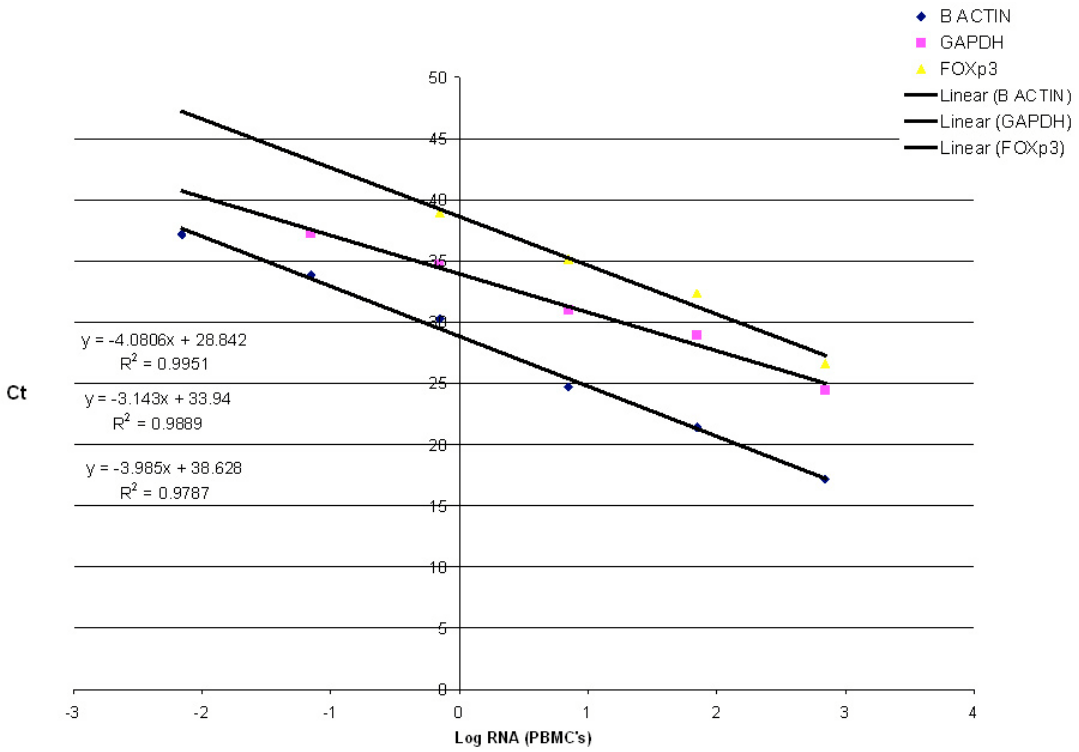

Figure 5. Reverse transcriptase real time PCR for Foxp3, GAPDH, and β-actin. This figure shows the linear range, sensitivity, and the slope of the standard curve for each of the genes under study in human PBMNCs.

The selection criteria for specific housekeeping genes used for normalizing gene expression targets in different cells and tissues measured by PCR are under study. The need for an appropriate housekeeping control gene for normalization has become increasingly clear. The normalizer gene to be selected should not be affected under the control, experimental, or study conditions of the study and should be analyzed across the same quantitative range as the target gene of interest under all conditions. These are essential requirements for a gene to be selected to function as a normalizer (Dr. John Pfeiffer, Applied Biosystems, Inc. personal communication, 2004). Normalizer reference genes are commonly used to normalize expression levels of other genes with the assumption that the expression of the normalizer gene is constant in different tissues and under different physiological and pathological conditions and this is under further investigation. Housekeeping gene expression has been reported to vary considerably and several systematic surveys have determined the variability of the housekeeping gene expression among different cells and tissues (12,13,27-31). Also of note is the fact that although β-actin mRNA encodes a cytoskeleton protein and is expressed among most all cell types, there is some evidence that its expression can be changed under various types of treatment and diseases e.g. in porcine immune cells and tissues (32.) Thus, the absence of a signal for β-actin in the neuronal amplified RNA could be due to the clinical condition of the patient as well as neuropathology.

We analyzed the expression of two genes in two different physiological environments (microdissected brain neurons and blood lymphocytes). We compared their levels of expression calculated by using different normalizer genes in order to optimize normalizer gene selection with regard to the sensitivity of the assay and the statistical significance of the fold-change in the level of gene expression. Gene expression data is expressed as the ratio of expression between experiment and control/reference samples for each gene by keeping the reference sample consistent, the resulting ratio (ΔΔCt) represents a precise estimate of the relative expression of each gene across the different conditions "only if" the normalizer gene is not affected by the treatment or any particular condition under study. In order to test the hypothesis that the levels of expression should be consistent for both the normalizer gene and the gene of interest (ΔCt in control samples) at basal/normal conditions and that the level of expression of the normalizer gene should not vary under the study conditions, we examined the changes in expression levels of two genes PS-1 and FOXp3 in different biological systems and under different experimental settings and compared different normalizer housekeeping genes. We found GAPDH the optimum normalizer gene consistent with some prior observations. Miller et al (33) showed that for RNAs prepared from brain tissue, SEs were lower for GAPDH compared to □2-microglobulin, neurofilament light polypeptide, and kynurenine formamidase. An earlier study by Johnston et al (27) also on brain tissue relied on GAPDH as a normalizer. Livak and Schmittgen (34) in a study of brain and kidney showed that □2-microglobulin is better than GADPH as the normalizer gene. Their study relied on the Syber green method that is less sensitive than the TaqMan method and is recommended neither for multiplex reactions nor for the amplification of rare transcripts. Data in a study by

