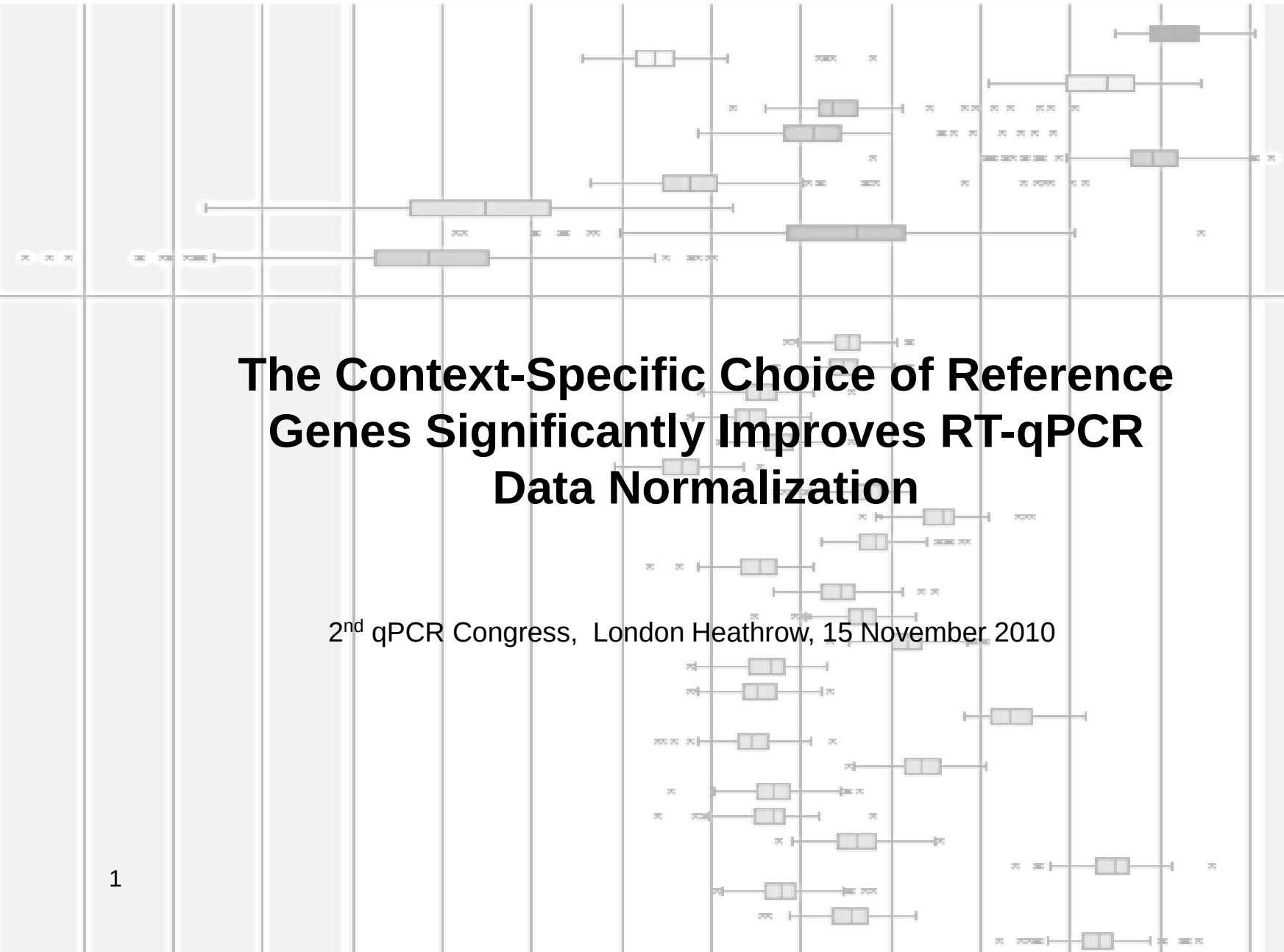


The Context-Specific Choice of Reference Genes Significantly Improves RT-qPCR Data Normalization

2nd qPCR Congress, London Heathrow, 15 November 2010

Fundamental question

- Which reference gene should I use in my RT-qPCR experiment?

EIF1?

GAPDH?

PPIA?

HPRT?

B2M?

ACTB?

28S?

