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Beta-2 microglobulin a robust reference housekeeping gene for RNA expression normalization in real time PCR on human leukocytes

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Background: Changes in gene expression are increasingly used to evaluate the effects of exposure to environmental agents. Housekeeping genes (Hk) are essential in these analyzes as internal controls to normalize expression levels assessed in real-time PCR (RT-PCR). Ideal Hk genes (i) are constitutively expressed; (ii) do not respond to external stimuli and (iii) show small or no variation between samples or from one assay to another. Previous studies indicate that some commonly used Hk genes, including glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and beta-actin, have differential expression in various cell lines. The objective of this study is to identify and validate the most appropriate housekeeping genes for RNA expression analysis of human primary peripheral blood mononuclear cells (PBMCs) in response to antigen like stimulation.

Results: Using reverse transcriptase RT-PCR protocol, we show that following activation only B2M remain unchanged in PBMCs. This result has been confirmed. In contrast, 18S, HPRT and GAPDH show significant variation in PBMC gene expression.

Conclusion: Although our results suggest that the relevance of Hk genes should be determined for each experimental condition, B2M appear to be excellent candidate as internal controls.

Keywords: Housekeeping genes, RT-PCR, Human primary PBMC.

BACKGROUND

Housekeeping (Hk) genes are essential for the interpretation of quantitative gene expression techniques. By definition, a Hk gene is constitutively expressed in a stable manner in all nucleated cell types, as they are essential for cell survival and functions(1). The use of Hk genes allows us to manage potential errors committed during RNA extraction or reverse transcription, as it is used as reference to normalize total RNA expression (2). Indeed, Hk gene expression should be constant in any cell type or activation state. If not, the normalization by a variable Hk gene will lead to potential errors in gene expression quantification. Moreover, if there is a variation in Hk gene expression, small differences in the expression of genes of interest will not be detected(2). However no known single gene has a constant expression level in all situations(3). According to the MIQE (Minimum Information for publication of Quantitative Real-Time PCR Experiments) guidelines, the normalization should be made on more than one reference gene (4).

Many studies show that some Hk genes previously recognized as a gold standard are not as stable as previously thought and that Hk gene transcription can vary significantly between different donors, activation states, development stages, cell origins or experimental conditions(3). No article reports that is the most trustworthy Hk genes for primary human immune cells. In this study, we have selected gene described to be good Hk genes in different cell lines to test them in PBMCs. The six genes carefully chosen are (i) *β -2Microglobulin (B2M)*, a component of major histocompatibility complex one (MHCI), (ii) *glycerolaldehydphosphate dehydrogenase (GAPDH)*, a glycolytic enzyme, (iii) *Hypoxanthine-guanine phosphoribosyl transferase (HPRT)*, involved in the metabolic salvation of purines, (iv) 18S ribosomal RNA (18S) which is part of ribosomal small subunit , (v) *Ubiquitin C (UbC)* involved in protein catabolism and finally (vi) *β -actin (ACTB)* a component of the microfilament part of the cytoskeleton (1,5–8).

Our aim is to identify the most reliable Hk gene to use it as reliable reference for RT-qPCR on human primary peripheral blood mononuclear cells (PBMC)

RESULTS

Housekeeping gene expression levels were measured by real-time PCR, and expressions stabilities were tested using both R and BestKeeper algorithms.

The ranking of these Hk genes, from the less to the most stable is *18S*, *ATCB*, *GAPDH*, *HPRT*, *UBC* and *B2M*.

Indeed, *18S* expression variation between donors is more than 3 Ct, and of 2 Ct regarding the activation state (Fig 1). *ATBC* is rather stable regarding different donors as variation is less than one Ct, but there is a higher difference, more than 2 Ct, between activated and non-activated PBMCs (Fig1). *GAPDH* follows the same trend with nearly no expression variation between donors but with more than 4 Ct of variation following activation (Fig 1). In our results, the rankings of *HPRT* and *UBC* is difficult because of their very similar profiles: both are very stable between donors but shows a variation of 2 Ct between activated and non-activated conditions (Fig 1). Nevertheless, statistical analysis by 2-way ANOVA show a weakly significative difference between donors for *HPRT* compared to the non-significative difference for *UBC*. Moreover, BestKeeper analysis show power of 1.88 for *UBC* and of 2.26 for *HPRT*, as power value have to be under 2 for good Hk genes, *UBC* seems to be better candidate. Finally, the most reliable Hk gene in this study is *B2M*. Indeed, regardless of the activation state or donor, the gene expression is very stable, without any significant differences in Ct outcome (Fig1). Moreover, regarding BestKeeper results, *B2M* is the only gene with a standard deviation under 1, as recommended ($SD = 0.25$ CP), and also the gene with the highest p-value (9). Finally the power is under 2 (Power [x-fold] = 1.07) which is recommended for a good Hk gene (9).

