

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

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



April 25, 2023

Contents

1	Introduction	1
2	Selection of most stable reference/housekeeping genes	1
3	Normalization by geometric averaging	6

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("N", 13)))
> res.BM <- selectHKgenes(vandesompele[tissue == "BM",], method = "Vandesompele", geneSymbol = "vandesompele")

```

```
#####
```

```
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 ):          0.07646901
```

```
#####
```

```
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 ):          0.113745
#####

```

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 = "RPL13A", E = 2)
> res.FIB <- selectHKgenes(vandesompele[tissue == "FIB",], method = "Vandesompele", geneSymbol = "UBC", E = 2)
> res.LEU <- selectHKgenes(vandesompele[tissue == "LEU",], method = "Vandesompele", geneSymbol = "YWHAZ", E = 2)
> res.NB <- selectHKgenes(vandesompele[tissue == "NB",], method = "Vandesompele", geneSymbol = "HPRT1", E = 2)

```

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.

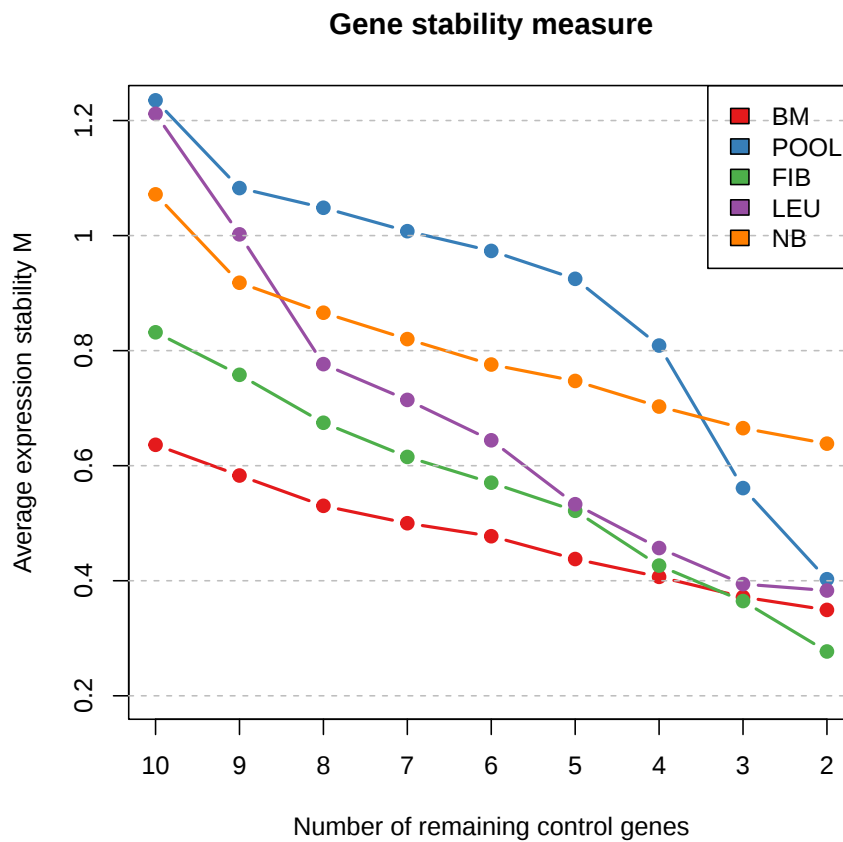
```

> 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).

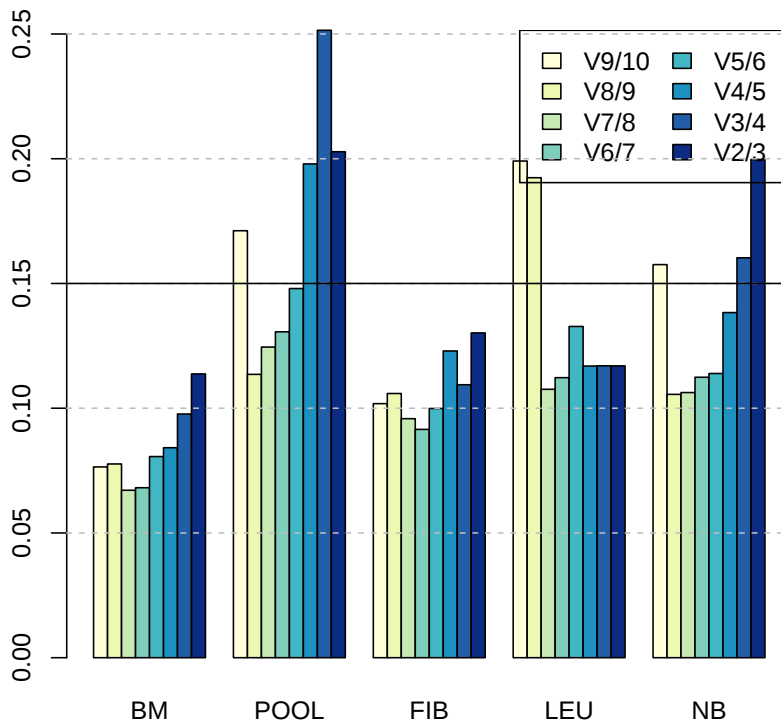
```
> library(RColorBrewer)
> mypalette <- brewer.pal(5, "Set1")
> matplot(cbind(res.BM$meanM, res.POOL$meanM, res.FIB$meanM, res.LEU$meanM, res.NB$meanM), t,
> axis(1, at = 1:9, labels = as.character(10:2))
> axis(2, at = seq(0.2, 1.2, by = 0.2), labels = as.character(seq(0.2, 1.2, by = 0.2)))
> box()
> 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),
> legend("topright", legend = c("V9/10", "V8/9", "V7/8", "V6/7", "V5/6", "V4/5", "V3/4", "V2/3"))
```

```
> 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>