

highSCREEN: High-Throughput Screening for Plate Based Assays

Ivo D. Shterev * Cliburn Chan Gregory D. Sempowski

August 22, 2018

1 Introduction

This vignette describes the use of the R extension package `highSCREEN` for high throughput screening of small molecule compounds with activities measured on multi-well plates. The plate-based assay raw results can be any continuous value - for example, optical density (OD; in nanometers). Package functionalities include small molecule compound library screening data extraction and normalization, plate quality control (QC), identifying compounds that are hits according to defined criteria and visualization of compounds and controls. The framework supports `96-well` and `384-well` plate formats¹. The package is also capable of handling any number of replicates of the data. Currently, `highSCREEN` implements three different within plate quality control (QC) procedures which determine plate pass or fail. The package implements three different normalization methods, namely the `b-score`, the `c-score` (also known as percent degranulation) and the `z-score` normalization methods [1]. The user can also plot the density and histogram of controls which can be helpful in tuning the QC procedures.

2 Data Format

2.1 Plate Layout

The following plate formats are supported:

- 96-well plate. This format represents an 8×12 matrix in which the first and last columns represent control wells and columns two to ten represent compound wells.
- 384-well plate. This format represents an 16×24 matrix. The first and last two columns represent control wells and columns three to twenty two represent compound wells.
- 384-well plate composed of four 96-well plates. This format represents an 16×24 matrix. The first and last two columns represent control wells and columns three to twenty two represent compound wells. From this 384-well plate, four 96-well plates can be constructed as shown in Fig. 1.

*Correspondence: i.shterev@duke.edu

¹See section 2 for additional details on supported plate layouts.

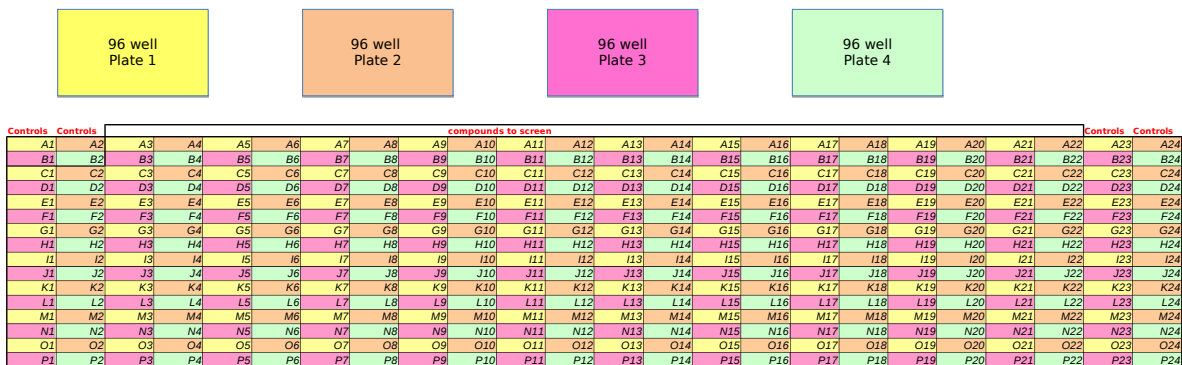


Figure 1: 384-well plate consisting of four 96-well plates.

2.2 Distribution of Controls

As mentioned in the previous subsection, it is assumed that the control wells are located either in the first and last plate columns (96-well plate), or in the first and last two plate columns (348-well plate) as shown in Fig. 1. The R package `highSCREEN` assumes that the plate contains positive and negative control wells. The package can also handle additional control types as specified by the user. The requirement is that in addition to the 384-well plate data the user also provides a control map. The map specifies the control type and its position in the control columns, and is used to identify the controls in the plate layout. There are two types of control maps, 96-well and 384-well plate control maps. The first column of the 384-well plate control map specifies the controls and their position in the first column of the 384-well plate. Similarly, the second, third and fourth control map columns correspond to the second, twenty third and twenty fourth 384-well plate columns, respectively. Similarly, the first and second columns of the 96-well plate control map specify the controls and their position in the first and second columns of the 96-well plate, respectively. In the example below, a 384-well plate contains five different types of controls, positive controls ("Control P"), negative controls ("Control N"), controls with low concentration ("Control low"), controls with medium concentration ("Control med"), and controls with high concentration ("Control high").

```
set.seed(1234)
library(highSCREEN)

## Loading required package: gplots
##
## Attaching package: 'gplots'
## The following object is masked from 'package:stats':
##
## lowess

nc = 24
nr = 16
# create a 384-well plate with compounds and controls
replicate = matrix(abs(rnorm(nr*nc)), nr, nc)
head(replicate)

##           [,1]      [,2]      [,3]      [,4]      [,5]      [,6]
## [1,] 1.2070657 0.5110095 0.7094400 0.5238281 0.007604756 0.1777900
## [2,] 0.2774292 0.9111954 0.5012581 0.4968500 1.777084448 0.1699941
## [3,] 1.0844412 0.8371717 1.6290935 1.8060313 1.138607737 1.3723019
## [4,] 2.3456977 2.4158352 1.1676193 0.5820759 1.367827179 0.1737872
## [5,] 0.4291247 0.1340882 2.1800396 1.1088896 1.329564791 0.8502323
## [6,] 0.5060559 0.4906859 1.3409932 1.0149620 0.336472797 0.6976087
```

```

##           [,7]      [,8]      [,9]      [,10]     [,11]     [,12]
## [1,] 1.1346080 1.10976723 0.6360998 0.5137628 0.8473501 1.12376279
## [2,] 0.8782036 0.84927420 0.2263015 0.3992718 0.2606394 3.04376589
## [3,] 0.9729168 0.02236253 1.0136903 1.6628564 0.4144197 0.23502131
## [4,] 2.1211171 0.83114062 0.2527501 0.2758934 0.1830508 0.03325861
## [5,] 0.4145235 1.24428785 1.1719483 0.5062726 0.4070561 2.73221952
## [6,] 0.4747185 0.16902641 0.6687143 0.3475520 0.6246331 0.09979059
##           [,13]     [,14]     [,15]     [,16]     [,17]     [,18]
## [1,] 0.7896469 0.03266396 0.2877097 0.05913517 1.4769696 0.6705594
## [2,] 0.4878146 1.11444896 0.6597701 0.41339889 1.2239038 0.9486326
## [3,] 2.1680325 0.41805782 2.9191401 1.09777217 0.2580684 2.0494030
## [4,] 0.5006946 0.40023524 0.6774155 0.71117526 0.4050028 0.6511136
## [5,] 0.6202102 1.49349310 0.6843203 0.71888873 0.9758033 0.8086193
## [6,] 0.9659032 1.60708094 0.1864921 0.25165107 0.3488767 0.9865806
##           [,19]     [,20]     [,21]     [,22]     [,23]     [,24]
## [1,] 1.5528590 0.02362661 0.14313216 0.27007961 0.7837751 0.04631853
## [2,] 0.1284340 0.64902822 0.02418865 1.61978988 0.2260540 2.25184180
## [3,] 0.9854434 0.50437422 0.50445152 0.21413117 1.5871030 0.60803373
## [4,] 0.1832475 1.61439150 1.58139681 0.81778246 0.5475242 1.50928817
## [5,] 1.7662292 0.44695981 0.03006642 0.05402292 1.8912270 0.23263177
## [6,] 0.6205337 0.76317676 0.71657670 0.33014161 0.8780771 0.03964870

# create 384-well plate control map
cmap = data.frame(X1=c(rep("Control P", floor(nr/3)), rep(c("Control low", "Control med", "Control high"),
(floor(nr/3)+nr-3*floor(nr/3))/3), rep("Control N", floor(nr/3))), X2=c(rep("Control P", floor(nr/3)),
rep(c("Control low", "Control med", "Control high"), (floor(nr/3)+nr-3*floor(nr/3))/3), rep("Control N",
(floor(nr/3))), X3=c(rep("Control N", floor(nr/3)), rep(c("Control low", "Control med", "Control high"),
(floor(nr/3)+nr-3*floor(nr/3))/3), rep("Control P", floor(nr/3))), X4=c(rep("Control N", floor(nr/3)),
rep(c("Control low", "Control med", "Control high"), (floor(nr/3)+nr-3*floor(nr/3))/3), rep("Control P",
floor(nr/3))))
cmap

##           X1           X2           X3           X4
## 1 Control P Control P Control N Control N
## 2 Control P Control P Control N Control N
## 3 Control P Control P Control N Control N
## 4 Control P Control P Control N Control N
## 5 Control P Control P Control N Control N
## 6 Control low Control low Control low Control low
## 7 Control med Control med Control med Control med
## 8 Control high Control high Control high Control high
## 9 Control low Control low Control low Control low
## 10 Control med Control med Control med Control med
## 11 Control high Control high Control high Control high
## 12 Control N Control N Control P Control P
## 13 Control N Control N Control P Control P
## 14 Control N Control N Control P Control P
## 15 Control N Control N Control P Control P
## 16 Control N Control N Control P Control P

# create 96-well plate control map
cmap = data.frame(X1=c(rep("Control P", floor(nr/3)), rep(c("Control low", "Control med", "Control high"),
(floor(nr/3)+nr-3*floor(nr/3))/3), rep("Control N", floor(nr/3))), X2=c(rep("Control N", floor(nr/3)),
rep(c("Control low", "Control med", "Control high"), (floor(nr/3)+nr-3*floor(nr/3))/3), rep("Control P",
floor(nr/3))))
cmap = cmap[seq(1,nr,2),]
cmap

##           X1           X2
## 1 Control P Control N
## 3 Control P Control N
## 5 Control P Control N
## 7 Control med Control med
## 9 Control low Control low
## 11 Control high Control high
## 13 Control N Control P
## 15 Control N Control P

