

Anaquin - Vignette

Ted Wong (t.wong@garvan.org.au)

April 15, 2025

Citation

- [1] Representing genetic variation with synthetic DNA standards. *Nature Methods*, 2017
- [2] Spliced synthetic genes as internal controls in RNA sequencing experiments. *Nature Methods*, 2016.
- [3] Reference standards for next-generation sequencing. *Nature Reviews*, 2017.
- [4] Anaquin: a software toolkit for the analysis of spike-in controls for next generation sequencing. *Bioinformatics*, 2017.

Website

Visit our website to learn more about sequins: www.sequin.xyz.

Overview

In this document, we show how to conduct statistical analysis that models the performance of sequin controls in next-generation-sequencing (NGS) experiment. We call the sequins **RnaQuin** for “RNA-Seq sequins”, **MetaQuin** for “metagenomic sequins”, **VarQuin** for “genomics variant sequins”, and the statistical framework **Anaquin**.

This vignette is written for R-usage. However, Anaquin is a framework covering the entire NGS workflow. Consequently, the R-package (and its documentation) is a subset of the overall Anaquin framework. We also distribute a detailed workflow guide on our website.

It is important to note Anaquin is both command-line tool and R-package. Our workflow guide has the details on how the command-line tool can be used with the R-package.

Sequins

Next-generation sequencing (NGS) enables rapid, cheap and high-throughput determination of sequences within a user’s sample. NGS methods have been applied widely, and have fuelled major advances in the life sciences and clinical health care over the past decade. However, NGS typically generates a large amount of sequencing data that must be first analyzed and interpreted with bioinformatics tools. There is no standard way to perform an analysis of NGS data; different tools provide different advantages in different situations. The complexity and variation of sequences further compound this problem, and there is little reference by which compare next-generation sequencing and analysis.

To address this problem, we have developed a suite of synthetic nucleic-acid sequins (sequencing spike-ins). Sequins are fractionally added to the extracted nucleic-acid sample prior to library preparation, so they are sequenced along with your sample of interest. We can use the sequins as an internal quantitative and qualitative control to assess any stage of the next-generation sequencing workflow.



Figure 1: NGS Workflow for sequins

Mixture

Sequins are combined together across a range of concentrations to formulate a mixture. Mixture file (CSV) is a text file that specifies the concentration of each sequin within a mixture. Mixture files are often required as input to enable Anaquin to perform quantitative analysis. Mixture file can be downloaded from our website.

Let's demonstrate RnaQuin mixture A with a simple example. Load the mixture file (you can also download the file directly from our website):

```
library('Anaquin')
```

```
## Loading required package: ggplot2
```

```
data("RnaQuinIsoformMixture")
```

```
head(RnaQuinIsoformMixture)
```

##	Name	Length	MixA	MixB
## 1	R1_101_1	719	11.329650	0.472075
## 2	R1_101_2	430	3.776550	1.416225
## 3	R1_102_1	1490	13.217925	7.553100
## 4	R1_102_2	1362	1.888275	52.871700
## 5	R1_103_1	1754	60.424806	453.186000
## 6	R1_103_2	1856	906.372094	30.212400

Each row represents a sequin. *Name* gives the sequin names, *Length* is the length of the sequins in nucleotide bases, *MixA* gives the concentration level in attoml/ul for Mixture A.

Imagine we have two RNA-Seq experiments; a well-designed experiment and a poorly-designed experiment. We would like to quantify their isoform expression.

Let's simulate the experiments:

```
set.seed(1234)
```

```
sim1 <- 1.0 + 1.2*log2(RnaQuinIsoformMixture$MixA) + rnorm(nrow(RnaQuinIsoformMixture),0,1)
```

```
sim2 <- c(1.0 + rnorm(100,1,3), 1.0 +  
          1.2*log2(tail(RnaQuinIsoformMixture,64)$MixA) +  
          rnorm(64,0,1))
```

In the first experiment, sequins are expected to correlate linearly with the measured FPKM. Indeed, the variables are strongly correlated:

```
names <- row.names(RnaQuinIsoformMixture)
```

```
input <- log2(RnaQuinIsoformMixture$MixA)
```

```
title <- 'Isoform expression (Good)'
```

```
xlab <- 'Input concentration (log2)'
```

```
ylab <- 'Measured FPKM (log2)'
```

```
plotLinear(names, input, sim1, title=title, xlab=xlab, ylab=ylab)
```

```
## Warning: The `<scale>` argument of `guides()` cannot be `FALSE`. Use "none" instead as  
## of ggplot2 3.3.4.
```

```
## i The deprecated feature was likely used in the Anaquin package.
```

```
## Please report the issue at <https://github.com/student-t/RAnaquin/issues>.
```

```
## This warning is displayed once every 8 hours.
```

```
## Call `lifecycle::last_lifecycle_warnings()` to see where this warning was  
## generated.
```

```
## Warning: Use of `data$x` is discouraged.
```

```
## i Use `x` instead.
```

```
## Warning: Use of `data$y` is discouraged.
```

```
## i Use `y` instead.
```

```
## Warning: Use of `data$x` is discouraged.
```

```
## i Use `x` instead.
```

