RNA-Sequencing Analysis using DESeq2

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Load RDS

```
Expression_data <-
  readRDS("D:/Google Drive/8_MDS/KW3/Course_MDS/example_KICH.rda")
```

Preparing the workspace

Install Bioconductor

```
if(!requireNamespace("BiocManager", quietly = TRUE))
  install.packages("BiocManager")
BiocManager::install(version = "3.14")
```

Install required BioConductor packages

```
BiocManager::install(c('DESeq2', 'glmpca', 'EnhancedVolcano'))
```

```
## Warning: package(s) not installed when version(s) same as current; use ‘force = TRUE‘ to
##   re-install: ’DESeq2’ ’glmpca’ ’EnhancedVolcano’
```

```
library(BiocManager)
library(ggplot2)
library(ggrepel)
library(genefilter)
library(dplyr)
library(tibble)
library(readr)
library(FactoMineR)
library(tidyr)
library(tidyverse)
library(DESeq2)
```

Perform Differential expression analysis

Generate a DESeq2 Data Set

```r
countMat <- Expression_data$countMat
ann <- Expression_data$ann
condition <- ann$condition
geneMat <- Expression_data$geneMat
```

```r
library(DESeq2)
```

```r
# Excluding low/ none expressed genes
keep <- rowSums(countMat > 0) >= 3

# BUILD DESEQ DATA
dds <- DESeqDataSetFromMatrix(countData = countMat[keep, ],
                              colData = ann,
                              design = ~ condition)
```

```
## converting counts to integer mode
```

```
## Warning in DESeqDataSet(se, design = design, ignoreRank): some variables in
## design formula are characters, converting to factors
```

```r
mcols(dds) <- DataFrame(mcols(dds), geneMat[keep, ])
```

```r
# GET COUNT
dds <- estimateSizeFactors(dds)
dds.counts <- counts(dds, normalized=TRUE)
```

```r
# get normalized rlogs
rld <- rlogTransformation(dds, blind=TRUE)
```

```
## rlog() may take a long time with 50 or more samples,
## vst() is a much faster transformation
```

```r
rlds <- assay(rld)
```

PCA Analyse

Dies dient der Visualisierung von Sample-Sample Abständen. Die Datenpunkte werden in einer 2D Projektion geplottet. Diese Richtungen können die Unterschiede der Proben erklären.

GLM-PCA

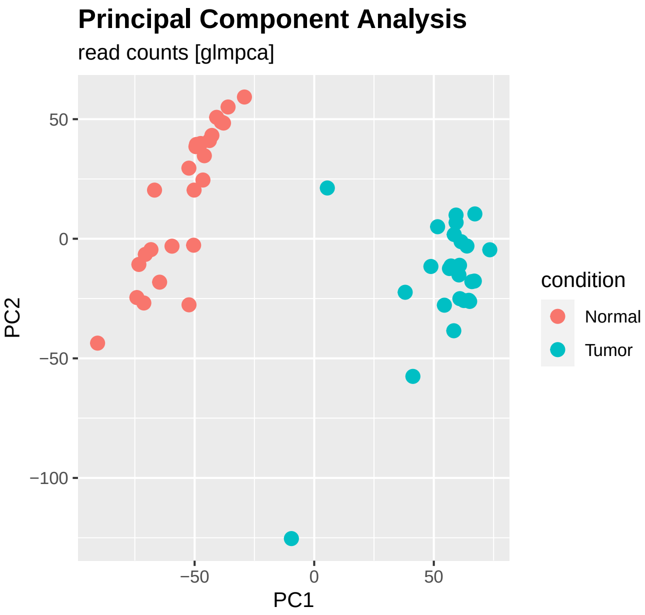
Steht für generalized PCA. Das Package heißt glmpca (Twones et al. 2019). Als Input werden die counts verwendet. Es vermeidet die Probleme, die bei der Normalisierng auftreten können.

```
library(glmpca)
```

```
gpca <- glmpca(counts(dds), L=2)
gpca.dat <- gpca$factors
gpca.dat$condition <- dds$condition
```

```
p <- ggplot(gpca.dat, aes(x = dim1, y = dim2, color = condition)) +
  geom_point(size=3) +
  xlab("PC1") +
  ylab("PC2") +
  coord_fixed() +
  ggtitle("Principal Component Analysis", subtitle = "read counts [glmpca]")

p + theme(plot.title=element_text(color="black", size=14, face="bold"))
```



```
#Save as PDF
ggsave(
  file = "PCA_readcounts.pdf",
  plot = last_plot(),
  path = NULL,
  scale = 1,
  width = 11.69,
  height = 8.27,
  units = "in",
  dpi = "retina",
  limitsize = TRUE,
  bg = NULL,
)
```

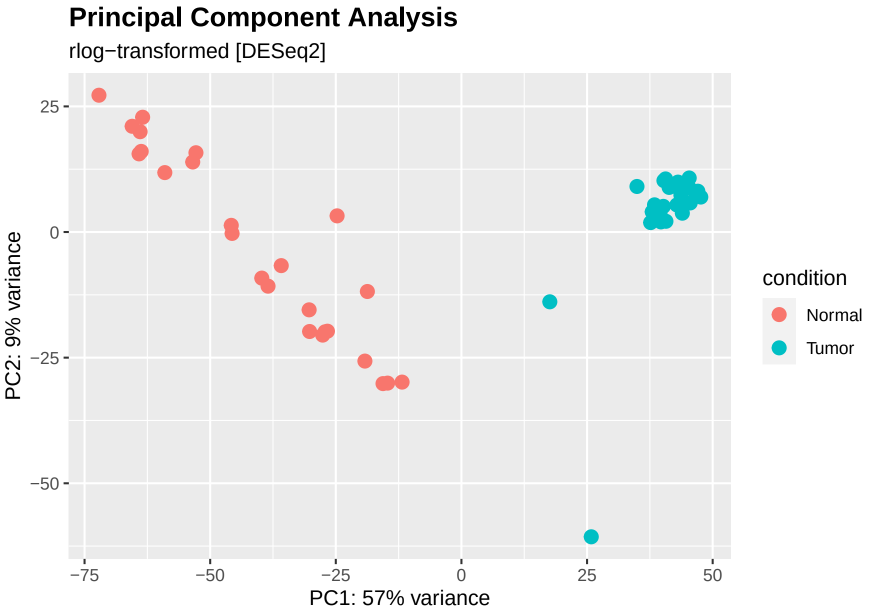
Die Proben des Normalgewebes (rot) unterscheiden sich voneinander auf der y-Achse (dim2). Sie unterscheiden sich vom Tumorgewebe (blau) auf der x-Achse (dim1). Eine Probe liegt in der Mitte zwischen beiden Gruppen, es gibt einen outlier bei den Tumor-Samples in dim2.

Die rlog-transformierten Daten können ebenfalls verwendet werden mittels der `plotPCA` Funktion des `DESeq2` Package:

```
pcaData <- plotPCA(rld, intgroup=c("condition"), returnData=TRUE)
percentVar <- round(100 * attr(pcaData, "percentVar"))
```

```
p <- ggplot(pcaData, aes(PC1, PC2, color=condition)) +
  geom_point(size=3) +
  xlab(paste0("PC1: ",percentVar[1],"% variance")) +
  ylab(paste0("PC2: ",percentVar[2],"% variance")) +
  coord_fixed() +
  ggtitle("Principal Component Analysis", subtitle = "rlog-transformed [DESeq2]")

p + theme(plot.title=element_text(color="black", size=14, face="bold"))
```



```
# Save as PDF
ggsave(
  file = "PCA_rlog.pdf",
  plot = last_plot(),
  path = NULL,
  scale = 1,
  width = 11.69,
  height = 8.27,
  units = "in",
  dpi = "retina",
  limitsize = TRUE,
  bg = NULL,
)
```

Perform differential expression analysis

```
# DIFFERENTIAL ANALYSIS
dds <- DESeq(dds, test = "Wald", fitType = "mean")
result_dss = results(dds)
```

```
summary(result_dss)
```

```
##
## out of 19317 with nonzero total read count
## adjusted p-value < 0.1
## LFC > 0 (up)       : 6597, 34%
## LFC < 0 (down)     : 6589, 34%
## outliers [1]       : 0, 0%
## low counts [2]     : 0, 0%
## (mean count < 0)
## [1] see 'cooksCutoff' argument of ?results
## [2] see 'independentFiltering' argument of ?results
```

