

SLqPCR: Functions for analysis of real-time quantitative PCR data at SIRS-Lab GmbH

Dr. Matthias Kohl
SIRS-Lab GmbH (Jena, Germany)



October 22, 2008

Contents

1	Introduction	1
2	Selection of most stable reference/housekeeping genes	1
3	Normalization by geometric averaging	7

1 Introduction

The package "SLqPCR" was designed for the analysis of real-time quantitative RT-PCR data. In this short vignette we describe and demonstrate the available functions.

2 Selection of most stable reference/housekeeping genes

We describe the selection of the best (most stable) reference/housekeeping genes using method and data set of Vandesompele et al (2002) [1] (in the sequel: Vand02). We load library and data

```
> library(SLqPCR)
> data(vandesompele)
> str(vandesompele)
```

```
'data.frame':      85 obs. of  10 variables:
 $ ACTB   : num  0.0425 0.0192 0.1631 0.5726 0.037 ...
```

```

$ B2M    : num  0.0576 0.0194 0.2956 1 0.0444 ...
$ GAPD   : num  0.1547 0.0703 0.7733 1 0.1192 ...
$ HMBS   : num  0.11 0.088 0.405 0.797 0.208 ...
$ HPRT1  : num  0.118 0.0708 0.5575 1 0.1304 ...
$ RPL13A : num  0.0742 0.0441 0.3481 0.5707 0.1078 ...
$ SDHA   : num  0.203 0.14 0.447 0.974 0.214 ...
$ TBP    : num  0.19 0.106 0.469 1 0.201 ...
$ UBC    : num  0.0992 0.0368 0.3401 0.598 0.0759 ...
$ YWHAZ  : num  0.1032 0.0393 0.3588 0.7863 0.1002 ...

```

We start by ranking the selected reference/housekeeping genes. The function `selectHKgenes` proceeds stepwise; confer Section “Materials and methods” in Vand02. That is, the gene stability measure M of all candidate genes is computed and the gene with the highest M value is excluded. Then, the gene stability measure M for the remaining gene is calculated and so on. This procedure is repeated until two respectively `minNrHK` is reached.

```

> tissue <- as.factor(c(rep("BM", 9), rep("POOL", 9), rep("FIB",
+   20), rep("LEU", 13), rep("NB", 34)))
> res.BM <- selectHKgenes(vandesompele[tissue == "BM", ], method = "Vandesompele",
+   geneSymbol = names(vandesompele), minNrHK = 2, trace = TRUE,
+   na.rm = TRUE)

```

```
#####
```

Step 1 :

gene expression stability values M :

HPRT1	YWHAZ	RPL13A	UBC	GAPD	SDHA	TBP	HMBS
0.5160313	0.5314564	0.5335963	0.5700961	0.6064919	0.6201470	0.6397969	0.7206013
B2M	ACTB						
0.7747634	0.8498739						

average expression stability M : 0.6362855

gene with lowest stability (largest M value): ACTB

Pairwise variation, (9 / 10): 0.076469

```
#####
```

Step 2 :

gene expression stability values M :

HPRT1	RPL13A	YWHAZ	UBC	GAPD	SDHA	TBP	HMBS
0.4705664	0.5141375	0.5271169	0.5554718	0.5575295	0.5738460	0.6042110	0.6759176
B2M							
0.7671985							

average expression stability M : 0.5828883

gene with lowest stability (largest M value): B2M

Pairwise variation, (8 / 9): 0.07765343

#####

Step 3 :

gene expression stability values M:

HPRT1	RPL13A	SDHA	YWHAZ	UBC	GAPD	TBP	HMBS
0.4391222	0.4733732	0.5243665	0.5253471	0.5403137	0.5560120	0.5622094	0.6210820

average expression stability M: 0.5302283

gene with lowest stability (largest M value): HMBS

Pairwise variation, (7 / 8): 0.067112

#####

Step 4 :

gene expression stability values M:

HPRT1	RPL13A	YWHAZ	UBC	SDHA	GAPD	TBP
0.4389069	0.4696398	0.4879728	0.5043292	0.5178634	0.5245346	0.5563591

average expression stability M: 0.4999437

gene with lowest stability (largest M value): TBP

Pairwise variation, (6 / 7): 0.06813202

#####

Step 5 :

gene expression stability values M:

HPRT1	RPL13A	UBC	YWHAZ	GAPD	SDHA
0.4292808	0.4447874	0.4594181	0.4728920	0.5012107	0.5566762

average expression stability M: 0.4773775

gene with lowest stability (largest M value): SDHA

Pairwise variation, (5 / 6): 0.08061944

#####

Step 6 :

gene expression stability values M:

UBC	RPL13A	HPRT1	YWHAZ	GAPD
0.4195958	0.4204997	0.4219179	0.4424631	0.4841646

average expression stability M: 0.4377282

gene with lowest stability (largest M value): GAPD

Pairwise variation, (4 / 5): 0.08416531

#####

Step 7 :

gene expression stability values M:

RPL13A	UBC	YWHAZ	HPRT1
0.3699163	0.3978736	0.4173706	0.4419220

average expression stability M: 0.4067706

gene with lowest stability (largest M value): HPRT1

Pairwise variation, (3 / 4): 0.09767827

#####

Step 8 :

gene expression stability values M:

UBC RPL13A YWHAZ
0.3559286 0.3761358 0.3827933

average expression stability M: 0.3716192

gene with lowest stability (largest M value): YWHAZ

Pairwise variation, (2 / 3): 0.1137450

#####

Step 9 :

gene expression stability values M:

RPL13A UBC
0.3492712 0.3492712

average expression stability M: 0.3492712

```
> res.POOL <- selectHKgenes(vandesompele[tissue == "POOL", ], method = "Vandesompele",
+   geneSymbol = names(vandesompele), minNrHK = 2, trace = FALSE,
+   na.rm = TRUE)
> res.FIB <- selectHKgenes(vandesompele[tissue == "FIB", ], method = "Vandesompele",
+   geneSymbol = names(vandesompele), minNrHK = 2, trace = FALSE,
+   na.rm = TRUE)
> res.LEU <- selectHKgenes(vandesompele[tissue == "LEU", ], method = "Vandesompele",
+   geneSymbol = names(vandesompele), minNrHK = 2, trace = FALSE,
+   na.rm = TRUE)
> res.NB <- selectHKgenes(vandesompele[tissue == "NB", ], method = "Vandesompele",
+   geneSymbol = names(vandesompele), minNrHK = 2, trace = FALSE,
+   na.rm = TRUE)
```

We obtain the following ranking of genes (cf. Table 3 in Vand02)

```
> ranks <- data.frame(c(1, 1:9), res.BM$ranking, res.POOL$ranking,
+   res.FIB$ranking, res.LEU$ranking, res.NB$ranking)
> names(ranks) <- c("rank", "BM", "POOL", "FIB", "LEU", "NB")
> ranks
```

	rank	BM	POOL	FIB	LEU	NB
1	1	RPL13A	GAPD	GAPD	UBC	GAPD
2	1	UBC	SDHA	HPRT1	YWHAZ	HPRT1
3	2	YWHAZ	HMBS	YWHAZ	B2M	SDHA
4	3	HPRT1	HPRT1	UBC	GAPD	UBC
5	4	GAPD	TBP	ACTB	RPL13A	HMBS
6	5	SDHA	UBC	TBP	TBP	YWHAZ
7	6	TBP	RPL13A	SDHA	SDHA	TBP