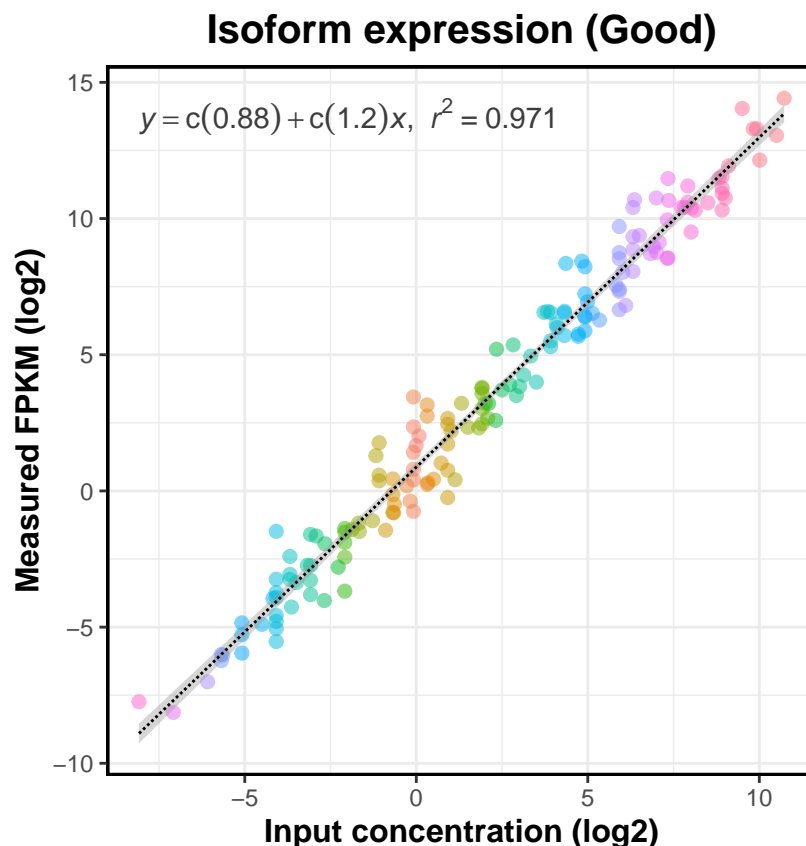
```
## Warning: Use of `data$y` is discouraged.
```

```
## i Use `y` instead.
```

```
## Warning: The following aesthetics were dropped during statistical transformation: label.
```

```
## i This can happen when ggplot fails to infer the correct grouping structure in  
## the data.
```

```
## i Did you forget to specify a `group` aesthetic or to convert a numerical  
## variable into a factor?
```