Data Frame transformations

```
#Transform results to Data-Frame
results.deseq2 <- as.data.frame(result_dss)
```

```
# Apply new column "Gene" from row Names
results.deseq2$Gene <- row.names(results.deseq2)
head(results.deseq2)
```

```
##                   baseMean log2FoldChange     lfcSE        stat       pvalue
## A1BG__1           44.53785     -0.7247072 0.3075293  -2.3565468 1.844575e-02
## A1CF__29974       43.10693     -8.1031350 0.6962427 -11.6383765 2.629736e-31
## A2BP1__54715      13.69221     -0.4792066 0.5697270  -0.8411162 4.002829e-01
## A2LD1__87769     549.07174     -3.9213580 0.3595323 -10.9068301 1.069198e-27
## A2ML1__144568      1.08798      1.8018951 0.6735547   2.6752025 7.468413e-03
## A2M__2         14525.75971     -1.0568956 0.1866217  -5.6633060 1.484840e-08
##                       padj          Gene
## A1BG__1       3.038946e-02       A1BG__1
## A1CF__29974   8.912037e-30   A1CF__29974
## A2BP1__54715  4.817910e-01  A2BP1__54715
## A2LD1__87769  2.817695e-26  A2LD1__87769
## A2ML1__144568 1.325134e-02 A2ML1__144568
## A2M__2        5.945824e-08        A2M__2
```

```
#Split both Values in the Gene column to "Gene_Name" and "Entrez_ID".
results.deseq2 <- separate(results.deseq2,
                           Gene,
                           sep = "__",
                           into = c("Gene_Name", "Entrez_ID"))
head(results.deseq2)
```

```
##                  baseMean log2FoldChange     lfcSE        stat       pvalue
## A1BG__1          44.53785     -0.7247072 0.3075293  -2.3565468 1.844575e-02
## A1CF__29974      43.10693     -8.1031350 0.6962427 -11.6383765 2.629736e-31
## A2BP1__54715     13.69221     -0.4792066 0.5697270  -0.8411162 4.002829e-01
## A2LD1__87769    549.07174     -3.9213580 0.3595323 -10.9068301 1.069198e-27
## A2ML1__144568     1.08798      1.8018951 0.6735547   2.6752025 7.468413e-03
## A2M__2        14525.75971     -1.0568956 0.1866217  -5.6633060 1.484840e-08
##                       padj Gene_Name Entrez_ID
## A1BG__1       3.038946e-02      A1BG         1
## A1CF__29974   8.912037e-30      A1CF     29974
## A2BP1__54715  4.817910e-01     A2BP1     54715
## A2LD1__87769  2.817695e-26     A2LD1     87769
## A2ML1__144568 1.325134e-02     A2ML1    144568
## A2M__2        5.945824e-08       A2M         2
```

```
# set Gene_Name to first column
results.deseq2 <- results.deseq2[, c(7,8,1,2,3,4,5,6)]
head(results.deseq2)
```

```
##               Gene_Name Entrez_ID    baseMean log2FoldChange     lfcSE
## A1BG__1            A1BG         1    44.53785     -0.7247072 0.3075293
## A1CF__29974        A1CF     29974    43.10693     -8.1031350 0.6962427
## A2BP1__54715      A2BP1     54715    13.69221     -0.4792066 0.5697270
## A2LD1__87769      A2LD1     87769   549.07174     -3.9213580 0.3595323
## A2ML1__144568     A2ML1    144568     1.08798      1.8018951 0.6735547
## A2M__2              A2M         2 14525.75971     -1.0568956 0.1866217
##                      stat       pvalue         padj
## A1BG__1        -2.3565468 1.844575e-02 3.038946e-02
## A1CF__29974   -11.6383765 2.629736e-31 8.912037e-30
## A2BP1__54715   -0.8411162 4.002829e-01 4.817910e-01
## A2LD1__87769  -10.9068301 1.069198e-27 2.817695e-26
## A2ML1__144568   2.6752025 7.468413e-03 1.325134e-02
## A2M__2         -5.6633060 1.484840e-08 5.945824e-08
```

```
# Order results by adjusted p value
results.deseq2.tmp <- results.deseq2[!is.na(results.deseq2$padj),]
results.deseq2.sig <- results.deseq2.tmp[which(results.deseq2.tmp$padj < 0.1),]
results.deseq2.sig.sort <- results.deseq2.sig[order(results.deseq2.sig$padj,
                                                     decreasing = F),]
head(results.deseq2.sig.sort)
```

```
##                Gene_Name Entrez_ID    baseMean log2FoldChange     lfcSE
## SFRP1__6422        SFRP1      6422   4259.7265      -7.504766 0.2645551
## CLDN19__149461    CLDN19    149461   1481.8347     -10.162496 0.3829832
## SLC9A3__6550      SLC9A3      6550   2226.0493      -9.419582 0.3586448
## UMOD__7369          UMOD      7369 114245.9944     -13.892978 0.5477335
## IRX1__79192         IRX1     79192    350.6543      -8.450470 0.3413448
## UGT3A1__133688    UGT3A1    133688    756.7011      -9.217647 0.3725829
##                     stat        pvalue          padj
## SFRP1__6422    -28.36750 5.092903e-177 9.837961e-173
## CLDN19__149461 -26.53509 3.816735e-155 3.686393e-151
## SLC9A3__6550   -26.26438 4.897083e-152 3.153232e-148
## UMOD__7369     -25.36448 6.220637e-142 3.004101e-138
## IRX1__79192    -24.75640 2.645200e-135 1.021947e-131
## UGT3A1__133688 -24.73985 3.986900e-135 1.283582e-131
```

Generate a `.xlsx` file for all DEG

```
library(openxlsx)
```

```
#Write xlsx file
write.xlsx(results.deseq2.sig.sort,
           file = "KICH_NormalvsTumor_DESeq.xlsx",
           asTable = F,
           firstRow = T,
           headerStyle = createStyle(textDecoration = 'bold'),
           keepNA = F,
           rowNames = F,
           overwrite = T)
```

Determine which genes are up- and down-regulated (log2FC > 0 or < 0, respectively)

```
#Upregulated Genes (log2 FC > 0)
results.deseq2.up <- filter(results.deseq2.sig.sort, log2FoldChange >0)
head(results.deseq2.up)
```

```
##                Gene_Name Entrez_ID  baseMean log2FoldChange     lfcSE     stat
## PHKA1__5255        PHKA1      5255  954.4815       3.002285 0.1444968 20.77752
## HAGHL__84264       HAGHL     84264 1209.9234       4.473427 0.2154308 20.76503
## KLK4__9622          KLK4      9622 1784.2420       9.562335 0.4644682 20.58771
## HCN2__610           HCN2       610 1552.6240       6.099920 0.3026359 20.15597
## KLK15__55554       KLK15     55554  504.6397       7.751174 0.3953849 19.60412
## SYNGR3__9143      SYNGR3      9143  891.9353       5.181701 0.2711818 19.10785
##                     pvalue         padj
## PHKA1__5255  6.914137e-96 8.347524e-93
## HAGHL__84264 8.967030e-96 1.018918e-92
## KLK4__9622   3.537152e-94 3.795953e-91
## HCN2__610    2.385464e-90 2.425263e-87
## KLK15__55554 1.425904e-85 1.101767e-82
## SYNGR3__9143 2.172297e-81 1.613933e-78
```

```
#Downregulated genes (log2 FC > 0)
results.deseq2.down <- filter(results.deseq2.sig.sort, log2FoldChange <0)
head(results.deseq2.down)
```

```
##                Gene_Name Entrez_ID    baseMean log2FoldChange     lfcSE
## SFRP1__6422        SFRP1      6422   4259.7265      -7.504766 0.2645551
## CLDN19__149461    CLDN19    149461   1481.8347     -10.162496 0.3829832
## SLC9A3__6550      SLC9A3      6550   2226.0493      -9.419582 0.3586448
## UMOD__7369          UMOD      7369 114245.9994     -13.892978 0.5477335
## IRX1__79192         IRX1     79192    350.6543      -8.450470 0.3413448
## UGT3A1__133688    UGT3A1    133688    756.7011      -9.217647 0.3725829
##                     stat        pvalue          padj
## SFRP1__6422    -28.36750 5.092903e-177 9.837961e-173
## CLDN19__149461 -26.53509 3.816735e-155 3.686393e-151
## SLC9A3__6550   -26.26438 4.897083e-152 3.153232e-148
## UMOD__7369     -25.36448 6.220637e-142 3.004101e-138
## IRX1__79192    -24.75640 2.645200e-135 1.021947e-131
## UGT3A1__133688 -24.73985 3.986900e-135 1.283582e-131
```

Export results

```
#Write xlsx of DEG up- and downregulated (with two sheets)
list_of_datasets <- list("upregulated" = results.deseq2.up,
                         "downregulated" = results.deseq2.down)

write.xlsx(list_of_datasets,
           file = "KICH_NormalvsTumor_DESeq_up_down.v2.xlsx",
           asTable = F,
           firstRow = T,
           headerStyle = createStyle(textDecoration = 'bold'),
           keepNA = F,
           rowNames = F,
           overwrite = T)
```

Volcano Plot

Plot basic Volcano

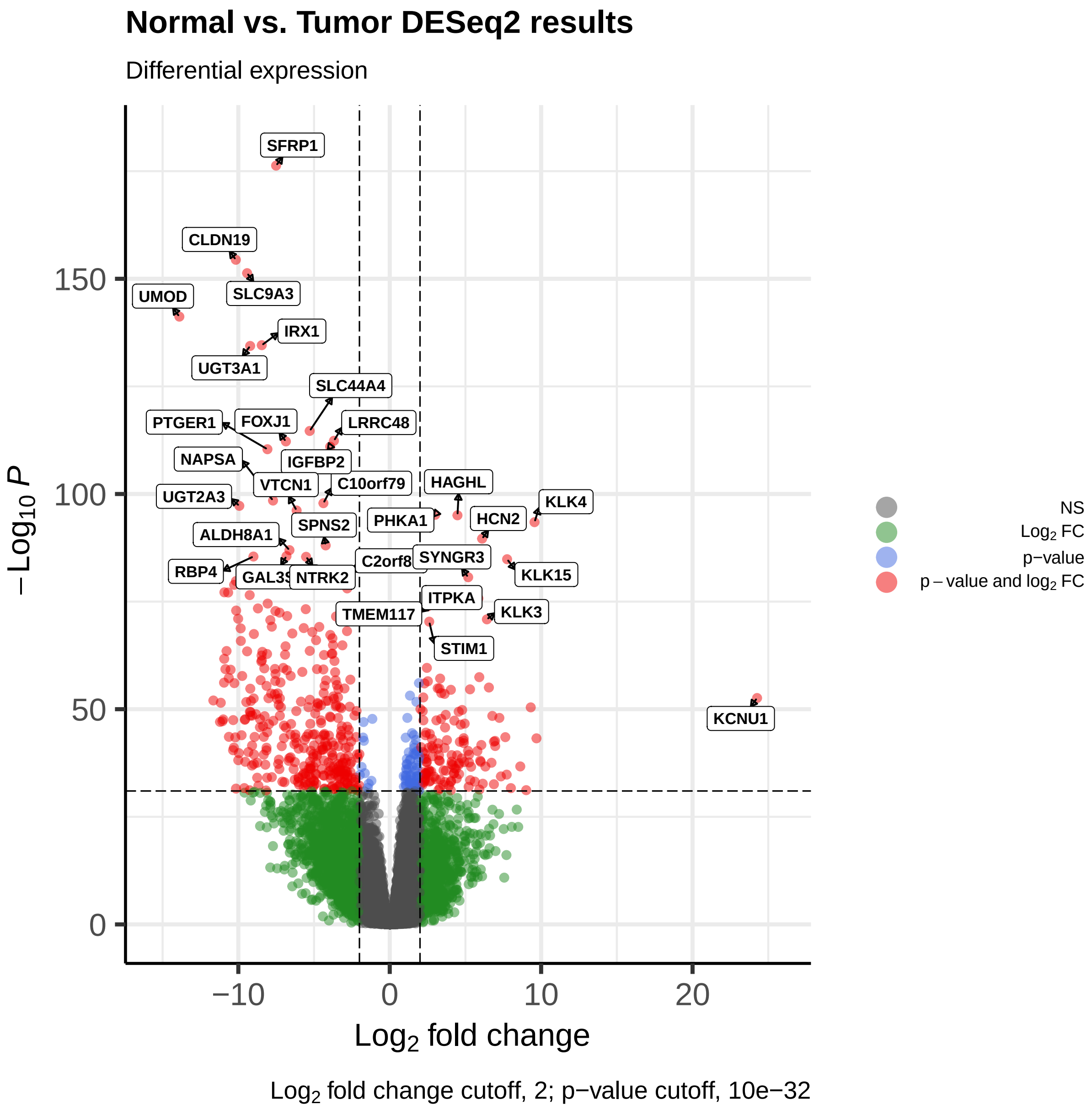
This was done using the `EnhancedVolcano` Package.

```
library(EnhancedVolcano)
```

```
## Registered S3 methods overwritten by 'ggalt':
##   method                  from
##   grid.draw.absoluteGrob  ggplot2
##   grobHeight.absoluteGrob ggplot2
##   grobWidth.absoluteGrob  ggplot2
##   grobX.absoluteGrob      ggplot2
##   grobY.absoluteGrob      ggplot2
```

```
p <- EnhancedVolcano(results.deseq2,
    lab = results.deseq2$Gene_Name,
    title = 'Normal vs. Tumor DESeq2 results',
    subtitle = "Differential expression",
    pointSize = 2.0,
    labSize = 3.0,
    FCcutoff = 2.0,
    drawConnectors = TRUE,
    widthConnectors = 0.5,
    colConnectors = 'black',
    boxedLabels = TRUE,
    pCutoff = 10e-32,
    labFace = 'bold',
    caption = bquote(~Log[2]~ "fold change cutoff, 2; p-value cutoff, 10e-32"),
    legendPosition = "right",
    legendLabSize = 10,
    x = 'log2FoldChange',
    y = 'pvalue') #+ coord_flip()
p
```

```
## Warning: ggrepel: 451 unlabeled data points (too many overlaps). Consider
## increasing max.overlaps
```



```
#Save as PDF
ggsave(
  file = "Volcanoplot.pdf",
  plot = last_plot(),
  path = NULL,
  scale = 1,
  width = 11.69,
  height = 8.27,
  units = "in",
  dpi = "retina",
  limitsize = TRUE,
  bg = NULL,
)
```