```

2.3 Distribution of Compounds

Some normalization methods implemented in `highSCREEN` assume that the compounds are distributed randomly in the plate. If there are different concentrations of the same compounds in the plate, some of the implemented normalization methods may not be biologically valid².

²See Section 4 for more details regarding within plate normalization methods and their applicability.

3 Assay and Activity Measurement

The package can handle single readings as well as multiple readings/replicates of 384-well plates. In the following example it is assumed that the OD data are collected at two different time instances t_0 and t_1 , respectively. Each time-specific data set consists of replicates. The user can specify which plate and replicate to extract from the input data sets. When extracting a specific plate and replicate, the output data are organized in the form of a list consisting of two elements. Elements `dat0` and `dat1` contain the plates (compounds and controls) of t_0 and t_1 specific data sets, respectively.

```
set.seed(1234)
library(highSCREEN)
nc = 24
nr = 16

# create 1st replicate of data matrix with compounds and controls
r1 = matrix(abs(rnorm(nr*nc)), nr, nc)

# create 2nd replicate of data matrix with compounds and controls
r2 = matrix(abs(rnorm(nr*nc)), nr, nc)

# create 3rd replicate of data matrix with compounds and controls
r3 = matrix(abs(rnorm(nr*nc)), nr, nc)

# combine all replicate to create t0-specific data set
replicates_t0 = list(r1, r2, r3)
names(replicates_t0) = c("R1", "R2", "R3")

# create 1st replicate of data matrix with compounds and controls
r1 = matrix(abs(rnorm(nr*nc)), nr, nc)

# create 2nd replicate of data matrix with compounds and controls
r2 = matrix(abs(rnorm(nr*nc)), nr, nc)

# create 3rd replicate of data matrix with compounds and controls
r3 = matrix(abs(rnorm(nr*nc)), nr, nc)

# combine all replicate to create t1-specific data set
replicates_t1 = list(r1, r2, r3)
names(replicates_t1) = c("R1", "R2", "R3")

# extract plate 3, replicate 2
extractplate(replicates_t0, replicates_t1, plate=3, replicate=2)

## $dat0
##           [,1]      [,2]      [,3]      [,4]      [,5]      [,6]
## [1,] 1.2150536  0.6040689  2.0624810  1.20673873  1.038110146  1.1271329
## [2,] 0.3157640  0.1426293  1.4114046  0.41970989  0.871449472  0.1955291
## [3,] 0.2055698  0.3741440  0.4071761  3.19590120  0.494249837  0.6391582
## [4,] 3.3960635  0.1660620  0.6512008  0.84079580  1.309190860  1.1296779
## [5,] 1.1024646  0.1139629  1.2016658  1.67432651  0.143056066  0.6886715
## [6,] 0.7893944  0.1155534  1.7970624  1.42836844  0.001313548  0.5944834
## [7,] 0.5388331  0.7418648  0.8028380  1.75726622  0.848196597  2.8643468
## [8,] 0.9164891  1.9188941  0.6979752  0.04314039  0.227479443  0.6152848
##           [,7]      [,8]      [,9]      [,10]     [,11]     [,12]
## [1,] 2.1624690  1.011654377  0.67308682  0.6112307  0.54721330  0.29972049
## [2,] 1.0137177  0.132361320  0.51439651  0.1904094  0.93329983  0.38118803
## [3,] 0.4746346  0.133177628  1.14801191  0.8268475  1.16388240  0.26767327
## [4,] 0.1070204  0.506401780  0.10293639  2.1653462  1.44963736  0.92749466
## [5,] 0.6021049  0.172793618  0.04123062  1.8519568  0.14006729  0.04230266
## [6,] 1.1544213  0.655928704  0.64400786  0.7476865  1.20643412  0.05275033
## [7,] 0.3650888  0.346083490  0.49084092  0.5915064  0.02509449  0.86320262
## [8,] 0.7659322  0.006265211  0.19069132  1.9185582  1.64235904  0.10369655
##
## $dat1
##           [,1]      [,2]      [,3]      [,4]      [,5]      [,6]
## [1,] 0.01153115  0.05004926  1.22189865  2.1789535  1.6602134  0.5518484
## [2,] 0.52816051  0.47547105  1.36490125  1.4207221  0.9658158  0.9542928
## [3,] 0.26237252  0.77404562  0.06520022  0.3104332  0.7513957  1.4439064
## [4,] 1.12361059  1.77287949  1.58770807  0.5319105  1.2270525  1.0217189
## [5,] 0.05532339  0.09447017  0.92751980  0.4115962  0.5543107  0.2311760
## [6,] 0.75817883  0.08743515  0.08046895  1.1067062  0.3800668  1.1217940
## [7,] 1.31672226  0.36062217  1.23444910  1.6680156  0.1701018  1.3002615
## [8,] 0.76800213  0.94641655  2.10159395  0.8925480  1.4429385  1.5986195
##           [,7]      [,8]      [,9]      [,10]     [,11]     [,12]
## [1,] 0.3600328  0.3260510  0.26010014  0.3803121  0.3632250  0.64218268
## [2,] 1.2979704  0.5867729  2.04793696  1.6628550  0.4512819  2.22606725
## [3,] 1.3448522  1.4756239  1.35294141  1.5699014  1.1655252  0.17283428
## [4,] 0.8948498  1.7675311  0.05831046  1.2859024  1.4498347  0.54745230
## [5,] 2.3064164  0.5932569  0.49850048  0.6323894  0.4032315  2.40240516
## [6,] 0.9404297  0.2082568  0.12624092  1.3501161  0.1291349  0.02339644
## [7,] 0.9438240  0.9430414  0.18652522  0.1146617  0.6742733  0.69777449
## [8,] 1.0216503  0.7475638  0.40275905  0.4027099  1.8020764  0.16960283
```

