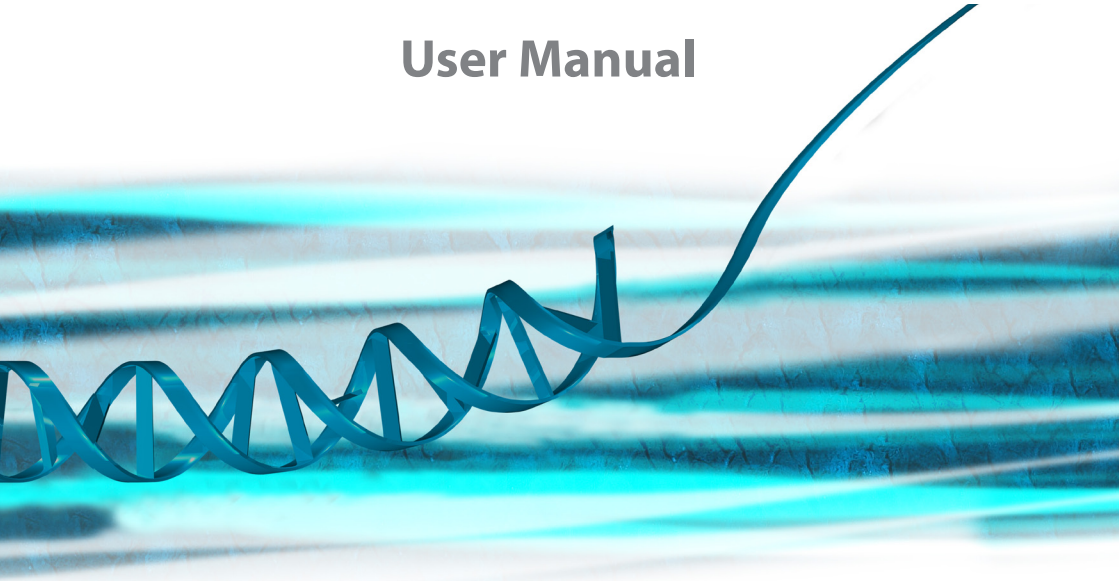
User Manual



Human Endogenous Control Gene Panel

Version 1.5 — March 2008

For use in quantitative real-time PCR

tataabiocenter

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Background

For accurate gene expression measurements it is necessary to normalize results of the expression of target genes to some reference, not affected by the parameters studied in the specific study. There exists to our know-ledge no reference gene that is unaffected in all conditions. Therefore it is necessary to find the optimal reference gene for your conditions and validate that this gene is indeed non-regulated.

For this reason we have developed the Human Endogenous Control Gene Panel which contains primersets for 12 potential reference genes. The expression of these genes can be measured on some representative samples, and from the results the most constantly expressed gene(s) can be determined.

This gene(s) is then used for subsequent normalization of target gene expression for more accurate results

Contents

- Positive Control DNA
- Primer solutions for 12 potential human reference genes. 100 rxns.
- Ready-to-use 2X mastermix (optional). 100rxns.
- GenEx Light software

Primers were designed to span exon-boundaries and for minimum amount of primer dimer formation. Efficiency of amplification was determined for each assay on a multitude of commercial kits using SYBR Green I.

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

*The B2M and 18S rRNA assays are designed within an exon and may amplify genomic DNA.

Additionally required materials and devices

• Real-time PCR instrumentation
This kit has been validated on several different instrument platforms. It is suitable for use on all real-time PCR instruments.

• Mastermix or mastermix components
This kit has been validated using most commercially available mastermixes, including Applied Biosystems SYBR Green PCR Kit, AbGene ABsolute SYBR Green QPCR mix, Qiagen QuantiTect SYBR Green PCR Kit, Eurogentec QPCR SYBR Green Mastermix, TaKaRa SYBR Premix Ex Taq, BioRad SYBR Supermix, Quantace SensiMix and Invitrogen Platinum SYBR Green qPCR SuperMix.

• Pipettes and tips

• Vortex and centrifuge

• Sample cDNA
It is recommended to use cDNA of high quality. Quality of RNA can be checked prior to cDNA synthesis using Agilent 2100 Bioanalyzer or BioRad Experion.

Storage

The contents of this kit can be stored at +4^{o}C for a period of 12 months. For long term storage -20^{o}C is recommended. Repeated freeze-thaw cycles are not recommended.

Amplification protocol

The amplification protocol below is recommended. Optionally a 2-step amplification protocol may be used. In this case we recommend 95°C 15s and 60°C 60s, repeated 40 cycles.