Ergebnis: KCNU1 is highly upregulated in the Tumor samples.

Heatmaps

Create a list with top 20 up-and downregulated genes

```
#Create date frame with significant genes (40)
total_up_down <- rbind(results.deseq2.up[1:20,],results.deseq2.down[1:20,])
#Add Gene column (used later to merge with count matrix)
total_up_down$Gene <- row.names(total_up_down)
total_up_down <- total_up_down %>% relocate(Gene, .before = Gene_Name)
head(total_up_down)
```

```
##                        Gene Gene_Name Entrez_ID  baseMean log2FoldChange
## PHKA1__5255   PHKA1__5255     PHKA1      5255  954.4815       3.002285
## HAGHL__84264 HAGHL__84264     HAGHL     84264 1209.9234       4.473427
## KLK4__9622     KLK4__9622      KLK4      9622 1784.2420       9.562335
## HCN2__610       HCN2__610      HCN2       610 1552.6240       6.099920
## KLK15__55554 KLK15__55554     KLK15     55554  504.6397       7.751174
## SYNGR3__9143 SYNGR3__9143    SYNGR3      9143  891.9353       5.181701
##                  lfcSE     stat       pvalue         padj
## PHKA1__5255  0.1444968 20.77752 6.914137e-96 8.347524e-93
## HAGHL__84264 0.2154308 20.76503 8.967030e-96 1.018918e-92
## KLK4__9622   0.4644682 20.58771 3.537152e-94 3.795953e-91
## HCN2__610    0.3026359 20.15597 2.385464e-90 2.425263e-87
## KLK15__55554 0.3953849 19.60412 1.425904e-85 1.101767e-82
## SYNGR3__9143 0.2711818 19.10785 2.172297e-81 1.613933e-78
```

Normalized count matrix

```
# Transform count matrix to Data frame
heatmap_norm.df <- as.data.frame(dds.counts)
# Change Colum Labels
names(heatmap_norm.df) <- ann$condition
# Generate unique columnames
colnames(heatmap_norm.df) <- make.unique(names(heatmap_norm.df))
# add rownames as column
heatmap_norm.df$Gene <- row.names(heatmap_norm.df)
heatmap_norm.df <- heatmap_norm.df %>% relocate(Gene, .before = Normal)
```

```r
# Merge total data frame and heatmap_norm.df by Gene column
data_frame_merge <- semi_join(heatmap_norm.df, total_up_down,
                              by = 'Gene', all = F)
```

```r
# Only add the Gene Name as Rowname, delete the Entrez ID for better overview
df <- separate(data_frame_merge,
               Gene,
               sep="__",
               into = c("Gene_Name", "Entrez_ID"))
data_frame <- df %>% remove_rownames %>% column_to_rownames(var="Gene_Name")
data_frame_noID <- select(data_frame, select = -c(Entrez_ID))
```

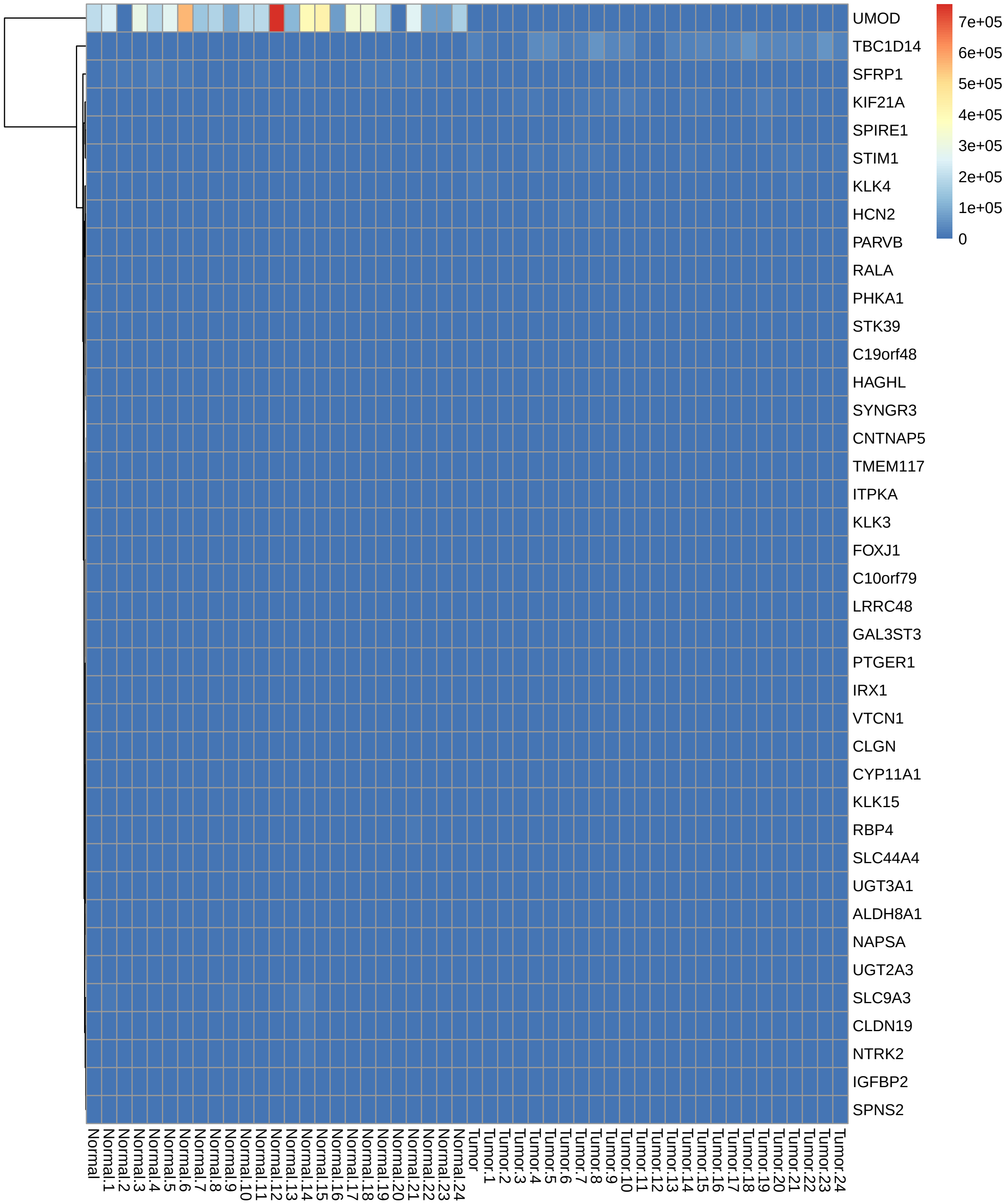
Merge

```r
#Load pheatmap
library(pheatmap)
```

```r
#Convert to Matrix
data_frame_merge <- as.matrix(data_frame_noID)
```

```r
#Plot
data_frame_merge %>% pheatmap(cluster_cols = F)
```