To complete these results a meta-analysis has been carried out on 27 patients with different profiles regarding *B2M* and *UBC*. Among them, 5 healthy donors, 12 patients with chronic hepatitis B and 10 hepatocellular carcinoma patients (10). This analysis confirms our results by showing a variation lower than 1Ct for *B2M* regardless of patients or activation state (figure 2A). Moreover, it also reinforces *UBC* results showing nearly no difference between donors but small variations regarding activation state (less than 2 Ct), especially patients suffering of hepatocellular carcinoma (figure 2 B)

Figure 1 : Housekeeping gene expression depending on donor or activation state (non-activated in white, activated with anti-CD3/anti-CD28 in grey) analysed by 2 ways ANOVA on R, Ns = no significant difference, * = $P \leq 0.05$, ** = $P \leq 0.01$, *** ≤ 0.001 .

Data of candidate housekeeping genes (n=3)						
	18S	ACTB	B2M	GAPDH	HPRT	UbC
N	16	16	16	16	16	16
Geo mean [CP]	21.72	18.12	16.62	17.78	23.69	24.42
Min [CP]	18.85	16.66	16.13	17.63	21.88	23.18
Max [CP]	26.94	20.49	17.32	22.35	25.6	25.7
Std dev [$\pm$ CP]	1.87	1.17	0.25	2.1	1.46	1.11
CV [%CP]	8.60	6.49	1.53	10.58	6.17	4.55
Power [x-fold]	2.72	1.90	1.07	3.34	2.26	1.88
p-value	0.001	0.001	0.188	0.001	0.001	0.001

Table 2: Results table from BestKeeper – Excel based tool.

Figure 2 : B2M (A) and UBC (B) expression among several patients PBMC.

DISCUSSION

Real-time PCR is a very sensitive and robust quantification method for gene expression analysis. Nonetheless, many factors can impact results, that's why robust Hk gene are essential for a reliable quantification of gene expression. Besides PBMCs, as it includes different cell type, hold lots of various information. It is important for us to identify strong Hk gene since PBMC are the more often used sample (as they are easy to collect by small blood collection) for clinical trial follow-up on immune system.

Some of these results are in agreement with the literature, *ATBC* variations are classified as unstable in chronic hepatitis patients' PBMCs (5). Large *ATCB* expression variation could have been predicted, as during activation there is a non-negligible variation of the nucleo-cytoplasmic ratio. The observations regarding *GAPDH* expression also correlate with other findings, which shows a higher variation in expression, more than 15-folds in 72 different human tissues (11). Furthermore, the activation mechanism chooses here (anti-CD3, anti-CD28) specifically target T lymphocyte activation by mimicking immunological synapse co-activators. Because of PBMC population heterogeneity, this kind of activation could act indirectly on other cell type activation, notably by cytokine secretion.

CONCLUSION

Here *B2M* is the best candidate as the expression level is stable regardless of the donor and activation state. This result is reinforced by statistical data obtain with Bestkeeper and by meta-analysis in PBMCs of cancer patients. Thus, *B2M* is the only one to complete all the recommendations and is the best candidate for internal control of gene expression in RT-qPCR. This result is coherent since *B2M* as part of MHC I is equally express on every cell, independently of immune cell activation. Nevertheless, UBC can be also used, as there is no significative difference between donors.

Although our results clearly indicate only one perfect candidate, the finding of one other Hk gene is still needed to get best accuracy for PBMC RT-PCR and match with the MIQE guideline recommendations

METHODS

PBMC Isolation and culture:

PBMC isolation was performed on two EDTA tubes of 8 mL from healthy donor blood (n=3), using a density gradient with lymphocyte separation medium (Eurobio). PBMCs were washed twice in phosphate buffered saline without calcium and magnesium (PBS -/-) before counting. PBMCs activated or not with anti-CD3 (Miltenyi Biotec) and anti-CD28 (Invitrogen) at 0.5mg/ml were cultured during 48 hours in RPMI 1640 medium complemented with 1% NEAA, 1% NaPy, HEPES, 0.1% Gentamycin, 0.1% β -Mercaptoethanol (Life technologies) and 10% human serum AB (SAB) (Sigma Aldrich). After 48 hours, PBMCs were harvested, 10 million are put in 1ml of TRIzol reagent (Ambion) and stored at -80°C until further use.