UNG step (optical)	according to mix instructions		
Enzyme activation	according to mix intructions		
Cycling (x40)	**denaturation**	20s	95°C
	annealing	20s	60°C
	elongation	20s	72°C

After amplification it is recommended to perform dissociation curve analysis from 65°C to 95°C. This is programmed according to the instrument manufaturers' intructions.

Pipetting protocol

We recommend that each sample is quantified in duplicate or triplicate for each gene in the panel. Approximately 5-10 samples of each type (healthy/sick, treated/non-treated etc.) should be evaluated for determination of the most constantly expressed gene.

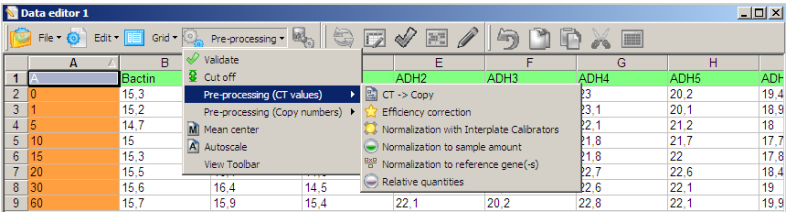
Make mastermixes for each gene according to the protocol below. Make at least 1 rxn extra so that you do not run out of mastermix during the pipetting.

Larger volume of cDNA may be added. In this case change the volume of water accordingly. 2µl of Positive Control DNA can be added as template as an additional control of the PCR reaction.

Component	1 rxn
PCR-Grade water	9,5µl
Primermix	1µl
Mastermix (2x)	12.5µl
cDNA	2µl
Final Volume	25µl

Selecting the most appropriate endogenous control gene using GenEx Light

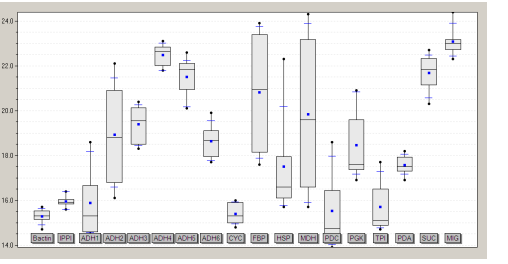
A number of methods have been proposed how to select the most appropriate control gene(s). Using the GenEx Light software you can easily determine the most stably expressed gene by using two commonly used methods. The GeNorm and Normfinder methods are both included in the software. GenEx Light also includes easy to use pre-processing tools to convert your data from Ct-values to relative quantities, normalization to reference genes or total RNA, the possibility to classify your samples in different groups (i.e. treated/non-treated) and much more. A number of other statistical methods to define the most appropriate reference genes from a panel, tested on a set of samples, are listed in the references.



	A	B			E	F	G	H	
1		Bactin			ADH2	ADH3	ADH4	ADH5	ADH
2	0	15,3					23	20,2	19,4
3	1	15,2					23,1	20,1	18,9
4	5	14,7					22,1	21,2	18
5	10	15					21,8	21,7	17,7
6	15	15,3					21,8	22	17,8
7	20	15,5					22,7	22,6	18,4
8	30	15,6	16,4	14,5			22,6	22,1	19
9	60	15,7	15,9	15,4	22,1	20,2	22,8	22,1	19,9

Install GenEx Light by inserting the included CD and running the file on your computer. Also included on the CD are tutorials showing different examples where GenEx Light is used, and example files where the functionality can be tested.

Before or after pre-processing, the data can be visualised using a number of built in functions.



GenEx Light is included as a time limited license when purchasing the Endogeneous Control Gene. The GenEx software is also available in Pro and Enterprise versions. Besides the functionality in GenEx Light, GenEx Pro also includes Hierachical clusering, Principle Component Analysis, Potential curves for classification, and basic statistical anlysis. GenEx Enterprise (available soon) also includes advanced Neural Netork modules. For upgrading your GenEx Light please contact us at order@tataa.com.

	GenEx Light	GenEx Pro
User friendly data pre-processing	X	X
GeNorm	X	X
Normfinder	X	X
Scatter plots and 3D visualization	X	X
Principle Component Analysis		X
Self Organizing Maps		X
Hierarchical Clustering		X
Trilinear Decomposition		X
Potential Curves		X
Pearson and Spearman correlation		X

Troubleshooting

• I do not get any amplification/signal?
The instrument may not have been programmed correctly or there may be a problem with your mastermix. Evaluate if the problem is in the detection or the amplification by running the samples on gel. Run a new test using the positive control DNA provided with the kit.