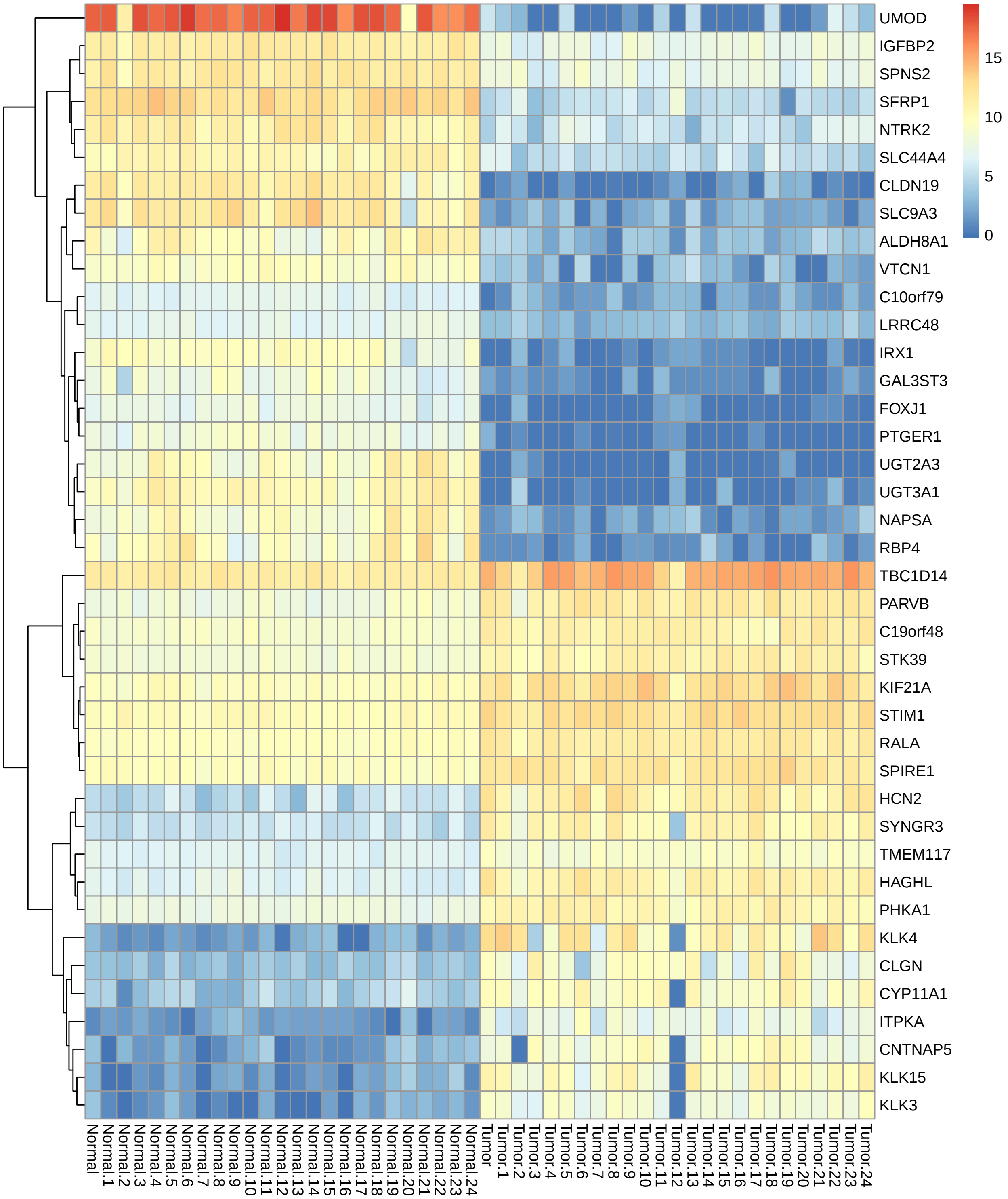
Visualization



```r
## Log2 tranformation
heatmap_data_log2 <- data_frame_merge %>% log2()
# Check if there are any INF values adn remove them
heatmap_data_log2[is.infinite(heatmap_data_log2)] <- 0
```

```r
#Plot log2 heatmap
heatmap_data_log2 %>% pheatmap(cluster_cols = F)
```

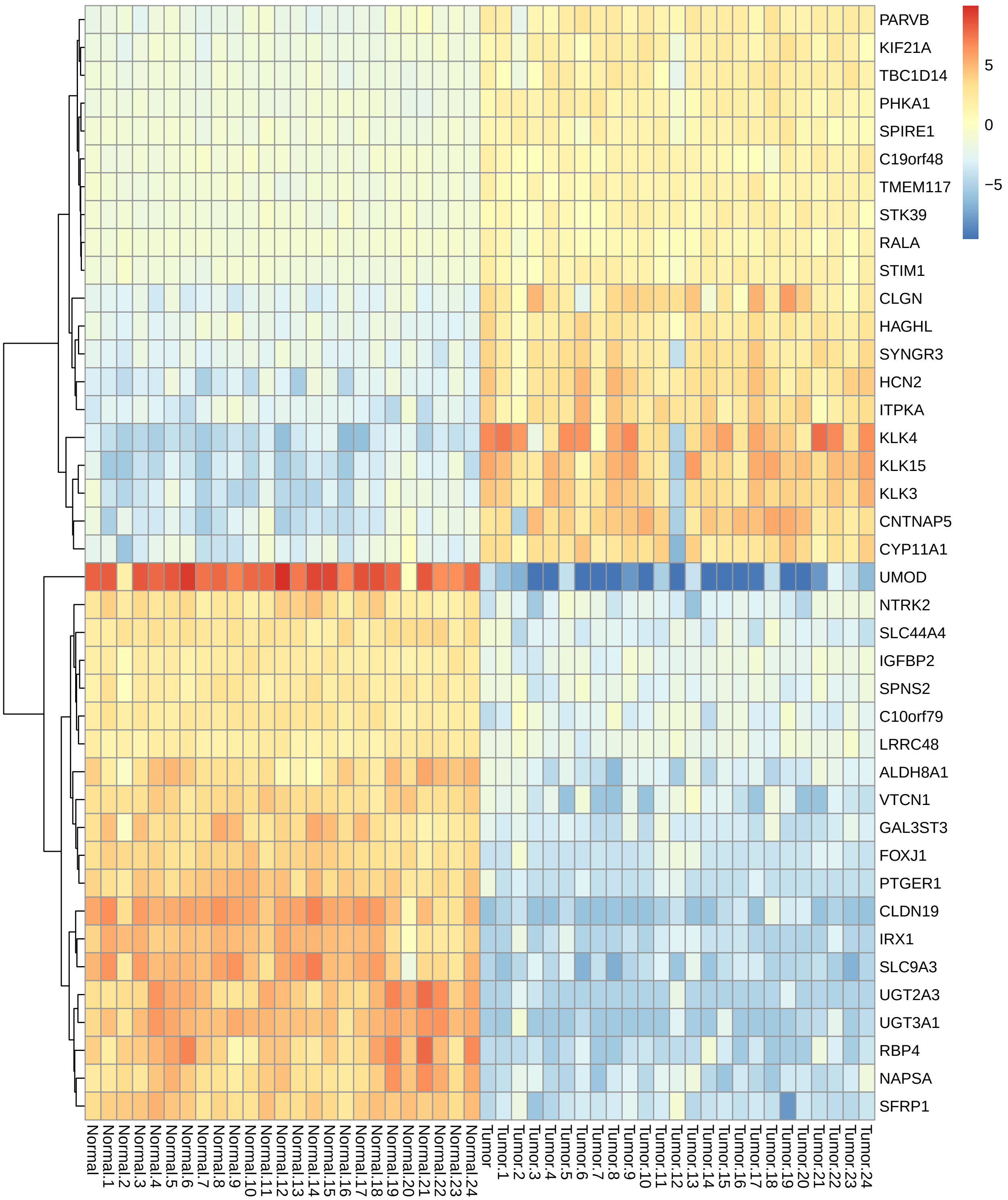
Log2 transformed



```
## Substract the mean
heatmap_data_meanSubstract <- heatmap_data_log2 - rowMeans((heatmap_data_log2))
```

```
#Plot  heatmap
heatmap_data_meanSubstract %>% pheatmap(cluster_cols = F)
```

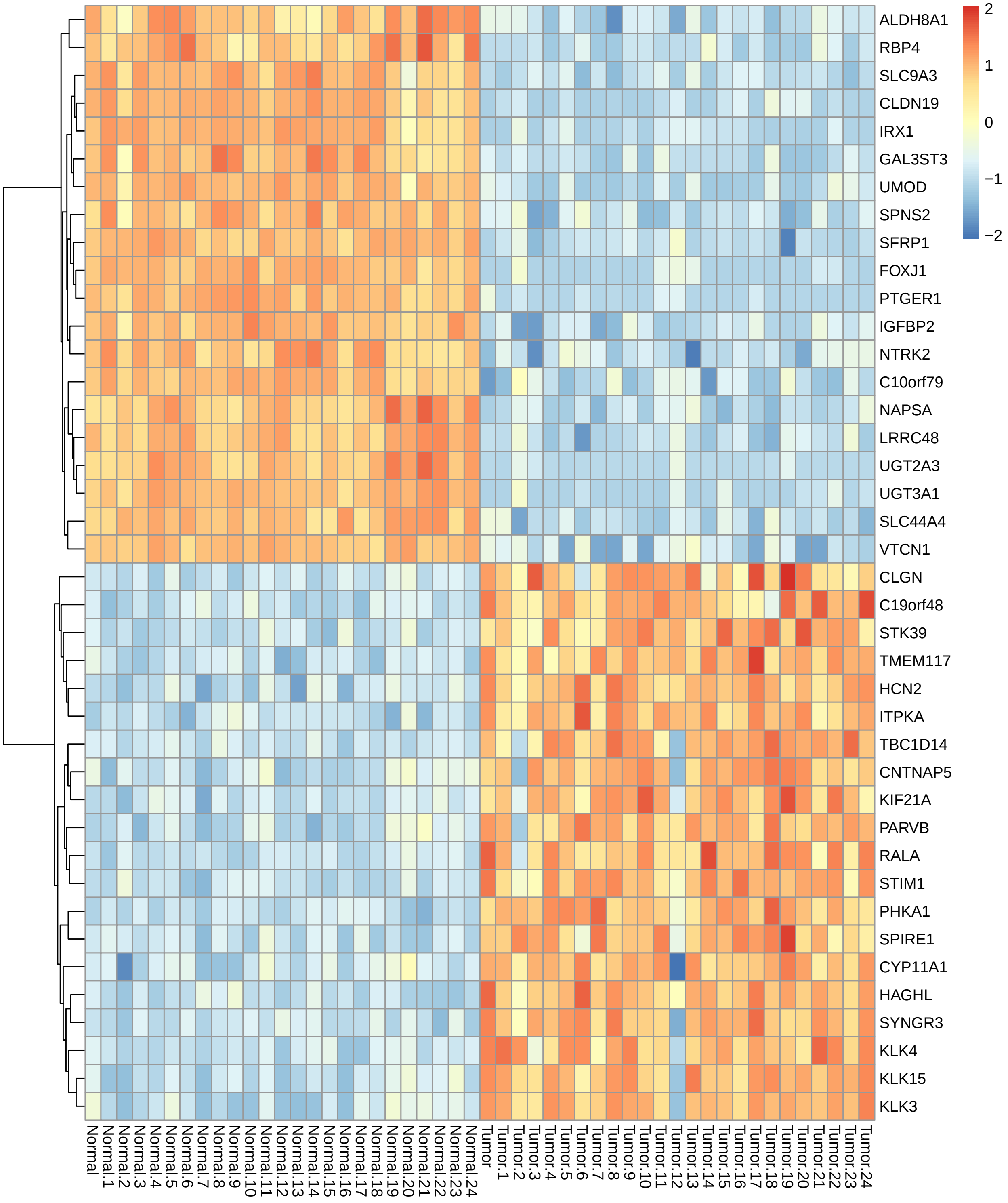
Substract Mean



```
heatmap_data_zscores <- heatmap_data_meanSubstract/
  rowSds(as.matrix(heatmap_data_log2))
```

```
heatmap_data_zscores %>%
  pheatmap(cluster_cols = F)
```

zScores (see also rlog transformed scaled)



rlog transformed count matrix

Die rlog Transformation ist eine Art der Count data transformation. Ähnlich wie bei der `log2()` Funktion, werden die count Values transformiert, aber es werden keine negativen Werte ausgegeben und die Werte werden zur library size normalisiert.

