

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

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



April 30, 2008

## Contents

<a href="#">1 Introduction</a>	1
<a href="#">2 Selection of most stable reference/housekeeping genes</a>	1
<a href="#">3 Normalization by geometric averaging</a>	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 0.0370 ...
```

```

$ B2M : num 0.0576 0.0194 0.2956 1.0000 0.0444 ...
$ GAPD : num 0.1547 0.0703 0.7733 1.0000 0.1192 ...
$ HMBS : num 0.110 0.088 0.405 0.797 0.208 ...
$ HPRT1 : num 0.1180 0.0708 0.5575 1.0000 0.1304 ...
$ RPL13A: num 0.0742 0.0441 0.3481 0.5707 0.1078 ...
$ SDHA : num 0.203 0.140 0.447 0.974 0.214 ...
$ TBP : num 0.190 0.106 0.469 1.000 0.201 ...
$ UBC : num 0.0992 0.0368 0.3401 0.5980 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:**

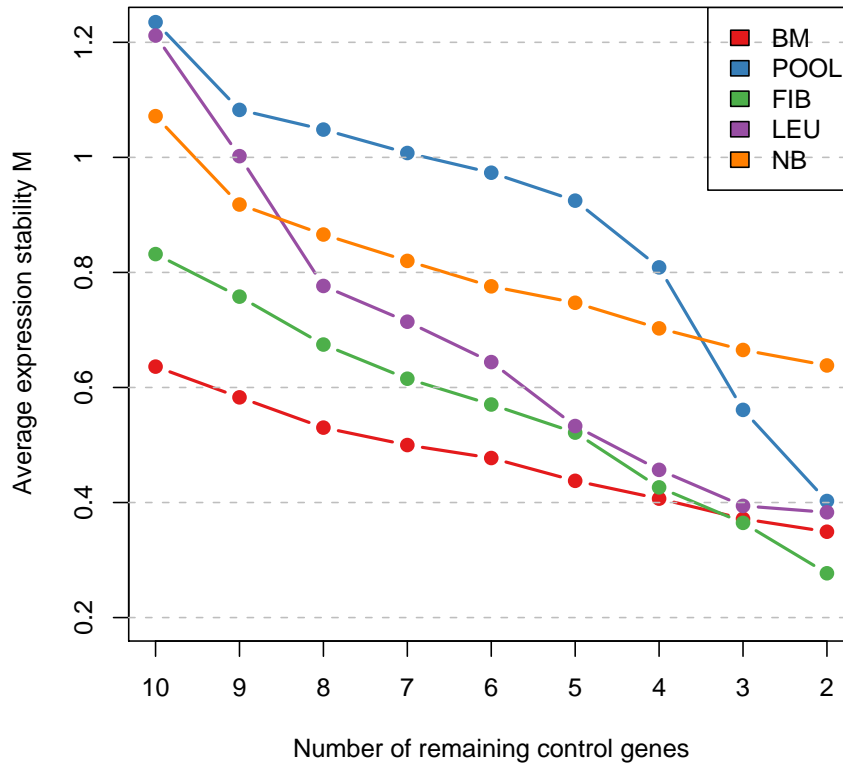
- (a) Since the computation is based on gene ratios, the two most stable control genes in each cell type cannot be ranked.
- (b) In praxis the selection of reference/housekeeping genes may require an additional step which is the computation of relative quantities via `relQuantPCR`; e.g.

```
> 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).

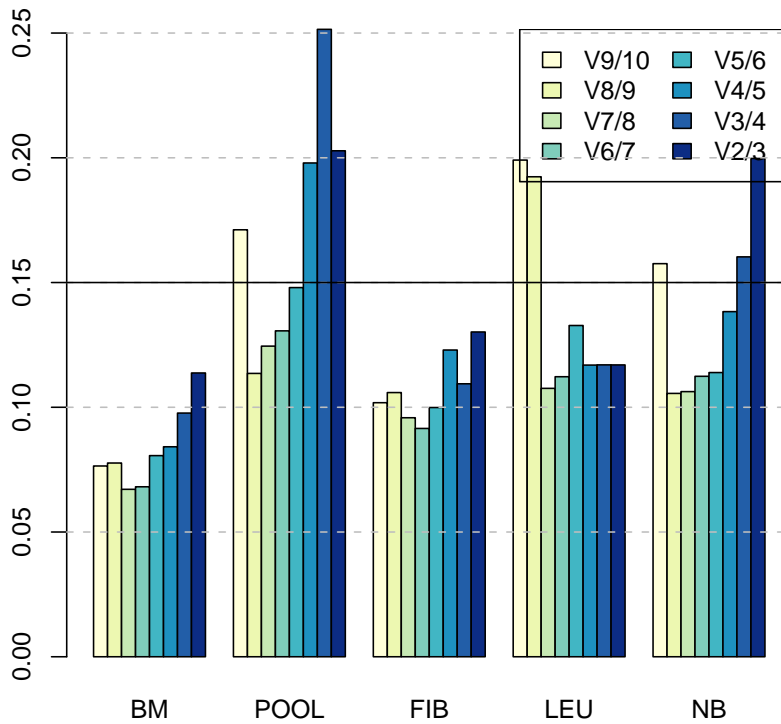
```
> library(RColorBrewer)
> mypalette <- brewer.pal(5, "Set1")
> matplot(cbind(res.BM$meanM, res.POOL$meanM, res.FIB$meanM, res.LEU$meanM,
+             res.NB$meanM), type = "b", ylab = "Average expression stability M",
+             xlab = "Number of remaining control genes", axes = FALSE,
+             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)
```

### Gene stability measure



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