Table 1. Ct values from different brain RNA sources for the quantification of ps1 expression

RNA	PS1	MEAN	SD	SE	β2-MICROGLOBULIN	MEAN	SD	SE	GAPDH	MEAN	SD	SE	18S	MEAN	SD	SE
Brain	30.41 30.64 30.81 30.41 30.57 30.81 31.01 31.43	30.76	0.34 **CV =** 1.11%	0.12	22.30 22.56 22.45 22.20 22.50 22.42 22.48 22.70	22.45	0.15 **CV =** 0.67%	0.05	19.48 19.68 19.15 19.77 19.51 19.45 19.77 19.74	19.57	0.21 **CV =** 1.07%	0.08	14.94 14.71 14.72 14.78 14.72 14.6 14.49 14.67	14.70	0.13 **CV =** 0.88%	0.05
Globus pallidus	29.14 29.60 28.96 29.99 29.67 29.95 30.18 30.47	29.75	0.51 **CV =** 1.71%	0.18	18.09 18.49 18.41 18.65 18.78 19.00 18.70 19.51	18.70	0.42 **CV =** 2.25%	0.15	17.49 17.59 17.08 17.50 17.24 17.96 17.36 17.36	17.45	0.26 **CV =** 1.49%	0.09	13.92 13.77 13.70 13.73 13.68 13.38 13.43 13.46	13.63	0.19 **CV =** .1.39%	0.07
Neurons	34.43 34.34 33.64 33.78 34.11	34.06	0.34 **CV =** 1.0%	0.15	20.61 20.92 20.44 20.96 20.78	20.74	0.22 **CV =** 1.06%	0.10	20.61 20.92 20.44 20.96 20.78	20.74	0.22 **CV =** 1.06%	0.10	19.38 19.15 19.51 19.25 19.12	19.28	0.16 **CV =** 0.83%	0.07

Table 2. Relative expression of ps1 in amplified RNA from neurons microdissected from basal ganglia (patient with HAD and HIVE)

ENDOGENOUS GENE	FOLD DIFFERENCE	T test	Nc + Ne	DF
PS1 gene expression IN 1,000 LCM NEURONS vs. Brain Total RNA				
B2-MICROGLOBULIN	0.03	38.3	8 + 9	15
GAPDH	3.07	0.64	8 + 9	15
18S	2.43	1.22	8 + 5	11
PS1 gene expression IN 1,000 LCM NEURONS vs. Globus pallidus Total RNA				
B2-MICROGLOBULIN	0.21	4.15	8 + 9	15
GAPDH	6.46	4.02	8 + 9	15
18S	2.38	0.94	8 + 5	11

Nc = control replicas Ne = experimental replicas DF = Nc+Ne -2

Table 3. Ct values before and after induction of FOXp3 in PBMNCs

	FOXp3	MEAN	SD	SE	GAPDH	MEAN	SD	SE	β-actin	MEAN	SD	SE
RNA Control	33.75 33.88 33.47 36.02 36.06 36.36 34.19 34.23 34.37 32.38 32.2 33.13 32.11 32.09 32.19	33.76	1.48 **CV =** 4.38%	0.38	21.42 21.43 21.52 20.77 21.96 20.74 21.13 20.56 21.51 21.04 21.07 22.73	21.32	0.59 **CV =** 2.76%	0.17	22.05 21.92 22.29 21.62 21.66 21.41 22.4 22.95 22.75 20.7 22.29 20.17	21.85	0.81 **CV =** 3.70%	0.23
1 Hour	32.34 32.46 32.71	32.50	0.19 **CV =** 0.58 %	0.11	21.42 21.43 21.52	21.46	0.06 **CV =** 0.28 %	0.03	22.05 21.92 22.29	22.09	0.19 **CV =** 0.86%	0.11
2 Hour	31.58 31.44 31.43	31.48	0.08 **CV =** 0.25%	0.05	20.77 21.96 20.74	21.16	0.70 **CV =** 3.3%	0.40	21.62 21.66 21.41	21.62	0.13 **CV =** 0.60%	0.08
6 Hour	30.44 30.75 30.98	30.72	0.27 **CV =** 0.88%	0.16	21.13 20.56 21.51	21.07	0.48 **CV =** 2.28%	0.28	18.22 18.33 18.56	18.33	0.17 **CV =** 0.93%	0.10