4 Within Plate Normalization

The user can normalize individual 96-well plates and replicates via the package function `normplate()`, which utilizes one of the implemented normalization methods. Currently, `highSCREEN` implements the **b-score**, the **c-score** and **z-score** normalization methods. It is worth emphasizing that the **b-score** and **z-score** normalization methods are biologically plausible if compounds are randomly distributed within a plate. If there are different concentrations of the same compounds, the **c-score** normalization method is more appropriate.

The input data for normalization can be taken from the output of the package function `extractplate()`. However, the user must provide a 96-well plate control map. The format of the control map and the normalized data are shown in the following example.

```
set.seed(1234)
library(highSCREEN)
nc = 24
nr = 16

# create control map
cmap = data.frame(X1=c(rep("Control P", floor(nr/3)), rep(c("Control low", "Control med", "Control high"),
(floor(nr/3)+nr-3*floor(nr/3))/3), rep("Control N", floor(nr/3))), X2=c(rep("Control N", floor(nr/3)),
rep(c("Control low", "Control med", "Control high"), (floor(nr/3)+nr-3*floor(nr/3))/3), rep("Control P", floor(nr/3))))
cmap = cmap[seq(1,nr,2),]
cmap

##           X1           X2
## 1 Control P Control N
## 3 Control P Control N
## 5 Control P Control N
## 7 Control med Control med
## 9 Control low Control low
## 11 Control high Control high
## 13 Control N Control P
## 15 Control N Control P

# create 1st replicate of data matrix with compounds and controls
r1 = matrix(abs(rnorm(nr*nc)), nr, nc)

# create 2nd replicate of data matrix with compounds and controls
r2 = matrix(abs(rnorm(nr*nc)), nr, nc)

# create 3rd replicate of data matrix with compounds and controls
r3 = matrix(abs(rnorm(nr*nc)), nr, nc)

# combine all replicate for the t0-specific data
replicates_t0 = list(r1, r2, r3)
names(replicates_t0) = c("R1", "R2", "R3")

# create 1st replicate of data matrix with compounds and controls
r1 = matrix(abs(rnorm(nr*nc)), nr, nc)

# create 2nd replicate of data matrix with compounds and controls
r2 = matrix(abs(rnorm(nr*nc)), nr, nc)

# create 3rd replicate of data matrix with compounds and controls
r3 = matrix(abs(rnorm(nr*nc)), nr, nc)

# combine all replicate for the t1-specific data
replicates_t1 = list(r1, r2, r3)
names(replicates_t1) = c("R1", "R2", "R3")

# extract plate 1, replicate 1
dat = extractplate(replicates_t0, replicates_t1, plate=1, replicate=1)

# normalize using c-score
head(normplate("Main Plate 1", dat[["dat0"]], dat[["dat1"]], cmap, plate=1, replicate=1, norm="cscore",
poscont="Control P", negcont="Control N"))

##      MainPlate Time Plate Replicate  Norm well row col      S
## 1 Main Plate 1    0    1          1 cscore  A1  A  1  1.207066
## 2 Main Plate 1    0    1          1 cscore  A2  A  2 165.388950
## 3 Main Plate 1    0    1          1 cscore  A3  A  3 402.215226
## 4 Main Plate 1    0    1          1 cscore  A4  A  4  21.920877
## 5 Main Plate 1    0    1          1 cscore  A5  A  5 190.136763
## 6 Main Plate 1    0    1          1 cscore  A6  A  6 118.852789
##      welltype
## 1 Control P
## 2 Compound
## 3 Compound
```

```

## 4 Compound
## 5 Compound
## 6 Compound

# normalize using b-score (medpolish)
head(normplate("Main Plate 1", dat[["dat0"]], dat[["dat1"]], cmap, plate=1, replicate=1, norm="bscore"))

## 1: 36.28535
## Final: 36.04823
## 1: 36.18311
## Final: 35.983
##      MainPlate Time Plate Replicate   Norm well row col      S
## 1 Main Plate 1    0    1         1 bscore  A1  A  1  1.2070657
## 2 Main Plate 1    0    1         1 bscore  A2  A  2 -1.0373700
## 3 Main Plate 1    0    1         1 bscore  A3  A  3 -1.2455040
## 4 Main Plate 1    0    1         1 bscore  A4  A  4  0.8786010
## 5 Main Plate 1    0    1         1 bscore  A5  A  5 -0.3495251
## 6 Main Plate 1    0    1         1 bscore  A6  A  6  0.2527863
##      welltype
## 1 Control P
## 2 Compound
## 3 Compound
## 4 Compound
## 5 Compound
## 6 Compound

# normalize using z-score
head(normplate("Main Plate 1", dat[["dat0"]], dat[["dat1"]], cmap, plate=1, replicate=1, norm="zscore"))

##      MainPlate Time Plate Replicate   Norm well row col      S
## 1 Main Plate 1    0    1         1 zscore  A1  A  1  1.2070657
## 2 Main Plate 1    0    1         1 zscore  A2  A  2 -0.1690751
## 3 Main Plate 1    0    1         1 zscore  A3  A  3 -1.2059072
## 4 Main Plate 1    0    1         1 zscore  A4  A  4  0.4590321
## 5 Main Plate 1    0    1         1 zscore  A5  A  5 -0.2774217
## 6 Main Plate 1    0    1         1 zscore  A6  A  6  0.0346615
##      welltype
## 1 Control P
## 2 Compound
## 3 Compound
## 4 Compound
## 5 Compound
## 6 Compound

```

5 Cross-Plate Normalization

Currently not implemented.

