Supplementary File S4

Experimental eCLIP data from wild-type cells

This document contains the required steps to generate the results for section *3.2.3: Experimental Scenario 3: RBFOX2 eCLIP data on wildtype cells.*

Please follow the instructions in materials and methods within the main manuscript to download and process the experimental data. Pull a Docker image with all the required packages installed (`docker pull ecvpaper2024/ecv_results`).

Data preparation.

Load required packages.

```
Loading required package: idr
Loading required package: mvtnorm
Loading required package: future
Loading required package: future.apply
Loading required package: MatrixGenerics
Loading required package: matrixStats

Attaching package: 'MatrixGenerics'
The following objects are masked from 'package:matrixStats':

    colAlls, colAnyNAs, colAnys, colAvgsPerRowSet, colCollapse,
    colCounts, colCummaxs, colCummins, colCumprods, colCumsums,
    colDiffs, colIQRDiffs, colIQRs, colLogSumExps, colMadDiffs,
    colMads, colMaxs, colMeans2, colMedians, colMins, colOrderStats,
    colProds, colQuantiles, colRanges, colRanks, colSdDiffs, colSds,
    colSums2, colTabulates, colVarDiffs, colVars, colWeightedMads,
    colWeightedMeans, colWeightedMedians, colWeightedSds,
    colWeightedVars, rowAlls, rowAnyNAs, rowAnys, rowAvgsPerColSet,
    rowCollapse, rowCounts, rowCummaxs, rowCummins, rowCumprods,
    rowCumsums, rowDiffs, rowIQRDiffs, rowIQRs, rowLogSumExps,
    rowMadDiffs, rowMads, rowMaxs, rowMeans2, rowMedians, rowMins,
    rowOrderStats, rowProds, rowQuantiles, rowRanges, rowRanks,
    rowSdDiffs, rowSds, rowSums2, rowTabulates, rowVarDiffs, rowVars,
    rowWeightedMads, rowWeightedMeans, rowWeightedMedians,
    rowWeightedSds, rowWeightedVars
```

```
 ------------------------------------------------------------------
|                        ______     __                         |
|                 ___   / ___\ \   / /                         |
|                / _ \ | |    \ \ / /                          |
|               |  __/ | |___  \ V /                           |
|                \___|  \____|  \_/                            |
|__________________________________________________________________|

        Enhanced Coefficient of Variation and IDR Extensions
                  for Reproducibility Assessment

This package provides extensions and alternative methods to  IDR  to
measure the reproducibility of omic data with an arbitrary number of
replicates. It introduces an enhanced Coefficient of Variation (eCV)
metric to assess the likelihood of omic features being reproducible.
Loading required package: tidyverse
-- Attaching packages --------------------------------------- tidyverse 1.3.2 --
v ggplot2 3.4.3     v purrr   1.0.2
v tibble  3.2.1     v dplyr   1.1.2
v tidyr   1.3.0     v stringr 1.5.0
v readr   2.1.2     v forcats 0.5.1
-- Conflicts ------------------------------------------ tidyverse_conflicts() --
x dplyr::count()  masks matrixStats::count()