> regularized logarithm or rlog, which incorporates a prior on the sample differences (Love, Huber, and Anders 2014). […] produce transformed data on the log2 scale which has been normalized with respect to library size or other normalization factors.

Quelle: https://bioconductor.org/packages/release/bioc/vignettes/DESeq2/inst/doc/DESeq2.html#data-transformations-and-visualization

```r
# Transform to count matrix to Data frame
heatmap_rlog.df <- as.data.frame(rlds)
# Change Colum Labels
names(heatmap_rlog.df) <- ann$condition
colnames(heatmap_rlog.df) <- make.unique(names(heatmap_rlog.df))
# add rownames as column
heatmap_rlog.df$Gene <- row.names(heatmap_rlog.df)
heatmap_rlog.df <- heatmap_rlog.df %>% relocate(Gene, .before = Normal)
```

```r
# Merge total data frame and heatmap_norm.df
data_frame_merge_rlog <- semi_join(heatmap_rlog.df, total_up_down,
                         by = 'Gene', all = F)
```

```r
# Only add the Gene Name as Rowname, delete the Entrez ID for better overview
df <- separate(data_frame_merge_rlog, Gene, sep="__", into = c("Gene_Name", "Entrez_ID"))
head(df)
```

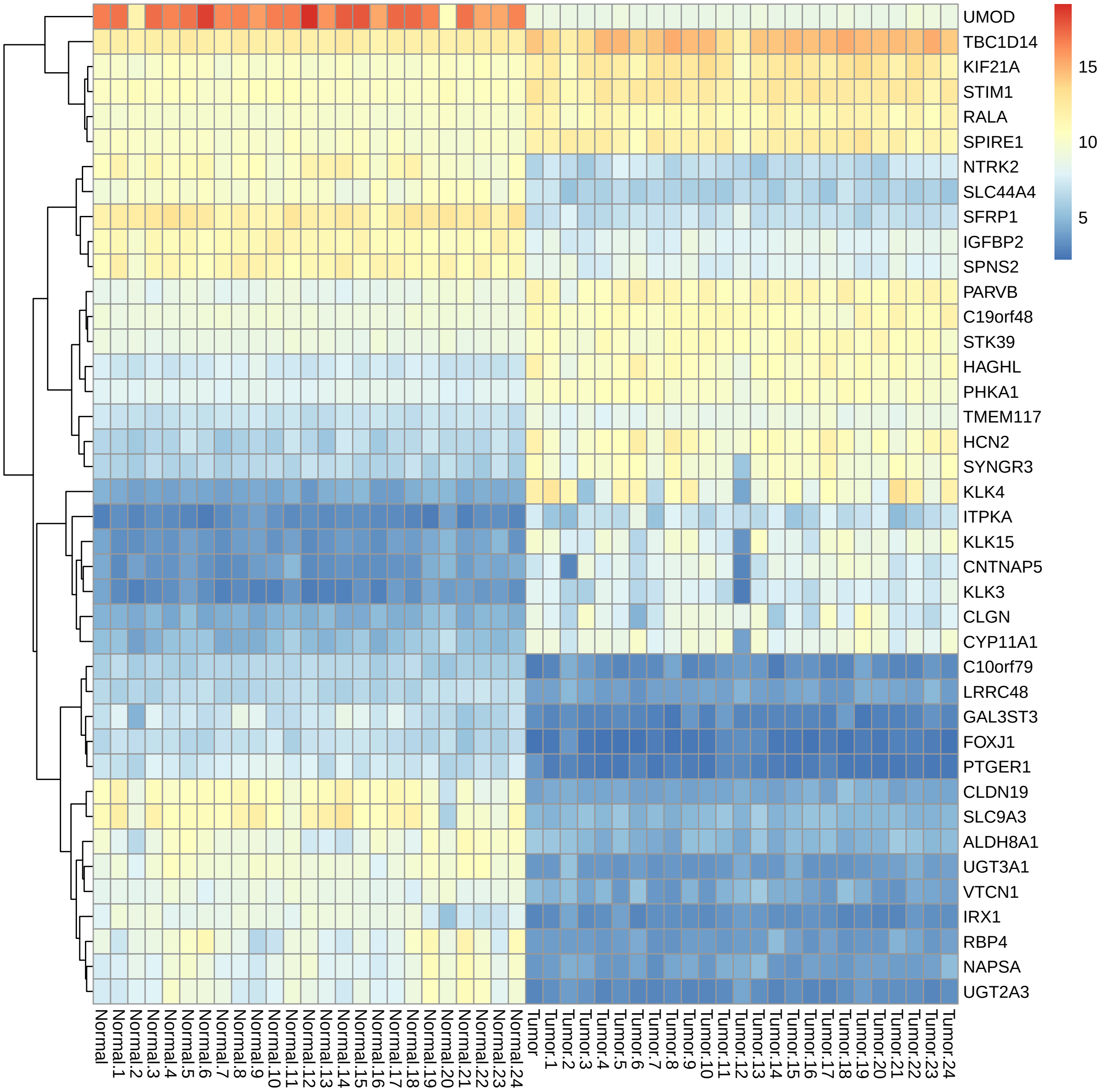
```
##                 Gene_Name Entrez_ID    Normal  Normal.1 Normal.2  Normal.3
## ALDH8A1__64577    ALDH8A1     64577  9.752661  8.208823 6.507364  8.895953
## C10orf79__80217  C10orf79     80217  6.007223  6.641022 5.757752  6.411329
## C19orf48__84798  C19orf48     84798  9.358023  8.855714 9.018381  9.247383
## CLDN19__149461     CLDN19    149461 10.652528 11.509810 8.958369 10.966079
## CLGN__1047           CLGN      1047  4.676399  4.569906 4.328707  4.826275
## CNTNAP5__129684   CNTNAP5    129684  4.309157  3.092744 3.975347  3.440519
##                  Normal.4  Normal.5  Normal.6  Normal.7  Normal.8  Normal.9
## ALDH8A1__64577  10.270395 10.490029  9.952188  8.974107  9.106849  9.154446
## C10orf79__80217  5.965141  5.847496  6.320047  6.257995  6.155887  6.543669
## C19orf48__84798  8.990570  9.258425  9.393197  9.593853  9.134907  9.316440
## CLDN19__149461  10.278012 10.530884 10.825129 10.672763 11.274600 10.749760
## CLGN__1047       4.124855  5.165932  4.154012  4.439149  4.716197  4.127614
## CNTNAP5__129684  3.457792  3.958423  3.484784  3.049073  3.308199  3.726265
##                 Normal.10 Normal.11 Normal.12 Normal.13 Normal.14 Normal.15
## ALDH8A1__64577   8.680069  9.300949  7.370703  7.616380  6.939930  8.596996
## C10orf79__80217  6.531131  6.326355  6.748434  6.515012  6.477849  6.546010
## C19orf48__84798  9.620044  9.184668  9.319852  8.953425  9.094395  8.974515
## CLDN19__149461  10.671679  9.577835 10.629324 10.843053 11.820812 10.648420
## CLGN__1047       4.613591  4.895635  4.465419  4.920513  4.224638  4.372640
## CNTNAP5__129684  4.000917  4.802432  3.109792  3.252585  3.465252  3.290189
##                 Normal.16 Normal.17 Normal.18 Normal.19 Normal.20 Normal.21
## ALDH8A1__64577   9.964163  9.000651  8.235884 10.356455  9.050292 11.071890
## C10orf79__80217  5.793326  6.379835  6.631040  5.723556  5.492353  6.048074
## C19orf48__84798  9.167426  8.867962  9.477509  9.350767  9.467134  9.386225
## CLDN19__149461  10.561049 11.176320 10.977627  9.816673  7.044606  9.978456
## CLGN__1047       5.040717  4.490351  4.450913  5.179722  5.450670  4.364874
## CNTNAP5__129684  3.254482  3.436069  3.458751  4.399250  4.844766  3.786258
##                 Normal.22 Normal.23 Normal.24     Tumor   Tumor.1   Tumor.2
## ALDH8A1__64577  10.443636 10.072136 10.478383  5.600884  5.518917  5.379627
## C10orf79__80217  5.746958  5.880867  5.853096  2.684253  2.877114  4.441801
## C19orf48__84798  9.060902  9.252636  9.121205 11.327029 10.831365 10.256760
## CLDN19__149461   8.581188  8.687754 10.154709  3.953725  4.226058  4.464997
## CLGN__1047       4.796753  4.860316  4.467864  8.866879  7.991721  6.345738
## CNTNAP5__129684  4.308638  4.121210  4.413901  7.261177  7.798898  2.888534
##                   Tumor.3   Tumor.4   Tumor.5   Tumor.6   Tumor.7   Tumor.8
## ALDH8A1__64577   4.874810  4.312124  5.153138  4.533395  4.357254  3.998048
## C10orf79__80217  3.717582  3.231375  2.878887  3.107987  3.126400  4.080819
## C19orf48__84798 10.169397 10.810658 11.035952 10.588345 10.314998 11.035775
## CLDN19__149461   4.108817  4.099665  4.344234  3.957247  3.965631  4.159181
## CLGN__1047      10.090916  8.369333  7.748462  4.602765  7.175427  8.871720
## CNTNAP5__129684  8.993410  7.700056  8.398900  6.747977  8.228159  8.455210
##                   Tumor.9  Tumor.10  Tumor.11  Tumor.12  Tumor.13  Tumor.14
## ALDH8A1__64577   5.106601  5.074073  4.881604  4.112023  5.571224  4.319967
## C10orf79__80217  2.877876  3.074698  3.661028  3.756344  3.632126  2.670580
## C19orf48__84798 10.979823 11.035654 11.282605 10.922901 10.965356 10.759264
## CLDN19__149461   3.936724  4.100250  4.213983  4.495662  4.115648  3.945928
## CLGN__1047       9.186208  9.025176  8.868916  8.570742  9.583484  5.595782
## CNTNAP5__129684  8.694670  9.296475  8.230658  2.911772  6.917589  8.711363
##                  Tumor.15  Tumor.16  Tumor.17 Tumor.18  Tumor.19  Tumor.20
## ALDH8A1__64577   5.024306  4.815860  5.069170 4.271532  4.625382  4.659988
## C10orf79__80217  3.491313  3.512009  2.966366 2.967965  4.078723  3.232499
## C19orf48__84798 10.589100 10.129957 10.145897 9.532940 11.445685 10.819990
## CLDN19__149461   4.352816  4.567294  3.980741 5.335018  4.675958  4.716344
## CLGN__1047       8.088709  6.278271 10.268451 7.712387 10.951436  9.472340
## CNTNAP5__129684  8.176833  8.933390  8.912588 9.731190  9.426789  9.020464
##                  Tumor.21  Tumor.22  Tumor.23  Tumor.24
## ALDH8A1__64577   5.706437  5.246541  4.886790  4.993905
## C10orf79__80217  2.944199  2.885161  3.699896  3.142887
## C19orf48__84798 11.564767 10.858825 10.870873 11.670125
## CLDN19__149461   3.969550  4.232046  4.149143  4.140266
## CLGN__1047       7.346713  7.291073  6.460496  7.909742
## CNTNAP5__129684  7.023985  7.820932  6.863534  7.705475
```

```r
data_frame <- df %>% remove_rownames %>% column_to_rownames(var="Gene_Name")
data_frame_merge_rlog <- select(data_frame, select = -c(Entrez_ID))
```

```r
#Convert to Matrix
data_frame_merge_rlog <- as.matrix(data_frame_merge_rlog)
```

```r
#Plot
data_frame_merge_rlog %>% pheatmap(cluster_cols = F)
```