In our second experiment, the weakly expressed isoforms exhibit stochastic behavior and are clearly not linear with the input concentration. Furthermore, there is a limit of quantification (LOQ); below which accuracy of the experiment becomes questionable.

```
names <- row.names(RnaQuinIsoformMixture)
input <- log2(RnaQuinIsoformMixture$MixA)

title <- 'Isoform expression (Bad)'
xlab <- 'Input concentration (log2)'
ylab <- 'Measured FPKM (log2)'

plotLinear(names, input, sim2, title=title, xlab=xlab, ylab=ylab)

## Warning: Use of `data$x` is discouraged.
## i Use `x` instead.

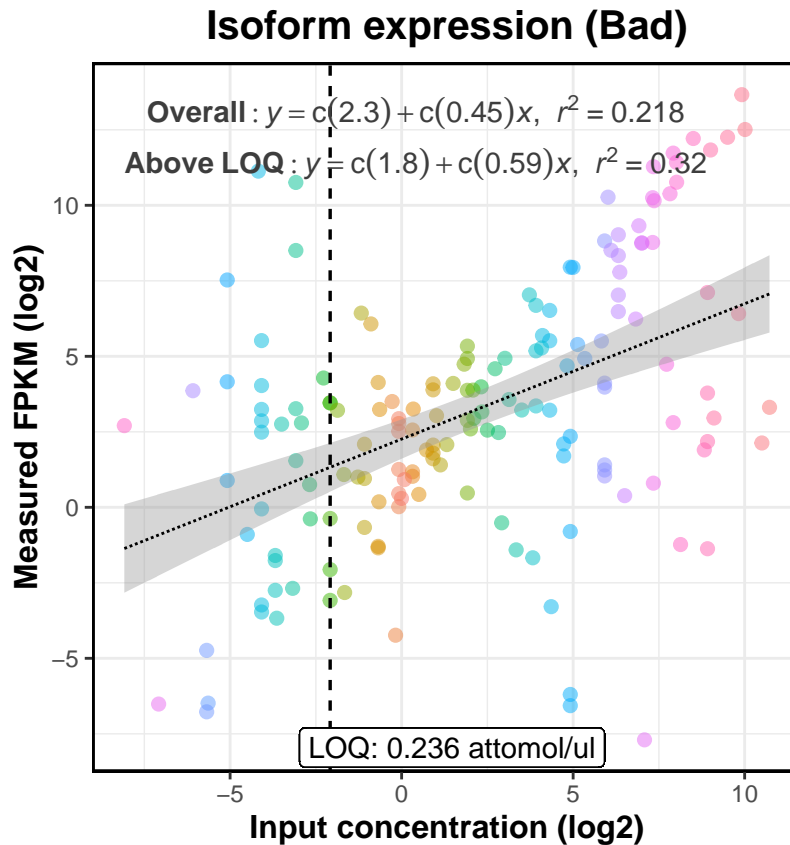
## Warning: Use of `data$y` is discouraged.
## i Use `y` instead.

## Warning: Use of `data$x` is discouraged.
## i Use `x` instead.

## Warning: Use of `data$y` is discouraged.
## i Use `y` instead.

## Warning in geom_label(aes_string(x = "max(LOQ$breaks$k)", y = "min(y)"), : All aesthetics have length
## i Please consider using `annotate()` or provide this layer with data containing
##   a single row.

## Warning: The following aesthetics were dropped during statistical transformation: label.
## i This can happen when ggplot fails to infer the correct grouping structure in
##   the data.
## i Did you forget to specify a `group` aesthetic or to convert a numerical
##   variable into a factor?
```



The primary observation is that the artificial scale imposed by sequins allow us to quantify our experiments.

Quantifying transcriptome assembly

To quantify RNA-Seq transcriptome assembly, we need to run a transcriptome assembler; a software that can assemble transcripts and estimates their abundances. Our workflow guide has the details.

Here, we use a data set generated by Cufflinks, described in **Section 5.4.5.1** in the user guide:

```
data(UserGuideData_5.4.5.1)
head(UserGuideData_5.4.5.1)
```

```
##           Input      Sn
## R1_101_1 10.0708 0.990264
## R1_101_2  5.0354 0.393023
## R1_102_1  0.8886 0.519463
## R1_102_2 14.2176 0.902349
## R1_103_1 107.4220 0.995439
## R1_103_2 859.3750 0.904095
```

