Normalisation example using Internal CTCF Peaks as a Standard

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Introduction

This is a worked example of our method to normalize ER ChIP-seq data using CTCF binding to provide an internal control.

Preprocessing

To prepare the data for the differential binding analysis, the data had to be aligned and CTCF and ER peaks called. As our CTCF control does not require any special alignment, data was aligned as for a standard ChIP-seq experiment and followed by removal of reads from blacklisted regions. Peak calling was done with macs2 using the script shown below.

```
#Calls peaks for all files in a directory against a single control. Change
# <filename> to the filename of your input sample.

 control=<filename>

 for bam in *bam
 do
   root=`basename $bam .bam`
   macs2 callpeak -t $bam -c $control -f BAM -n $root -g hs
 done
```

As macs2 cannot differentiate between a peak arising from CTCF binding or ER binding, we used samples prepared with only CTCF antibody to establish a CTCF binding consensus: one treated and one untreated. These peaks were then merged as follows.

```
cat  SLX-14438.D701_D504.HKN27BBXX.s_8.r_1.fq.gz.bam_peaks.xls \
      SLX-14438.D702_D503.HKN27BBXX.s_8.r_1.fq.gz.bam_peaks.xls > CTCF_merged.bed

grep -v \# CTCF_merged.bed  | grep -v start > CTCF_merged_filtered.bed
sort -k1,1 -k2,2n CTCF_merged_filtered.bed > CTCF_merged_sorted_filtered.bed

bedtools merge -i CTCF_merged_sorted_filtered.bed  > CTCF_union.bed
```

We then used this CTCF consensus to establish specific CTCF or ER binding in the experimental samples, either by taking the intersection of the CTCF consensus and the peak file for a particluar sample to provide a list of CTCF binding sites in the sample, or by subtratracting the consensus CTCF peaks from the macs2 output to provide a list of ER binding sites for that sample.

```
mkdir CTCF
for f in *.narrowPeak
do
    echo $f
    bedtools intersect -a $f -b CTCF_union.bed  > CTCF/$f
```

```
done

mkdir ER
for f in *.narrowPeak
do
    echo $f
    bedtools subtract -b CTCF_union.bed -a $f -A > ER/$f
done
```

The peak list files were then used as the basis for two DiffBind sample sheets as described in the DiffBind vignette. One uses the CTCF peak list generated from the above scripts; this is the control sample sheet. The second uses the ER peaks; this is the experimental sample sheet. These two sample sheets are then provided as the input in the analysis below.

Load convenience functions

Brundle provies a series of functions we developed for this pipeline that are used to normalise ChIP-seq data. The usage of these functions are explained in the example that follows.

```
source('../package/brundle.R')
```

Apply settings

The intial settings for the analysis are minimal. The variable jg.controlMinOverlap is set to 5 as this is 1 less than the total sample count and we want a strong consensus of CTCF peaks to normalise against. The control and experimental sample sheets are as described above. The conditions reflect the treated and untreated conditions we have used when writing our sample sheet.

```
jg.controlMinOverlap      <- 5
jg.controlSampleSheet     <- "samplesheet/samplesheet_SLX14438_hs_CTCF_DBA.csv"
jg.experimentSampleSheet  <- "samplesheet/samplesheet_SLX14438_hs_ER_DBA.csv"
jg.treatedCondition       =  "Fulvestrant"
jg.untreatedCondition     =  "none"
```

Load control and experimental DiffBind object

Typically we would load the raw data using DiffBind as we are planning to return our normalised data as a DiffBind object.

In this example, as the BAM files are greater in size than the GitHub file size limit, we have included the data as the DiffBind object that these functions would output and instead of loading from the raw data directly. If the code does not find the R data object, it will try to regenerate it from the raw data.

```
filename<-"Rdata/example_001_SLX-14438_dba_human_ER_CTCF.rda"
if(!file.exists(filename)){
  dbaExperiment <- jg.getDba(jg.experimentSampleSheet, bRemoveDuplicates=TRUE)
  dbaControl    <- jg.getDba(jg.controlSampleSheet, bRemoveDuplicates=TRUE)
  save(dbaExperiment,dbaControl,file=filename)
} else {
  load(filename)
}
```

DiffBind allows us to extract the counts in each peak. We use this function to obtain the data in a form that is easier to normalise.

```
#  Extract Peak set from DiffBind

jg.experimentPeakset <- jg.dbaGetPeakset(dbaExperiment)
jg.controlPeakset    <- jg.dbaGetPeakset(dbaControl)
```