Heatmap of rlog transformed values



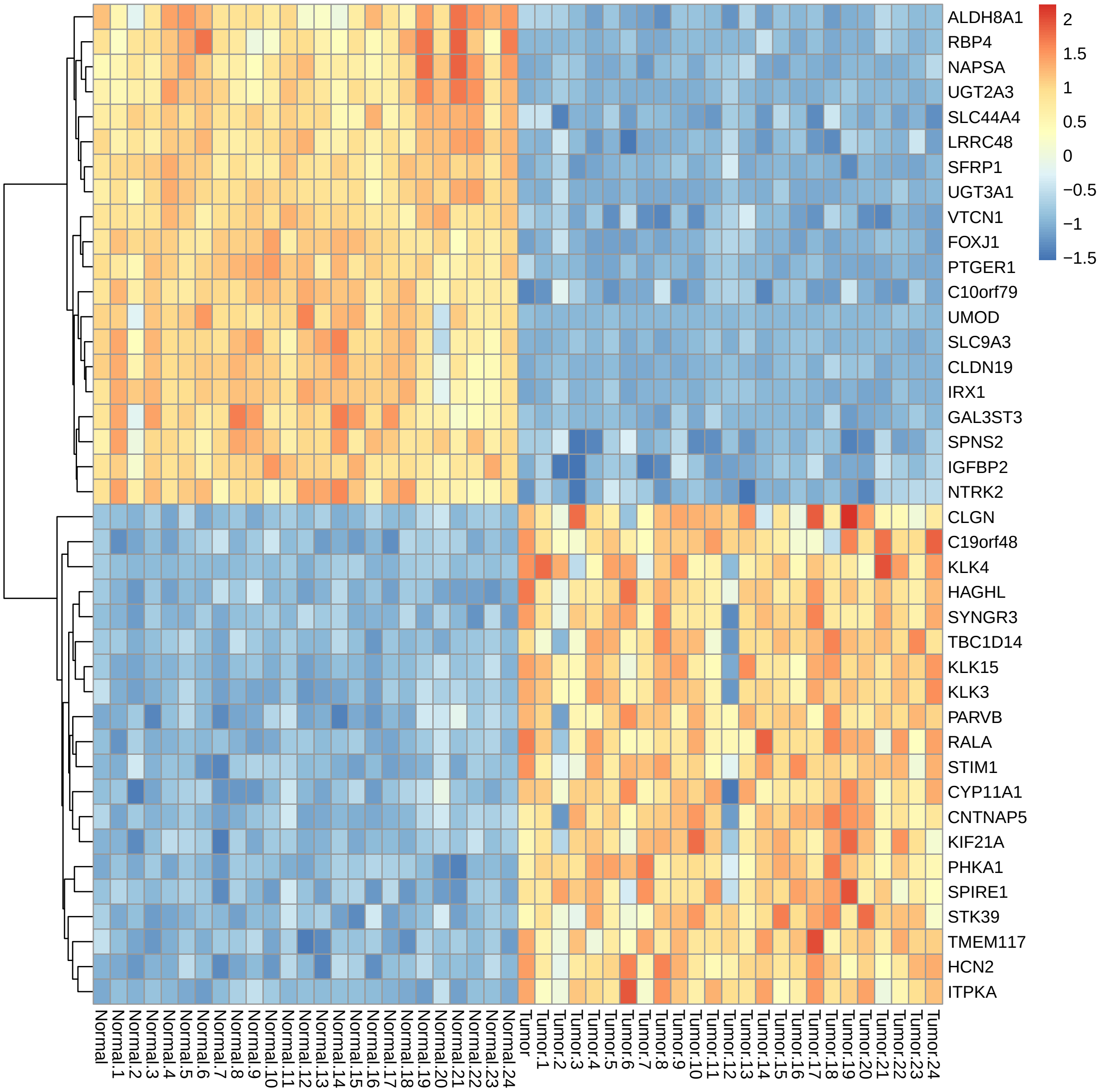
rlog scaled

Erzeugung einer rlog_scaled Matrix:

```
data_frame_merge_rlog_scaled <- t(scale(t(data_frame_merge_rlog),
                                        center = T,
                                        scale =T))
```

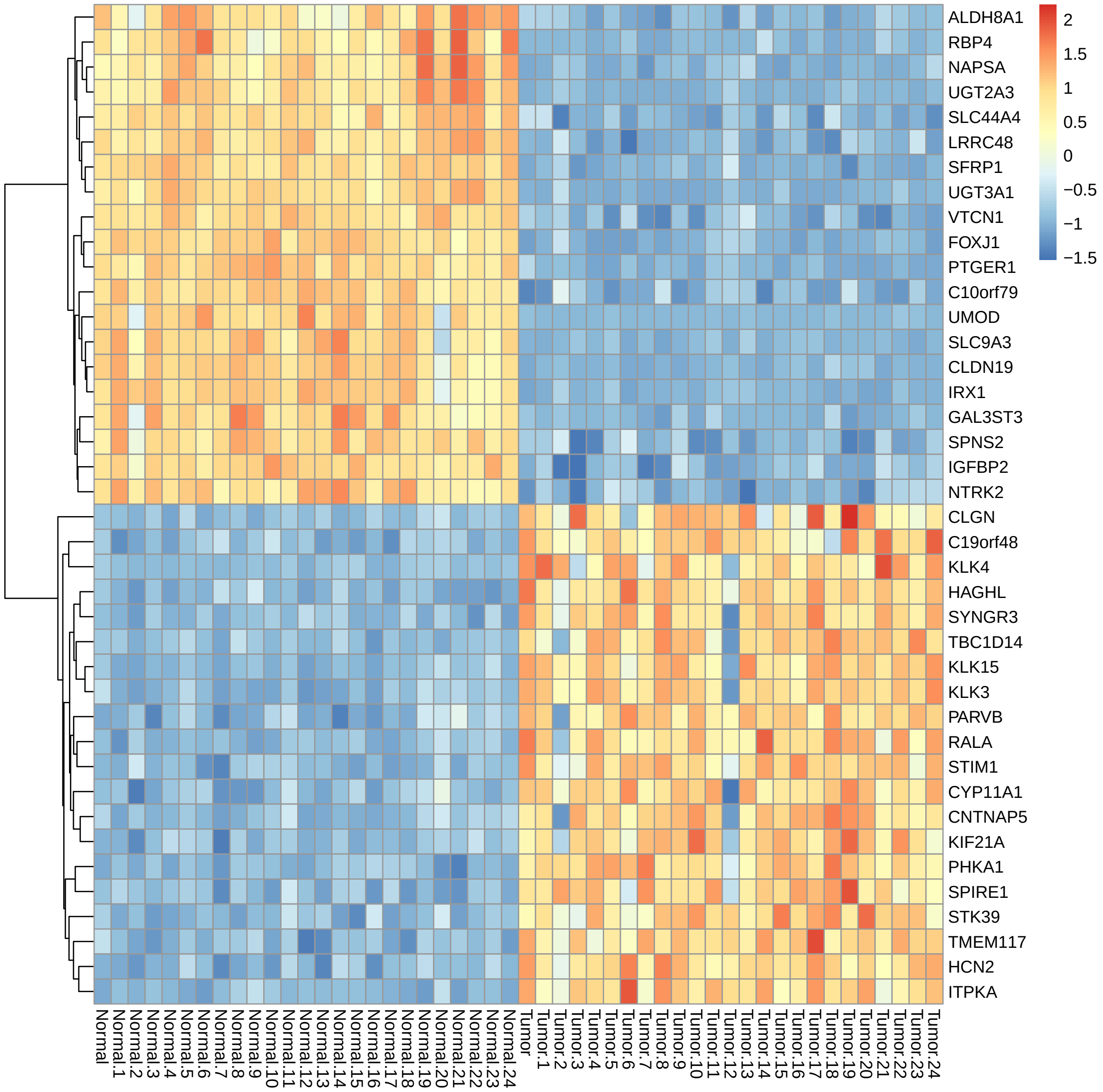
Erzeugung der Heatmap

```
#Plot
data_frame_merge_rlog_scaled  %>%
  pheatmap(cluster_cols = F)
```