The first column gives the input concentration for each sequin in attomol/ul. The second column is the measured sensitivity. Run the following R-code to generate a sensitivity plot.

```
title <- 'Assembly Plot'
xlab  <- 'Input Concentration (log2)'
ylab  <- 'Sensitivity'

# Sequin names
names <- row.names(UserGuideData_5.4.5.1)
```

```

# Input concentration
x <- log2(UserGuideData_5.4.5.1$Input)

# Measured sensitivity
y <- UserGuideData_5.4.5.1$Sn

plotLogistic(names, x, y, title=title, xlab=xlab, ylab=ylab, showLOA=TRUE)

## Warning: Use of `data$x` is discouraged.
## i Use `x` instead.

## Warning: Use of `data$y` is discouraged.
## i Use `y` instead.

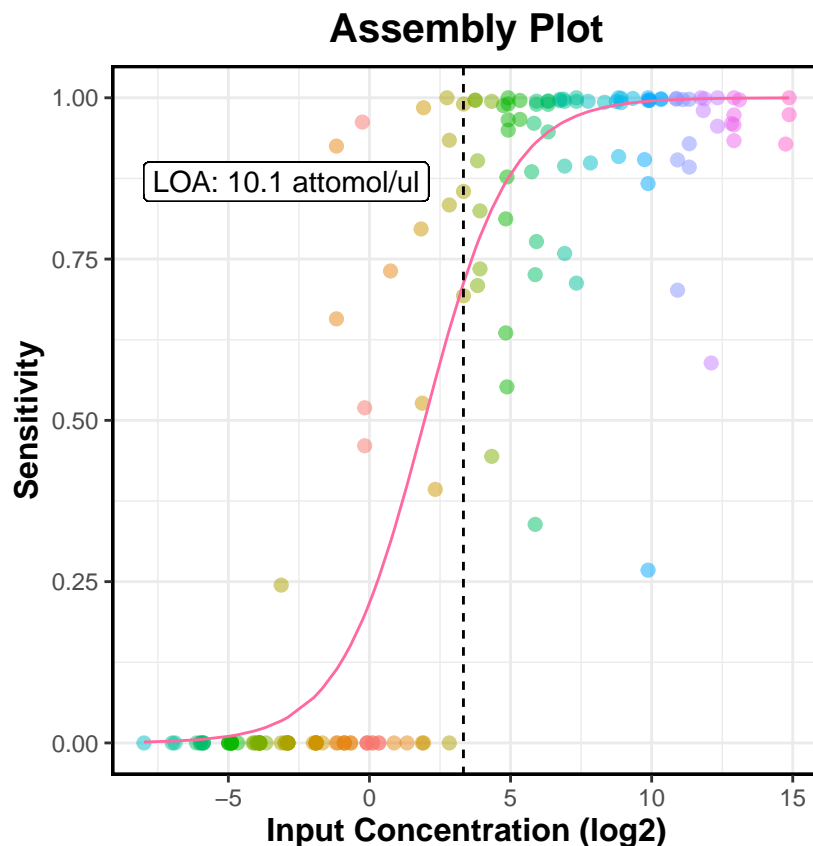
## Warning in geom_label(aes(x = min(data$x), y = max(data$y) - 0.1), label = label, : All aesthetics h
## i Please consider using `annotate()` or provide this layer with data containing
##   a single row.

## Warning: Use of `data$x` is discouraged.
## i Use `x` instead.

## Warning: Use of `data$y` is discouraged.
## i Use `y` instead.

## Warning in geom_label(aes(x = min(data$x), y = max(data$y) - 0.1), label = label, : All aesthetics h
## i Please consider using `annotate()` or provide this layer with data containing
##   a single row.

```



The fitted logistic curve reveals clear relationship between input concentration and sensitivity. Unsurprisingly,

the assembler has higher sensitivity with highly expressed isoforms. The limit-of-assembly (LOA) is defined as the intersection of the curve to sensitivity of 0.70.

Quantifying gene expression

Quantifying gene/isoform expression involves building a linear model between input concentration and measured FPKM. In this section, we consider a dataset generated by Cufflinks, described in **Section 5.4.5.1** of the user guide.

Load the data set:

```
data(UserGuideData_5.4.6.3)
head(UserGuideData_5.4.6.3)
```

	Input	Observed1	Observed2	Observed3
## R1_101	15.1062	0.958838	1.456650	0.960190
## R1_102	15.1062	0.806596	0.604539	0.652783
## R1_103	966.7970	2.650470	2.890570	3.211090
## R1_11	241.6990	3.876010	3.919950	4.246390
## R1_12	30.2124	0.779118	0.898644	0.733175
## R1_13	7734.3800	1305.710000	1328.950000	1358.970000

The first column gives input concentration for each sequin in attomol/ul. The other columns are the FPKM values for each replicate (three replicates in total). The following code will quantify the first replicate:

```
title <- 'Gene Expression'
xlab <- 'Input Concentration (log2)'
ylab <- 'FPKM (log2)'

# Sequin names
names <- row.names(UserGuideData_5.4.6.3)

# Input concentration
x <- log2(UserGuideData_5.4.6.3$Input)

# Measured FPKM
y <- log2(UserGuideData_5.4.6.3$Observed1)

plotLinear(names, x, y, title=title, xlab=xlab, ylab=ylab, showLOQ=TRUE)
```

```
## Warning: Use of `data$x` is discouraged.
## i Use `x` instead.
```

```
## Warning: Use of `data$y` is discouraged.
## i Use `y` instead.
```

