Identification of Suitable qPCR Reference Genes during IL-5 Induced Cord Blood Eosinophilopoeisis

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Background and Objective

Quantitative real time polymerase chain reaction (qPCR) has become a widely used and often preferred tool to study gene expression in a variety of applications. In order to use this sensitive technique reliably however, a set of unaffected reference genes is necessary to normalize the target gene expression. Traditionally, many studies used "house-keeping genes" like *GAPDH* or *ACTB* for this matter. These genes were thought to be expressed evenly throughout any chosen condition.
Recently, it has become very apparent that the use of one or more "house-keeping genes" could be unsuitable under certain conditions as their expression does not remain unaffected and needs to be determined for each application individually.

We therefore aim to evaluate a panel of twelve human reference genes that will be useful to study gene expression during Interleukin (IL-5) induced cord blood eosinophilopoeisis.

Methods

Cord Blood Samples: Two random cord blood samples of presumably healthy mothers undergoing Caesarean sections were collected at Kingston General Hospital after written consent was given.
Isolation of Mono-nuclear cells (MNCs): After their collection the cord blood samples were processed immediately as follows. All samples were depleted of red blood cells via unit gravity sedimentation in 1 % Dextran, followed by an AccuPrep® density gradient to isolate MNCs. The resulting MNC populations were frozen at -80 °C for temporary storage.
IL-5 induced eosinophilopoeisis: All samples were thawed, depleted of adherent cells and reseeded at 5-10 million cells per condition. Non-adherent MNCs (NAMNCs) were then stimulated with recombinant human IL-5 to induce eosinophilopoeisis.
RNA isolation and reverse transcription: NAMNCs were collected at 0, 24, 50 and 75 hours post stimulation and RNA was isolated using the RNeasy® Plus Mini kit (QIAGEN). All samples were then reverse-transcribed using the QuantiTect® Reverse Transcription kit (QIAGEN).
qPCR: Firstly, a panel of twelve human reference genes (*GAPDH, TUBB, PPIA, ACTB, YWHAZ, RRN18S, B2M, UBC, TBP, RPLP, GUSB and HPRT1*) was investigated in all cDNA samples obtained using the QuantiTect® SYBR Green PCR kit (QIAGEN). Fully evaluated primer pairs for the above listed genes were obtained from Tataabiocenter, their sequence however is unknown (also see Table 1). Utilizing the statistic software 'GenEx' (including 'NormFinder') we selected reference genes suitable for this assay.
Secondly, we intend to design and evaluate our own primer pairs and probes for the identified genes to be used in multiplex PCR.

Gene	Full name	Expression level	PCR product size [bp]
RRN18S	*18S rRNA*	Very High	120
ACTB	*Actin, beta*	High	188
GAPDH	*Glyceraldehyde-3-phosphate dehydrogenase*		220
TUBB	*Tubulin, beta polypeptide*		119
B2M	*Beta-2-microglobulin*	Medium	161
RPLP	*60S acidic ribosomal protein P0*		150
TBP	*TATAA-box binding protein*		174
GUSB	*Beta-glucuronidase*	Low	165
HPRT1	*Hypoxanthine-guanine phosphoribsyltransferase*		94
PPIA	*Cyclophilin A*		114
UBC	*Ubiquitin C*		239
YWHAZ	*Tyrosine 3/tryptophan 5 -monooxygenase activation protein, zeta polypeptide*		248

Table 1: The Tataabiocenter 'Reference Gene Panel Human' was chosen to identify optimal reference genes during IL-5 induced cord blood eosinophilopoeisis. Listed are the genes examined, their predicted expression levels and PCR product sizes (modified from product brochure).