Table 4. Relative expression of FOXp3 in PBMC'S

Endogenous Gene	Fold Difference	T test	Nc + Ne	DF
GAPDH				
1 Hour	1.27	-0.28	15 + 3	16
2 Hour	2.28	1.03	15 + 3	16
6 Hour	3.42	3.9	15 + 3	16
Overnight	5.05	3.46	15 + 3	16
B - ACTIN				
1 Hour	1.12	-0.7	3 + 2	3
2 Hours	0.8	-0.51	3 + 3	4
6 Hours	10.51	9.29	3 + 3	4
7 Hours	12.67	12.51	3 + 3	4

Nc = control replicas Ne = experimental replicas DF = Nc+Ne -2

Table 5. Simulation analysis for degrees of freedom (number of replicas) for FOXP3

Endogenous gene GAPDH	Fold difference	T test	DF
1 Hour	1.27	-0.28	16
1 Hour		0.01	20
2 Hour	2.28	1.03	16
6 Hour	3.42	3.9	16
Overnight	5.05	3.46	16
β - ACTIN			
1 Hour	0.75	-0.7	4
2 Hours	0.8	-0.26	4
		-0.02	16
		0.003	18
3 Hours	2.91	0.31	4
4 Hours	15.93	-24.95	4
		-1.12	14
		0.15	16
5 Hours	6.7	4.36	4
6 Hours	10.51	9.29	4
7 Hours	12.67	12.51	4

The purpose of this table is to demonstrate that a large number of replicas would be required to bring beta actin to an acceptable level of significance.

Vandesompele et al (31), using a different method of statistical analysis involving geometric averaging of multiple internal control genes, was reanalyzed by Szabo et al (35). This re-analysis placed GAPDH ahead of other housekeeping genes. However, data produced by Szabo et al (35) found variation among different surgically removed tumor tissues and they did not conclude that GAPDH had any advantage compared to other genes in their own data. Similarly, Jin et al (12) questioned the use of single genes for normalization and promote the use of multiple normalizer genes. However, they do not utilize the ΔΔCt method of analysis. A study of cytokines in pigs using real time PCR similarly found that GAPDH showed lower variation than β-actin or hypoxanthine phospho-ribosyl-transferase mRNA (32).

The limitations of sample sources result in restrictions in experimental design. It is thus crucial in gene expression analysis to determine if hypothetical differences in expression levels between control and experimental specimens are statistically significant. Important differences may be obscured by biological variability and experimental error that produce difficulties to distinguish statistically significant differences from random variability. Furthermore, statistical analysis is necessary especially when observed differences are small compared to experimental imprecision and biological variability. When using the ΔΔCt methodology and in order for results to be analyzed accurately it is required that a statistical equation to test the results should be selected (36).

6. CONCLUSIONS

The findings in this paper using two divergent biological systems (PS-1 in brain micro-dissected neurons and FOXp3 in PBMNCs) demonstrate GAPDH as the optimum normalizer for the study of levels of gene expression for relative quantitation compared to other normalizers. It should be noted that normalizer reference genes are used as common denominators whereas the expression of test genes are described as the relative ratio as described in the ΔΔCt methodology. We conclude that a contribution to the high CV range when it occurs is produced by fluctuations in the normalizer gene signal in the denominators as occurs with 1 cycle (Ct +1). The contributions of such differences to the standard deviation of the experiment can be substantial. Furthermore, simple observations of substantial changes in Ct values across different study conditions for an endogenous gene is indicative of fluctuations for such gene that will eventually influence the data generated.

For most of the gene expression analysis one should consider that if the occurrence of the transcript of interest is extremely low, it is ideal to normalize its signal for RNA mass. The best normalizer is then one in which the ratio of that gene to RNA mass does not fluctuate from sample to sample in basal conditions (normal biological replicas). Each normalizer gene should be run in different RNA samples of the same or the closest source of material and the gene that shows the least fluctuation is most likely the best normalizer for that system (minimal standard deviation in the Ct data).

When quantitating gene expression, another factor we consider important to take into consideration is the inclusion of 'reverse transcription' as a separate step. Although the cDNA preparation step offers advantages in terms of numbers of genes that can be studied and stability over time, the conversion of mRNA to cDNA requires the conversion of heterogeneous mRNA populations into a high-quality cDNA where the different original transcripts are represented and may not reflect the identical concentrations as in the original RNA sample due to methodology. The efficiency of RNA transcription may have CV's ranging from 12% - 28% (37). Thus "one-steps RT-PCR" reaction offers a more accurate assessment of genes and their expression levels especially when the signal of a gene of interest is normalized to a housekeeping/endogenous gene. Each particular gene is captured from a more intact pool of total RNA via a specific reverse primer and the efficiency of converting total RNA to cDNA is comparable among the different gene targets. This may further attain a lower limit of detection of rare transcripts. Thus, the comparative Ct

method is more accurate and more comparable to the efficiencies obtained through the detailed standard curves approach. The efficiencies for the cDNA step when run as separate reactions are usually unknown and cannot be accounted for during subsequent statistical analysis. We show that the GAPDH gene is the less variant throughout the biological treatments examined and thus the most suitable for normalizing purposes in the systems studied. We conclude that for a more accurate determination of levels of gene expression it is preponderant to establish which one of the housekeeping genes will provide with a more precise system in which registered fluctuations are the result of true biological variations.

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