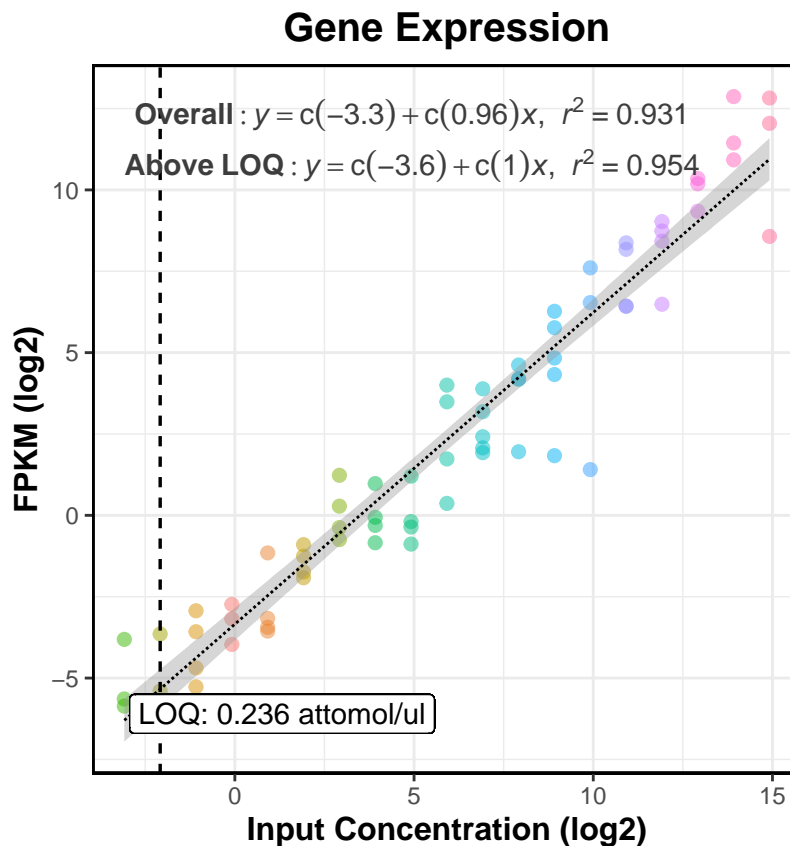
```
## Warning: Use of `data$x` is discouraged.
## i Use `x` instead.
```

```
## Warning: Use of `data$y` is discouraged.
## i Use `y` instead.
```

```
## Warning in geom_label(aes_string(x = "max(LOQ$breaks$k)", y = "min(y)"), : All aesthetics have length 1
## i Please consider using `annotate()` or provide this layer with data containing a single row.
```

```
## Warning: The following aesthetics were dropped during statistical transformation: label.
## i This can happen when ggplot fails to infer the correct grouping structure in
```

```
## the data.
## i Did you forget to specify a `group` aesthetic or to convert a numerical
## variable into a factor?
```



Coefficient of determination is over 0.90; over 90% of the variation (e.g. technical bias) can be explained by the model. LOQ is 3.78 attomol/ul, this is the estimated empirical detection limit.