x dplyr::filter() masks stats::filter()
x dplyr::lag()    masks stats::lag()

Attaching package: 'reshape2'


The following object is masked from 'package:tidyr':

    smiths


Loading required package: stats4

Loading required package: BiocGenerics


Attaching package: 'BiocGenerics'


The following objects are masked from 'package:dplyr':

    combine, intersect, setdiff, union


The following objects are masked from 'package:stats':

    IQR, mad, sd, var, xtabs


The following objects are masked from 'package:base':

    anyDuplicated, append, as.data.frame, basename, cbind, colnames,
    dirname, do.call, duplicated, eval, evalq, Filter, Find, get, grep,
    grepl, intersect, is.unsorted, lapply, Map, mapply, match, mget,
    order, paste, pmax, pmax.int, pmin, pmin.int, Position, rank,
    rbind, Reduce, rownames, sapply, setdiff, sort, table, tapply,
    union, unique, unsplit, which.max, which.min


Loading required package: S4Vectors


Attaching package: 'S4Vectors'


The following objects are masked from 'package:dplyr':

    first, rename


The following object is masked from 'package:tidyr':

    expand


The following objects are masked from 'package:base':

    expand.grid, I, unname


Loading required package: IRanges


Attaching package: 'IRanges'


The following objects are masked from 'package:dplyr':

    collapse, desc, slice


The following object is masked from 'package:purrr':

    reduce


Loading required package: GenomeInfoDb

Loading required package: AnnotationDbi

Loading required package: Biobase

Welcome to Bioconductor

    Vignettes contain introductory material; view with
    'browseVignettes()'. To cite Bioconductor, see
    'citation("Biobase")', and for packages 'citation("pkgname")'.
```

```

Attaching package: 'Biobase'

The following object is masked from 'package:MatrixGenerics':

    rowMedians

The following objects are masked from 'package:matrixStats':

    anyMissing, rowMedians


Attaching package: 'AnnotationDbi'

The following object is masked from 'package:dplyr':

    select

Loading required package: Biostrings

Loading required package: XVector

Attaching package: 'XVector'

The following object is masked from 'package:purrr':

    compact


Attaching package: 'Biostrings'

The following object is masked from 'package:base':

    strsplit

Loading required package: rtracklayer

Type 'citation("pROC")' for a citation.

Attaching package: 'pROC'

The following objects are masked from 'package:IRanges':
    cov, var


The following objects are masked from 'package:S4Vectors':

    cov, var


The following object is masked from 'package:BiocGenerics':

    var


The following objects are masked from 'package:stats':

    cov, smooth, var
Warning: replacing previous import 'GenomicRanges::union' by 'dplyr::union' when
loading 'Sierra'
Warning: replacing previous import 'GenomicRanges::intersect' by
'dplyr::intersect' when loading 'Sierra'
Warning: replacing previous import 'GenomicRanges::setdiff' by 'dplyr::setdiff'
when loading 'Sierra'
```