Commonly used reference genes

OpenArray™ Human Housekeeping Panel Kit

Gene expression analysis is routinely performed by most research labs. In order to quantify the expression levels of various or unknown genes, a comparison to the expression of a standard gene is made. The most common standard genes are known as housekeeping genes. These genes are expressed constitutively in most cells because they are genes that encode proteins that

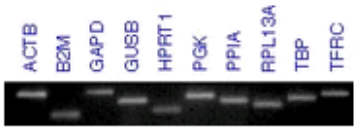
ASSAY	REF SEQ ID	GENE ID	GENE
1	NM_000688.4	ALAS	delta-aminolevulinate synthase
2	NM_004048.2	B2M	beta-2-microglobulin
3	NM_000981.3	RPL19	ribosomal protein L19
4	NM_021130.3	PPIA	peptidylprolyl isomerase
5	NM_001402.5	EF1A	elongation factor-1 alph
6	NM_002046.3	GAPDH	glyceraldehyde-3-phosp
7	NM_000181.2	GusB	Glucuronidase, beta
8	NM_000194.1	HPRT	Hypoxanthine phospho
9	NM_001002.3	RPLP0	ribosomal protein, large
10	NM_007359.4	MLN51	metastatic lymph node
11	NM_000937.2	POLR2A	DNA directed RNA poly
12	NM_000291.2	PGK	Protein kinase, cGMP-d
13	NM_003194.3	TBP	TATA box binding prote

Human Housekeeping Gene Primer Set

Item# HHK-1

$79.95

Add to cart



Human Endogenous Control Gene Panel

Background

For all gene expression studies using quantitative PCR it is necessary to compensate for differences between samples due to material losses, differences in RT yields and PCR inhibition. Normalization should include an endogenous control gene, but can also be complemented by identical sample input amounts. The endogenous control gene should have constant expression in all the samples compared. There is no universal control gene, expressed at a constant level under all conditions and in all tissues.

The best way to choose the proper reference gene is by running a panel of potential genes on a number of representative test samples. The gene(s) most appropriate for normalization are chosen in each case.

The Human Endogenous Control Panel consists of 12 validated qPCR assays for the most common endogenous control genes for gene expression studies, and provides a rapid and cost efficient way to identify your control genes.

Endogenous Control Panel

The genes included in the Human Endogenous Control Panel are commonly used in gene expression studies. Genes with varying cellular function and expression level have been selected. Primers have been designed to be exon-spanning where possible and to give low levels of primer-dimers.

Gene	Full name
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
TUBB	Tubulin, beta polypeptide
PPIA	Cyclophilin A
ACTB	Actin, beta
YWHAZ	Tyrosine 3/tryptophan 5 -monooxygenase activator protein, zeta polypeptide
RRN18S	18S rRNA
B2M	Beta-2-microglobulin
UBC	Ubiquitin C
TBP	TATA-box binding protein
RPLP	60S acidic ribosomal protein P0
GUSB	Beta-glucuronidase
HPRT1	Hypoxanthine-guanine phosphoribosyltransferase

Product Description

Normalize real time PCR results to multiple housekeepers rather than one for more accurate quantitation

Perform up to 1000 assays (based on 20 µl assay volume) for each housekeeping gene

Includes primer sets for ACTB, B2M, GAPD, GUSB, HPRT1, PGK, PP1A, RPL13A, TBP and TFRC.

Problems with housekeeping gene panels

β-Actin and GAPDH housekeeping gene expression in asthmatic airways is variable and not suitable for normalising mRNA levels

E M Glare, M Divjak, M J Bailey, E H Walters

Thorax 2002;57:765–770

Identification and Validation of Endogenous Reference Genes for Expression Profiling of T Helper Cell Differentiation by Quantitative Real-Time RT-PCR[1]

H. K. Hamalainen,*,[2] J. C. Tubman,† S. Vikman,* T. Kyrölä,* E. Ylikoski,* J. A. Warrington,‡ and R. Lahesmaa*

"Our initial plan to use the expression level of GAPDH in normalizing the results failed, because the mRNA expression of GAPDH underwent significant changes during the cell culture."

Validation of housekeeping genes for normalizing RNA expression in real-time PCR

Keertan Dheda[1], Jim F. Huggett[1], Stephen A. Bustin[2], Margaret A. Johnson[1], Graham Rook[1], and Alimuddin Zumla[1]

BioTechniques 37:112-119 (July 2004)