We can also quantify multiple replicates:

```
title <- 'Gene Expression'
xlab <- 'Input Concentration (log2)'
ylab <- 'FPKM (log2)'

# Sequin names
names <- row.names(UserGuideData_5.4.6.3)

# Input concentration
x <- log2(UserGuideData_5.4.6.3$Input)

# Measured FPKM
y <- log2(UserGuideData_5.4.6.3[,2:4])

plotLinear(names, x, y, title=title, xlab=xlab, ylab=ylab, showLOQ=TRUE)
```

```
## Warning: Use of `data$x` is discouraged.
## i Use `x` instead.

## Warning: Use of `data$y` is discouraged.
## i Use `y` instead.
```



```
## Warning: Use of `data$x` is discouraged.
## i Use `x` instead.

## Warning: Use of `data$y` is discouraged.
## i Use `y` instead.

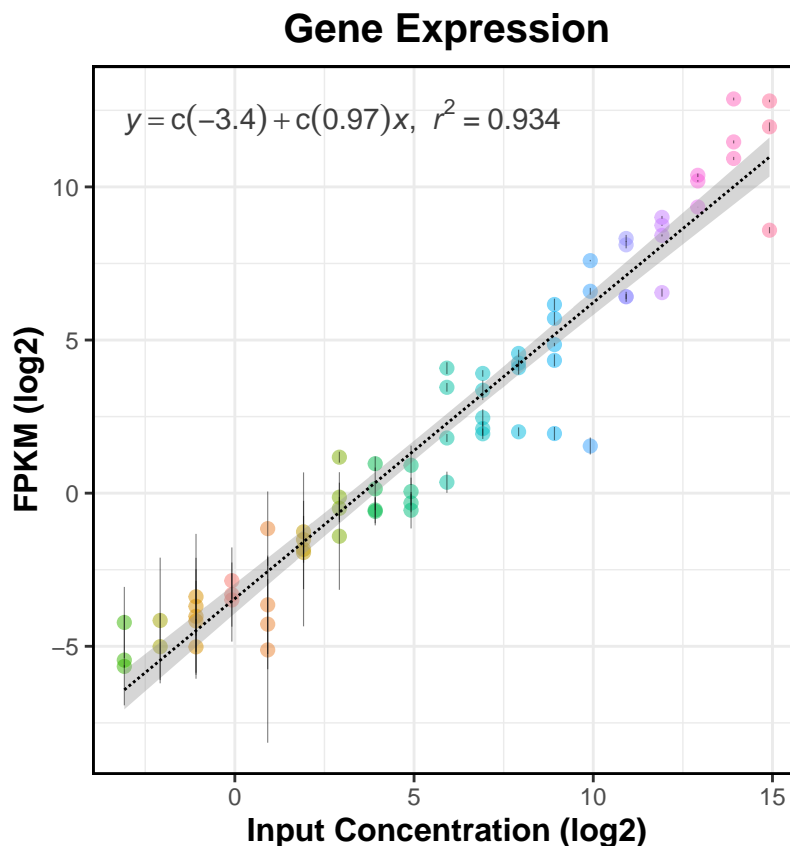
## Warning: Use of `data$x` is discouraged.
## i Use `x` instead.

## Warning: Use of `data$ymin` is discouraged.
## i Use `ymin` instead.

## Warning: Use of `data$x` is discouraged.
## i Use `x` instead.

## Warning: Use of `data$ymax` is discouraged.
## i Use `ymax` instead.

## Warning: The following aesthetics were dropped during statistical transformation: label.
## i This can happen when ggplot fails to infer the correct grouping structure in
##   the data.
## i Did you forget to specify a `group` aesthetic or to convert a numerical
##   variable into a factor?
```



Differential analysis

In this section, we show how to quantify differential expression analysis between expected fold-change and measured fold-change. We apply our method to a data set described in **Section 5.6.3** of the user guide.

```
data(UserGuideData_5.6.3)
head(UserGuideData_5.6.3)
```

	ExpLFC	ObsLFC	SD	Pval	Qval	Mean	Label
## R1_101	-3	-1.890122	0.701723	7.069675e-03	2.056337e-02	9.953556	TP
## R1_102	-4	-2.051777	0.546374	1.731616e-04	7.646243e-04	17.285262	TP
## R1_103	-1	3.837784	0.377602	2.883289e-24	6.534028e-23	1221.301532	TP
## R1_11	-4	-2.431582	0.591352	3.924117e-05	1.974336e-04	47.174250	TP
## R1_12	1	1.542757	0.425562	2.887104e-04	1.214989e-03	73.008720	TP
## R1_13	0	0.717701	0.242493	3.079564e-03	1.000416e-02	44053.259914	FP

For each of the sequin gene, we have expected log-fold change, measured log-fold change, standard deviation, p-value, q-value and mean. The estimation was done by DESeq2.

Run the following code to construct a folding plot:

```
title <- 'Gene Fold Change'
xlab <- 'Expected fold change (log2)'
ylab <- 'Measured fold change (log2)'
```

```
# Sequin names
```

```
names <- row.names(UserGuideData_5.6.3)
```

```
# Expected log-fold
```

```
x <- UserGuideData_5.6.3$ExpLFC
```

```
# Measured log-fold
```

```
y <- UserGuideData_5.6.3$ObsLFC
```

```
plotLinear(names, x, y, title=title, xlab=xlab, ylab=ylab, showAxis=TRUE,
            showLOQ=FALSE)
```

```
## Warning: Use of `data$x` is discouraged.
```

```
## i Use `x` instead.
```

```
## Warning: Use of `data$y` is discouraged.
```

```
## i Use `y` instead.
```

```
## Warning: Use of `data$x` is discouraged.
```

```
## i Use `x` instead.
```

