Supplementary File S3

Experimental miR-eCLIP data from wild-type cells

This document contains the required steps to generate the results for section *3.2.2: Experimental Scenario 2: miR-eCLIP data from wild-type 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: 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
```

Set color palette.

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

Upload miR-eCLIP data and keep only chromosome 1.

```r
(mireclip_data <-
  read_tsv(
    file = "CH010_merged_peaks.miR_chim_peak_table.tsv",
    col_types = cols()) %>%
   dplyr::filter(chrom == "chr1")
  )
# A tibble: 137,259 x 16
   chrom start   end strand gene                        ensg  feature Symbol_miRNA
   <chr> <dbl> <dbl> <chr>  <chr>                       <chr> <chr>   <chr>
 1 chr1  89749 89840 -      ENSG00000238009|ENSG0000~ ENSG~ noncod~ hsa-miR-17-~
 2 chr1  89749 89840 -      ENSG00000238009|ENSG0000~ ENSG~ noncod~ hsa-miR-193~
 3 chr1  89749 89840 -      ENSG00000238009|ENSG0000~ ENSG~ noncod~ hsa-miR-20a~
 4 chr1  89749 89840 -      ENSG00000238009|ENSG0000~ ENSG~ noncod~ hsa-miR-223~
 5 chr1  89749 89840 -      ENSG00000238009|ENSG0000~ ENSG~ noncod~ hsa-miR-25-~
 6 chr1  89749 89840 -      ENSG00000238009|ENSG0000~ ENSG~ noncod~ hsa-miR-27a~
 7 chr1  89749 89840 -      ENSG00000238009|ENSG0000~ ENSG~ noncod~ hsa-miR-92a~
 8 chr1  89749 89840 -      ENSG00000238009|ENSG0000~ ENSG~ noncod~ hsa-miR-92b~
 9 chr1  89840 89922 -      ENSG00000239945|ENSG0000~ ENSG~ noncod~ hsa-miR-17-~
10 chr1  89840 89922 -      ENSG00000239945|ENSG0000~ ENSG~ noncod~ hsa-miR-20a~
# i 137,249 more rows
# i 8 more variables: CH010_1_IP1_S17_L001_R1_001 <dbl>,
#   CH010_2_IP2_S18_L001_R1_001 <dbl>, CH010_3_IP3_S19_L001_R1_001 <dbl>,
#   CH010_4_IP4_S20_L001_R1_001 <dbl>, CH010_5_IP5_S21_L001_R1_001 <dbl>,
#   CH010_6_IP6_S22_L001_R1_001 <dbl>, CH010_7_IP7_S23_L001_R1_001 <dbl>,
#   CH010_8_IP8_S24_L001_R1_001 <dbl>
```

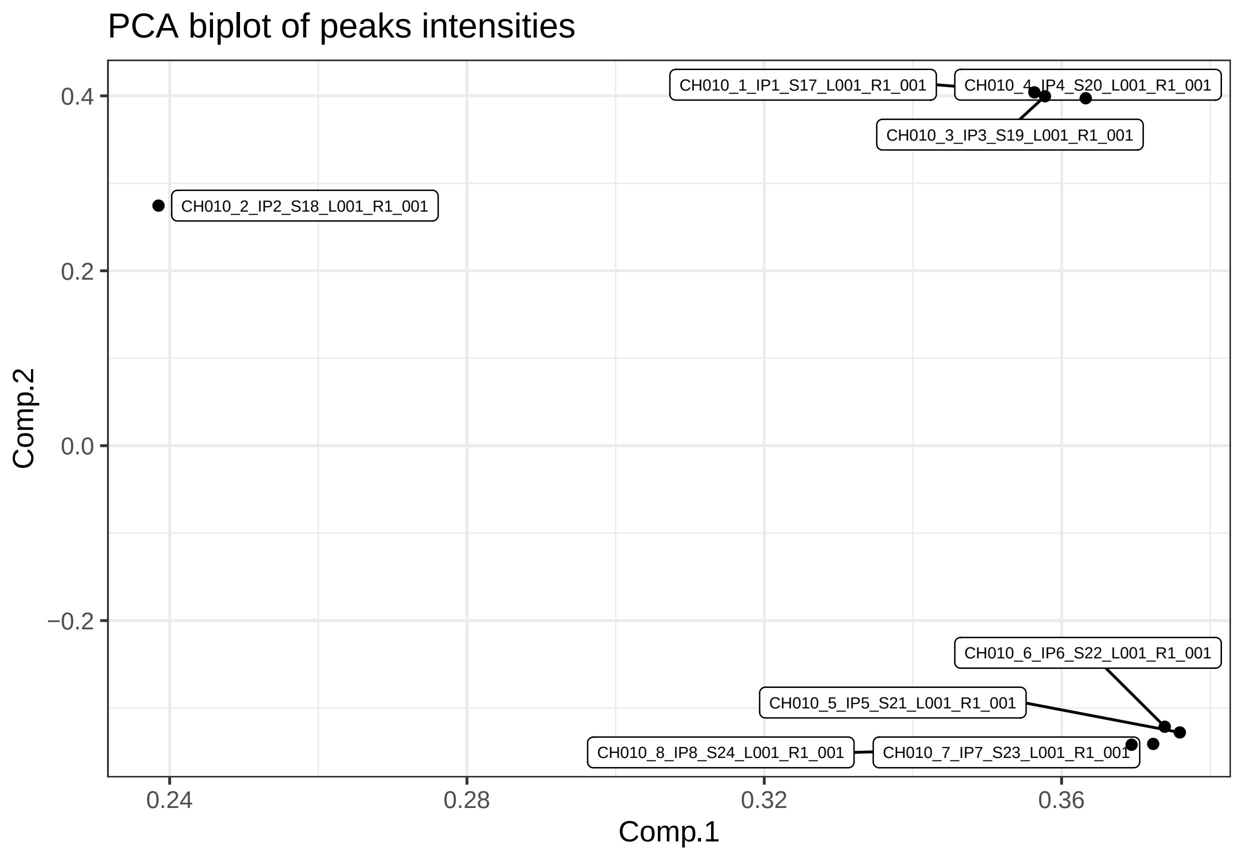
Data analysis.

Filter out peaks without enough counts.