6 Reformatting Normalized Data of Replicates

The package allows for reformatting the normalized data for easier interpretation via the function `formatRESULT()`. In the following example the normalized data of replicates are combined and reformatted for easier visualization.

```

set.seed(1234)
library(highSCREEN)
nc = 24
nr = 16

# create 1st replicate of data matrix with compounds and controls
r1 = matrix(abs(rnorm(nr*nc)), nr, nc)

# create control map
cmap = data.frame(X1=c(rep("Control P", floor(nr/3)), rep(c("Control low", "Control med", "Control high"),
(floor(nr/3)+nr-3*floor(nr/3))/3), rep("Control N", floor(nr/3))), X2=c(rep("Control N", floor(nr/3)),
rep(c("Control low", "Control med", "Control high"), (floor(nr/3)+nr-3*floor(nr/3))/3), rep("Control P", floor(nr/3))))
cmap = cmap[seq(1,nr,2),]

# create 2nd replicate of data matrix with compounds and controls
r2 = matrix(abs(rnorm(nr*nc)), nr, nc)

# create 3rd replicate of data matrix with compounds and controls
r3 = matrix(abs(rnorm(nr*nc)), nr, nc)

# combine all replicates for the t0-specific data
replicates_t0 = list(r1, r2, r3)

```

```

names(replicates_t0) = c("R1", "R2", "R3")

# create 1st replicate of data matrix with compounds and controls
r1 = matrix(abs(rnorm(nr*nc)), nr, nc)

# create 2nd replicate of data matrix with compounds and controls
r2 = matrix(abs(rnorm(nr*nc)), nr, nc)

# create 3rd replicate of data matrix with compounds and controls
r3 = matrix(abs(rnorm(nr*nc)), nr, nc)

# combine all replicate for the t1-specific data
replicates_t1 = list(r1, r2, r3)
names(replicates_t1) = c("R1", "R2", "R3")

# extract plate 1, all replicates
dat1 = extractplate(replicates_t0, replicates_t1, plate=1, replicate=1)
dat2 = extractplate(replicates_t0, replicates_t1, plate=1, replicate=2)
dat3 = extractplate(replicates_t0, replicates_t1, plate=1, replicate=3)

# normalize data of all replicates
res1 = normplate("Main Plate 1", dat1[["dat0"]], dat1[["dat1"]], cmap, plate=1, replicate=1, norm="zscore")
res2 = normplate("Main Plate 1", dat2[["dat0"]], dat2[["dat1"]], cmap, plate=1, replicate=2, norm="zscore")
res3 = normplate("Main Plate 1", dat3[["dat0"]], dat3[["dat1"]], cmap, plate=1, replicate=3, norm="zscore")

# reformat data of all replicates
head(formatRESULT(rbind(res1, res2, res3), replicate="Replicate", score="S", t="Time"))

##
## ID MainPlate Plate Norm well row col welltype
## 1 Main Plate 1_1_A1 Main Plate 1 1 zscore A1 A 1 Control P
## 2 Main Plate 1_1_A2 Main Plate 1 1 zscore A2 A 2 Compound
## 3 Main Plate 1_1_A3 Main Plate 1 1 zscore A3 A 3 Compound
## 4 Main Plate 1_1_A4 Main Plate 1 1 zscore A4 A 4 Compound
## 5 Main Plate 1_1_A5 Main Plate 1 1 zscore A5 A 5 Compound
## 6 Main Plate 1_1_A6 Main Plate 1 1 zscore A6 A 6 Compound
## SO_R1 SO_R2 SO_R3 S1_R1 S1_R2 S1_R3
## 1 1.2070657 0.83812938 0.3734610 0.3898951 0.05987781 0.30662212
## 2 -0.1690751 0.51125068 0.2421926 2.5087697 1.16922097 -0.30944605
## 3 -1.2059072 -0.74947348 -0.3606832 -0.5092629 -0.96487503 -1.26814744
## 4 0.4590321 -0.09137493 -1.0919842 -0.3357455 0.26074171 -0.56183908
## 5 -0.2774217 -1.23745258 -1.0676386 0.3536054 -1.10089981 -0.04345365
## 6 0.0346615 -0.37110086 0.6776791 1.7928550 -0.46648819 0.22701845

```

7 QC

The package implements several QC procedures to determine if a plate is eligible for further analyses. Currently, all implemented quality checks are within plate QC procedures. Across plates QC procedures are currently not supported.

The package implements three QC procedures via the function `qcplate()`. The first QC procedure (QC1) checks if all control replicates from t_0 -specific data set are above a pre-defined threshold value. If any control replicate falls below that threshold, it is determined that the plate fails QC1. The second QC procedure (QC2) computes the mean of all positive controls for a given replicate. The plate passes QC2 iff all of the three means are below a pre-defined threshold value. The third QC procedure (QC3) assumes that there are in total five different types of controls, negative controls (hypothetically denoted as "Control N"), positive controls ("Control P") and an additional control that is represented in three different concentrations ("Control low", "Control med" and "Control high"). The QC3 procedure computes the means of all t_1 -specific replicates of a given control and concentration, and compares them. In order for the plate to pass QC3, the following must be satisfied³:

$$\text{mean}(\text{Control N}) < \text{mean}(\text{Control low}) < \text{mean}(\text{Control med}) < \text{mean}(\text{Control high}) < \text{mean}(\text{Control P}). \quad (1)$$

A plate passes the overall QC iff it passes all individual QC procedures. This provides a

³The controls (except positive and negative) need to be specified as an input to `qcplate()` in the same order as they appear in the QC3 condition (1).

conservative QC control. The code below demonstrates the use of the package QC capability on a single 96-well plate.

```

set.seed(1234)
library(highSCREEN)
nc = 24
nr = 16

# create 1st replicates of data matrix with compounds and controls
r1 = matrix(abs(rnorm(nr*nc)), nr, nc)

# create control map
cmap = data.frame(X1=c(rep("Control P", floor(nr/3)), rep(c("Control low", "Control med", "Control high"),
(floor(nr/3)+nr-3*floor(nr/3)/3), rep("Control N", floor(nr/3))), X2=c(rep("Control N", floor(nr/3)),
rep(c("Control low", "Control med", "Control high"), (floor(nr/3)+nr-3*floor(nr/3)/3), rep("Control P", floor(nr/3))))
cmap = cmap[seq(1,nr,2),]

# create 2nd replicate of data matrix with compounds and controls
r2 = matrix(abs(rnorm(nr*nc)), nr, nc)

# create 3rd replicate of data matrix with compounds and controls
r3 = matrix(abs(rnorm(nr*nc)), nr, nc)

# combine all replicate for the t0-specific data
replicates_t0 = list(r1, r2, r3)

# create 1st replicate of data matrix with compounds and controls
r1 = matrix(abs(rnorm(nr*nc)), nr, nc)

# create 2nd replicate of data matrix with compounds and controls
r2 = matrix(abs(rnorm(nr*nc)), nr, nc)

# create 3rd replicate of data matrix with compounds and controls
r3 = matrix(abs(rnorm(nr*nc)), nr, nc)

# combine all replicates for the t1-specific data
replicates_t1 = list(r1, r2, r3)
names(replicates_t1) = c("R1", "R2", "R3")

# extract plate 1, replicate 1
dat11 = extractplate(replicates_t0, replicates_t1, plate=1, replicate=1)

# extract plate 1, replicate 2
dat12 = extractplate(replicates_t0, replicates_t1, plate=1, replicate=2)

# extract plate 1, replicate 3
dat13 = extractplate(replicates_t0, replicates_t1, plate=1, replicate=3)

# no normalization (norm="raw")
res11 = normplate("Main Plate 1", dat11[["dat0"]], dat11[["dat1"]], cmap, plate=1, replicate=1, norm="raw")

## [1] "raw"

res12 = normplate("Main Plate 1", dat12[["dat0"]], dat12[["dat1"]], cmap, plate=1, replicate=2, norm="raw")

## [1] "raw"

res13 = normplate("Main Plate 1", dat13[["dat0"]], dat13[["dat1"]], cmap, plate=1, replicate=3, norm="raw")

## [1] "raw"

# combine 3 replicates
res1 = rbind(res11, res12, res13)

# reformat result
res1 = formatRESULT(res1, replicate="Replicate", score="S", t="Time")

# perform QC
qcplate(res1, poscont="Control P", negcont="Control N", qc1.val=0.225, qc2.val=2,
addcont=c("Control low", "Control med", "Control high"), welltype="welltype")

## passQC1 passQC2 passQC3 passQC
## 1 FALSE FALSE FALSE FALSE