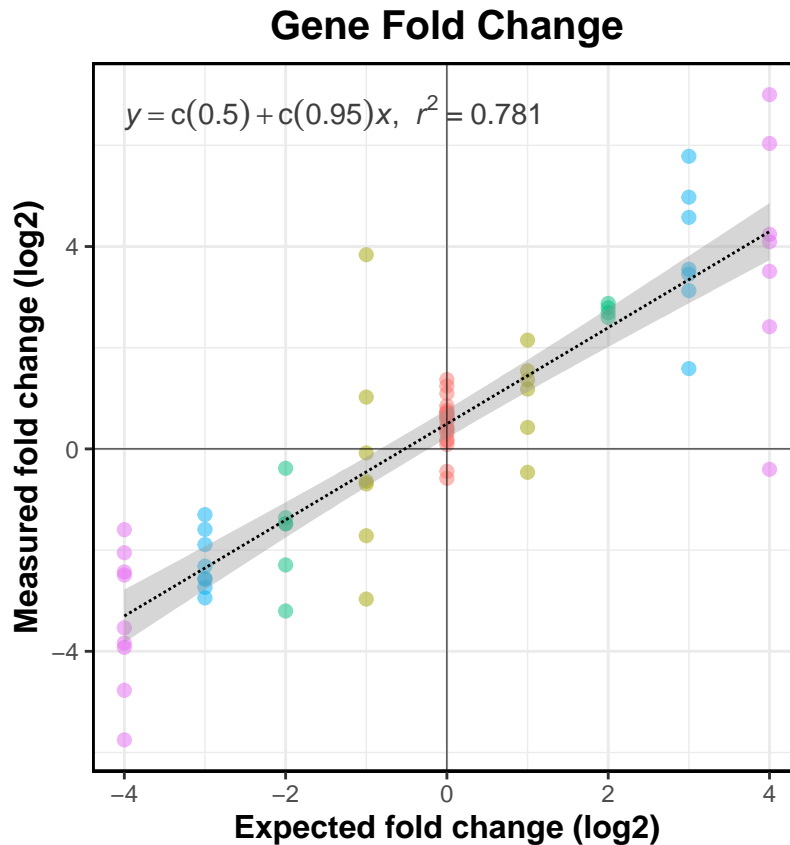
```
## Warning: Use of `data$y` is discouraged.
```

```
## i Use `y` instead.
```

```
## Warning: The following aesthetics were dropped during statistical transformation: label.
```

```
## i This can happen when ggplot fails to infer the correct grouping structure in
## the data.
```

```
## i Did you forget to specify a `group` aesthetic or to convert a numerical
## variable into a factor?
```



Outliers are obvious throughout the reference scale. Overall, DESeq2 is able to account for 78% of the variation.

We can also construct a ROC plot. [1] has details on how the true-positives and false-positives are defined.

```
title <- 'ROC Plot'

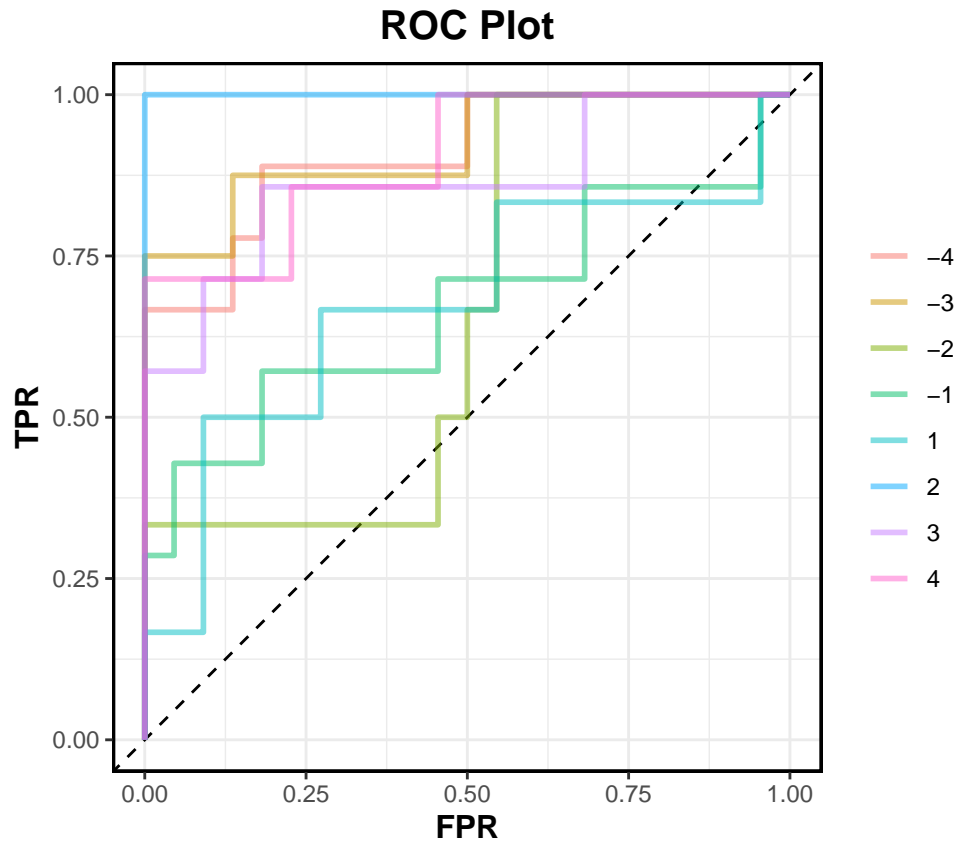
# Sequin names
seqs <- row.names(UserGuideData_5.6.3)

# Expected ratio
ratio <- UserGuideData_5.6.3$ExpLFC

# How the ROC points are ranked (scoring function)
score <- 1-UserGuideData_5.6.3$Pval

# Classified labels (TP/FP)
label <- UserGuideData_5.6.3$Label

plotROC(seqs, score, ratio, label, title=title, refGroup=0)
```



