Non-parametric analysis of thermal proteome profiles

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Contents

1 Introduction

This workflow shows how to reproduce the analysis described by Childs, Bach, Franken et al. (2018): Non-parametric analysis of thermal proteome profiles reveals novel drug-binding proteins.

2 Preparation

Load necessary packages:

```r
library(tidyverse)
library(broom)
library(knitr)
```

3 Data import

First we load the data from the different TPP experiments. All data have been downloaded from the supplements of the respective publications (Franken et al. 2015, Reinhard et al. (2015), Savitski et al. (2014)), converted into tidy format, and concatenated into one table. This table will be made available as supplementary material to the paper. Until then, it can be found in the same folder as this vignette.

```r
tppData <- readRDS("tppData.Rds")
```

Let's take a look at the first lines of the imported data:

```r
tppData %>% head %>% kable()
```

dataset	uniqueID	relAbundance	temperature	compoundConcentration	replicate	unique
ATP	12 KDA PROTEIN._IPI00879767	NA	25	2000	1	
ATP	12 KDA PROTEIN._IPI00879767	NA	41	2000	1	
ATP	12 KDA PROTEIN._IPI00879767	NA	44	2000	1	
ATP	12 KDA PROTEIN._IPI00879767	NA	47	2000	1	
ATP	12 KDA PROTEIN._IPI00879767	NA	50	2000	1	
ATP	12 KDA PROTEIN._IPI00879767	NA	53	2000	1	

We can see that the data contains the following columns:

- `dataset`: The dataset containing the measurements of several TMT-10 experiments. In each experiment, cells were treated with a vehicle or with the compound in one or two concentrations, and measured at ten different temperatures.
- `uniqueID`: The unique identifier for each protein. Depending on the dataset, it either contains the gene symbol, or the gene symbol concatenated by IPI id.
- `relAbundance`: The relative signal intensity of the protein in each experiment, scaled to the intensity at the lowest temperature.
- `temperature`: The temperatures corresponding to each of the ten measurements in a TMT experiment.
- `compoundConcentration` The concentration of the administered compound in μM.

- `replicate`: The replicate number in each experimental group. Each pair of vehicle and treatment experiments was conducted in two replicates.
- `uniquePeptideMatches`: The number of unique peptides with which a protein was identified.

And a data summary:

```
tppData %>%
  mutate(compoundConcentration = factor(compoundConcentration),
         replicate = factor(replicate),
         dataset = factor(dataset)) %>%
  summary()
##           dataset         uniqueID          relAbundance     temperature
##  ATP          :268000   Length:1432280     Min.   :  0.0    Min.   :25.00
##  Dasatinib 0.5:308520   Class :character   1st Qu.:  0.1    1st Qu.:44.00
##  Dasatinib 5  :308520   Mode  :character   Median :  0.6    Median :52.50
##  Panobinostat :240160                      Mean   :  0.6    Mean   :51.86
##  Staurosporine:307080                      3rd Qu.:  1.0    3rd Qu.:59.00
##                                            Max.   :577.6    Max.   :67.00
##                                            NA's   :372809
##  compoundConcentration replicate  uniquePeptideMatches
##  0   :716140           1:716140   Min.   :  0.0
##  0.5 :154260           2:716140   1st Qu.:  2.0
##  1   :120080                      Median :  5.0
##  5   :154260                      Mean   :  8.3
##  20  :153540                      3rd Qu.: 10.0
##  2000:134000                      Max.   :351.0
##                                   NA's   :337020
```

4 Data preprocessing

Remove all decoy proteins remaining in the panobinostat data. They can be recognized by the prefix `###`, which was assigned by the quantification software `isobarQuant`.

```
tppData <- tppData %>% filter(!grepl("###[[:alnum:]]*###", uniqueID))
```

Remove all proteins that were not found with at least one unique peptide.

```
tppData <- filter(tppData, uniquePeptideMatches >= 1)
```

Re remove all proteins that only contain missing values.

```
tppData <- tppData %>% filter(!is.na(relAbundance))
```

Remove all proteins not reproducibly observed with full melting curves in both replicates and treatment groups per dataset. A full melting curve is defined by the presence of measurements at all 10 temperatures for the given experimental group.

```
tppData <- tppData %>%
  group_by(dataset, uniqueID) %>%
  mutate(n = n()) %>%
  group_by(dataset) %>%
  mutate(max_n = max(n)) %>%
  filter(n == max_n) %>%
  dplyr::select(-n, -max_n) %>%
  ungroup
```

4.1 Reproduce Table 1 of the paper

Count the numbers of proteins remaining in each dataset. They coincide with the values reported in Table 1.

```
tppData %>%
  distinct(dataset, uniqueID) %>%
  distinct %>%
  group_by(dataset) %>%
  tally %>%
  kable()
```

dataset	n
ATP	4177
Dasatinib 0.5	4625
Dasatinib 5	4154
Panobinostat	3649
Staurosporine	4505

5 Illustrative example

We first illustrate the principles of nonparametric analysis of response curves (NPARC) on an example protein (STK4) from the staurosporine dataset. The same protein is shown in Figures 1 and 2 of the paper.

5.1 Select data

We first select all data entries belonging to the desired protein and dataset:

```
stk4 <- filter(tppData, dataset == "Staurosporine", uniqueID == "STK4_IPI00011488")
```

The table `stk4` has 40 rows that contain measurements observed in four experimental groups. They consist of two treatment groups (vehicle: 0 muM staurosporine, treatment: 20 muM staurosporine) with two replicates each. Let us look at the treatment group of replicate 1 for an exaple:

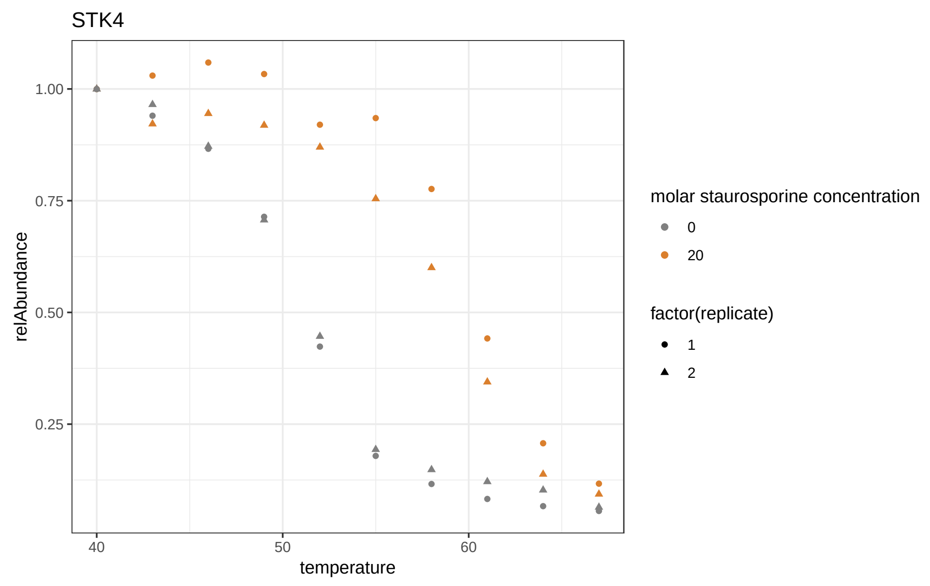
```
stk4 %>% filter(compoundConcentration == 20, replicate == 1) %>% kable()
```

dataset	uniqueID	relAbundance	temperature	compoundConcentration	replicate	uniquePeptide
Staurosporine	STK4_IPI00011488	0.9996869	40	20	1	
Staurosporine	STK4_IPI00011488	1.0299191	43	20	1	
Staurosporine	STK4_IPI00011488	1.0591140	46	20	1	
Staurosporine	STK4_IPI00011488	1.0333794	49	20	1	
Staurosporine	STK4_IPI00011488	0.9200177	52	20	1	
Staurosporine	STK4_IPI00011488	0.9348346	55	20	1	
Staurosporine	STK4_IPI00011488	0.7761524	58	20	1	
Staurosporine	STK4_IPI00011488	0.4417615	61	20	1	
Staurosporine	STK4_IPI00011488	0.2072024	64	20	1	
Staurosporine	STK4_IPI00011488	0.1168182	67	20	1	