```

8 Plate Statistical Effect Size

The package provides additional plate-based assessment, by computing z-factor and strictly standardized mean difference (ssmd) of a 96-well plate. The following example computes z-factor and ssmd of a 96-well plate replicate via the package function `zfactor.ssmd()`.


```

set.seed(1234)
library(highSCREEN)

nc = 24
nr = 16

# create 1st replicate of data matrix with compounds and controls
r1 = matrix(abs(rnorm(nr*nc)), nr, nc)

# create control map
cmap = data.frame(X1=c(rep("Control P", floor(nr/3)), rep(c("Control low", "Control med", "Control high"),
(floor(nr/3)+nr-3*floor(nr/3))/3), rep("Control N", floor(nr/3))), X2=c(rep("Control N", floor(nr/3)),
rep(c("Control low", "Control med", "Control high"), (floor(nr/3)+nr-3*floor(nr/3))/3), rep("Control P", floor(nr/3))))
cmap = cmap[seq(1,nr,2),]

# create 2nd replicate of data matrix with compounds and controls
r2 = matrix(abs(rnorm(nr*nc)), nr, nc)

# create 3rd replicate of data matrix with compounds and controls
r3 = matrix(abs(rnorm(nr*nc)), nr, nc)

# combine all replicates for the t0-specific data
replicates_t0 = list(r1, r2, r3)
names(replicates_t0) = c("R1", "R2", "R3")

# create 1st replicate of data matrix with compounds and controls
r1 = matrix(abs(rnorm(nr*nc)), nr, nc)

# create 2nd replicate of data matrix with compounds and controls
r2 = matrix(abs(rnorm(nr*nc)), nr, nc)

# create 3rd replicate of data matrix with compounds and controls
r3 = matrix(abs(rnorm(nr*nc)), nr, nc)

# combine all replicates for the t1-specific data
replicates_t1 = list(r1, r2, r3)
names(replicates_t1) = c("R1", "R2", "R3")

# extract plate 1, replicate 1
dat1 = extractplate(replicates_t0, replicates_t1, plate=1, replicate=1)
# extract plate 1, replicate 2
dat2 = extractplate(replicates_t0, replicates_t1, plate=1, replicate=2)
# extract plate 1, replicate 3
dat3 = extractplate(replicates_t0, replicates_t1, plate=1, replicate=3)

# no normalization
datraw1 = normplate("Main Plate 1", dat1[["dat0"]], dat1[["dat1"]], cmap, plate=1, replicate=1, norm="raw")

## [1] "raw"

datraw2 = normplate("Main Plate 1", dat2[["dat0"]], dat2[["dat1"]], cmap, plate=1, replicate=2, norm="raw")

## [1] "raw"

datraw3 = normplate("Main Plate 1", dat3[["dat0"]], dat3[["dat1"]], cmap, plate=1, replicate=3, norm="raw")

## [1] "raw"

# combine 3 replicates
datraw = rbind(datraw1, datraw2, datraw3)

# reformat result
datraw = formatRESULT(datraw, replicate="Replicate", score="S", t="Time")

# compute z-factor and ssmd for each raw compound, replicate 1
zfactor.ssmd(datraw, "Control P", "Control N", "Main Plate 1", 1)

##      MainPlate replicate ZFactor0 ZFactor1 SSMD0 SSMD1
## 1 Main Plate 1          1 -8.242231 -3.029124 0.3901324 -0.9424345

```

9 Identifying Hits

The user can identify hits via the package function `hits()`, based on specific threshold values. After identifying candidate hits, the user can rank the hits via the package function `rankhits()`, using different selection rules.

9.1 Criteria

Currently, the package implements three criteria for identifying hits. Firstly, the compounds identified as hits should pass QC1 based on the mean of t_0 -specific raw replicates. Secondly, the mean of t_0 -specific normalized replicates should be smaller than the mean of t_1 -specific normalized replicates. Thirdly, the mean of t_1 -specific normalized replicates should be larger than a pre-defined threshold value. The output of `hits()` contains columns IND2 and IND3, which specify which compound passes (TRUE) or fails (FALSE) the second and third criteria respectively. Only compounds that pass QC1 and belong to plates that passed overall QC are included in the output of `hits()`.

```
set.seed(1234)
library(highSCREEN)

nc = 24
nr = 16

# create 1st replicate of data matrix with compounds and controls
r1 = matrix(abs(rnorm(nr*nc)*0.01), nr, nc)

# create control map
cmap = data.frame(X1=c(rep("Control P", floor(nr/3)), rep(c("Control low", "Control med", "Control high"),
(floor(nr/3)+nr-3*floor(nr/3))/3), rep("Control N", floor(nr/3))), X2=c(rep("Control N", floor(nr/3)),
rep(c("Control low", "Control med", "Control high"), (floor(nr/3)+nr-3*floor(nr/3))/3), rep("Control P", floor(nr/3))))
cmap = cmap[seq(1,nr,2),]

# create 2nd replicate of data matrix with compounds and controls
r2 = matrix(abs(rnorm(nr*nc)*0.01), nr, nc)

# create 3rd replicate of data matrix with compounds and controls
r3 = matrix(abs(rnorm(nr*nc)*0.01), nr, nc)

# combine all replicates for the t0-specific data
replicates_t0 = list(r1, r2, r3)
names(replicates_t0) = c("R1", "R2", "R3")

# create 1st replicate of data matrix with compounds and controls
r1 = matrix(abs(rnorm(nr*nc)), nr, nc)

# create 2nd replicate of data matrix with compounds and controls
r2 = matrix(abs(rnorm(nr*nc)), nr, nc)

# create 3rd replicate of data matrix with compounds and controls
r3 = matrix(abs(rnorm(nr*nc)), nr, nc)

# combine all replicate for the t1-specific data
replicates_t1 = list(r1, r2, r3)
names(replicates_t1) = c("R1", "R2", "R3")

# extract plate 1, replicate 1
dat1 = extractplate(replicates_t0, replicates_t1, plate=1, replicate=1)

# extract plate 1, replicate 2
dat2 = extractplate(replicates_t0, replicates_t1, plate=1, replicate=2)

# extract plate 1, replicate 3
dat3 = extractplate(replicates_t0, replicates_t1, plate=1, replicate=3)

# no normalization
datraw1 = normplate("Main Plate 1", dat1[["dat0"]], dat1[["dat1"]], cmap, plate=1, replicate=1, norm="raw")

## [1] "raw"

datraw2 = normplate("Main Plate 1", dat2[["dat0"]], dat2[["dat1"]], cmap, plate=1, replicate=2, norm="raw")

## [1] "raw"

datraw3 = normplate("Main Plate 1", dat3[["dat0"]], dat3[["dat1"]], cmap, plate=1, replicate=3, norm="raw")

## [1] "raw"

# combine 3 replicates
datraw = rbind(datraw1, datraw2, datraw3)

# reformat result
datraw = formatRESULT(datraw, replicate="Replicate", score="S", t="Time")

# c-score normalization
datnorm1 = normplate("Main Plate 1", dat1[["dat0"]], dat1[["dat1"]], cmap, plate=1, replicate=1, norm="cscore",
poscont="Control P", negcont="Control N")
```

```

datnorm2 = normplate("Main Plate 1", dat2[["dat0"]], dat2[["dat1"]], cmap, plate=1, replicate=2, norm="cscore",
poscont="Control P", negcont="Control N")
datnorm3 = normplate("Main Plate 1", dat3[["dat0"]], dat3[["dat1"]], cmap, plate=1, replicate=3, norm="cscore",
poscont="Control P", negcont="Control N")

# combine 3 replicates
datnorm = rbind(datnorm1, datnorm2, datnorm3)

# reformat result
datnorm = formatRESULT(datnorm, replicate="Replicate", score="S", t="Time")

# identify hits
head(hits(datraw, datnorm, qc.mainplates="Main Plate 1", qc1.val=0.225, hit.val=3))

##
##      ID      MainPlate Plate  Norm well row col welltype
## 5 Main Plate 1_1_A5 Main Plate 1  1 cscore A5  A  5 Compound
## 6 Main Plate 1_1_A6 Main Plate 1  1 cscore A6  A  6 Compound
## 7 Main Plate 1_1_A7 Main Plate 1  1 cscore A7  A  7 Compound
## 8 Main Plate 1_1_A8 Main Plate 1  1 cscore A8  A  8 Compound
## 11 Main Plate 1_1_A11 Main Plate 1  1 cscore A11 A  11 Compound
## 23 Main Plate 1_1_B11 Main Plate 1  1 cscore B11 B  11 Compound
##
##      SO_R1      SO_R2      SO_R3      S1_R1      S1_R2      S1_R3 IND2 IND3
## 5 190.1368 -279.73258 322.36378 75.93379 129.55053 145.46611 TRUE TRUE
## 6 118.8528 23.69479 46.05115 186.03993 88.95740 339.23544 TRUE TRUE
## 7 138.3241 -43.64655 -28.22197 135.15912 -54.62282 71.21409 TRUE TRUE
## 8 307.6970 340.59177 -12.29621 191.15576 28.91436 879.95351 TRUE TRUE
## 11 356.4831 -89.51039 79.66726 18.25135 138.49332 1479.93856 TRUE TRUE
## 23 234.5600 -107.33533 278.90875 61.25786 125.86286 1491.08000 TRUE TRUE