AUC statistics for LFC 3 and 4 are higher than LFC 1 and 2. Overall, all LFC ratios can be correctly classified relative to LFC 0.

Furthermore, we can construct limit of detection ratio (LOD) curves:

```
xlab <- 'Average Counts'
ylab <- 'P-value'
title <- 'LOD Curves'

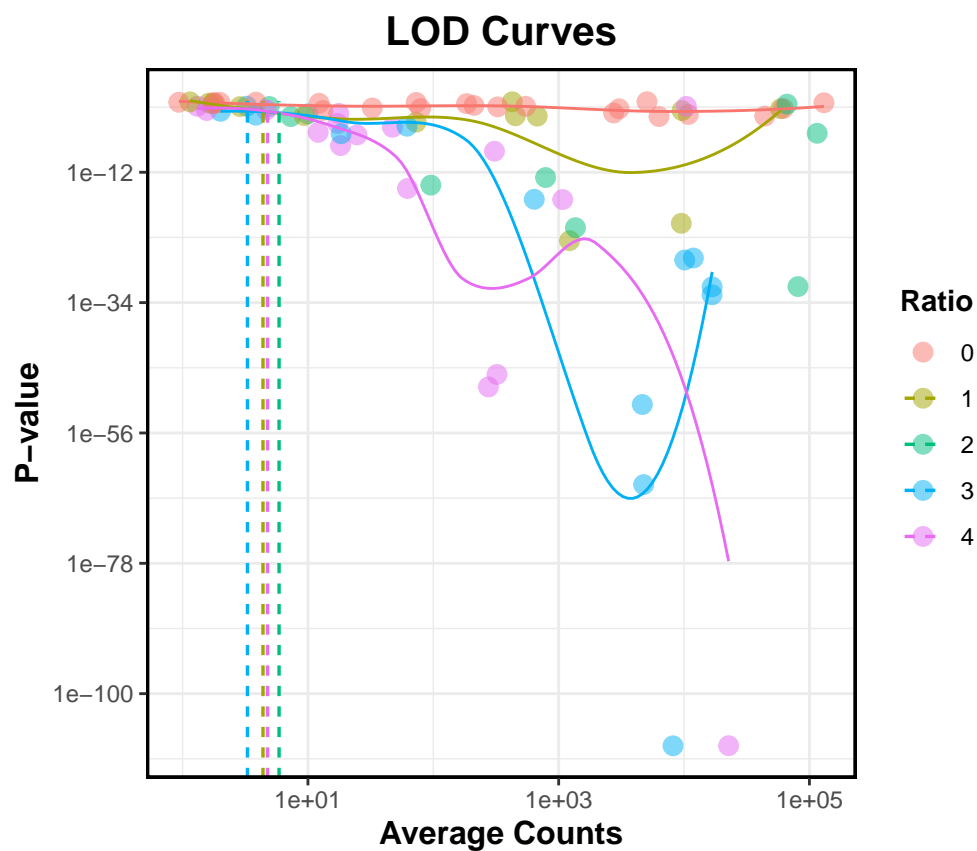
# Measured mean
mean <- UserGuideData_5.6.3$Mean

# Expected log-fold
ratio <- UserGuideData_5.6.3$ExpLFC

# P-value
pval <- UserGuideData_5.6.3$Pval

qval <- UserGuideData_5.6.3$Qval

plotLOD(mean, pval, abs(ratio), qval=qval, xlab=xlab, ylab=ylab, title=title, FDR=0.05)
```



Unsurprisingly, p-value is inverse quadratically related with average counts. All the LFC ratios systematically outperform LFC 0. The function also estimates the empirical detection limits, [1] has the details.