Set color palette.

```
res_colors <-
  c(IDR = "tan",
    gIDR = "#30B7BC", # bright teal
    eCV = "#AF275F", # light magenta
    mIDR = "#DE653A" # medium teal
  )
```

Download genome.

```
wget https://ftp.ebi.ac.uk/pub/databases/gencode/Gencode_human/release_41/GRCh38.p13.genome.fa.gz
gunzip GRCh38.p13.genome.fa.gz
```

Download GTF file.

```
wget https://ftp.ebi.ac.uk/pub/databases/gencode/Gencode_human/release_41/gencode.v41.chr_patch_hapl_sca
gunzip gencode.v41.chr_patch_hapl_scaff.annotation.gtf.gz
```

Upload eCLIP data and keep only chromosome one for faster computations.

```
(eclip_data <-
  read_tsv(
    file = "RBFOX2_new_reagents.peak_table.tsv",
    col_types = cols()) %>%
   dplyr::filter(chrom == "chr1"))
New names:
* `` -> `...1`
# A tibble: 25,391 x 13
```

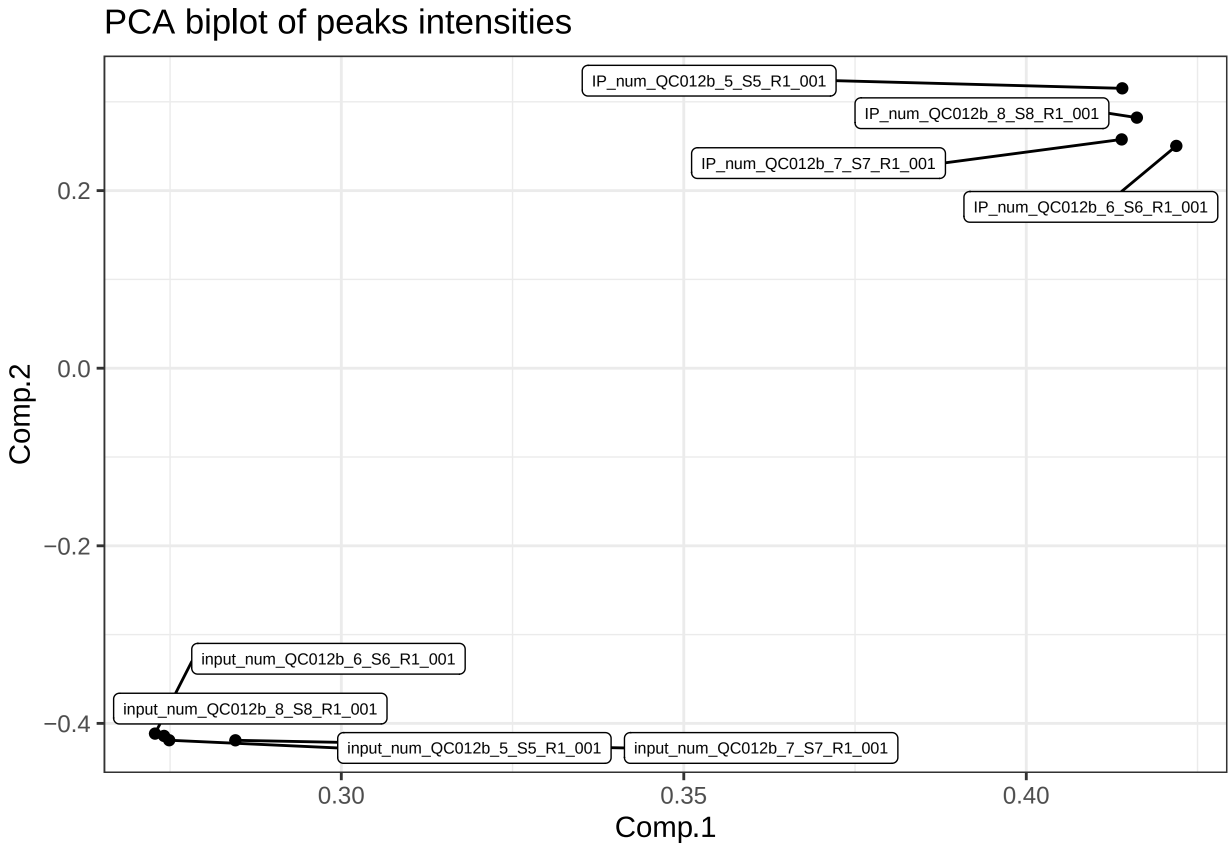
```
    ...1 chrom  start    end strand IP_num_QC012b_5_S5_R1_001
   <dbl> <chr>  <dbl>  <dbl> <chr>                      <dbl>
 1      0 chr1   16239  16316 -                              2
 2      1 chr1   17435  17527 -                              9
 3      2 chr1  185698 185733 -                              1
 4      3 chr1  186700 186754 -                              1
 5      4 chr1  186866 186925 -                              1
 6      5 chr1  187457 187537 -                              2
 7      6 chr1  188049 188114 -                              4
 8      7 chr1  189416 189524 -                              3
 9      8 chr1  190076 190138 -                              1
10      9 chr1  190810 190867 -                              2
# i 25,381 more rows
# i 7 more variables: IP_num_QC012b_6_S6_R1_001 <dbl>,
#   IP_num_QC012b_7_S7_R1_001 <dbl>, IP_num_QC012b_8_S8_R1_001 <dbl>,
#   input_num_QC012b_5_S5_R1_001 <dbl>, input_num_QC012b_6_S6_R1_001 <dbl>,
#   input_num_QC012b_7_S7_R1_001 <dbl>, input_num_QC012b_8_S8_R1_001 <dbl>
```

Extract peaks intensities.

```
eclip_inten <-
  eclip_data %>%
  dplyr::select(contains("num_QC"))
```

