

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

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

```

```
#####
```

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)
> res.FIB <- selectHKgenes(vandesompele[tissue == "FIB",], method = "Vandesompele", geneSymbol)
> res.LEU <- selectHKgenes(vandesompele[tissue == "LEU",], method = "Vandesompele", geneSymbol)
> res.NB <- selectHKgenes(vandesompele[tissue == "NB",], method = "Vandesompele", geneSymbol)

```

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",
> 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"), fill = mypalette)
```

```
> 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>