Evaluation of 10 feline reference genes for the investigation of viral RNA loads in tissues and blood samples

Kessler Yvonne[1], Hungerbuehler Anne Katrin[1], Meli Marina[1], Cattori Valentino[1], Ossent Pete[2], Riond Barbara[1], Lutz Hans[1], and Hofmann-Lehmann Regina[1]

[1] Clinical Laboratory,Vetsuisse Faculty, University of Zurich, Zurich, Switzerland

[2] Institute of Veterinary Pathology, Vetsuisse Faculty, University of Zurich, Zurich, Switzerland

University of Bern | University of Zurich
vetsuisse-faculty

Introduction

Gene expression analysis is a very important tool in today's research, with quantitative real-time reverse transcriptase PCR as the method of choice for reliable and fast quantification of mRNA transcription levels. Accurate quantification of mRNA levels as well as of viral RNA loads in tissues of FeLV-infected cats require stable reference genes as an internal control. The expression levels of these genes should ideally not undergo tissue-specific and experimental-dependent variation. Several publications in the recent past emphasize the need for more than one reference gene for exact analysis of mRNA transcription levels because so far no single reference gene has been found to be stable in all tissues and under all experimental conditions.

Aims of the study

- to develop and optimize Taqman real time PCR assays for potential reference gene assays for the feline species
- to evaluate the assays for different tissues and for blood samples
- to apply these assays for the quantification of FeLV RNA loads in tissues from FeLV-infected cats

Material and methods

Commonly used reference genes in the human, canine and feline species were point of origin for further investigation of their suitability as internal control genes in the cat. Eight new assays were designed and investigated:

- Primer and probe sets spanning different exons
- Presence of pseudogenes
- Detection of genomic DNA
- Optimization of the assays
- Application in blood, salivary gland, lymphoid, gastrointestinal, hepatic, neuronal, hormonal, muscular and neoplastic tissues
- Analysis of gene expression with the software programs geNorm and NormFinder

Table 1: Details of assays

Symbol	Gene Name	Gene Function	Accession Number	Amplicon size (bp)	Detection of gDNA	Exon Fv	Exon Rv	Exon Pr	Intron size (bp)	Pseudo-genes
ABL	v-abl Abelson murine leukemia viral oncogene homolog	Protein kinase; regulation of cell cycle, mismatch repair, DNA damage response	ENSFCAT00000005306	83	Yes	ex2	ex3	ex2/3	12000 [1]	No
ACTB	ß - actin	Cytoskeletal structural protein	AB051104.1	127	Yes	ex3	ex4	ex4	442 [3]	Yes
B2M	ß-2-microglobulin	Major histocompatibility complex antigen class I receptor activity	NM_001009876	84	No	ex1	ex2	ex1	3187 [1]	No
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	Glycolytic enzyme	AF097177	82	Yes	ex1	ex2	ex1/2	NA	Yes
GUSB	ß- glucuronidase	Glycoside hydrolase (carbohydrate metabolism)	NM_001009310	80	Yes	ex4	ex5	ex5	450 [2]	Yes
HMBS	Hydroxymethyl-bilane synthase *Alias:* Porphobilinogen deaminase (PBGD)	Heme synthesis, porphyrin metabolism	ENSFCAG00000001160	94	Yes	ex3	ex4	ex3/4	554 [2]	Yes
HPRT	Hypoxanthine phosphoribosyltransferase	Purin synthesis in salvage pathway	EF453697	100	Yes	ex4/5	ex6	ex6	>3400 [3]	Yes
RPS7	Ribosomal protein S7	Ribosomal protein	NM 001009832	74	Yes	ex4/5	ex5	ex5	2207[3]	Yes
YWHAZ	Tyrosine 3-monooxygenase *Alias:* tryptophan 5-monooxygenase activation protein, zeta polypeptide *Alias:* Phospholipase A2	Mediator of signal transduction	EF458621	84	Yes	ex4	ex5	ex4/5	>2800 [2]	Yes
18s rRNA		Ribosomal RNA	X03205	187	Yes	NA	NA	NA	NA	NA

[1] according to Ensemble
[2] according to human sequences
[3] according to Penning et al.; 2007
[4] TaqMan® Gene Expression Assay (Applied Biosystems)

Results - validation of gene expression in blood

geNorm: pairwise comparison approach, ranks the genes according to the similarity of their expression profiles

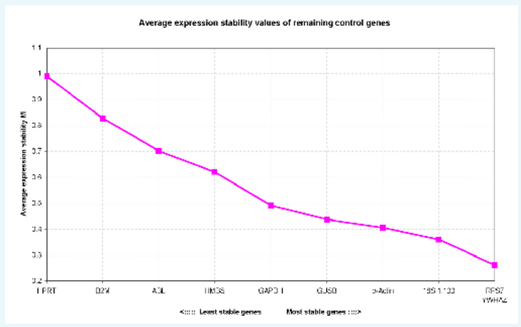

Fig 1: geNorm; 7 blood samples from SPF cats

M= gene expression stability; genes with the lowest M values have the most stable expression

a RPS7/YWHAZ are the two most stable genes in blood

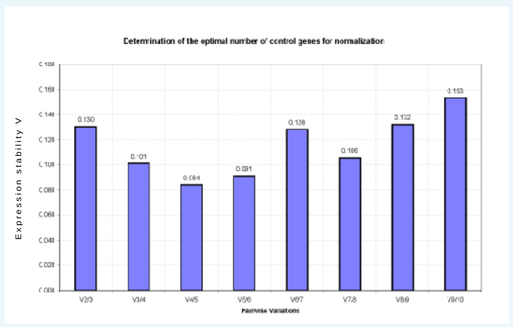

Fig 2: geNorm; 7 blood samples from SPF cats

V= pairwise variation between the genes; the lower variable V, the less variation

a The calculated normalization factor for blood should at least contain the best two reference genes

NormFinder: model-based approach, top ranks the reference genes with minimal estimated intra- and intergroup variation

Table 2: NormFinder; Blood samples from 7 SPF cats

Ranking order	Gene	Stability value
1	b-Actin	0.103
2	GAPDH	0.117
3	18S	0.129
4	YWHAZ	0.199
5	B2M	0.237
6	GUSB	0.280
7	ABL	0.299
8	HMBS	0.622
9	HPRT	0.755
10	RPS7	0.774

A low stability value corresponds to a high expression stability of the respective gene.