Get PCA of peak intensities.

```
eclip_inten <- eclip_inten %>% ceiling()
pca <- princomp(log2(eclip_inten + 1))
ggplot(pca$loadings[1:8,TRUE] %>%
         as.data.frame() %>%
         rownames_to_column("Sample"),
       aes(x=Comp.1, y=Comp.2)) +
  ggrepel::geom_label_repel(aes(label=Sample),size=2) + geom_point() +
  theme_bw() + ggtitle("PCA biplot of peaks intensities")
```



Get normalized IP intensities.

```
eclip_inten <- (eclip_inten %>% dplyr::select(contains("IP_num")) %>%
                  mutate_all(~ . + 1)) /
  (eclip_inten %>% dplyr::select(-contains("IP_num")) %>% mutate_all(~ . + 1))
```

Filter peaks with normalized intensity higher than 1.

```
tmp <- rowSums(eclip_inten >=
                   1) > 0
eclip_data <- eclip_data[tmp,]
eclip_inten <- eclip_inten[tmp,]
```

```
eclip_inten <-
      eclip_inten %>%
              mutate_all(~ . + 1.1) %>%
               as.matrix() %>%
  log()
```

Create subset with different number of replicates.

```
# Subset of j replicates.
eclip_inten_reps <-
  lapply(c(2, 3, 4), function(j) eclip_inten[,seq_len(j)])
```

```r
names(eclip_inten_reps) <- paste0("n_rep=", c(2,3,4))

# Check dimensions.
lapply(eclip_inten_reps, dim)
$`n_rep=2`
[1] 24703     2

$`n_rep=3`
[1] 24703     3

$`n_rep=4`
[1] 24703     4
```

Set initial values for IDR, gIDR, and mIDR and number of iterations for eCV.

```r
# Set parameters for each model.
methods_params <- list(
  eCV = list(max.ite = 1e4),
  IDR = list(
    mu = 2.5,
    sigma = 1,
    rho = 0.8,
    p = 0.5,
    eps = 1e-3,
    max.ite = 100
  ),
  gIDR = list(
    mu = 2.5,
    sigma = 1,
    rho = 0.8,
    p = 0.5,
    eps = 1e-3,
    max.ite = 100
  ),
  mIDR = list(
    mu = 2.5,
    sigma = 1,
    rho = 0.8,
    p = 0.5,
    eps = 1e-3,
    max.ite = 100
  )
)
```

Data analysis.

FOX2 motif enrichment.

Create peak IDs.

```
eclip_data <-
  eclip_data %>%
  mutate(ID = paste0(chrom,":",start,"-",end,strand),
         score = 0)
```

Export table as a bed file.

```
eclip_data %>%
  dplyr::select(chrom, start, end, ID, score, strand) %>%
  write_tsv(file = "eclip_data.bed", col_names = FALSE)
```

Detect peaks matching FOX2 binding motif with Homer.

```
bin/findMotifsGenome.pl "eclip_data.bed" \
      GRCh38.p13.genome.fa \
      .aux \
      -find FOX2_all.motif -rna -size 75 \
      > "eclip_data_homer_res.bed"
rm -f -r .aux
```

Load Homer's results.

```
eclip_data <-
  eclip_data %>%
  mutate(motif=eclip_data$ID %in%
           read_delim(file = "eclip_data_homer_res.bed",
                      col_types = cols())$PositionID)
```

Features distribution.

```
gtf <-
  import.gff("gencode.v41.chr_patch_hapl_scaff.annotation.gtf")
```

make TxDb.

```
txdb <-
  makeTxDbFromGFF("gencode.v41.chr_patch_hapl_scaff.annotation.gtf")
Import genomic features from the file as a GRanges object ... OK
Prepare the 'metadata' data frame ... OK
Make the TxDb object ...
Warning in .get_cds_IDX(mcols0$type, mcols0$phase): The "phase" metadata column contains non-NA values
  stop_codon. This information was ignored.
OK
```

```
feature_dist <-
  eclip_data %>%
  makeGRangesFromDataFrame() %>%
  annotate_gr_from_gtf(gtf_gr = gtf,
                       gtf_TxDb = txdb,
                     transcriptDetails = TRUE)

Annotating 3' UTRs
Annotating 5' UTRs
Annotating introns
Annotating exons
Annotating CDS
```

Correlation analysis of impossed reproducible features.

```
tmp <- eclip_data$motif & (feature_dist$UTR3 == "YES" | feature_dist$intron == "YES")

R<- cor(eclip_inten[tmp, ])
r <- mean(R[upper.tri(R)])

CorCI(r,sum(tmp))
      cor    lwr.ci    upr.ci
0.7195490 0.6988817 0.7390157
```

Assess reproducibility with each method.