8	7	HMBS	YWHAZ	RPL13A	HPRT1	ACTB
9	8	B2M	ACTB	B2M	HMBS	RPL13A
10	9	ACTB	B2M	HMBS	ACTB	B2M

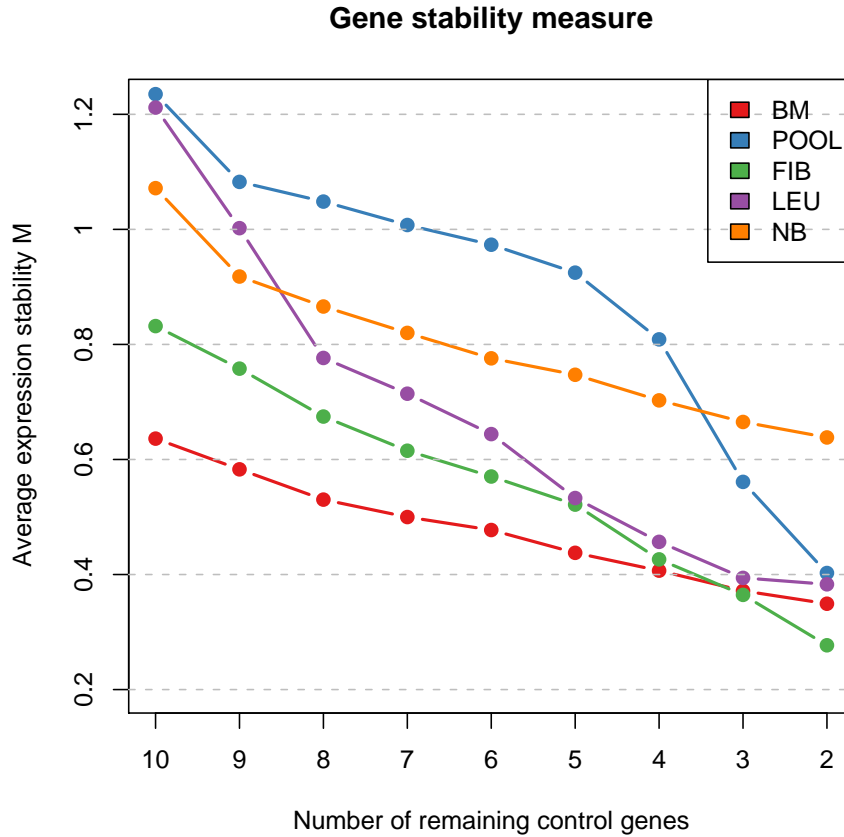
Remark 1:

- (a) Since the computation is based on gene ratios, the two most stable control genes in each cell type cannot be ranked.
- (b) In praxis the selection of reference/housekeeping genes may require an additional step which is the computation of relative quantities via `relQuantPCR`; e.g.

```
> exa1 <- apply(vandesompele[tissue == "BM", ], 2, relQuantPCR,
+             E = 2)
```

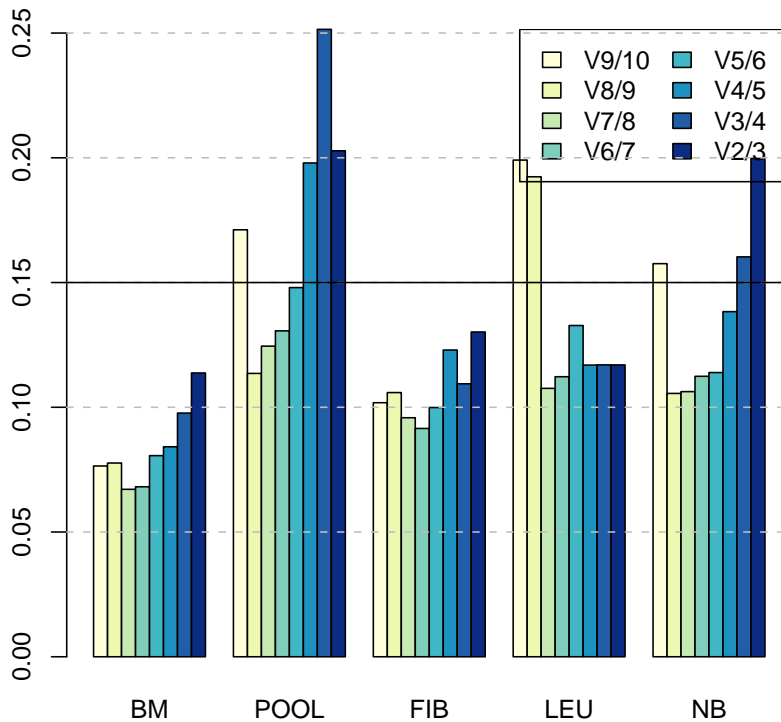
We plot the average expression stability M for each cell type (cf. Figure 2 in Vand02).

```
> library(RColorBrewer)
> mypalette <- brewer.pal(5, "Set1")
> matplot(cbind(res.BM$meanM, res.POOL$meanM, res.FIB$meanM, res.LEU$meanM,
+   res.NB$meanM), type = "b", ylab = "Average expression stability M",
+   xlab = "Number of remaining control genes", axes = FALSE,
+   pch = 19, col = mypalette, ylim = c(0.2, 1.22), lty = 1,
+   lwd = 2, main = "Gene stability measure")
> axis(1, at = 1:9, labels = as.character(10:2))
> axis(2, at = seq(0.2, 1.2, by = 0.2), labels = as.character(seq(0.2,
+   1.2, by = 0.2)))
> box()
> abline(h = seq(0.2, 1.2, by = 0.2), lty = 2, lwd = 1, col = "grey")
> legend("topright", legend = c("BM", "POOL", "FIB", "LEU", "NB"),
+   fill = mypalette)
```