To normalise, we need to separate samples out by condition. This is done using the following function. The peakset contains the reads in peaks; the sample sheet provides the metadata needed and the jg.treatedCondition/jg.untreatedCondition specifies which condition we want to get the counts for.

```
#Get counts for each condition
jg.controlCountsTreated<-jg.getControlCounts(jg.controlPeakset,
                                             jg.controlSampleSheet,
                                             jg.treatedCondition)
jg.controlCountsUntreated<-jg.getControlCounts(jg.controlPeakset,
                                               jg.controlSampleSheet,
                                               jg.untreatedCondition)
```

At the same time we can extract the samples names for each condition to use later.

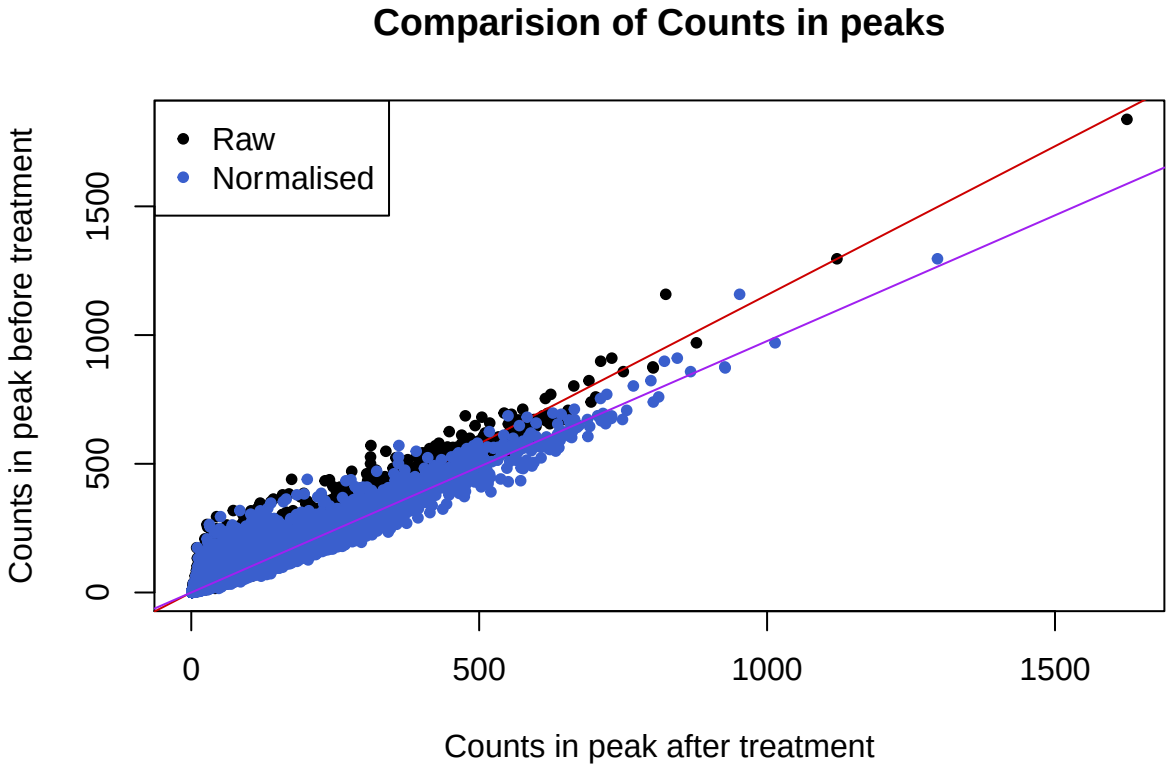
```
#Get sample names for conditions
jg.untreatedNames <- names(jg.controlCountsUntreated)
jg.treatedNames   <- names(jg.controlCountsTreated)
```

The normalisation factor is generated by a linear regression from the mean number of counts for each peak before and after treatment. The gradient of the linear fit should be equal to 1 given the assumption that, before and after treatment, the majority of the control peaks do not change.