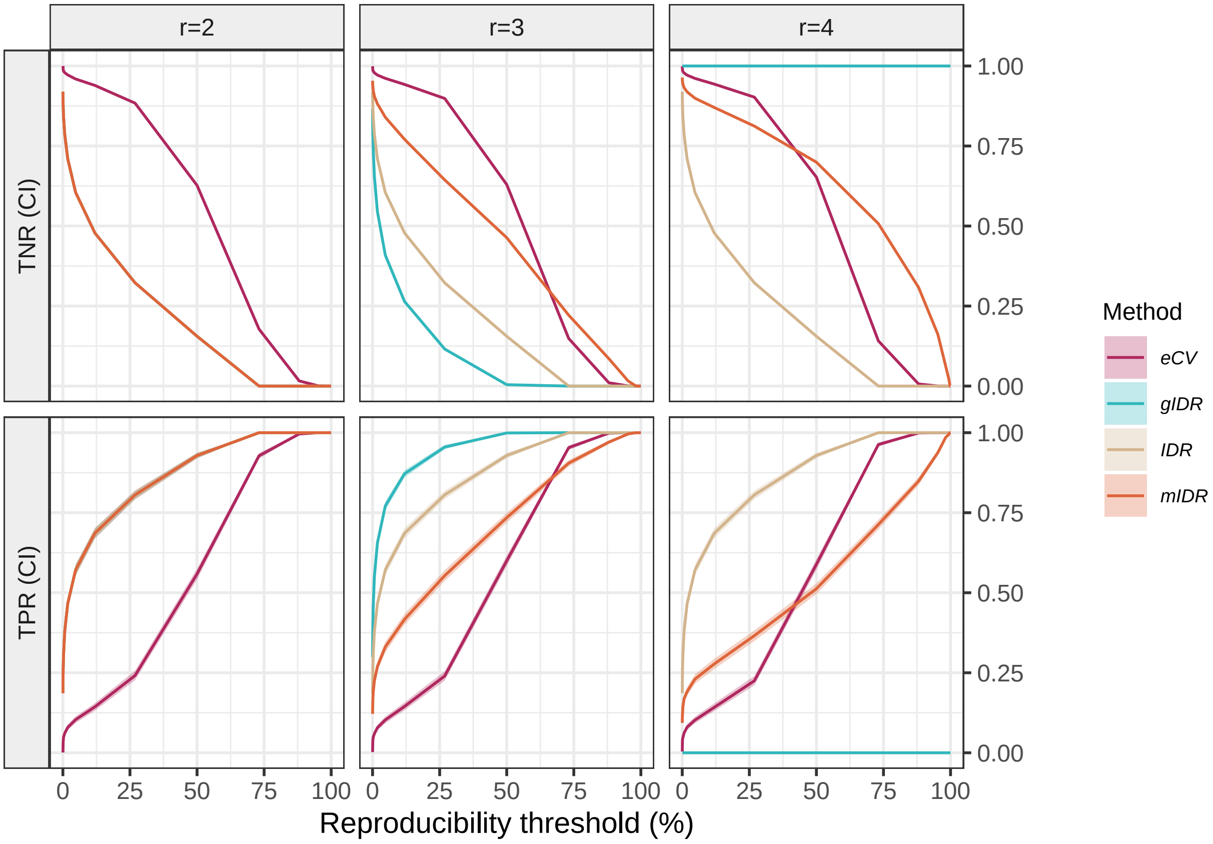
Create a table with all combinations of parameters.

```
(par_settings <- expand.grid(n_rep =  paste0("n_rep=",c(4,3,2)),
                            method=c("gIDR","mIDR","IDR","eCV"),
                            stringsAsFactors = FALSE) %>%
  mutate(par_com = seq_along(n_rep)))
     n_rep method par_com
1  n_rep=4   gIDR       1
2  n_rep=3   gIDR       2
3  n_rep=2   gIDR       3
4  n_rep=4   mIDR       4
5  n_rep=3   mIDR       5
6  n_rep=2   mIDR       6
7  n_rep=4    IDR       7
8  n_rep=3    IDR       8
9  n_rep=2    IDR       9
10 n_rep=4    eCV      10
11 n_rep=3    eCV      11
12 n_rep=2    eCV      12
```

```
# Set parallel scheduler.
future::plan(multisession, workers = 4)
perf_res <- NULL

for (i in par_settings$par_com) {
  set.seed(42)
  print(par_settings[i,])
  n_rep <- par_settings$n_rep[i]
  method <- par_settings$method[i]
  if(method != "eCV") {
    X <- preprocess(eclip_inten_reps[[n_rep]],
                         value_transformation = "identity",
                         jitter=1e-4)
  } else {
    X <- eclip_inten_reps[[n_rep]]
  }

  rep_index <- mrep_assessment(
          x = X,
          method = method,
          param = methods_params[[method]],
          n_threads = 4
        )$rep_index

   tmp <-  eclip_data$motif & (feature_dist$UTR3 == "YES" | feature_dist$intron == "YES")

   print(perf <- roc(tmp, rep_index, quiet = TRUE))
   perf_thr <- ci.coords(perf,conf.level=0.90,
                          x =  1/(1 + exp(-c(1:20) + 10)),
                          ret=c("threshold","tpr","tnr"))
    perf_thr <- rbind(perf_thr$tpr %>%
    as.data.frame() %>%
    mutate(threshold= 1/(1 + exp(-c(1:20) + 10)),
           perf="TPR (CI)") ,
        perf_thr$tnr %>%
    as.data.frame() %>%
    mutate(threshold= 1/(1 + exp(-c(1:20) + 10)),
           perf="TNR (CI)"))
  perf_thr$n_rep <- n_rep
  perf_thr$method <- method
  perf_res <- rbind(perf_res, perf_thr)
}

# Close me buddies.
future::plan(sequential)

# Save results.
saveRDS(perf_res,file="perf_resRealRBP.rds")
```