RNA extraction and Reverse transcription:

PBMC total RNA was extracted using TRIzol reagent method according to manufacturer's instructions. RNA concentrations were measured using a Nanodrop (ThermoScientific). Reverse transcription was performed using 1 μ g of total RNA.

Selection of interest housekeeping genes:

Gene symbol	Gene Name	Sequence
<i>HPRT</i>	Hypoxanthine-guanine phosphoribosyl transferase	5' CCCTGGCGTCGTGATTAG 3' 3' ATGGCCTCCCATCTCCTT 5'
<i>GAPDH</i>	Glyceraldehyde-3-phosphate dehydrogenase	5' GCCAAGGTCATCCATGACAACCTTTGG 3' 3' GCCTGCTTCACCACCTTCTTGATGTC 5'
<i>18S</i>	18S ribosomal RNA	5' TCAAGAACGAAAGTCGGAGG 3' 3' GGACATCTAAGGGCATCACA 5'
<i>UBC</i>	Ubiquitin C	5'CCGACCACAGTGGCTATGC 3' 3' CCTCTTTTAATATCTCCAGGCTTGA 5'
<i>B2M</i>	Beta-2 Microglobulin	5'GCTCGCGCTACTCTCTCTTT 3' 3' TCTGAATGCTCCACTTTTTCAA 5'
<i>ATBC</i>	Beta Actin	5' CACGGCATCGTCACCAACT 3' 3' AGCCACACGCAGCTCATTG 5'

Table 1 : Sequences of the selected housekeeping genes

The selection of five housekeeping genes (*HPRT*, *GAPDH*, *18S*, *UBC*, β 2M) is based on the literature (Table 1). They belong to different functional classes and are reported to be used in genomic studies of immune cells. Primers were designed using FASTA sequences of each gene in Primer Blast (NCBI-NIH) and synthesized by Eurogentech (Liège, Belgium)

Real time PCR:

The RT-PCR reactions were performed, for selected Hk genes (Table 1), according to the manufacturer's instructions using 2X MESA GREEN qPCR MasterMix Plus for SYBR® 258 Assay (Eurogentech), 96 well qPCR plate (Sarstedt), optical seal (Dutcher) and the Mx3005PTM sequence detection system (Agilent technologies). In each reaction, 10ng of reverse transcribed RNA (based on initial RNA concentration) was used. All primers were used at 400nM in a 20µL reaction. Quantitative analysis was made based on the cycle threshold (Ct) value for each well and calculated using MxPro software (Agilent).

Statistics:

The validation of expression stability was statistically tested by R and by BestKeeper methods. BestKeeper algorithm uses raw Ct values and calculates the standard variations (SD and CV). Unstable genes show SD > 1. The most stable HK gene is determined with the correlation coefficient (r) of their expression compared to the BestKeeper Index, which is the geometric mean of Ct values of the highly correlated candidate reference genes.(8,9)

R statistical analysis were done using two Way ANOVA tests.

Meta-analysis:

The study shows data obtained in GPL570 data set from Gene Expression Omnibus (GEO).

Abbreviations

Hk: Housekeeping

RT-PCR: Real Time polymerase chain reaction

MIQE: Minimum Information for publication of Quantitative Real-Time PCR Experiments

B2M: *β-2Microglobulin*,

GAPDH: *glycerolaldehydphosphate*

HPRT: *Dehydrogenase Hypoxanthine-guanine phosphoribosyl transferase*

18S: 18S ribosomal RNA

UbC: *Ubiquitin C*

ACTB: *β-actin*

PBMC: primary peripheral blood mononuclear cells

Declarations

Ethics approval and consent to participate

Ethics approval was obtained for this paper. Human blood samples were collected from adult healthy donors after obtaining written consent in accordance with the approval of the Institutional Review Board at the Biology Institute of Lille (DC-2013-1919).

Consent for publication

Not applicable

Availability of data and materials

Not applicable

Competing interests

The authors declare that they have not competing interests

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Authors' contributions

CM performed all experimentations and participated to the writing of the publication ; CI participated to experimental supervision ; Dr OM and Pr ND participated to the design of the study and to the writing of the publication. All authors have read and approved the manuscript.

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Not Applicable

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Figures

Figure 1

Housekeeping gene expression depending on donor or activation state (non-activated in white, activated with anti-CD3/anti-CD28 in grey) analysed by 2 ways ANOVA on R, Ns = no significant difference, * = $P \leq 0.05$, ** = $P \leq 0.01$, *** ≤ 0.001 .

Figure 2

B2M (A) and UBC (B) expression among several patients PBMC.