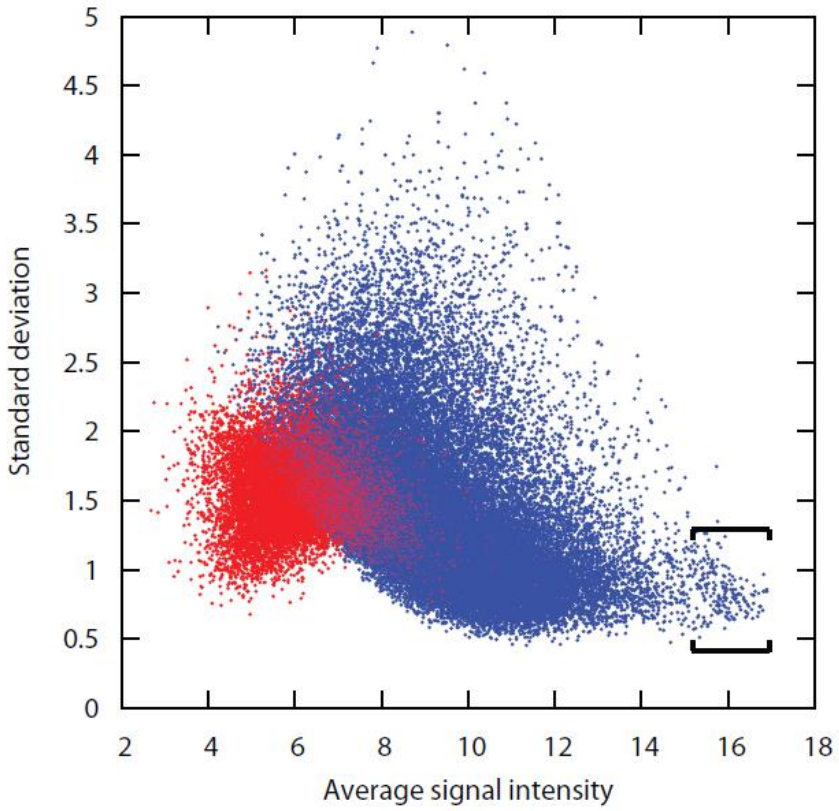
"None of the commonly used housekeeping genes (…) were found to be suitable as internal references, as they were highly variable (>30-fold maximal variability)."

Does “expression stability“ really exist?

- How variable can transcriptional regulation be?
- Is expression stability a global property or is it locally defined? (e.g. under given biological contexts or controlled conditions)
- How conserved is expression stability across related tissues?
- How conserved is expression stability between related species?

Hypothesis 1 - non-unversality

- No genes are „universally stable“

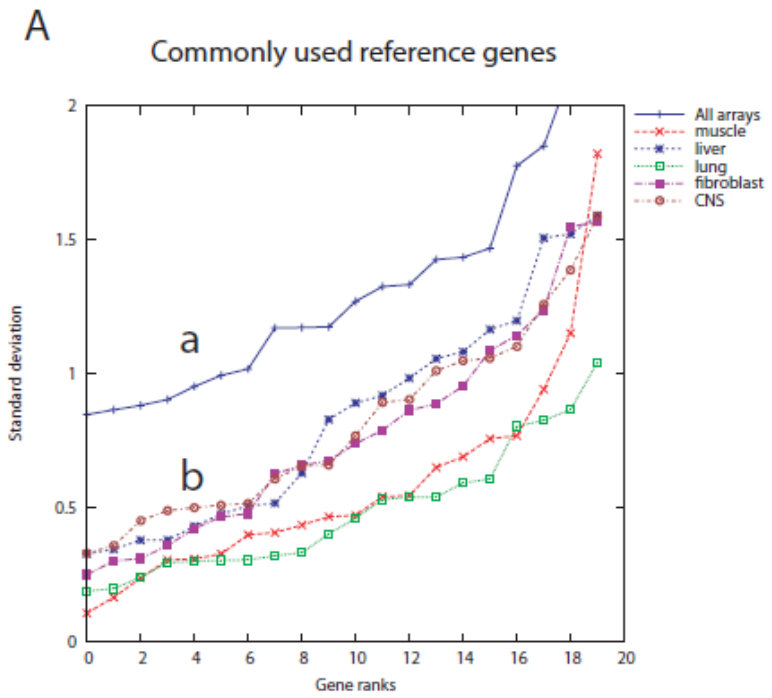


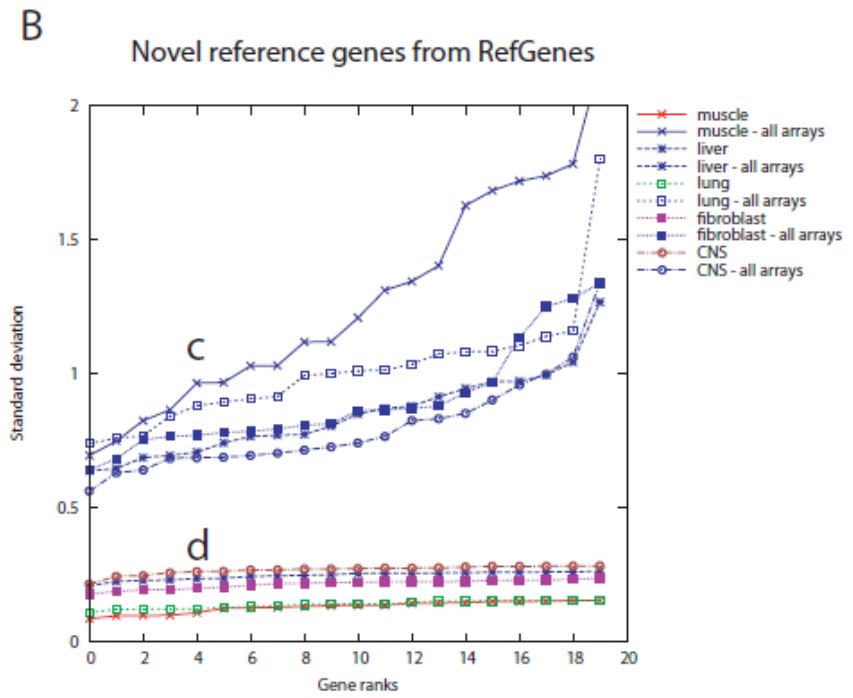
5014 microarrays
(Affymetrix Human133 2.0)