To obtain a first impression of the measurements in each experimental group, we generate a plot of the measurements:

```
stk4_plot <- ggplot(stk4, aes(x = temperature, y = relAbundance)) +
  geom_point(aes(shape = factor(replicate), color = factor(compoundConcentration))) +
  theme_bw() +
  ggtitle("STK4") +
  scale_color_manual("molar staurosporine concentration",
                     values = c("#808080", "#da7f2d"))

print(stk4_plot)
```



We will show how to add the fitted curves to this plot in the following steps.

5.2 Define function for model fitting

To assess whether there is a significant difference between both treatment groups, we will fit a null model and an alternative models to the data. The null model fits a sigmoid melting curve through all data points irrespective of experimental condition. The alternative model fits separate melting curves per experimental group .

Because we have to repeat the fitting several times in this workflow, we define a function that we can call repeatedly:

```
fitSingleSigmoid <- function(x, y, start=c(Pl = 0, a = 550, b = 10)){
  try(nls(formula= y ~ (1 - Pl)  / (1+exp((b - a/x))) + Pl,
          start=start,
          data=list(x=x, y=y),
          na.action = na.exclude,
          algorithm = "port",
          lower = c(0.0, 1e-5, 1e-5),
          upper = c(1.5, 15000, 250),
          control = nls.control(maxiter=50)),
      silent = TRUE)
}
```

5.3 Fit null models

Now, we can use the function defined in the previous Section to fit the null model:

```
nullFit <- fitSingleSigmoid(x = stk4$temperature, y = stk4$relAbundance)
```

The function returns an object of class `nls` and we can display the results by the `summary()` function:

```
summary(nullFit)
##
## Formula: y ~ (1 - Pl)/(1 + exp((b - a/x))) + Pl
##
## Parameters:
##    Estimate Std. Error t value Pr(>|t|)
## Pl   0.0000     0.1795   0.000  1.00000
## a  692.6739   226.9106   3.053  0.00419 **
## b   12.5048     4.4989   2.780  0.00851 **
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
##
## Residual standard error: 0.1814 on 37 degrees of freedom
##
## Algorithm "port", convergence message: relative convergence (4)
```

The underlying data is contained in a nested list. The function `augment` from the `broom` package provides a convenient way to obtain the predictions and residuals at each temperature in tabular format. By appending the returned predictions and residuals to our measurements, we ensure that relevant data is collected in the same table and can be added to the plot for visualization. The residuals will be needed later for construction of the test statistic:

```
nullPredictions <- broom::augment(nullFit)
```

Let us look at the values returned by `augment` at two consecutive temperatures. Note that, while the predictions will be the same for each experiment at a given temperature, the residuals will differ because they were computed by comparing the predictions to the actual measurements:

```
nullPredictions %>% filter(x %in% c(46, 49)) %>% kable()
```

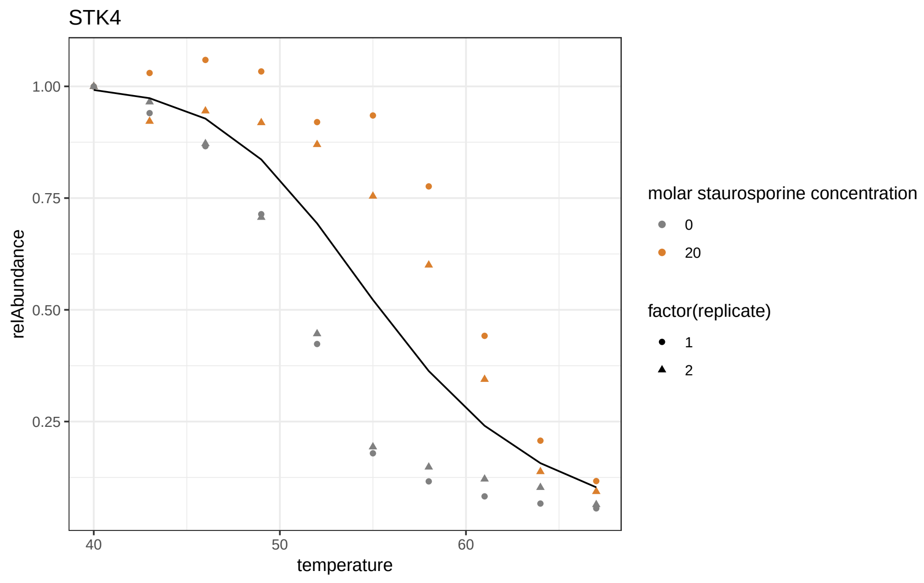
x	y	.fitted	.resid
46	1.0591140	0.9278000	0.1313139
49	1.0333794	0.8363683	0.1970111
46	0.9449568	0.9278000	0.0171568
49	0.9187253	0.8363683	0.0823571
46	0.8661451	0.9278000	-0.0616550
49	0.7139894	0.8363683	-0.1223788
46	0.8717407	0.9278000	-0.0560594
49	0.7068211	0.8363683	-0.1295471

Now we can append these values to our data frame and show the predicted curve in the plot:

```
stk4$nullPrediction <- nullPredictions$.fitted
stk4$nullResiduals <- nullPredictions$.resid

stk4_plot <- stk4_plot + geom_line(data = stk4, aes(y = nullPrediction))

print(stk4_plot)
```



5.4 Fit alternative models

Next we fit the alternative model. Again, we compute the predicted values and the corresponding residuals by the `broom::augment()` function. To take the compound concentration as a factor into account, we iterate over both concentrations and fit separate models to each subset. We implement this by first grouping the data using the function `dplyr::group_by()`, and starting the model fitting by `dplyr::do()`.

```
alternativePredictions <- stk4 %>%
# Fit separate curves per treatment group:
  group_by(compoundConcentration) %>%
  do({
    fit = fitSingleSigmoid(x = .$temperature,
                           y = .$relAbundance)
    broom::augment(fit)
  }) %>%
  ungroup %>%
  # Rename columns for merge to data frame:
  dplyr::rename(alternativePrediction = .fitted,
                alternativeResiduals = .resid,
                temperature = x,
                relAbundance = y)
```

Add the predicted values and corresponding residuals to our data frame:

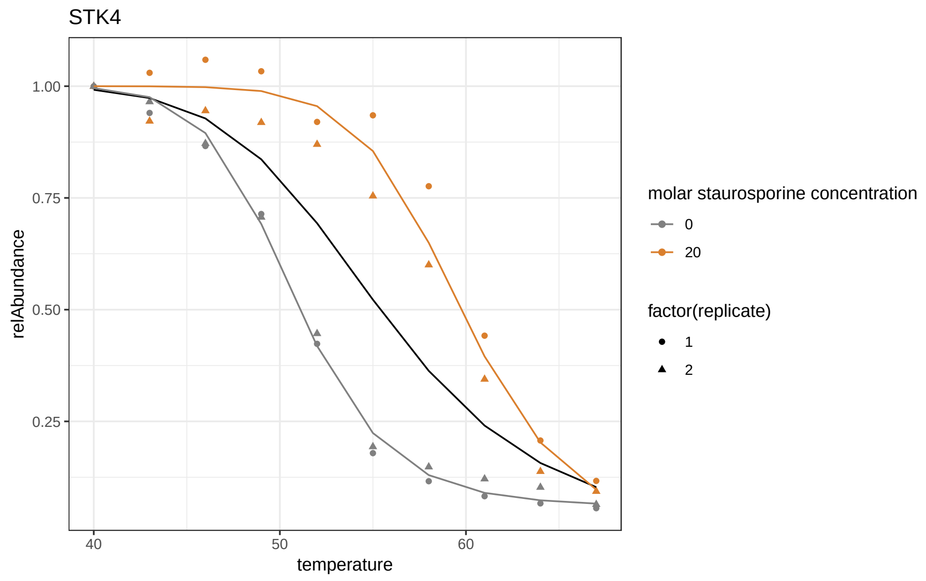
```
stk4 <- stk4 %>%
  left_join(alternativePredictions,
            by = c("relAbundance", "temperature",
                   "compoundConcentration")) %>%
  distinct()
```

5.5 Reproduce Figure 2 (A)/(B) of the paper

Add the curves predicted by the alternative model to the plot:

```
stk4_plot <- stk4_plot +
  geom_line(data = distinct(stk4, temperature, compoundConcentration, alternativePrediction),
            aes(y = alternativePrediction, color = factor(compoundConcentration)))

print(stk4_plot)
```



This plot corresponds to Figures 2(A) and 2(B) in the paper.

5.6 Compute RSS values

In order to quantify the improvement in goodness-of-fit of the alternative model relative to the null model, we compute the sum of squared residuals (RSS):

```
rssPerModel <- stk4 %>%
  summarise(rssNull = sum(nullResiduals^2),
            rssAlternative = sum(alternativeResiduals^2))

kable(rssPerModel, digits = 4)
```

rssNull	rssAlternative
1.2181	0.0831

These values will be used to construct the F-statistic according to

$$F = \frac{\mathrm{DOF}_2}{\mathrm{DOF}_1} \cdot \frac{\mathrm{RSS}^0 - \mathrm{RSS}^1}{\mathrm{RSS}^1}. \quad (1)$$

To compute this statistic and to derive a p-value, we need the degrees of freedom DOF_1 and DOF_2. As described in the paper, they cannot be analytically derived due to the correlated nature of the measurements. The paper describes how to estimate these values from the RSS-values of all proteins in the dataset. In the following Section, we illustrate how to repeat the model fitting for all proteins of a dataset and how to perform hypothesis testing on these models.

6 Extending the analysis to all proteins

In order to analyze all datasets as described in the paper, we fit null and alternative models to all proteins in each dataset, as shown in the following.

Before starting the model fitting, we combine both dasatinib datasets into one dataset with four replicates of the vehicle experiments, and two replicates in each of two treatment groups. In one treatment group, dasatinib was administered with 0.5 μM concentration, and in the other group with $5 \mu M$.

```
# Remove suffix from dataset names that distinguishes both dasatinib datasets
tppData <- tppData %>%
  mutate(replicate = ifelse(dataset == "Dasatinib 5",
                            yes = replicate + 2,
                            no = replicate)) %>%
  mutate(dataset = gsub(" 0.5| 5", "", dataset))

# Check result: List all dataset names and the administered drug concentrations
tppData %>%
  distinct(dataset, replicate, compoundConcentration) %>%
  filter(compoundConcentration > 0) %>%
  dplyr::rename(`drug concentration (treatment groups)` = compoundConcentration) %>%
  kable()
```

dataset	drug concentration (treatment groups)	replicate
ATP	2e+03	1
ATP	2e+03	2
Dasatinib	5e-01	1
Dasatinib	5e-01	2
Dasatinib	5e+00	3
Dasatinib	5e+00	4
Panobinostat	1e+00	1
Panobinostat	1e+00	2
Staurosporine	2e+01	1
Staurosporine	2e+01	2

6.1 Define functions

We fit the models by the same function as illustrated on the STK4 example above. In order to iterate over all proteins and treatment groups, we split the data by the `dplyr::group_by()` function, and loop over all subsets by the `dplyr::do()` function. For each model, we retrieve the residuals by the function `residuals()` and compute the sum of their squared values (RSS). We encapsulate this code into a function that we can re-use for the null and alternative model fits of each protein. It will also make debugging easier if the code lives within a separate function.

For a few proteins, the nonlinear least-squares optimization will not converge with the given start parameters. For some of these proteins, however, convergence can be obtained after adding a small random noise to the start paramters. To this purpose, we write a wrapper around fitSingleSigmoid()‘ that starts the optimization repeatedly with randomly perturbed start parameters for such proteins:

```
repeatFits <- function(x, y, seed = NULL, alwaysPermute = FALSE, maxAttempts = 100){

  start <- c(Pl = 0, a = 550, b = 10)
  i <- 0
  doFit <- TRUE
  doVaryPars <- alwaysPermute

  if (!is.null(seed)){
    set.seed(seed)
  }

  while (doFit){
    startTmp <- start * (1 + doVaryPars*runif(1, -0.5, 0.5))
    m <- fitSingleSigmoid(x = x, y = y, start = startTmp)
    i <- i + 1
    doFit <- inherits(m, "try-error") & i < maxAttempts
    doVaryPars <- TRUE
  }

  return(m)
}
```

```
computeRSS <- function(x, y, seed = NULL, alwaysPermute = FALSE, maxAttempts = 100){

  # Start model fitting
  fit <- repeatFits(x = x, y = y, seed = seed,
                    alwaysPermute = alwaysPermute,
                    maxAttempts = maxAttempts)

  if (!inherits(fit, "try-error")){
    # If model fit converged, obtain data frame containing predicted values and residuals
    resid <- residuals(fit)
    rss <- sum(resid^2, na.rm = TRUE)
    fittedValues <- sum(!is.na(resid))
  } else {
    # If model fit did not converge, return default values
    rss <- NA
    fittedValues <- 0
  }

  return(data.frame(rss = rss, fittedValues = fittedValues))
}
```

6.2 Fit null models

Now we can fit the null models to each protein in each dataset:

```
nullRSS <- tppData %>%
  group_by(dataset, uniqueID) %>%
  do(
    computeRSS(x = .$temperature, y = .$relAbundance, seed = 123)
    ) %>%
  ungroup
```

Show a data summary:

```
nullRSS %>%
  mutate(dataset = factor(dataset), fittedValues = factor(fittedValues)) %>%
  summary()
##          dataset       uniqueID              rss            fittedValues
##  ATP          :4177   Length:17120       Min.   :    0.005   0 :   30
##  Dasatinib    :4789   Class :character   1st Qu.:    0.077   38:  162
##  Panobinostat :3649   Mode  :character   Median :    0.159   40:12952
##  Staurosporine:4505                      Mean   :    4.586   78: 3976
##                                          3rd Qu.:    0.390
##                                          Max.   :16290.763
##                                          NA's   :30
```