```

9.2 Selection Rules

After identifying candidate hits, the next step is to rank them according to certain criteria/rules. The package incorporates several criteria for ranking candidate hits. One of the criteria is based on the mean of the replicates. The candidate hits are sorted according to decreasing value of the mean. Additionally, the package computes for each candidate hit, the standard deviation (SD) based on the replicates, the coefficient of variation (CV) as the ratio of the mean and standard deviation, and other parameters such as whether a compound CV is within $1.5 * IQR$, where IQR is the inter-quartile range computed from all candidate hit CVs. These additional parameters can be helpful to the user in developing their own customized hit selection rules. An example of ranking candidate hits based on the mean of replicate scores is shown below.

```

set.seed(1234)
library(highSCREEN)

nc = 24
nr = 16

# create 1st replicate of data matrix with compounds and controls
r1 = matrix(abs(rnorm(nr*nc)*0.01), nr, nc)

# create control map
cmap = data.frame(X1=c(rep("Control P", floor(nr/3)), rep(c("Control low", "Control med", "Control high"),
(floor(nr/3)+nr-3*floor(nr/3))/3), rep("Control N", floor(nr/3))), X2=c(rep("Control N", floor(nr/3)),
rep(c("Control low", "Control med", "Control high"), (floor(nr/3)+nr-3*floor(nr/3))/3), rep("Control P", floor(nr/3))))
cmap = cmap[seq(1, nr, 2),]

# create 2nd replicate of data matrix with compounds and controls
r2 = matrix(abs(rnorm(nr*nc)*0.01), nr, nc)

# create 3rd replicate of data matrix with compounds and controls
r3 = matrix(abs(rnorm(nr*nc)*0.01), nr, nc)

# combine all replicates for the t0-specific data
replicates_t0 = list(r1, r2, r3)
names(replicates_t0) = c("R1", "R2", "R3")

# create 1st replicate of data matrix with compounds and controls
r1 = matrix(abs(rnorm(nr*nc)), nr, nc)

# create 2nd replicate of data matrix with compounds and controls
r2 = matrix(abs(rnorm(nr*nc)), nr, nc)

# create 3rd replicate of data matrix with compounds and controls
r3 = matrix(abs(rnorm(nr*nc)), nr, nc)

# combine all replicates for the t1-specific data

```

```

replicates_t1 = list(r1, r2, r3)
names(replicates_t1) = c("R1", "R2", "R3")

# extract plate 1, replicate 1
dat1 = extractplate(replicates_t0, replicates_t1, plate=1, replicate=1)
# extract plate 1, replicate 2
dat2 = extractplate(replicates_t0, replicates_t1, plate=1, replicate=2)
# extract plate 1, replicate 3
dat3 = extractplate(replicates_t0, replicates_t1, plate=1, replicate=3)

# no normalization
datraw1 = normplate("Main Plate 1", dat1[["dat0"]], dat1[["dat1"]], cmap, plate=1, replicate=1, norm="raw")

## [1] "raw"

datraw2 = normplate("Main Plate 1", dat2[["dat0"]], dat2[["dat1"]], cmap, plate=1, replicate=2, norm="raw")

## [1] "raw"

datraw3 = normplate("Main Plate 1", dat3[["dat0"]], dat3[["dat1"]], cmap, plate=1, replicate=3, norm="raw")

## [1] "raw"

# combine 3 replicates
datraw = rbind(datraw1, datraw2, datraw3)

# reformat result
datraw = formatRESULT(datraw, replicate="Replicate", score="S", t="Time")

# c-score normalization
datnorm1 = normplate("Main Plate 1", dat1[["dat0"]], dat1[["dat1"]], cmap, plate=1, replicate=1, norm="cscore",
  poscont="Control P", negcont="Control N")
datnorm2 = normplate("Main Plate 1", dat2[["dat0"]], dat2[["dat1"]], cmap, plate=1, replicate=2, norm="cscore",
  poscont="Control P", negcont="Control N")
datnorm3 = normplate("Main Plate 1", dat3[["dat0"]], dat3[["dat1"]], cmap, plate=1, replicate=3, norm="cscore",
  poscont="Control P", negcont="Control N")

# combine 3 replicates
datnorm = rbind(datnorm1, datnorm2, datnorm3)

# reformat result
datnorm = formatRESULT(datnorm, replicate="Replicate", score="S", t="Time")

# identify hits
h = hits(datraw, datnorm, qc.mainplates="Main Plate 1", qc1.val=0.225, hit.val=3)

# rank hits in descending order of mean of i1-specific replicate scores "ma"
head(rankhits(h))

##
## ID MainPlate Plate Norm well row col welltype
## 93 Main Plate 1_1_H9 Main Plate 1 1 cscore H9 H 9 Compound
## 23 Main Plate 1_1_B11 Main Plate 1 1 cscore B11 B 11 Compound
## 11 Main Plate 1_1_A11 Main Plate 1 1 cscore A11 A 11 Compound
## 66 Main Plate 1_1_F6 Main Plate 1 1 cscore F6 F 6 Compound
## 63 Main Plate 1_1_F3 Main Plate 1 1 cscore F3 F 3 Compound
## 68 Main Plate 1_1_F8 Main Plate 1 1 cscore F8 F 8 Compound
## SO_R1 SO_R2 SO_R3 S1_R1 S1_R2 S1_R3 IND2 IND3
## 93 301.9929 -174.38692 185.015900 14.567208 -21.31639 1728.583 TRUE TRUE
## 23 234.5600 -107.33533 278.908749 61.257857 125.86286 1491.080 TRUE TRUE
## 11 356.4831 -89.51039 79.667265 18.251352 138.49332 1479.939 TRUE TRUE
## 66 -170.3251 -193.59575 7.090824 55.847542 89.44584 1485.904 TRUE TRUE
## 63 -293.8078 -205.33634 250.975582 17.017140 115.50113 1419.763 TRUE TRUE
## 68 297.0844 170.26893 -2.107865 -9.687373 21.84226 1454.098 TRUE TRUE
## diff m0 s0 rs0 m1 s1 rs1
## 93 469.7373 104.20729 248.2578 2.3823461 573.9446 1000.1071 1.742515
## 23 424.0224 135.37780 211.3621 1.5612762 559.4002 807.5047 1.443519
## 11 430.0144 115.54664 225.1511 1.9485736 545.5611 811.4250 1.487322
## 66 662.6757 -118.94333 109.7672 -0.9228529 543.7324 816.1173 1.500954
## 63 600.1499 -82.72286 292.3573 -3.5341778 517.4270 782.9957 1.513248
## 68 333.6690 155.08182 150.1732 0.9683482 488.7508 836.1635 1.710817
## ind_below ind_above ind
## 93 TRUE TRUE TRUE
## 23 TRUE TRUE TRUE
## 11 TRUE TRUE TRUE
## 66 TRUE TRUE TRUE
## 63 TRUE TRUE TRUE
## 68 TRUE TRUE TRUE