GAPDH, ACTB, B2M, PPIA, EIF1, ACTG1,
UBC, EEF1G, TUBA1B, EEF1A1, TPT1

Hypothesis 2 – context specificity

- For each experimental context, a set of genes exists that is more stable in this context than genes that are most stable over all conditions





HSP90AB1, TFRC, B2M, NONO, GUSB, UBC, ACTB, H2AFZ, POLR2A, TUBB4, HIST2H2AA1, RPL22, GAPDH, YWHAZ, CANX, CYC1, SDHA, EIF4A2, ATP5B, and EEF1E1

Hypothesis 2 – context specificity

- Experimental validation of context-specific reference genes

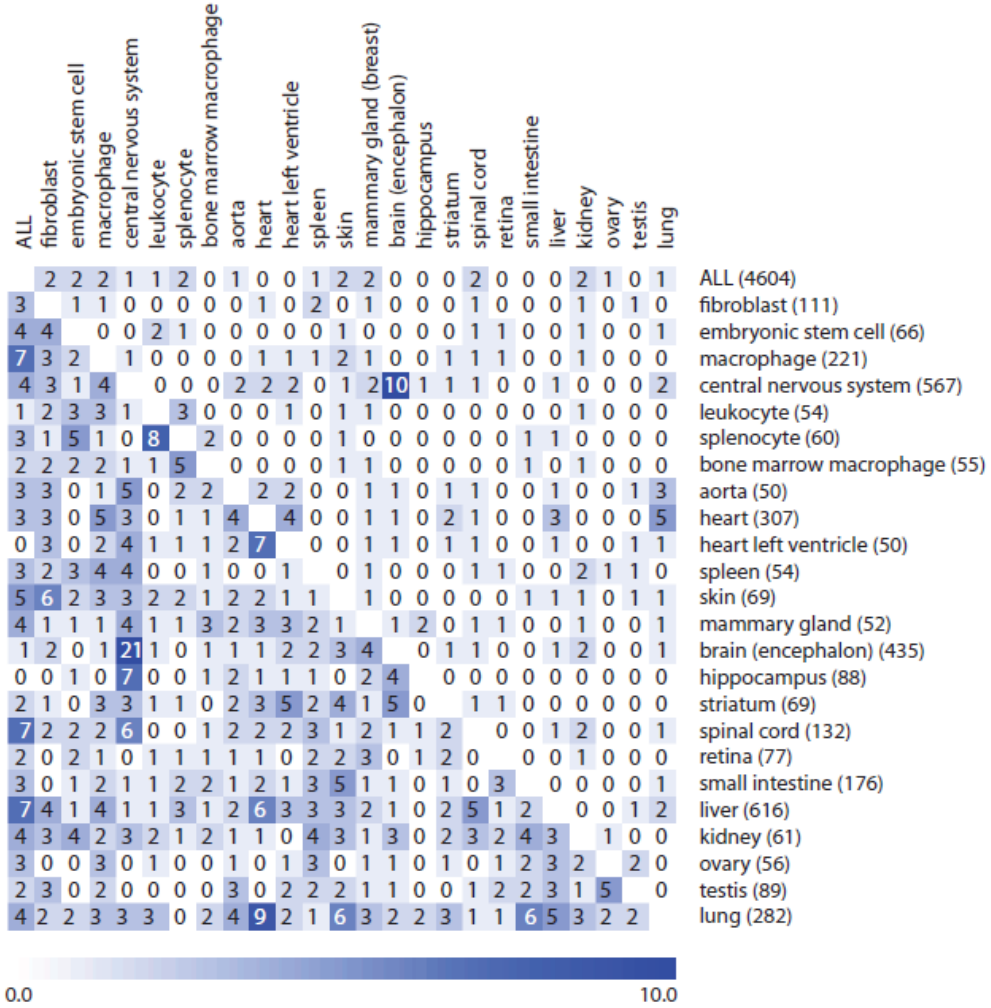
		Rank of the average expression stability values of remaining reference genes								Mean values for		
	Samples	1	2	3	4	5	6	7	8	Top 3 genes	RefGenes candidates	Common ref. genes
SPECIFIC TISSUES												
Mouse liver	16	GAK	SRP72	mRpL16	VPS4A	ACTB	HPRT	GAPDH	TUBB			
GeNorm (Avg M)		*0.15*	*0.15*	*0.17*	*0.19*	*0.21*	*0.24*	*0.27*	*0.30*	*0.16*	*0.17*	*0.26*
Mean Ct		25.02	24.68	26.56	26.91	20.47	25.09	19.50	24.41	25.42	25.79	22.37
Arabidopsis seedling	16	At3g24160	At1g13320	At3g27820	GADPH	ACTB	UBQ10					
GeNorm (Avg M)		*0.19*	*0.19*	*0.22*	*0.25*	*0.28*	*0.32*			*0.20*	*0.20*	*0.28*
Mean Ct		20.23	21.04	21.47	17.74	17.51	17.73			20.91	20.91	17.66
Arabidopsis leaf	16	At3g01150	GAPDH	At3g61710	ACTB	At1g32050	UBQ10					
GeNorm (Avg M)		*0.16*	*0.16*	*0.31*	*0.42*	*0.50*	*0.63*			*0.21*	*0.32*	*0.40*
Mean Ct		26.65	21.07	25.80	21.66	20.03	23.63			24.51	*24.16*	22.12
Arabidopsis apex	10	At2g17390	AT3G17920	At5g51880	ACTB	GADPH	UBQ10					
GeNorm (Avg M)		*0.11*	*0.11*	*0.15*	*0.20*	*0.22*	*0.49*			*0.12*	*0.12*	*0.30*
Mean Ct		18.89	23.14	22.22	17.91	17.92	21.86			21.42	21.42	19.23
RELATED TISSUES FROM SAME ORGANISM *(RefGenes search included B-lymphocytes and related tissues; qRT-PCR done on B-lymphocytes)*												
Human LCL + related	16	EIF4EBP2	INTS4	SDHA	GAPD	YWHAZ	B2M	ZNF410	BUD13			
GeNorm (Avg M)		*0.08*	*0.08*	*0.12*	*0.14*	*0.16*	*0.17*	*0.18*	*0.20*	*0.09*	*0.14*	*0.15*
Mean Ct		23.64	26.10	23.52	16.65	21.10	15.79	24.38	26.07	*24.42*	*25.05*	*19.27*
SAME TISSUE FROM RELATED ORGANISM *(RefGenes identified genes from mouse liver data; orthologs were used for qRT-PCR in other species)*												