Determine the maximum number of measurements possible per protein in each dataset. We will need this information to detect those proteins for which the model converged in each experimental group.

```
maxFitted <- nullRSS %>%
  group_by(dataset) %>%
  summarise(maxDataPoints = max(fittedValues, na.rm = TRUE))
```

6.3 Fit alternative models

Next we fit the alternative models:

```
alternativeRSS <- tppData %>%
  group_by(dataset, uniqueID, compoundConcentration) %>%
  do(
    computeRSS(x = .$temperature, y = .$relAbundance, seed = 123)
    ) %>%
  ungroup
```

Show a data summary:

```
alternativeRSS %>%
  mutate(dataset = factor(dataset), fittedValues = factor(fittedValues)) %>%
  summary()
##          dataset        uniqueID         compoundConcentration
##  ATP          : 8354   Length:38230       Min.   :   0.0
##  Dasatinib    :13568   Class :character   1st Qu.:   0.0
##  Panobinostat : 7298   Mode  :character   Median :   0.5
##  Staurosporine: 9010                      Mean   : 221.6
##                                           3rd Qu.:   5.0
##                                           Max.   :2000.0
##
```

```
##       rss             fittedValues
##  Min.   :    0.001   0 :   92
##  1st Qu.:    0.026   19: 4295
##  Median :    0.056   20:29868
##  Mean   :    2.101   39: 3975
##  3rd Qu.:    0.143
##  Max.   :14939.823
##  NA's   :92
```

Count lengths of fitted curves occuring per experiment:

```
alternativeRSS %>%
  group_by(dataset, fittedValues, compoundConcentration) %>%
  tally() %>%
  kable()
```

dataset	fittedValues	compoundConcentration	n
ATP	0	0e+00	9
ATP	0	2e+03	3
ATP	20	0e+00	4168
ATP	20	2e+03	4174
Dasatinib	0	0e+00	20
Dasatinib	0	5e-01	10
Dasatinib	0	5e+00	21
Dasatinib	19	0e+00	162
Dasatinib	19	5e+00	4133
Dasatinib	20	0e+00	632
Dasatinib	20	5e-01	4615
Dasatinib	39	0e+00	3975
Panobinostat	0	0e+00	15
Panobinostat	0	1e+00	4
Panobinostat	20	0e+00	3634
Panobinostat	20	1e+00	3645
Staurosporine	0	0e+00	7
Staurosporine	0	2e+01	3
Staurosporine	20	0e+00	4498
Staurosporine	20	2e+01	4502

Compute the final RSS values per protein as the sum of the RSS values in each treatment group:

```
alternativeRSSCollated <- alternativeRSS %>%
  group_by(dataset, uniqueID) %>%
  summarise(rss = sum(rss, na.rm = TRUE),
            fittedValues = sum(fittedValues))
```

6.4 Combine results from both model fits

Combine the RSS values of all proteins for which the models converged in all groups:

```r
# Make unique columns for merge to a common data frame:
dat1 <- nullRSS %>%
  dplyr::rename(rss0 = rss, n0 = fittedValues)

dat2 <- alternativeRSSCollated %>%
  dplyr::rename(rss1 = rss, n1 = fittedValues)

# Perform merge of both tables:
allRSS <- full_join(dat1, dat2,
                    by = c("dataset", "uniqueID")) %>%
  # Select proteins for which models converged in each treatment group:
  left_join(maxFitted, by = "dataset") %>%
  mutate(allConverged = (n0 == maxDataPoints &
                           n1 == maxDataPoints)) %>%
  dplyr::select(-maxDataPoints)
```

In order to quantify the improvement in goodness-of-fit of the alternative model relative to the null model, we compute the difference in RSS between both models for each protein. Proteins for which RSS^1 is not smaller than RSS^0 are excluded from p-value calculation.

```r
allRSS <- allRSS %>%
  mutate(rssDiff = rss0 - rss1) %>%
  mutate(rssDiff = ifelse(rssDiff < 0, NA, rssDiff)) %>%
  mutate(applicableForTesting = allConverged & !is.na(rssDiff))
```

Data summary of proteins for which we could compute valid RSS differences per dataset:

```r
allRSS %>%
  filter(applicableForTesting) %>%
  mutate(dataset = factor(dataset), n0 = factor(n0), n1 = factor(n1)) %>%
  summary()
##          dataset       uniqueID              rss0            n0
##  ATP          :4161   Length:16173       Min.   :    0.005   40:12272
##  Dasatinib    :3901   Class :character   1st Qu.:    0.075   78: 3901
##  Panobinostat :3619   Mode  :character   Median :    0.152
##  Staurosporine:4492                      Mean   :    3.446
##                                          3rd Qu.:    0.362
##                                          Max.   :16290.763
##       rss1            n1        allConverged      rssDiff
##  Min.   :    0.004   40:12272   Mode:logical   Min.   : 0.00000
##  1st Qu.:    0.062   78: 3901   TRUE:16173     1st Qu.: 0.00486
##  Median :    0.126                             Median : 0.01438
##  Mean   :    3.380                             Mean   : 0.06552
##  3rd Qu.:    0.307                             3rd Qu.: 0.04081
##  Max.   :16290.423                             Max.   :33.97808
##  applicableForTesting
##  Mode:logical
##  TRUE:16173
##
##
##
```

```
##
```

6.5 Compute test statistics

6.5.1 Why we need to estimate the degrees of freedom

In order to compute F-statistics per protein and dataset according to Equation (1), we need to know the degrees of freedom of the corresponding null distribution. If we could assume independent and identically distributed (iid) residuals, we could compute them from the number of fitted values and model parameters. In the following, we will show why this simple equation is not appropriate for the curve data we are working with.

First, we compute the degrees of freedom that we would assume for iid residuals:

```
DOF <- allRSS %>%
  filter(applicableForTesting) %>%
  mutate(paramsNull = 3,
         paramsAlternative = ifelse(n1 > 40, yes = 9, no = 6)) %>%
  mutate(DOF1 = paramsAlternative - paramsNull,
         DOF2 = n1 - paramsAlternative)
```

Let us take a look at the computed degrees of freedom:

```
DOF %>%
  filter(allConverged) %>%
  distinct(dataset, n0, n1, paramsNull, paramsAlternative, DOF1, DOF2) %>%
  kable()
```

dataset	n0	n1	paramsNull	paramsAlternative	DOF1	DOF2
ATP	40	40	3	6	3	34
Dasatinib	78	78	3	9	6	69
Panobinostat	40	40	3	6	3	34
Staurosporine	40	40	3	6	3	34