```

10 Visualization

highSCREEN incorporates several capabilities for compounds and controls visualization. This capability is useful when determining threshold values for QC procedures. The user can plot the density of a particular control via the function `plotcont()`. In the following example the density of positive control OD values, the density of negative control OD values and the density of low, medium and high concentration control OD values are plotted in three separate plots. Additionally, the user can plot single plate heat maps using the function `plotplate()` as shown in the next example.

```
set.seed(1234)
library(highSCREEN)
library(gplots)

nc = 24
nr = 16

# create 1st replicate of data matrix with compounds and controls
r1 = matrix(abs(rnorm(nr*nc)), nr, nc)

# create control map
cmap = data.frame(X1=c(rep("Control P", floor(nr/3)), rep(c("Control low", "Control med", "Control high"),
(floor(nr/3)+nr-3*floor(nr/3))/3), rep("Control N", floor(nr/3))), X2=c(rep("Control N", floor(nr/3)),
rep(c("Control low", "Control med", "Control high"), (floor(nr/3)+nr-3*floor(nr/3))/3), rep("Control P", floor(nr/3))))
cmap = cmap[seq(1,nr,2),]

# create 2nd replicate of data matrix with compounds and controls
r2 = matrix(abs(rnorm(nr*nc)), nr, nc)

# create 3rd replicate of data matrix with compounds and controls
r3 = matrix(abs(rnorm(nr*nc)), nr, nc)

# combine all replicates for the t0-specific data
replicates_t0 = list(r1, r2, r3)
names(replicates_t0) = c("R1", "R2", "R3")

# create 1st replicate of data matrix with compounds and controls
r1 = matrix(abs(rnorm(nr*nc)), nr, nc)

# create 2nd replicate of data matrix with compounds and controls
r2 = matrix(abs(rnorm(nr*nc)), nr, nc)

# create 3rd replicate of data matrix with compounds and controls
r3 = matrix(abs(rnorm(nr*nc)), nr, nc)

# combine all replicates for the t1-specific data
replicates_t1 = list(r1, r2, r3)
names(replicates_t1) = c("R1", "R2", "R3")

# extract plate 1, replicate 1
dat11 = extractplate(replicates_t0, replicates_t1, plate=1, replicate=1)
# extract plate 1, replicate 2
dat12 = extractplate(replicates_t0, replicates_t1, plate=1, replicate=2)
# extract plate 1, replicate 3
dat13 = extractplate(replicates_t0, replicates_t1, plate=1, replicate=3)

# no normalization (norm="raw")
res11 = normplate("Main Plate 1", dat11[["dat0"]], dat11[["dat1"]], cmap, plate=1, replicate=1, norm="raw")
## [1] "raw"

res12 = normplate("Main Plate 1", dat12[["dat0"]], dat12[["dat1"]], cmap, plate=1, replicate=2, norm="raw")
## [1] "raw"

res13 = normplate("Main Plate 1", dat13[["dat0"]], dat13[["dat1"]], cmap, plate=1, replicate=3, norm="raw")
## [1] "raw"

# combine 3 replicates
res1 = rbind(res11, res12, res13)
# reformat result
res1 = formatRESULT(res1, replicate="Replicate", score="S", t="Time")

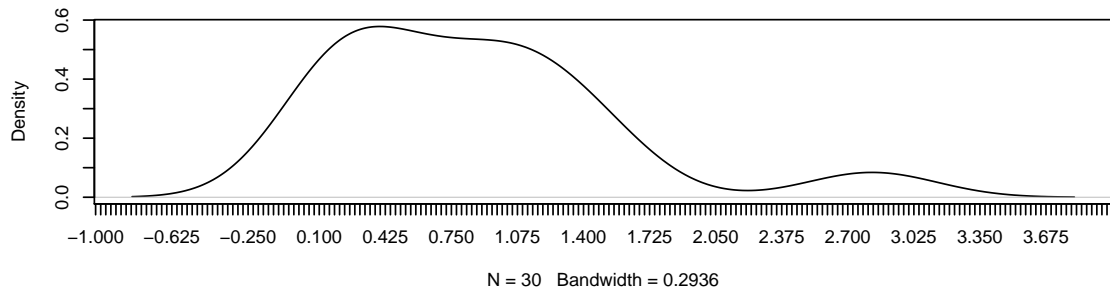
layout(matrix(c(1,2,3), 3, 1, byrow = TRUE))

# plot density of all positive controls
plotcont(subset(res1, welltype=="Control P"), main="Density of Positive Controls", xaxis.marks=seq(-1,5,0.025))

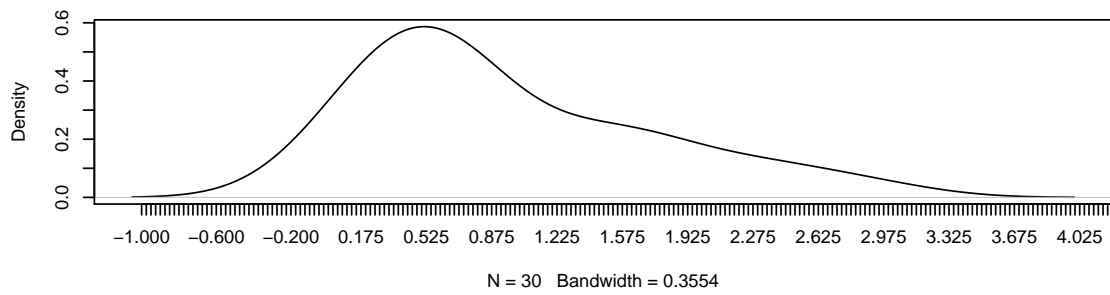
# plot density of all negative controls
plotcont(subset(res1, welltype=="Control N"), main="Density of Negative Controls", xaxis.marks=seq(-1,5,0.025))

# plot density of controls with low, medium and high concentrations
plotcont(subset(res1, welltype=="Control low" | welltype=="Control med" | welltype=="Control high"), main="Density of Controls with Low,
Medium and High Concentrations", xaxis.marks=seq(-1,5,0.025))
```