This plot is not needed to calculate the normalisation factor but it does provide a visual interpretation of the process.

```
# Plot showing normalisation calculation (Optional)

jg.plotNormalization(jg.controlCountsTreated,
                     jg.controlCountsUntreated)
```




```
## rowMeans(jg.controlCountsTreated)
##                            1.155732
```

There are two parts to the normalisation coefficent we use in the final correction. The first is the the coefficent to make the levels of CTCF binding before and after treament equal. The second is because DiffBind normalises against the total read count for each sample, we therefore generated a correction factor to control for this.

```
# Get Normalisation Coefficient

jg.coefficient<-jg.getNormalizationCoefficient(jg.controlCountsTreated,
                                               jg.controlCountsUntreated)
jg.correctionFactor<-jg.getCorrectionFactor(jg.experimentSampleSheet,
                                            jg.treatedNames,
                                            jg.untreatedNames)
```

Once we have these two peices of information, we apply both the normalisation coeffficient and the correction factor to our data.

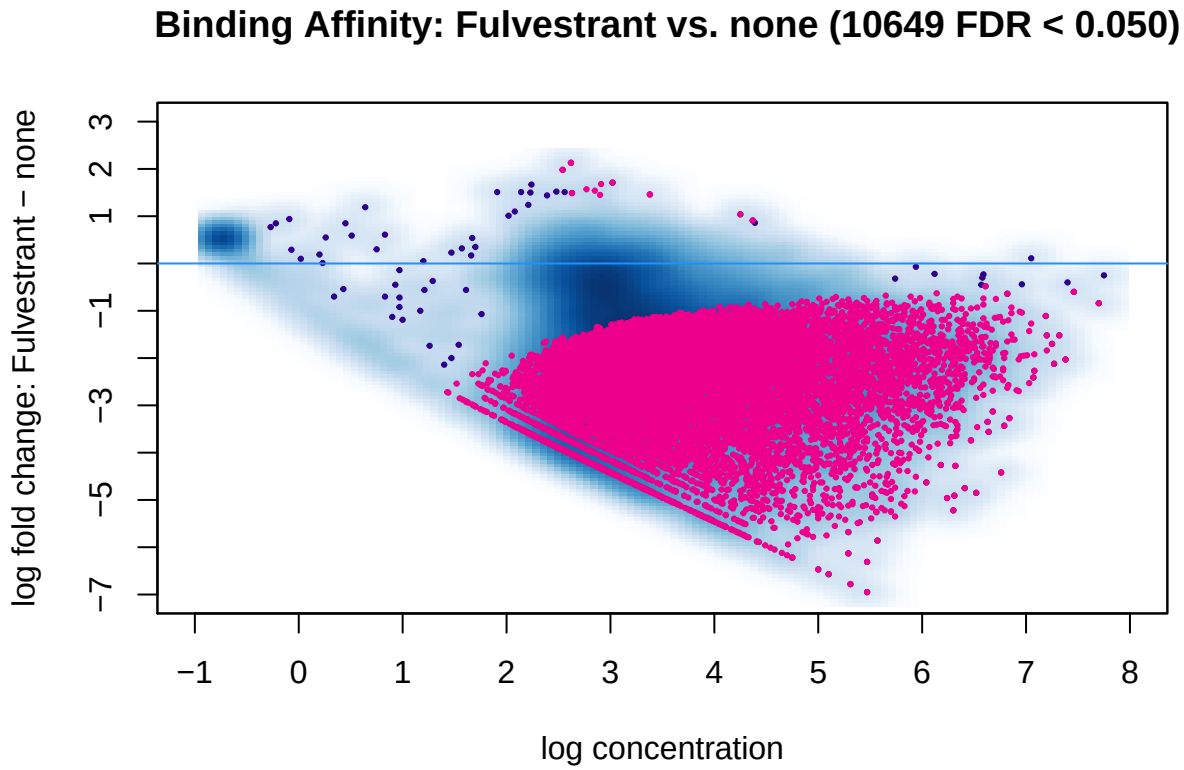
```
#Apply coefficent and control factor
jg.experimentPeaksetNormalised<-jg.applyNormalisation(jg.experimentPeakset,
                                                      jg.coefficient,
                                                      jg.correctionFactor,
                                                      jg.treatedNames)
```

Finally we return the correct values into the DiffBind object. This requires a modified version DiffBind as included in the original manuscript. It is expected that this will be supported natively by future versions of DiffBind.

```
#Return values to DiffBind and plot normalised result.
jg.dba <- DiffBind:::pv.resetCounts(dbaExperiment,
                                    jg.experimentPeaksetNormalised)
```

We can then visualise the data as normal using DiffBind.

```
jg.dba_analysis<-dba.analyze(jg.dba)
dba.plotMA(jg.dba_analysis,bFlip=TRUE)
```



Save results

```
write.csv(dba.report(jg.dba_analysis),file="results/Example_001.csv")
```