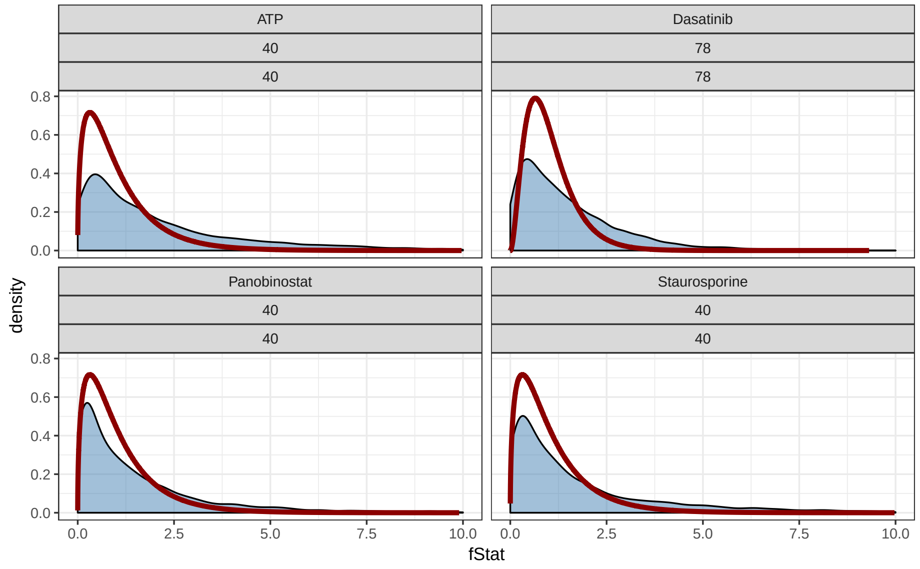
No we calculate the F-statistics per protein and compare them to the corresponding F-distribution to derive p-values:

```
testResults <- DOF %>%
  mutate(fStat = (rssDiff/DOF1) / (rss1/DOF2),
         pVal = 1 - pf(fStat, df1 = DOF1, df2 = DOF2),
         pAdj = p.adjust(pVal, "BH"))
```

We plot the F-statistics against the theoretical F-distribution to check how well the null distribution is approximated now:

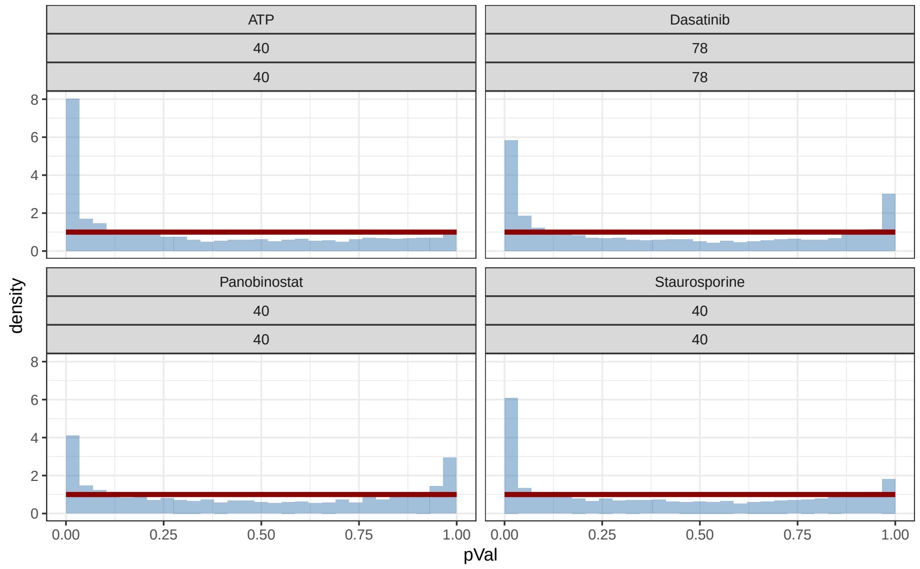
```
ggplot(testResults) +
  geom_density(aes(x = fStat), fill = "steelblue", alpha = 0.5) +
  geom_line(aes(x = fStat, y = df(fStat, df1 = DOF1, df2 = DOF2)), color = "darkred", size = 1.5) +
  facet_wrap(~ dataset + n0 + n1) +
  theme_bw() +
  # Zoom in to small values to increase resolution for the proteins under H0:
```

```
  xlim(c(0, 10))
## Warning: Removed 597 rows containing non-finite values (stat_density).
## Warning: Removed 174 rows containing missing values (geom_path).
```



The densities of the theoretical F-distribution (red) do not fit the observed values (blue) very well. While the theoretical distribution tends to overestimate the number of proteins with test statistics smaller than 2.5, it appears to underestimate the amount of proteins with larger values. This would imply that even for highly specific drugs, we observe many more significant differences than we would expect by chance. This hints at an anti-conservative behaviour of our test with the calculated degree of freedom parameters. This is reflected in the p-value distributions. If the distribution assumptions were met, we would expect the null cases to follow a uniform distribution, with a peak on the left for the non-null cases. Instead, we observe a tendency to obtain fewer values than expected in the middle range (around 0.5), but distinct peaks to the left.

```
ggplot(testResults) +
  geom_histogram(aes(x = pVal, y = ..density..), fill = "steelblue", alpha = 0.5, boundary = 0) +
  geom_line(aes(x = pVal, y = dunif(pVal)), color = "darkred", size = 1.5) +
  facet_wrap(~ dataset + n0 + n1) +
  theme_bw()
## `stat_bin()` using `bins = 30`. Pick better value with `binwidth`.
```



6.5.2 How to estimate the degrees of freedom

In the paper, we describe an alternative way to estimate the degrees of freedom by fitting χ^2 distributions to the numerator and denominator across all proteins in a dataset. To enable fitting of the distributions, we first need to re-scale the variables by a scaling factor. Because the scaling factors are characteristic for each dataset (it depends on the variances of the residuals in the respective dataset), we estimate them from the data according to:

$$\sigma_0^2 = \frac{1}{2}\frac{V}{M}, \quad (2)$$

where V is the variance of the distribution, and M is the mean of the distribution.

We estimate V and M from the empirical distributions of the RSS differences $(\mathrm{RSS}^1 - \mathrm{RSS}^0)$. To increase robustness, we estimate M and V by their D-estimates Marazzi (2002) (median and median absolute deviation).

```
scalingFactors <- allRSS %>%
  filter(applicableForTesting) %>%
  group_by(dataset) %>%
  summarise(M = median(rssDiff, na.rm = T), V = mad(rssDiff, na.rm = T)^2) %>%
  ungroup %>%
  mutate(s0_sq = 1/2 * V/M)

scalingFactors %>% kable()
```

dataset	M	V	s0_sq
ATP	0.0122212	0.0002103	0.0086058
Dasatinib	0.0325668	0.0011668	0.0179144
Panobinostat	0.0098916	0.0001496	0.0075610
Staurosporine	0.0096553	0.0001298	0.0067206

We scale the numerator and denominator of the F-statistic by these scaling factors and estimate the degree of freedom parameters by fitting unscaled χ^2 distributions.

First we add the scaling factors to the filtered RSS data as a separate column:

```r
rssScaled <- scalingFactors %>%
  dplyr::select(dataset, s0_sq) %>%
  left_join(allRSS, by = "dataset") %>%
  mutate(rssDiff = rssDiff/s0_sq,
         rss1 = rss1/s0_sq)
```

Then we fit the degrees of freedom parameters numerically. This estimation proves to be fairly robust regarding the choice of the initial values, so we choose a small value of 1 for each optimization.

```r
newDOF <- rssScaled %>%
  filter(applicableForTesting) %>%
  group_by(dataset) %>%
  do(
    data.frame(
      DOF1 = MASS::fitdistr(x = .$rssDiff, densfun = "chi-squared", start = list(df = 1))[["estimate"]],
      DOF2 = MASS::fitdistr(x = .$rss1, densfun = "chi-squared", start = list(df = 1))[["estimate"]]
    ))

newDOF %>% kable()
```