Results

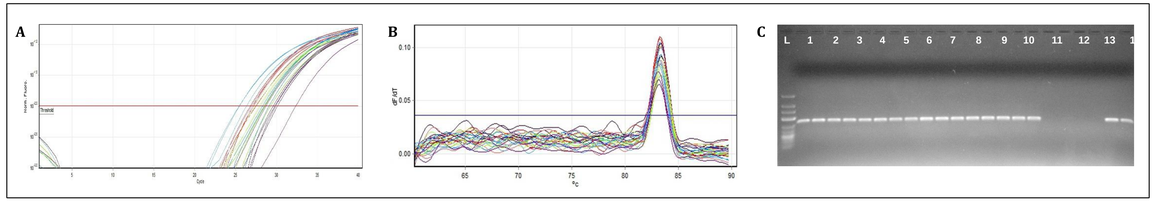

Figure 1: Representative quantification data for cycling SYBR Green (A), melt curve (B) and gel electrophoresis (C) for each of the four time points (0, 24, 50 and 75 hours post IL-5 stimulation). All primer pairs (Tataabiocenter) used appeared to be very specific. Shown are the averaged quantification data (A) and melt curves (B) of two biological replicas (R017 and R018) for *UBC* with a n=3 each per condition. Gel electrophoresis (C) was performed to partially confirm the qPCR results. The samples were loaded as followed: (L) O'Gene Ruler™ DNA ladder, low range (Fermentas); (1-2) 0 hours, R017; (3-4) 24 hours, R017; (5-6) 50 hours, R017; (7-8) 75 hours, R017; (9-10) 0 hours, R018; (11-12) 24 hours, R018; (13-14) 50 hours, R018; (15-16) 75 hours, R018; (17-18) NO_RT; (19-20) NTC; (21-22) positive control DNA

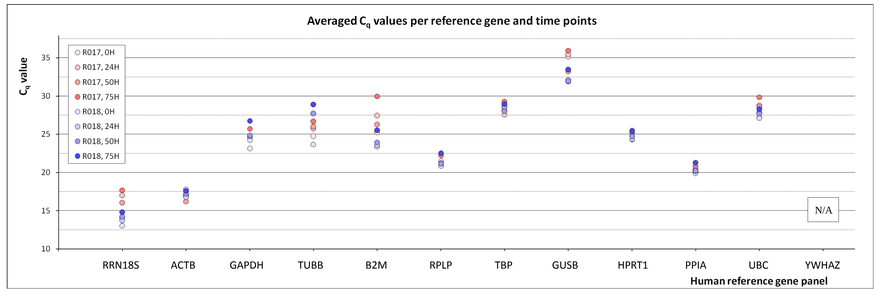

Figure 2: The expression of *ACTB, RPLP, TBP, HPRT1, PPIA* and *UBC* appears to be less influenced during IL-5 induced eosinophilopoeisis than the expression of *RRN18S, GAPDH, TUBB, B2M* and *GUSB*.
Two random, individual human cord blood samples were examined, R017 (red) and R018 (blue). Shown are the average C_q values for each cDNA sample at four time points (0, 24, 50 and 75 hours post IL-5 stimulation) for *RRN18S, ACTB, GAPDH, TUBB, B2M, RPLP, TBP, GUSB, HPRT1, PPIA, UBC* and *YWHAZ*. The expression levels of *YWHAZ* were below the detection limit of the qPCR assay and could not be determined in these samples.

A

Gene	Intragroup variation R017	Intragroup variation R018	Intergroup variation R017	Intergroup variation R018
RRN18S	1.098	0.021	0.798	-0.798
ACTB	0.731	0.774	-0.615	0.615
GAPDH	0.293	0.166	-0.615	0.615
TUBB	0.120	3.589	-0.929	0.929
B2M	1.892	0.063	1.230	-1.230
RPLP	0.378	0.007	-0.344	0.344
TBP	0.599	0.233	-0.365	0.365
GUSB	1.106	0.119	0.967	-0.967
HPRT1	0.028	0.048	0.063	-0.063
PPIA	0.289	0.007	-0.311	0.311
UBC	0.233	0.069	0.118	-0.118
YWHAZ	N/A	N/A	N/A	N/A

B

Gene	Variability
RRN18S	1.100
ACTB	1.092
GAPDH	0.907
TUBB	1.379
B2M	1.534
RPLP	0.567
TBP	0.758
GUSB	1.306
HPRT1	0.199
PPIA	0.514
UBC	0.374
YWHAZ	N/A

Best gene (Variability):
HPRT1 (0.199)

Best combination of two genes (Variability):
UBC and HPRT1 (0.247)

Figure 3: Utilizing the software GenEx (including NormFinder) a combination of two reference genes – *HPRT1* and *UBC* – was identified as optimal under the chosen conditions. *HPRT1* expression showed the smallest intra- and intergroup variation (A) and variability (B) of expression in NAMNCs at 0, 24, 50 and 75 hours of IL-5 stimulation.