Arrange results for figure creation.

```
p <- perf_res %>%
mutate(n_rep = paste0("r=",str_remove(n_rep,"n_rep="))) %>%
  ggplot(aes(x=threshold,y=`50%`,color=method)) +
  facet_grid(perf~n_rep,switch ="y") +
   geom_ribbon(color = NA,
    aes(x = threshold, ymin = `5%`, ymax = `95%`,fill=method),
     alpha = 0.3)  +
   geom_line() +
  scale_color_manual(values=alpha(res_colors, 1)) +
  scale_linetype_manual(values=c("miR1"="solid", "miR124"="dashed")) +
  scale_fill_manual(values=alpha(res_colors, 0.6)) +
    scale_y_continuous(position = "right")+
   theme_bw() + theme(
                     legend.title = element_text(size=9),
                     legend.text = element_text(size=7, face = "italic"),
                     strip.background = element_rect(fill=alpha("gray", 0.25)),
                  legend.background = element_rect(fill = alpha("white", 0.1))) +
   guides(color=guide_legend(ncol=1,override.aes = list(size = 2))) +
   labs(y="",fill="Method",linetype="Transfection",
       color="Method",x="Reproducibility threshold (%)") +
   scale_x_continuous(breaks=c(0, 0.25, 0.5, 0.75, 1),
                     labels = c("0","25","50","75","100"))
p
```



```
ggsave(filename = "Figure6.tiff",plot = p,device = "tiff",
       dpi=300,units = "in",width = 7,height = 5,scale = 0.85)
```