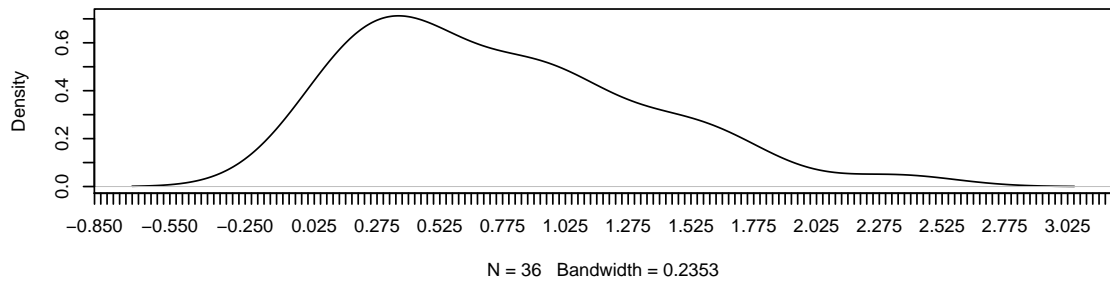
Density of Positive Controls



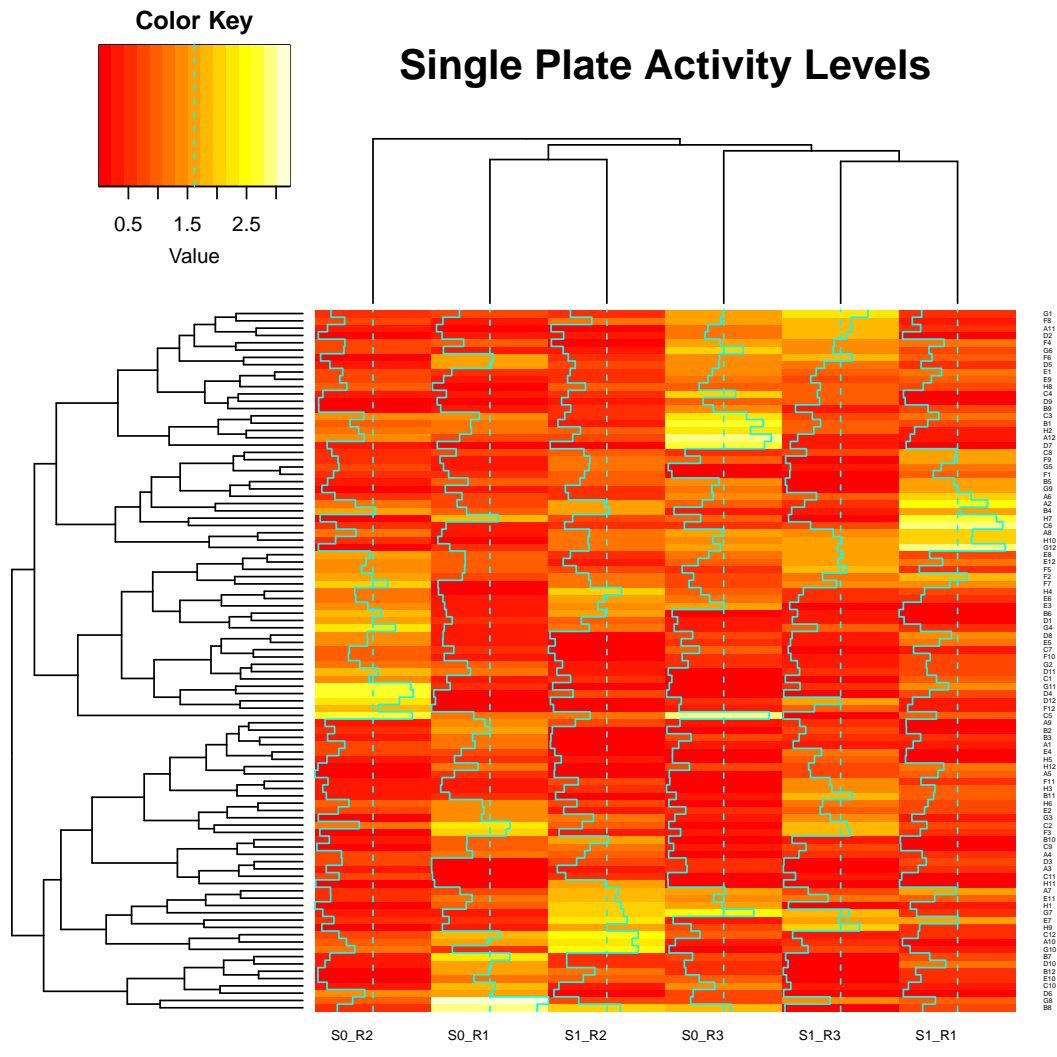
Density of Negative Controls



Density of Controls with Low, Medium and High Concentrations



```
# plot single plate activity levels  
plotplate(res1, main="Single Plate Activity Levels")
```



```

sessionInfo()

## R version 3.4.4 (2018-03-15)
## Platform: i686-pc-linux-gnu (32-bit)
## Running under: Ubuntu 18.04.1 LTS
##
## Matrix products: default
## BLAS: /usr/lib/i386-linux-gnu/blas/libblas.so.3.7.1
## LAPACK: /usr/lib/i386-linux-gnu/lapack/liblapack.so.3.7.1
##
## locale:
## [1] LC_CTYPE=en_US.UTF-8      LC_NUMERIC=C
## [3] LC_TIME=en_US.UTF-8      LC_COLLATE=C
## [5] LC_MONETARY=en_US.UTF-8  LC_MESSAGES=en_US.UTF-8
## [7] LC_PAPER=en_US.UTF-8     LC_NAME=C
## [9] LC_ADDRESS=C             LC_TELEPHONE=C
## [11] LC_MEASUREMENT=en_US.UTF-8 LC_IDENTIFICATION=C
##
## attached base packages:
## [1] stats      graphics  grDevices  utils      datasets  methods   base
##
## other attached packages:
## [1] highSCREEN_0.3  gplots_3.0.1
##
## loaded via a namespace (and not attached):
## [1] compiler_3.4.4  magrittr_1.5    tools_3.4.4
## [4] KernSmooth_2.23-15 stringi_1.2.4   highr_0.7
## [7] gdata_2.18.0    knitr_1.20     caTools_1.17.1.1

```

```
## [10] stringr_1.3.1    bitops_1.0-6      gtools_3.8.1
## [13] evaluate_0.11
```


11 Acknowledgement

This project was funded by the Division of Allergy, Immunology, and Transplantation, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Department of Health and Human Services, under contract No. HHSN272201400054C entitled “Adjuvant Discovery For Vaccines Against West Nile Virus and Influenza”, awarded to Duke University and lead by Drs. Herman Staats and Soman Abraham.

References

- [1] N. Malo, J. A. Hanley, S. Cerquozzi, J. Pelletier, and R. Nadon. Statistical practice in high-throughput screening data analysis. *Nature Biotechnology*, 24(2):167–175, 2006.