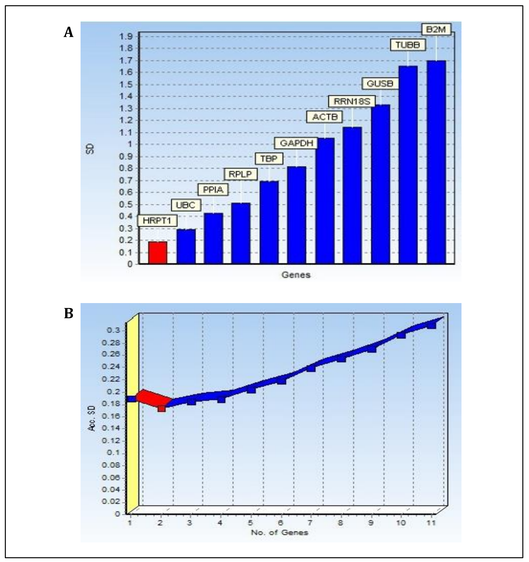

Figure 4: During eosinophilopoeisis, the expression of *HPRT1* and *UBC* exhibited only minor changes. With a standard deviation (SD) of 0.189 *HPRT1* was observed to be the least affected (A). Using the accumulated standard deviation (Acc. SD) a combination of two reference genes (Acc. SD = 0.173) was determined to be most beneficial (B).

Discussion

The study of umbilical cord blood for potential biomarkers of future atopy is of increasing interest to researchers; our laboratory in particular is interested in mRNA expression of genes important to eosinophil-lineage commitment. In this study we evaluated a panel of twelve human genes that can be used in gene expression analyses concerning IL-5 induced cord blood eosinophilopoeisis. Although this project addresses a very basic research question, the results obtained here are important to accurately examine gene expression in future studies. Using an unsuitable set of reference genes can lead to misinterpretation of gene expression data, which in turn could impact the identification of putative biomarkers and potential treatment approaches including drug development. The outcomes of such studies could range from false diagnosis to incorrect therapy and could be devastating to individuals affected by allergic/immune diseases.

We identified the combination of *HPRT1* and *UBC* as ideal reference genes during IL-5 induced eosinophilopoeisis of human NAMNCs using the GenEx software (including NormFinder). NormFinder, an algorithm designed to select the optimal reference gene(s) out of a panel of candidate genes, takes grouping information of cDNA samples into account and can result in multiple references genes that accomodate each others expression. For example, one gene might be over-expressed in one group, the other however is proportionally under-expressed within the same group.
Furthermore, the expression levels of the two genes identified in our cDNA samples are medium to low, making it an excellent reference gene combination when studying low abundant targets.

Summary

The potential of twelve different human genes (primer panel was obtained from Tataabiocenter) have been evaluated as putative reference genes for mRNA expression studies concerning IL-5 induced umbilical cord blood eosinophilopoeisis in NAMNCs.

For each gene, we investigated the mRNA expression levels at four timepoints (0, 24, 50 and 75 hours of IL-5 stimulation) via qPCR, and confirmed the obtained data using gel electrophoresis (Figure 1). *ACTB, RPLP, TBP, HPRT1, PPIA* and *UBC* appeared to be less affected during IL-5 induced eosinophilopoeisis than the expression of *RRN18S, GAPDH, TUBB, B2M* and *GUSB* (Figure 2). The final analysis using the software GenEx revealed the combination of HPRT1 and UBC as ideal reference genes for this particular application (Figures 3 and 4).

Following up on these results we will design and evaluate our own primer pairs and probes for human *HPRT1* and *UBC* to be used in multiplex qPCR studies to identify putative biomarkers of future atopy.

References

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Acknowledgements

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