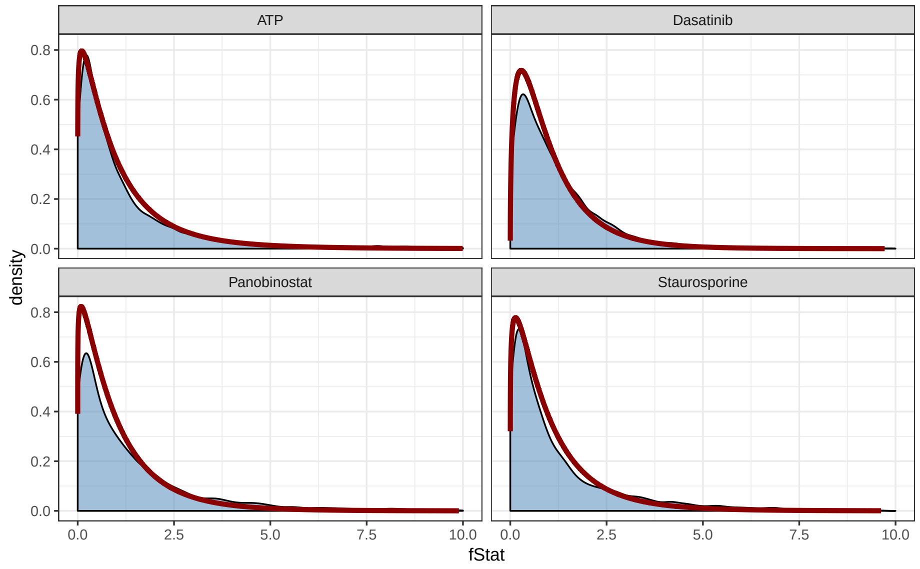
dataset	DOF1	DOF2
ATP	2.278906	11.89375
Dasatinib	2.885937	25.20000
Panobinostat	2.211719	22.32500
Staurosporine	2.355469	17.66875

Finally, we can compute the test statistics according to Equation (1) and compare them to the F-distribution:

```r
newFStatistics <- rssScaled %>%
  filter(applicableForTesting) %>%
  left_join(newDOF, by = "dataset") %>%
  mutate(fStat = (rssDiff/DOF1) / (rss1/DOF2),
         pVal = 1 - pf(fStat, df1 = DOF1, df2 = DOF2)) %>%
  group_by(dataset) %>%
  mutate(pAdj = p.adjust(pVal, "BH"))
```

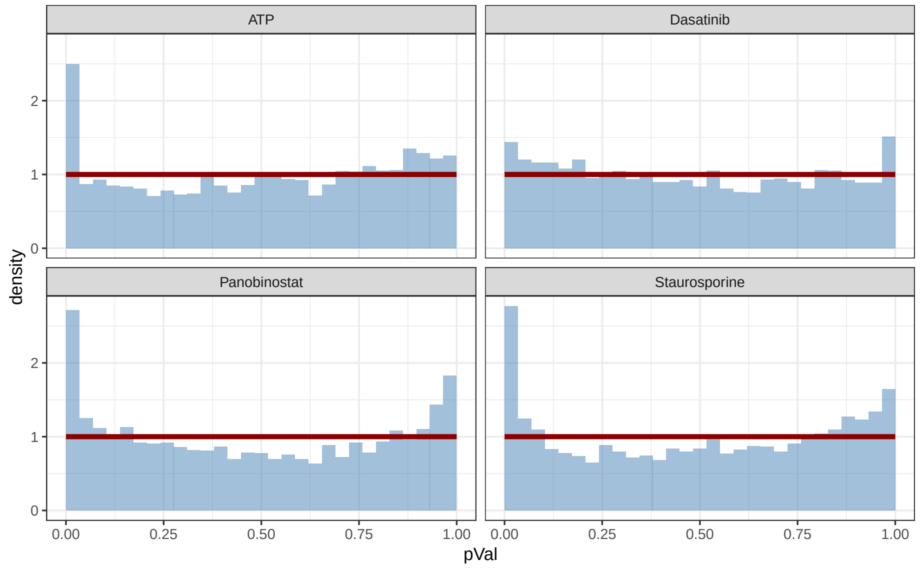
We plot the F-statistics against the theoretical F-distribution to check how well the null distribution is approximated now:

```r
ggplot(newFStatistics) +
  geom_density(aes(x = fStat), fill = "steelblue", alpha = 0.5) +
  geom_line(aes(x = fStat, y = df(fStat, df1 = DOF1, df2 = DOF2)), color = "darkred", size = 1.5) +
  facet_wrap(~ dataset) +
  theme_bw() +
  # Zoom in to small values to increase resolution for the proteins under H0:
  xlim(c(0, 10))
## Warning: Removed 307 rows containing non-finite values (stat_density).
## Warning: Removed 108 rows containing missing values (geom_path).
```



Also check the p-value histograms. We expect the null cases to follow a uniform distribution, with a peak on the left for the non-null cases:

```
ggplot(newFStatistics) +
  geom_histogram(aes(x = pVal, y = ..density..), fill = "steelblue", alpha = 0.5, boundary = 0) +
  geom_line(aes(x = pVal, y = dunif(pVal)), color = "darkred", size = 1.5) +
  facet_wrap(~ dataset) +
  theme_bw()
## `stat_bin()` using `bins = 30`. Pick better value with `binwidth`.
```



The F-statistics and p-values approximate the expected distributions substantially closer when based on the estimated degrees of freedom than when based on the theoretical degrees of freedom.

6.6 Detect significantly shifted proteins

Finally, we can select proteins that are significantly shifted by putting a threshold on the Benjamini-Hochberg corrected p-values.

```
topHits <- newFStatistics %>%
  filter(pAdj <= 0.01) %>%
  dplyr::select(dataset, uniqueID, fStat, pVal, pAdj) %>%
  arrange(-fStat) %>%
  nest(-dataset)
```

The table `topHits` contains a list-column `data` with separate data frames for all proteins with Benjamini-Hochberg corrected p-values ≤ 0.01.

How many proteins were found per dataset?

```
topHits %>%
  mutate(n = map(data, nrow)) %>%
  unnest(n)
## # A tibble: 4 x 3
##   dataset       data                  n
##   <chr>         <list>            <int>
## 1 Staurosporine <tibble [80 x 4]>    80
## 2 Panobinostat  <tibble [15 x 4]>    15
## 3 Dasatinib     <tibble [6 x 4]>      6
## 4 ATP           <tibble [69 x 4]>    69
```

Let us look at the targets detected in each dataset. The same proteins as shown in Fig. S3, S4, S6, and S7 of the paper.

```
lapply(topHits$data %>% set_names(topHits$dataset), kable)
```