Cattle liver	42	VPS4A	GAK	ACTB	PMPCA	UBQ	GAPDH					
GeNorm (Avg M)		*0.25*	*0.25*	*0.27*	*0.29*	*0.32*	*0.35*			*0.26*	*0.26*	*0.31*
Mean Ct		16.20	17.05	11.99	17.45	11.52	13.32			15.08	16.90	12.28
Pig liver	48	Histone H3	UBQ	VPS4A	GAK	GAPDH	PMPCA					
GeNorm (Avg M)		*0.29*	*0.29*	*0.30*	*0.32*	*0.34*	*0.36*			*0.29*	*0.33*	*0.31*
Mean Ct		13.10	9.68	17.41	16.98	16.87	18.08			13.40	17.49	13.22

Take home message 1

There are no universal reference genes.
For each condition, a set of specific reference genes exists which are more suitable for normalization than “housekeeping” genes.

Hypothesis 3 – stability in related tissues

- Expression stability is similar between related tissues in the same organism



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Hypothesis 3 – stability in related tissues

- Expression stability is similar between related tissues in the same organism

		Rank of the average expression stability values of remaining reference genes								Mean values for		
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Mean Ct		25.02	24.68	26.56	26.91	20.47	25.09	19.50	24.41	25.42	25.79	22.37
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GeNorm (Avg M)		*0.19*	*0.19*	*0.22*	*0.25*	*0.28*	*0.32*			*0.20*	*0.20*	*0.28*
Mean Ct		20.23	21.04	21.47	17.74	17.51	17.73			20.91	20.91	17.66
Arabidopsis leaf	16	At3g01150	GAPDH	At3g61710	ACTB	At1g32050	UBQ10					
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Mean Ct		26.65	21.07	25.80	21.66	20.03	23.63			24.51	*24.16*	22.12
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Mean Ct		18.89	23.14	22.22	17.91	17.92	21.86			21.42	21.42	19.23
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Mean Ct		23.64	26.10	23.52	16.65	21.10	15.79	24.38	26.07	*24.42*	*25.05*	*19.27*
SAME TISSUE FROM RELATED ORGANISM *(RefGenes identified genes from mouse liver data; orthologs were used for qRT-PCR in other species)*												
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Mean Ct		16.20	17.05	11.99	17.45	11.52	13.32			15.08	16.90	12.28
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Mean Ct		13.10	9.68	17.41	16.98	16.87	18.08			13.40	17.49	13.22