Best suited gene for blood: b-Actin

Results - validation of gene expression in liver

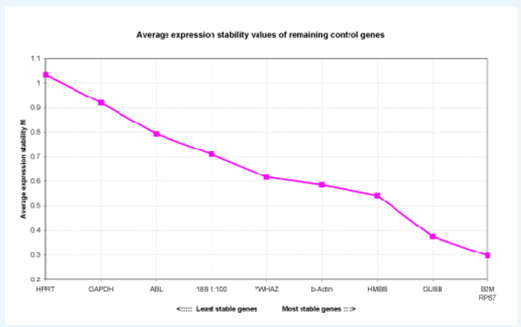

Fig 3: geNorm; liver tissue from 8 cats

B2M/RPS7 are the two most stable genes in liver tissue

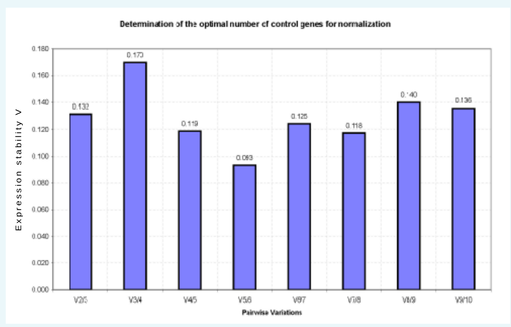

Fig 4: geNorm; liver tissue from 8 cats

The calculated normalization factor for liver tissue should at least contain the best two reference genes, the optimal number would be 5

Table 3: NormFinder; liver tissue from 8 cats

Ranking order	Gene	Stability value
1	B2M	0.192
2	RPS7	0.204
3	HMBS	0.251
4	b-Actin	0.334
5	GUSB	0.401
6	YWHAZ	0.403
7	ABL	0.586
8	18S	0.609
9	GAPDH	0.896
10	HPRT	0.928

A low stability value corresponds to a high expression stability of the respective gene.

Best suited gene for liver tissue: B2M

Results - validation of gene expression all tissues combined

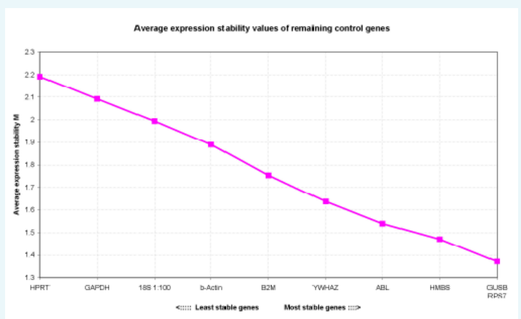

Fig 5 geNorm; all tissues combined (15 cats)

Table 4: NormFinder; all tissues combined (15 cats)

Ranking order	Gene	Stability value
1	RPS7	0.425
2	HMBS	0.808
3	GUSB	0.836
4	ABL	0.878
5	YWHAZ	0.919
6	B2M	1.081
7	b-Actin	1.285
8	GAPDH	1.361
9	18S	1.405
10	HPRT	1.491

RPS7/GUSB are the two most stable genes in all tissues combined

Best suited gene for all tissues combined: RPS7

Conclusions:

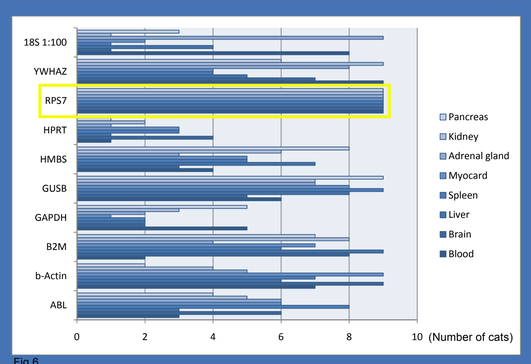

Fig 6

- Every tissue has to be analyzed separately, as there exists no single reference gene which is suitable for each and every tissue!
- If only one reference gene can be applied, RPS7 seems to yield the best results

References

Andersen CL, Jensen JL, Ørntoft TF.
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 Res. 2004 Aug 1;64(15):5245-50.
PMID: 15289330 [PubMed - indexed for MEDLINE]

Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A, Speleman F.
Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes.
Genome Biol. 2002 Jun 18;3(7):RESEARCH0034. Epub 2002 Jun 18.
PMID: 12184808 [PubMed - indexed for MEDLINE]

Lyng MB, Laenkholm AV, Pallisgaard N, Ditzel HJ.
Identification of genes for normalization of real-time RT-PCR data in breast carcinomas.
BMC Cancer. 2008 Jan 22;8:20.
PMID: 18211679 [PubMed - indexed for MEDLINE]

Contact: ykessler@vetclinics.uzh.ch; Winterthurerstr. 260, CH-8057 Zurich; phone n°: +41- 446358267