$Staurosporine

uniqueID	fStat	pVal	pAdj
CDK5_IPI00023530	369.70353	0.0000000	0.0000000
MAP2K2_IPI00003783	148.48125	0.0000000	0.0000000
CSK_IPI00013212	138.66150	0.0000000	0.0000000
PMPCA_IPI00166749	137.15524	0.0000000	0.0000000
AURKA_IPI00298940	131.15558	0.0000000	0.0000000
FECH_IPI00554589	128.64839	0.0000000	0.0000000
IRAK4_IPI00007641	122.25268	0.0000000	0.0000000
CAMKK2_IPI00290239	116.68423	0.0000000	0.0000000
PAK4_IPI00014068	113.34324	0.0000000	0.0000000
STK4_IPI00011488	102.39278	0.0000000	0.0000000
STK38_IPI00027251	99.69296	0.0000000	0.0000000
PDPK1_IPI00002538	96.91349	0.0000000	0.0000000
GSK3B_IPI00216190	93.03510	0.0000000	0.0000001
ADRBK1_IPI00012497	82.42851	0.0000000	0.0000002
BMP2K_IPI00337426	74.64797	0.0000000	0.0000003
FER_IPI00029263	70.40135	0.0000000	0.0000005
MAP2K1_IPI00219604	62.25164	0.0000000	0.0000012
MAP2K7_IPI00302112	59.76483	0.0000000	0.0000015
MAP4K2_IPI00149094	57.38529	0.0000000	0.0000020
MAPK12_IPI00296283	55.76645	0.0000000	0.0000023
PRKCE_IPI00024539	54.55703	0.0000000	0.0000025
ADK_IPI00290279	54.49588	0.0000000	0.0000025
STK3_IPI00411984	53.30688	0.0000000	0.0000029
MAPK8_IPI00220306	50.13069	0.0000000	0.0000042
MAPKAPK5_IPI00160672	50.11063	0.0000000	0.0000042
PKN1_IPI00412672	50.01186	0.0000000	0.0000042
PTK2_IPI00413961	49.33391	0.0000000	0.0000043
AAK1_IPI00916402	49.32396	0.0000000	0.0000043
CHEK2_IPI00423156	46.53685	0.0000000	0.0000065
CDK2_IPI00031681	45.28064	0.0000001	0.0000077
MAP2K4_IPI00024674	44.27708	0.0000001	0.0000086
CPOX_IPI00093057	44.23314	0.0000001	0.0000086
PHKG2_IPI00012891	43.01355	0.0000001	0.0000103
TNIK_IPI00514275	42.43947	0.0000001	0.0000111
VRK1_IPI00019640	41.36259	0.0000001	0.0000130
MAPKAPK2_IPI00026054	38.00061	0.0000002	0.0000237
MARK2_IPI00555838	37.78614	0.0000002	0.0000240
CAMK2G_IPI00908444	36.17242	0.0000003	0.0000322
GSK3A_IPI00292228	36.03375	0.0000003	0.0000323
PRKAR2B_IPI00554752	35.79923	0.0000003	0.0000330
RIOK2_IPI00306406	35.27343	0.0000003	0.0000358
PRKACA_IPI00396630	33.88421	0.0000004	0.0000461
RPS6KA3_IPI00020898	33.84602	0.0000004	0.0000461
MAPK3_IPI00018195	33.27453	0.0000005	0.0000509
STK24_IPI00872754	32.20468	0.0000006	0.0000629
MARK3_IPI00183118	30.04718	0.0000010	0.0001006
TTK_IPI00151170	29.46182	0.0000012	0.0001130
MKNK1_IPI00304048	26.02004	0.0000028	0.0002613
PDCD10_IPI00298558	25.80161	0.0000030	0.0002711
OSBPL3_IPI00023555	25.52905	0.0000032	0.0002856
HEBP1_IPI00148063	25.11716	0.0000035	0.0003126
RPS6KA1_IPI00477982	24.34095	0.0000044	0.0003789
PIK3CD_IPI00384817	24.06929	0.0000047	0.0004008
MAP3K2_IPI00513803	23.84959	0.0000050	0.0004183
SGK3_IPI00655852	23.33736	0.0000058	0.0004746
PRKCB_IPI00219628	23.00379	0.0000064	0.0005129

$Panobinostat

uniqueID	fStat	pVal	pAdj
HDAC1	86.89568	0.00e+00	0.0000001
TTC38	78.75205	0.00e+00	0.0000001
HDAC6	65.80324	0.00e+00	0.0000003
HDAC2	45.77831	0.00e+00	0.0000061
H2AFV|H2AFZ	38.27073	0.00e+00	0.0000245
ZFYVE28	27.15088	6.00e-07	0.0003749
HDAC8	23.28485	2.10e-06	0.0010877
HDAC10	18.86740	1.02e-05	0.0046122
GNB1L	17.71987	1.60e-05	0.0059064
SMTN	17.66667	1.63e-05	0.0059064
C5orf51	17.15533	2.01e-05	0.0066012
WDR26	16.38570	2.76e-05	0.0078195
NUP93	16.34231	2.81e-05	0.0078195
GTF2B	15.95300	3.31e-05	0.0085613
RNASEH2C	15.54337	3.95e-05	0.0095282

$Dasatinib

uniqueID	fStat	pVal	pAdj
CRKL_IPI00004839	84.00830	0.0e+00	0.0000000
YES1_IPI00013981	46.29650	0.0e+00	0.0000005
MAPK14_IPI00221141	40.19671	0.0e+00	0.0000016
BTK_IPI00029132	22.45262	3.0e-07	0.0003285
AKAP9_IPI00220628	16.45990	4.8e-06	0.0037208
GAB2_IPI00749276	15.28195	8.6e-06	0.0056102

$ATP

uniqueID	fStat	pVal	pAdj
NARS_IPI00306960	73.88431	0.0000001	0.0002186
PRKCQ_IPI00029196	71.73357	0.0000002	0.0002186
ABCF1_IPI00873899	71.46904	0.0000002	0.0002186
RPS6KB1_IPI00216132	54.88794	0.0000007	0.0006361
MARK3_IPI00183118	51.45883	0.0000009	0.0006361
EHD2_IPI00100980	51.23744	0.0000010	0.0006361
RFK_IPI00099995	50.32031	0.0000011	0.0006361
PRKCA_IPI00385449	46.12280	0.0000017	0.0008873
RIOK1_IPI00171336	44.44750	0.0000021	0.0009603
KIF3A_IPI00867739	42.67870	0.0000026	0.0010526
MCM5_IPI00018350	42.05890	0.0000028	0.0010526
VPS4A_IPI00411356	40.15753	0.0000035	0.0012309
ABCF2_IPI00068506	37.88793	0.0000048	0.0015404
EHD4_IPI00005578	35.99389	0.0000063	0.0018671
SYK_IPI00018597	34.83491	0.0000074	0.0020637
DARS2_IPI00100460	34.13168	0.0000083	0.0020790
HARS_IPI00021808	33.94944	0.0000085	0.0020790
HSPA9_IPI00007765	33.42727	0.0000092	0.0021261
RFC2_IPI00017412	32.65686	0.0000104	0.0022213
RIOK2_IPI00306406	32.46723	0.0000107	0.0022213
MAP2K5_IPI00158248	31.79940	0.0000119	0.0023520
GALK1_IPI00019383	30.45855	0.0000148	0.0027939
HARS2_IPI00027445	29.53588	0.0000173	0.0030230
RECQL_IPI001784312	29.47413	0.0000174	0.0030230
RG9MTD1_IPI00099996	28.68023	0.0000200	0.0033288
EHD1_IPI00017184	27.93468	0.0000228	0.0036516
MARK2_IPI00555838	27.67958	0.0000239	0.0036810
MAP2K3_IPI00218858	27.36263	0.0000253	0.0037405
NSUN2_IPI00306369	27.19723	0.0000261	0.0037405
RFC5_IPI00031514	26.69304	0.0000286	0.0038822
DDX19A_IPI00008943	26.48886	0.0000297	0.0038822
RFC4_IPI00017381	26.43355	0.0000300	0.0038822
RPS6KB2_IPI00217069	26.29950	0.0000308	0.0038822
IARS2_IPI00017283	25.59923	0.0000352	0.0043044
MAP2K4_IPI00024674	25.11405	0.0000386	0.0043980
RIOK3_IPI00298199	25.08341	0.0000389	0.0043980
NEK7_IPI00152658	24.96306	0.0000398	0.0043980
PRKAA1_IPI00410287	24.68849	0.0000420	0.0043980
MCM7_IPI00299904	24.62801	0.0000425	0.0043980
STK25_IPI00893500	24.56923	0.0000430	0.0043980
GAPDH_IPI00788737	24.52063	0.0000434	0.0043980
PFKFB2_IPI00305589	24.41119	0.0000444	0.0043980
TRIP13_IPI00003505	24.11473	0.0000471	0.0045593
MCM4_IPI00018349	23.60989	0.0000522	0.0046796
MYO6_IPI00816452	23.60924	0.0000522	0.0046796
ACSM3_IPI00297635	23.60327	0.0000523	0.0046796
MAP2K1_IPI00219604	23.55062	0.0000529	0.0046796
FER_IPI00029263	23.28989	0.0000558	0.0048359
PRKCD_IPI00329236	23.15240	0.0000574	0.0048747
VPS4B_IPI00182728	22.11836	0.0000715	0.0059516
NUBPL_IPI00384517	21.90644	0.0000749	0.0061100
IDH1_IPI00027223	21.79555	0.0000767	0.0061396
CCNB1_IPI00745793	21.62588	0.0000796	0.0062526
DSTYK_IPI00465346	21.50325	0.0000818	0.0063054
MCM3_IPI00013214	21.28267	0.0000859	0.0065020
KIF2C_IPI00290435	21.19502	0.0000876	0.0065124