• My negative controls give a positive amplification?
Since the assays are based on SYBR Green detection, all dsDNA is detected, including primer dimers. Depending on which mix is used, varying amounts of primer dimers are formed. Typically negative controls give Ct-values well above 35 cycles. Evaluate whether amplification is due to primer dimers or contamination using melt-curve analysis.

• My samples have same/higher Ct-value than my negative controls?
This indicates that you have added too little cDNA. Add more cDNA and try again. The cDNA may be of low quality. Check the quality of the RNA before doing cDNA synthesis.

• My replicates are not very tight?
With good quality cDNA and good pipetting technique, very high reproducibility is possible. Low amounts of cDNA can lead to higher variation. Also, low quality cDNA can lead to big differences between replicates. Check the accuracy and reproducibility of your pipettes.

• The signal for 18S rRNA comes very early and does not have a good baseline?
In some cases the amount of 18S is too high which can result in erroneous background substraction. Try diluting the cDNA sample.

• I get a positive amplification from genomic DNA
Where possible the assays in the Endogenous Control Gene Panel have been designed to span exon-boundaries. However, when intron-less pseudogenes are present in the genome, genomic DNA may still give a positive amplification. Try removing DNA contamination by DNase treatment of the RNA sample.

References

Vandesompele J. et al (2002) Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes Genome Biology 3(7) 0034.I - 0034.II

Szabo A. et al (2004) Statistical modeling for selecting housekeeper genes Genome Biology 5:R59

Andersen C.L. et al (2004) Normalization of Real-Time Quantitative Reverse Transcription-PCR Data: A Model-Based Variance Estimation Approach to Identify Genes Suited for Normalization, Applied to Bladder and Colon Cancer Data Sets Cancer Research 64, 5245–5250

Stålberg A. et al (2005) Quantiative real-time PCR for cancer detection: the lymphoma case Exp. Rev. Mol. Diagn. 5(2) 2005

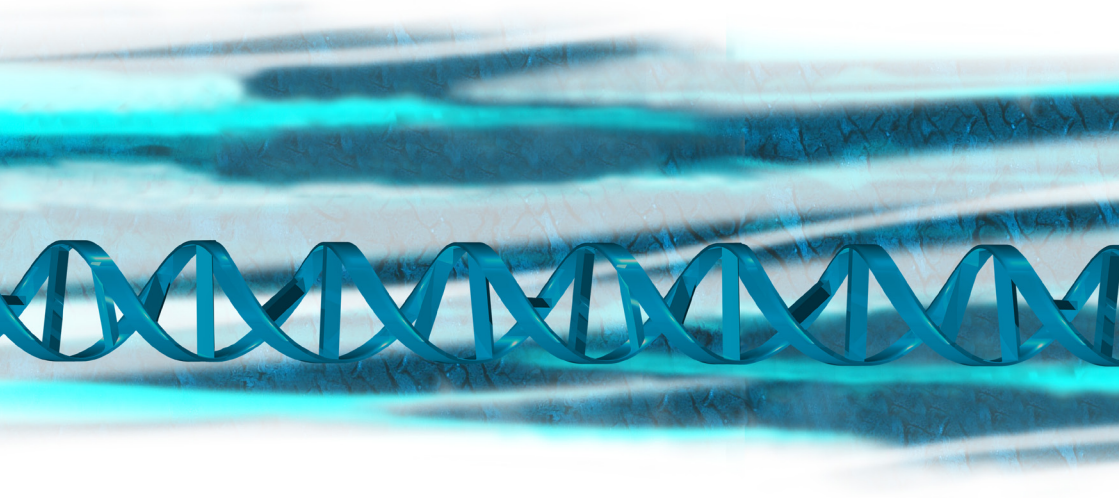
Contact

For more information about the product and on endogenous control genes please contact us at info@tataa.com

License information

PCR is covered by several patents owned by Hoffman-La Roche Inc. and Hoffman-LaRoche, Ltd. Purchase of the Human Endogenous Control Gene Panel does not include or provide a license with respect to any PCR-related patent owned by Hoffman-La Roche or others. TATAA Biocenter does not encourage or support the unauthorised or unlicensed use of the PCR process.

Express your genius



TATAA Biocenter, with offices in Gothenburg, San Franscisco, Prague and Munich, is the leading provider of real-time PCR services and the prime organizer of real-time PCR workshops globally. TATAA Biocenter conducts commissioned research and training within field of molecular diagnostics and gene expression analysis, along with developing real-time PCR expression panels. TATAA Biocenter have great experience and expertise in high resolution gene expression profiling, pathogen detection, and small sample/single cell analysis.

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