Hypothesis 4 – stability of gene orthologs

- Expression stability is similar between gene orthologs from related species

		Rank of the average expression stability values of remaining reference genes								Mean values for		
	Samples	1	2	3	4	5	6	7	8	Top 3 genes	RefGenes candidates	Common ref. genes
SPECIFIC TISSUES												
Mouse liver	16	GAK	SRP72	mRpL16	VPS4A	ACTB	HPRT	GAPDH	TUBB			
GeNorm (Avg M)		*0.15*	*0.15*	*0.17*	*0.19*	*0.21*	*0.24*	*0.27*	*0.30*	*0.16*	*0.17*	*0.26*
Mean Ct		25.02	24.68	26.56	26.91	20.47	25.09	19.50	24.41	25.42	25.79	22.37
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Mean Ct		26.65	21.07	25.80	21.66	20.03	23.63			24.51	*24.16*	22.12
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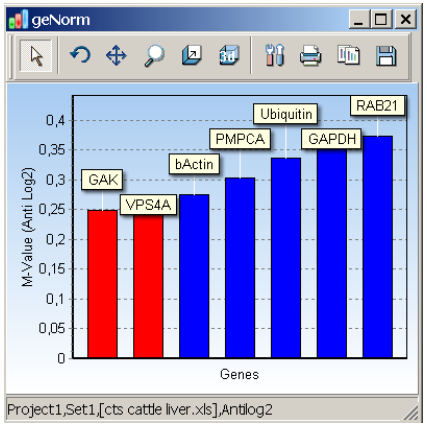
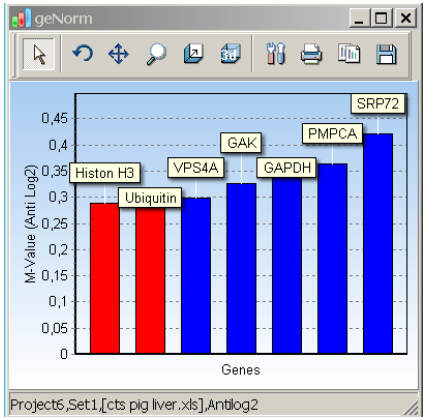
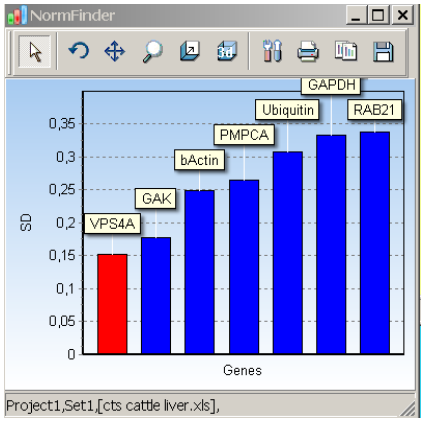
Mean Ct		13.10	9.68	17.41	16.98	16.87	18.08			13.40	17.49	13.22

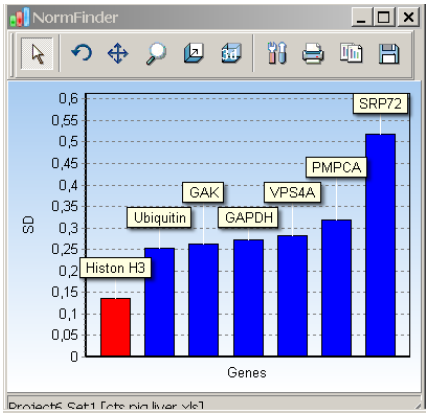
Hypothesis 4 – validation in bovine and pig