7 Session info

```
devtools::session_info()
## Session info ----------------------------------------------------------------
##  setting  value
##  version  R version 3.5.1 (2018-07-02)
##  system   x86_64, darwin15.6.0
##  ui       X11
##  language (EN)
##  collate  en_US.UTF-8
##  tz       Europe/Berlin
##  date     2018-08-16
## Packages --------------------------------------------------------------------
##  package    * version date       source
##  assertthat   0.2.0   2017-04-11 CRAN (R 3.5.0)
##  backports    1.1.2   2017-12-13 CRAN (R 3.5.0)
##  base       * 3.5.1   2018-07-05 local
##  bindr        0.1.1   2018-03-13 CRAN (R 3.5.0)
##  bindrcpp   * 0.2.2   2018-03-29 CRAN (R 3.5.0)
##  BiocStyle  * 2.9.3   2018-06-13 Bioconductor
##  bookdown     0.7     2018-02-18 CRAN (R 3.5.0)
##  broom      * 0.5.0   2018-07-17 cran (@0.5.0)
##  cellranger   1.1.0   2016-07-27 CRAN (R 3.5.0)
##  cli          1.0.0   2017-11-05 CRAN (R 3.5.0)
##  codetools    0.2-15  2016-10-05 CRAN (R 3.5.1)
##  colorspace   1.3-2   2016-12-14 CRAN (R 3.5.0)
##  compiler     3.5.1   2018-07-05 local
##  crayon       1.3.4   2017-09-16 CRAN (R 3.5.0)
##  datasets   * 3.5.1   2018-07-05 local
##  devtools     1.13.6  2018-06-27 CRAN (R 3.5.0)
##  digest       0.6.15  2018-01-28 CRAN (R 3.5.0)
##  dplyr      * 0.7.6   2018-06-29 CRAN (R 3.5.1)
##  evaluate     0.11    2018-07-17 cran (@0.11)
##  fansi        0.2.3   2018-05-06 cran (@0.2.3)
##  forcats    * 0.3.0   2018-02-19 CRAN (R 3.5.0)
##  ggplot2    * 3.0.0   2018-07-03 CRAN (R 3.5.0)
##  glue         1.3.0   2018-07-17 cran (@1.3.0)
##  graphics   * 3.5.1   2018-07-05 local
##  grDevices  * 3.5.1   2018-07-05 local
##  grid         3.5.1   2018-07-05 local
##  gtable       0.2.0   2016-02-26 CRAN (R 3.5.0)
##  haven        1.1.2   2018-06-27 CRAN (R 3.5.0)
##  hms          0.4.2   2018-03-10 CRAN (R 3.5.0)
##  htmltools    0.3.6   2017-04-28 CRAN (R 3.5.0)
##  httr         1.3.1   2017-08-20 CRAN (R 3.5.0)
##  jsonlite     1.5     2017-06-01 CRAN (R 3.5.0)
##  knitr      * 1.20    2018-02-20 CRAN (R 3.5.0)
##  labeling     0.3     2014-08-23 CRAN (R 3.5.0)
##  lattice      0.20-35 2017-03-25 CRAN (R 3.5.1)
##  lazyeval     0.2.1   2017-10-29 CRAN (R 3.5.0)
## lubridate    1.7.4   2018-04-11 CRAN (R 3.5.0)
## magrittr     1.5     2014-11-22 CRAN (R 3.5.0)
## MASS         7.3-50  2018-04-30 CRAN (R 3.5.1)
## memoise      1.1.0   2017-04-21 CRAN (R 3.5.0)
## methods    * 3.5.1   2018-07-05 local
## modelr       0.1.2   2018-05-11 CRAN (R 3.5.0)
## munsell      0.5.0   2018-06-12 CRAN (R 3.5.0)
## nlme         3.1-137 2018-04-07 CRAN (R 3.5.1)
## pillar       1.3.0   2018-07-14 cran (@1.3.0)
## pkgconfig    2.0.1   2017-03-21 CRAN (R 3.5.0)
## plyr         1.8.4   2016-06-08 CRAN (R 3.5.0)
## purrr      * 0.2.5   2018-05-29 CRAN (R 3.5.0)
## R6           2.2.2   2017-06-17 CRAN (R 3.5.0)
## Rcpp         0.12.18 2018-07-23 CRAN (R 3.5.0)
## readr      * 1.1.1   2017-05-16 CRAN (R 3.5.0)
## readxl       1.1.0   2018-04-20 CRAN (R 3.5.0)
## rlang        0.2.1   2018-05-30 CRAN (R 3.5.0)
## rmarkdown    1.10    2018-06-11 CRAN (R 3.5.0)
## rprojroot    1.3-2   2018-01-03 CRAN (R 3.5.0)
## rstudioapi   0.7     2017-09-07 CRAN (R 3.5.0)
## rvest        0.3.2   2016-06-17 CRAN (R 3.5.0)
## scales       0.5.0   2017-08-24 CRAN (R 3.5.0)
## stats      * 3.5.1   2018-07-05 local
## stringi      1.2.4   2018-07-20 CRAN (R 3.5.0)
## stringr    * 1.3.1   2018-05-10 CRAN (R 3.5.0)
## tibble     * 1.4.2   2018-01-22 CRAN (R 3.5.0)
## tidyr      * 0.8.1   2018-05-18 CRAN (R 3.5.0)
## tidyselect   0.2.4   2018-02-26 CRAN (R 3.5.0)
## tidyverse  * 1.2.1   2017-11-14 CRAN (R 3.5.0)
## tools        3.5.1   2018-07-05 local
## utf8         1.1.4   2018-05-24 CRAN (R 3.5.0)
## utils      * 3.5.1   2018-07-05 local
## withr        2.1.2   2018-03-15 CRAN (R 3.5.0)
## xfun         0.3     2018-07-06 CRAN (R 3.5.0)
## xml2         1.2.0   2018-01-24 CRAN (R 3.5.0)
## yaml         2.2.0   2018-07-25 CRAN (R 3.5.0)
```

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