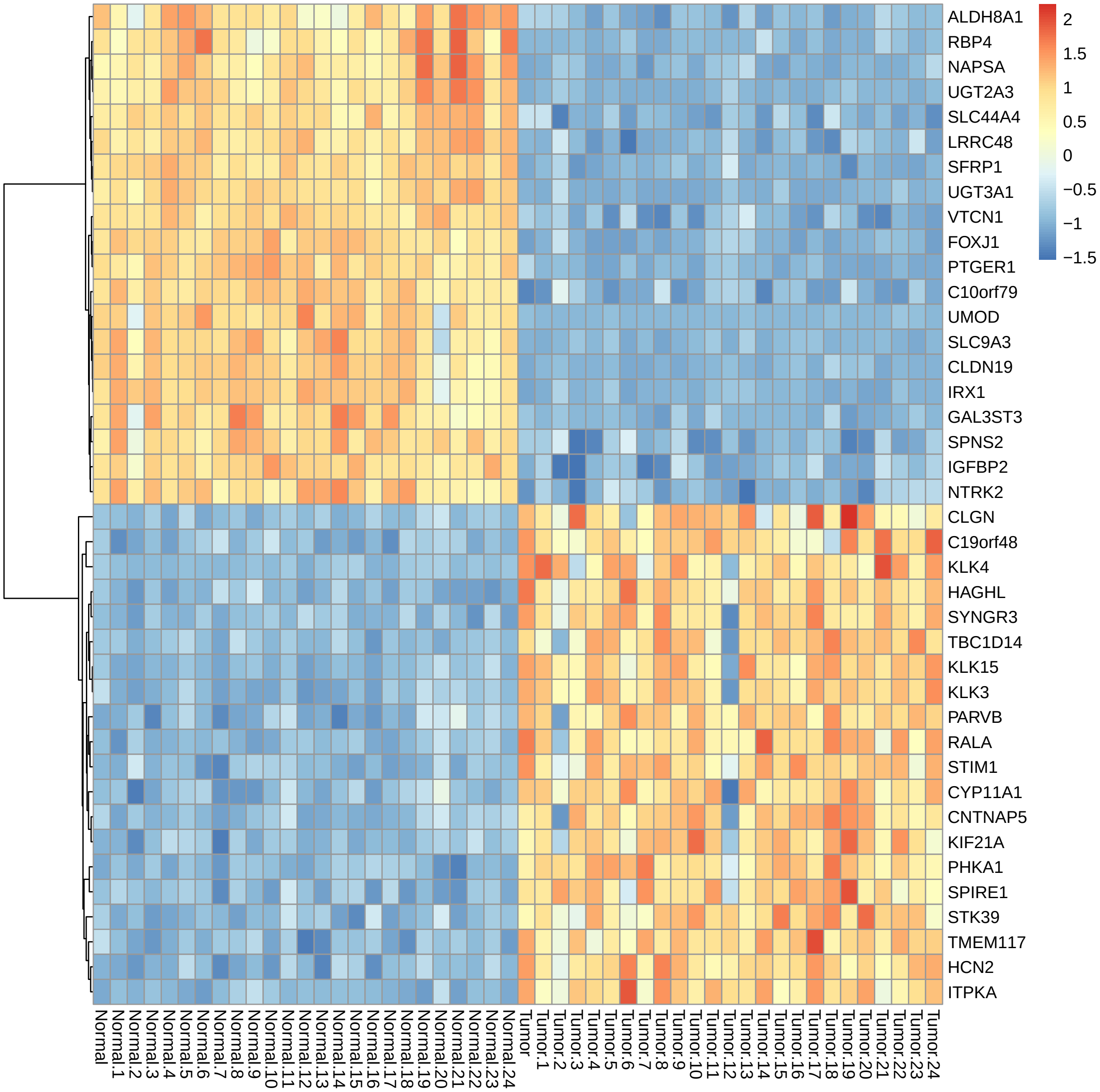
```
# Definition of row clusters (not necessary, is already pheatmap default)
heatmap_rowClusters <- data_frame_merge_rlog_scaled %>%
  dist() %>%
  hclust()
```

```
#Plot
data_frame_merge_rlog_scaled %>%
  pheatmap(cluster_cols = F,
           cluster_rows = heatmap_rowClusters)
```



```r
#Generate correlation matrix
rows_cor <- data_frame_merge_rlog_scaled %>%
  t() %>%
  cor()
# Convert to distance
heatmap_rowClusters_cor <- as.dist(1-rows_cor) %>%
  hclust()
```

```r
#Plot
data_frame_merge_rlog_scaled %>%
  pheatmap(cluster_cols = F,
           cluster_rows = heatmap_rowClusters_cor)
```



```
#Define breaks
symmetric_breaks <- seq(-max(abs(data_frame_merge_rlog_scaled)),
                        max(abs(data_frame_merge_rlog_scaled)),
                        length.out = 101)
```

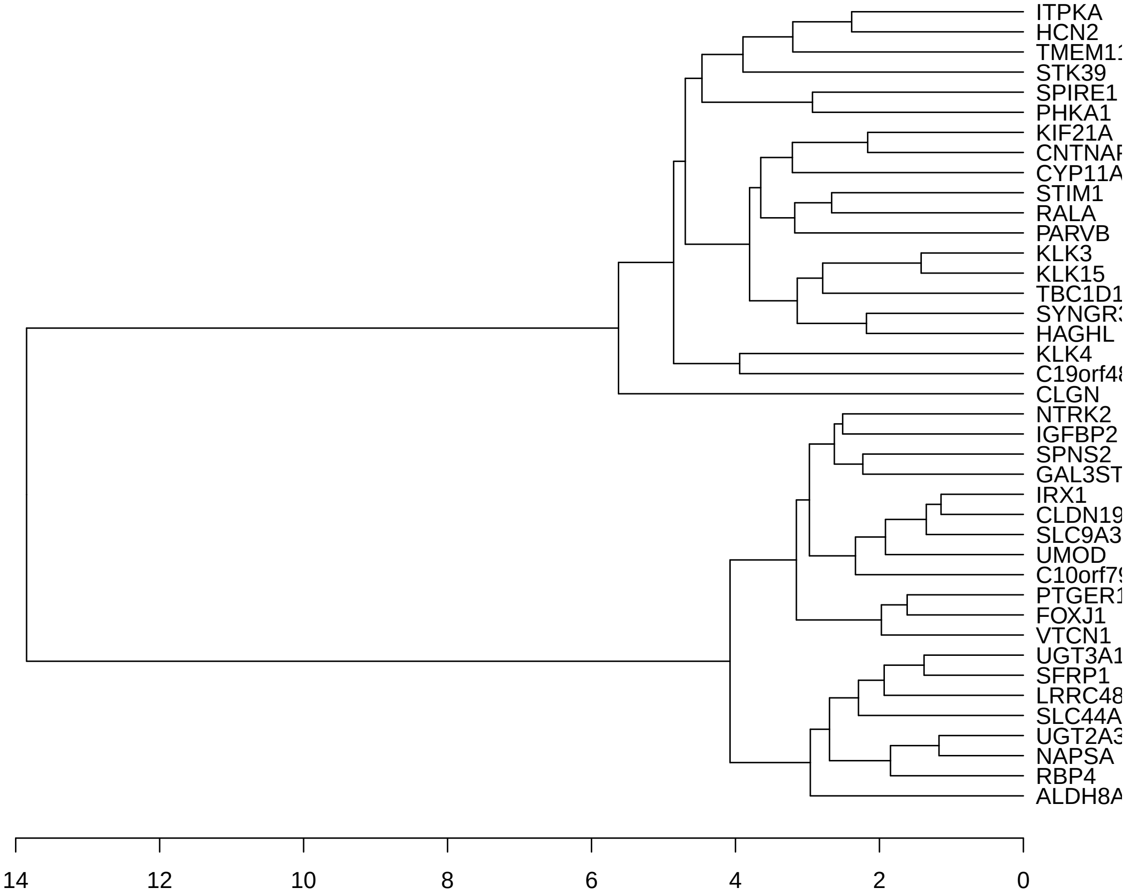
```
# Cluster genes using "dendextend" dendrogramm
my_hclust_gene <- hclust(dist(data_frame_merge_rlog_scaled),
                         method = "complete")

# install if necessary
#install.packages("dendextend")

# load package
```

```
library(dendextend)
```