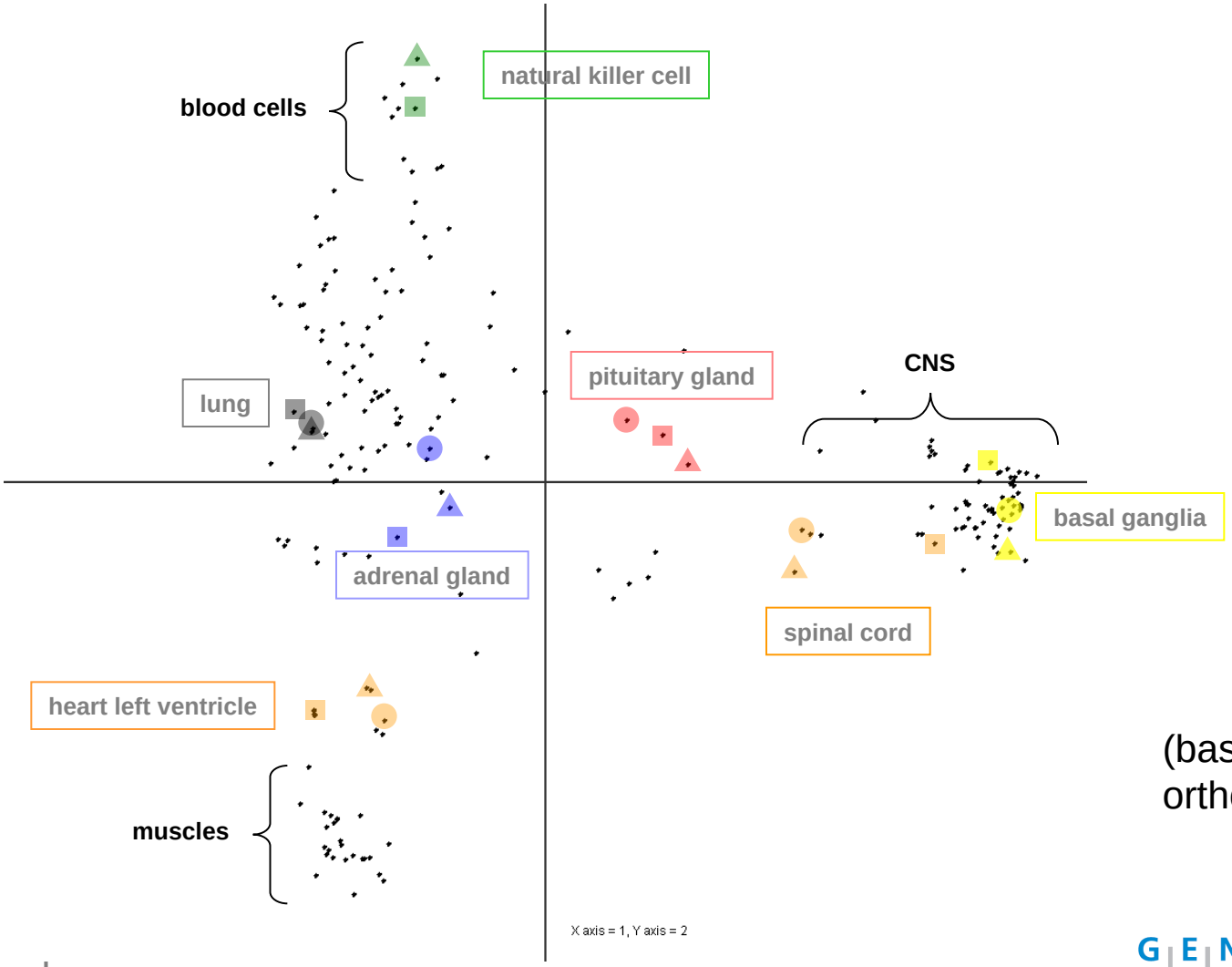


Bovine



Pig

Tissue specific gene expression



rat
mouse
human

(based on 693 orthologous genes)