Second, we plot the pairwise variation for each cell type (cf. Figure 3 (a) in Vand02)

```
> mypalette <- brewer.pal(8, "YlGnBu")
> barplot(cbind(res.BM$variation, res.POOL$variation, res.FIB$variation,
+   res.LEU$variation, res.NB$variation), beside = TRUE, col = mypalette,
+   space = c(0, 2), names.arg = c("BM", "POOL", "FIB", "LEU",
+   "NB"))
> legend("topright", legend = c("V9/10", "V8/9", "V7/8", "V6/7",
+   "V5/6", "V4/5", "V3/4", "V2/3"), fill = mypalette, ncol = 2)
> abline(h = seq(0.05, 0.25, by = 0.05), lty = 2, col = "grey")
> abline(h = 0.15, lty = 1, col = "black")
```



Remark 2:

Vand02 recommend a cut-off value of 0.15 for the pairwise variation. Below this bound the inclusion of an additional housekeeping gene is not required.

3 Normalization by geometric averaging

To normalize your data by geometric averaging of multiple reference/housekeeping genes you can proceed as follows

```
> data(SLqPCRdata)
> SLqPCRdata
```

	Gene1	Gene2	HK1	HK2
A1	26.6	25.6	12.8	18.5
A2	26.9	25.8	13.2	19.2
A3	27.4	26.1	13.1	19.2

```

A4  27.7  26.6  13.4  19.5
B1  26.7  25.8  12.9  18.8
B2  24.4  21.5  13.1  18.7
B3  26.5  24.6  12.9  18.7
B4  25.6  23.5  13.8  19.4
C1  28.8  26.6  13.1  19.1
C2  24.4  19.2  13.2  18.5
C3  28.3  25.1  12.9  18.6
C4  25.3  20.6  13.3  19.1
D1  29.3  26.5  12.9  19.0
D2  24.7  18.8  12.7  18.4
D3  27.3  21.1  13.0  18.6
D4  27.3  21.3  13.1  18.4

```

```
> (relData <- apply(SLqPCRdata, 2, relQuantPCR, E = 2))
```

	Gene1	Gene2	HK1	HK2
A1	0.21763764	0.008974206	0.9330330	0.9330330
A2	0.17677670	0.007812500	0.7071068	0.5743492
A3	0.12500000	0.006345722	0.7578583	0.5743492
A4	0.10153155	0.004487103	0.6155722	0.4665165
B1	0.20306310	0.007812500	0.8705506	0.7578583
B2	1.00000000	0.153893052	0.7578583	0.8122524
B3	0.23325825	0.017948412	0.8705506	0.8122524
B4	0.43527528	0.038473263	0.4665165	0.5000000
C1	0.04736614	0.004487103	0.7578583	0.6155722
C2	1.00000000	0.757858283	0.7071068	0.9330330
C3	0.06698584	0.012691444	0.8705506	0.8705506
C4	0.53588673	0.287174589	0.6597540	0.6155722
D1	0.03349292	0.004809158	0.8705506	0.6597540
D2	0.81225240	1.000000000	1.0000000	1.0000000
D3	0.13397168	0.203063099	0.8122524	0.8705506
D4	0.13397168	0.176776695	0.7578583	1.0000000

```
> geneStabM(relData[, c(3, 4)])
```

	HK1	HK2
	0.2574717	0.2574717

```
> (exprData <- normPCR(SLqPCRdata, c(3, 4)))
```

	Gene1	Gene2
A1	1.728585	1.663601

A2 1.689720 1.620623
A3 1.727684 1.645714
A4 1.713602 1.645553
B1 1.714500 1.656708
B2 1.558954 1.373669
B3 1.706201 1.583870
B4 1.564586 1.436241
C1 1.820707 1.681626
C2 1.561410 1.228651
C3 1.826986 1.620401
C4 1.587369 1.292483
D1 1.871526 1.692677
D2 1.615795 1.229836
D3 1.755636 1.356920
D4 1.758402 1.371940

References

- [1] Jo Vandesompele, Katleen De Preter, Filip Pattyn, Bruce Poppe, Nadine Van Roy, Anne De Paepe and Frank Speleman (2002). Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biology* 2002, 3(7):research0034.1-0034.11 <http://genomebiology.com/2002/3/7/research/0034/> 1