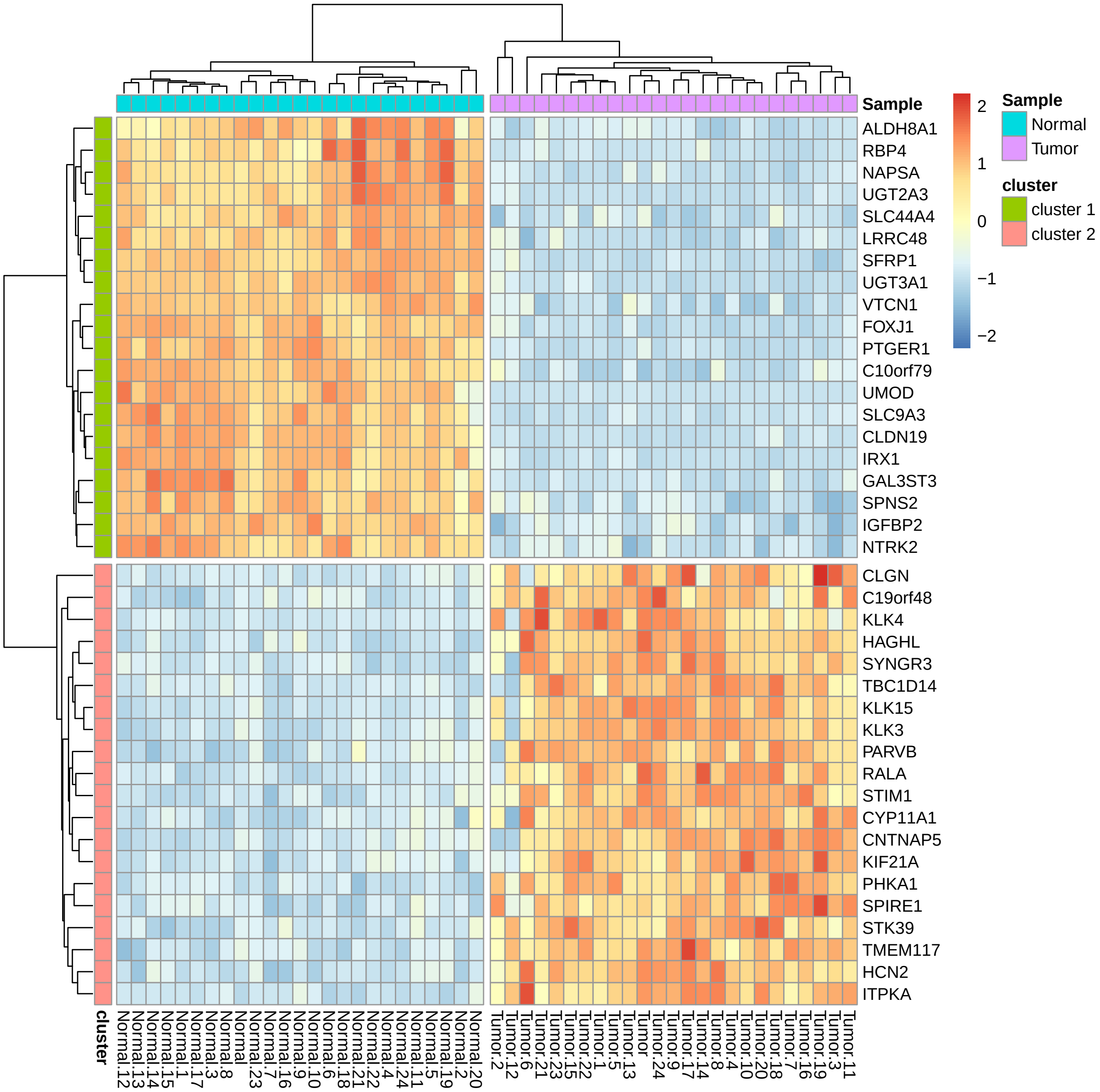
```
as.dendrogram(my_hclust_gene) %>%
  plot(horiz = TRUE)
```



```
#Row annotation using dendrogramm:
my_gene_col <- cutree(tree = as.dendrogram(my_hclust_gene), k = 2)
#Cluster annotation (one or two)
my_gene_col <- data.frame(cluster = ifelse(test = my_gene_col == 1,
                                           yes = "cluster 1",
                                           no = "cluster 2"))
```

```
# Column annotation using Sample type
my_sample_col <- data.frame(Sample = rep(c("Normal", "Tumor"), c(25,25)))
row.names(my_sample_col) <- colnames(data_frame_merge_rlog_scaled)
```

```
#Create final Heatmap
pheatmap(data_frame_merge_rlog_scaled,
         annotation_row = my_gene_col,
         annotation_col = my_sample_col,
         breaks = symmetric_breaks,
         cluster_cols = T,
         cutree_rows = 2,
         cutree_cols = 2)
```



```
#Create final Heatmap pdf
pheatmap(data_frame_merge_rlog_scaled,
         annotation_row = my_gene_col,
         annotation_col = my_sample_col,
         breaks = symmetric_breaks,
         cluster_cols = T,
         cutree_rows = 2,
         cutree_cols = 2,
         filename = "heatmap.pdf",
         width = 11.69,
         height = 8.27)
```

Erklärung

Scaling (oder Standardization) transformiert die Daten, der Mean/Durchschnitt wird auf 0 und die Standard-Abweichung auf 1 gesetzt. Dies entspricht den z scores. Dies eignet sich be Werten mit hoher Varianz und Extremweren, da diese sonst die Heatmap dominieren (z.B. UMOD Gen).

Citations

```
citation("DESeq2")
```

```
##
##   Love, M.I., Huber, W., Anders, S. Moderated estimation of fold change
##   and dispersion for RNA-seq data with DESeq2 Genome Biology 15(12):550
##   (2014)
##
## Ein BibTeX-Eintrag für LaTeX-Benutzer ist
##
##   @Article{,
##     title = {Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2},
##     author = {Michael I. Love and Wolfgang Huber and Simon Anders},
##     year = {2014},
##     journal = {Genome Biology},
##     doi = {10.1186/s13059-014-0550-8},
##     volume = {15},
##     issue = {12},
##     pages = {550},
##   }
```

```
citation("EnhancedVolcano")
```

```
##
## To cite package 'EnhancedVolcano' in publications use:
##
##   Kevin Blighe, Sharmila Rana and Myles Lewis (2021). EnhancedVolcano:
##   Publication-ready volcano plots with enhanced colouring and labeling.
##   R package version 1.12.0.
##   https://github.com/kevinblighe/EnhancedVolcano
##
## Ein BibTeX-Eintrag für LaTeX-Benutzer ist
##
##   @Manual{,
##     title = {EnhancedVolcano: Publication-ready volcano plots with enhanced colouring and
## labeling},
##     author = {Kevin Blighe and Sharmila Rana and Myles Lewis},
##     year = {2021},
##     note = {R package version 1.12.0},
##     url = {https://github.com/kevinblighe/EnhancedVolcano},
##   }
```

Session info

```
sessionInfo()
```

```
## R version 4.1.2 (2021-11-01)
## Platform: x86_64-w64-mingw32/x64 (64-bit)
## Running under: Windows 10 x64 (build 19042)
##
## Matrix products: default
##
## locale:
## [1] LC_COLLATE=German_Germany.1252  LC_CTYPE=German_Germany.1252
## [3] LC_MONETARY=German_Germany.1252 LC_NUMERIC=C
## [5] LC_TIME=German_Germany.1252
##
## attached base packages:
## [1] stats4    stats     graphics  grDevices utils     datasets  methods
## [8] base
##
## other attached packages:
##  [1] dendextend_1.15.2           pheatmap_1.0.12
##  [3] EnhancedVolcano_1.12.0      openxlsx_4.2.4
##  [5] glmpca_0.2.0                DESeq2_1.34.0
##  [7] SummarizedExperiment_1.24.0 Biobase_2.54.0
##  [9] MatrixGenerics_1.6.0        matrixStats_0.61.0
## [11] GenomicRanges_1.46.1        GenomeInfoDb_1.30.0
## [13] IRanges_2.28.0              S4Vectors_0.32.3
## [15] BiocGenerics_0.40.0         forcats_0.5.1
## [17] stringr_1.4.0               purrr_0.3.4
## [19] tidyverse_1.3.1             tidyr_1.1.4
## [21] FactoMineR_2.4              readr_2.1.1
## [23] tibble_3.1.6                dplyr_1.0.7
## [25] genefilter_1.76.0           ggrepel_0.9.1
## [27] ggplot2_3.3.5               BiocManager_1.30.16
##
## loaded via a namespace (and not attached):
##   [1] ggbeeswarm_0.6.0       colorspace_2.0-2       ellipsis_0.3.2
##   [4] XVector_0.34.0         fs_1.5.1               rstudioapi_0.13
##   [7] farver_2.1.0           DT_0.20                bit64_4.0.5
##  [10] AnnotationDbi_1.56.2   fansi_0.5.0            lubridate_1.8.0
##  [13] xml2_1.3.3             splines_4.1.2          leaps_3.1
##  [16] extrafont_0.17         cachem_1.0.6           geneplotter_1.72.0
##  [19] knitr_1.36             jsonlite_1.7.2         Rttf2pt1_1.3.9
##  [22] broom_0.7.10           annotate_1.72.0        cluster_2.1.2
##  [25] dbplyr_2.1.1         png_0.1-7              compiler_4.1.2
##  [28] httr_1.4.2           backports_1.4.0        assertthat_0.2.1
##  [31] Matrix_1.3-4         fastmap_1.1.0          cli_3.1.0
##  [34] htmltools_0.5.2      tools_4.1.2            gtable_0.3.0
##  [37] glue_1.5.1           GenomeInfoDbData_1.2.7 maps_3.4.0
##  [40] Rcpp_1.0.7           cellranger_1.1.0       vctrs_0.3.8
##  [43] Biostrings_2.62.0    ggalt_0.4.0            extrafontdb_1.0
##  [46] xfun_0.28            rvest_1.0.2            lifecycle_1.0.1
##  [49] XML_3.99-0.8         zlibbioc_1.40.0        MASS_7.3-54
##  [52] scales_1.1.1         ragg_1.2.1             hms_1.1.1
##  [55] proj4_1.0-10.1       parallel_4.1.2         RColorBrewer_1.1-2
##  [58] yaml_2.2.1           gridExtra_2.3          memoise_2.0.1
##  [61] ggrastr_1.0.1        stringi_1.7.6          RSQLite_2.2.9
##  [64] highr_0.9            zip_2.2.0              BiocParallel_1.28.2
##  [67] rlang_0.4.12         pkgconfig_2.0.3        systemfonts_1.0.3
##  [70] bitops_1.0-7         evaluate_0.14          lattice_0.20-45
##  [73] htmlwidgets_1.5.4    labeling_0.4.2         bit_4.0.4
##  [76] tidyselect_1.1.1     magrittr_2.0.1         R6_2.5.1
##  [79] generics_0.1.1       DelayedArray_0.20.0    DBI_1.1.1
##  [82] pillar_1.6.4         haven_2.4.3            withr_2.4.3
##  [85] ash_1.0-15           survival_3.2-13        KEGGREST_1.34.0
##  [88] scatterplot3d_0.3-41 RCurl_1.98-1.5         modelr_0.1.8
##  [91] crayon_1.4.2         KernSmooth_2.23-20     utf8_1.2.2
##  [94] tzdb_0.2.0           rmarkdown_2.11         viridis_0.6.2
##  [97] locfit_1.5-9.4       grid_4.1.2             readxl_1.3.1
## [100] blob_1.2.2           reprex_2.0.1           digest_0.6.28
## [103] flashClust_1.01-2    xtable_1.8-4           textshaping_0.3.6
## [106] munsell_0.5.0        viridisLite_0.4.0      beeswarm_0.4.0
## [109] vipor_0.4.5
```