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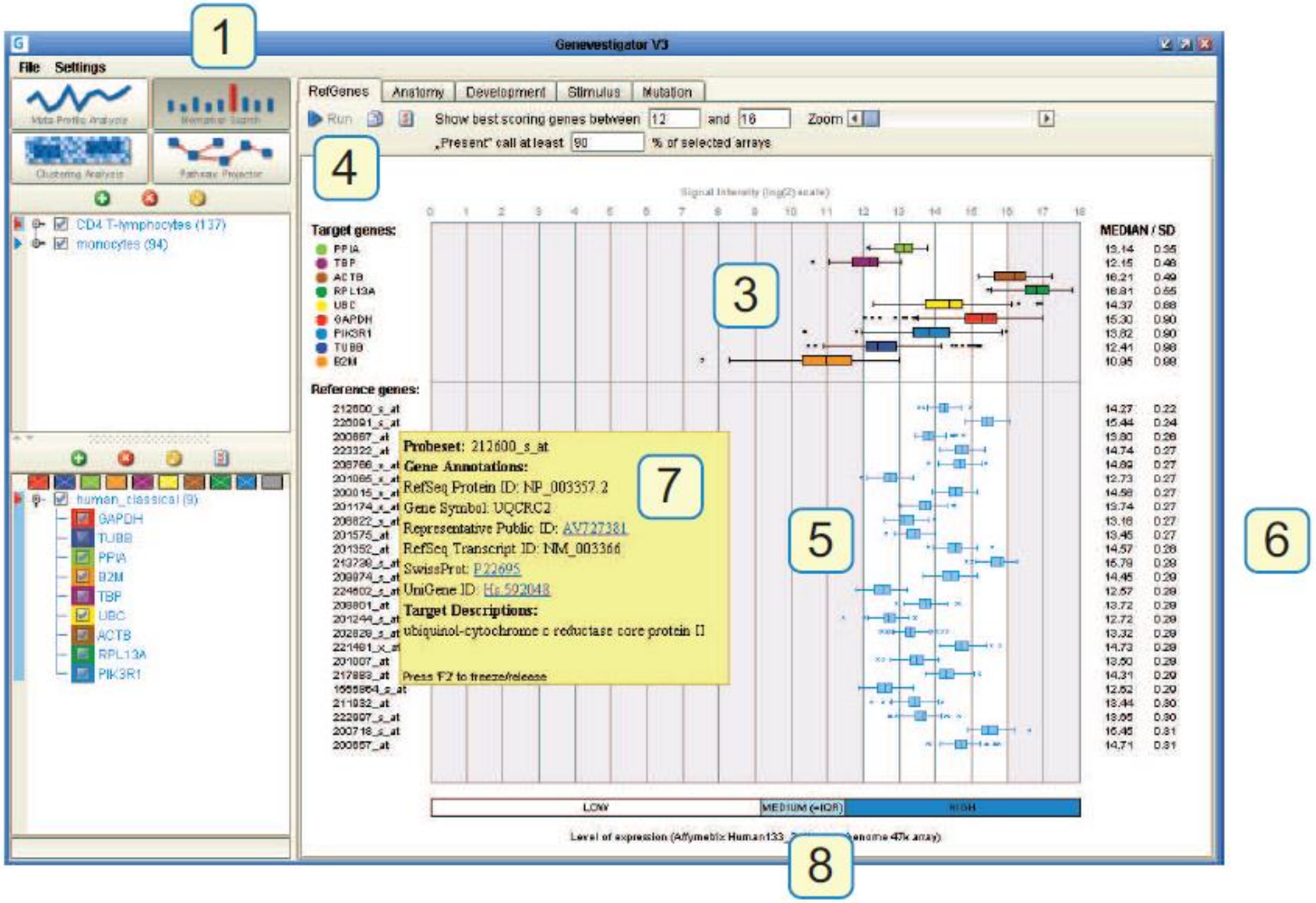
Take home message 2

The choice of reference genes should be based on

expression evidence collected from similar biological contexts

and not on general assumptions about the stability of genes


1
2
3
4
5
6
7
8
Genevestigator V3
File
Settings
RefGenes
Anatomy
Development
Stimulus
Mutation
Run
Show best scoring genes between
12
and
16
Zoom
„Present" call at least
90
% of selected arrays
CD4 T-lymphocytes (137)
monocytes (94)
human_classical (9)
GAPDH
TUBB
PPIA
B2M
TBP
UBC
ACTB
RPL13A
PIK3R1
Target genes:
PPIA
TBP
ACTB
RPL13A
UBC
GAPDH
PIK3R1
TUBB
B2M
Reference genes:
MEDIAN / SD
Probeset: 212600_s_at
Gene Annotations:
RefSeq Protein ID: NP_003357.2
Gene Symbol: UQCRC2
Representative Public ID: AV727381
RefSeq Transcript ID: NM_003366
SwissProt: P22695
UniGene ID: Hs.592048
Target Descriptions:
ubiquinol-cytochrome c reductase core protein I
Press 'F2' to freeze/release
LOW
MEDIUM (+IQR)
HIGH

Acknowledgements

- ETH Zurich
  - Prof. Wilhelm Gruissem
  - Prof. Peter Widmayer
  - Tomas Hruz
  - Markus Wyss
  - Lorenzo Borghi
  - Philip Zimmermann
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  - Stefan Bleuler
  - Oliver Laule
- KTI/CTI
  - Swiss Commission for Technology and Innovation
- TU München
  - Michael Pfaffl
  - Sabine Masanetz
- University of Geneva
  - Mylène Docquier
  - Patrick Descombes
- University of Western Australia
  - Phebe Verbrugge
  - Luba Kalayjieva

KTI/CTI
DIE FÖRDERAGENTUR FÜR INNOVATION
L'AGENCE POUR LA PROMOTION DE L'INNOVATION
L'AGENZIA PER LA PROMOZIONE DELL'INNOVAZIONE
THE INNOVATION PROMOTION AGENCY

UNIVERSITÉ DE GENÈVE

NEBION

TECHNISCHE UNIVERSITÄT MÜNCHEN

THE UNIVERSITY OF WESTERN AUSTRALIA

ETH
Eidgenössische Technische Hochschule Zürich
Swiss Federal Institute of Technology Zurich

GENEVESTIGATOR
shaping biological discovery

Additional slides

Validation of reference genes using Genevestigator