```
tmp <-
  (mireclip_data %>%
  dplyr::select(contains("CH010")) %>% rowMeans()) > 3
tmp <-
mireclip_data <- mireclip_data[tmp, ]
```

Get PCA of peak intensities.

```
pca <-
  mireclip_data %>%
  dplyr::select(contains("CH010")) %>%
  ceiling() %>% mutate_all(~ log(. + 1)) %>%
  princomp()
ggplot(pca$loadings[,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")
```



Create subset with different number of replicates and use four replicates within the same condition.

```r
# Subset replicates.
mireclip_data <- mireclip_data %>%
  dplyr::select(-rownames(pca$loadings[pca$loadings[, 2] > 0, ]))
```

Use PCs to obtain number of independent groups of peaks for multiple comparisons adjustment.

```r
pc <- pca$scores

# Function to convert binary vector to decimal number
binary_to_decimal <- function(binary_vector) {
  decimal_number <- sum(binary_vector * 2^(rev(seq_along(binary_vector) - 1)))
      return(decimal_number)
}
# We take scores at, or above, the third Quartile as "informative".
# The remaining scores are taken as "uninformative".
# Number of clusters is estimated by turning binary rows into
# single decimals numbers.
decimal_numbers <- apply(
      apply(pc, 2, function(x) as.numeric(x >= quantile(x, probs = 0.75))),
      1, binary_to_decimal
)

# The estimated number of independent tests is used to get.
# The number of independent tests is estimated by taking the number of
```

```
    # different decimal numbers.
n_ind_peaks <- sum(unique(decimal_numbers) != 0)
```

Seed matching analysis.

Create a genomic range object to extract sequence information.

```
# Create a variable with formatted information
mireclip_data <- mireclip_data %>%
  mutate(genomic_info = paste0(chrom,
                               ":",
  start,
  "-",
  end,
  strand),)
gr <- makeGRangesFromDataFrame(mireclip_data,
                               keep.extra.columns = TRUE)
unique_peaks <- unique(gr)
```

Extract peaks sequences.

```
(peak_seqs <- getSeq(BSgenome.Hsapiens.UCSC.hg38, unique_peaks))
DNAStringSet object of length 1897:
       width seq
   [1]    91 ATACCACCAATCAATACTCATCATTAATAATC...ATAGCCCCCTTTCACTTCTGAGTCCCAGAGGT
   [2]    84 CTGTAGGCTCATTCATTTCTCTAACAGCAGTA...TTGAGAAGCCTTCGCTTCGAAGCGAAAAGTCC
   [3]   100 AACCACCCAACTATCTATAAACCTAGCCATGG...TTCGCTCTAAGATTAAAAATGCCCTAGCCCAC
   [4]    72 AATTCTACTGACTATCCTAGAAATCGCTGTCG...CAAGCCTACGTTTTTACACTTCTAGTAAGCCT
   [5]   117 CCTGAACTTCCCTGAGATCAAACGAAGGAAGA...AACAGCTCTGAAGAGGACGACACCGAGGGATT
   ...   ... ...
[1893]    29 GACAAGACATCACAGTGGTCTGGGCTGGA
[1894]   114 AGGAACTGAACCCCTCAGGATCCCCGCCGACC...ATTCTCCAGCTCACTCCCAATCCCAGGCTCAC
[1895]    91 GTTGCCAATTGTATCTGTTTTTATGAAATGTT...ATGGGGAGACCATAGAAGAATGATCCAAGGAG
[1896]   106 AAGGATGTTCTGGGAAACCTCTCCCGATTCAC...AACTAATCTTCTCATACTTACATTTTGCAGAT
[1897]   115 ATGCTTCAGGGAGTACCACATCCGGTGACATC...TGGCAGTTGAGCACTTCTGTTTTGTGTTGGAA
names(peak_seqs) <- unique_peaks$genomic_info
```

Extract peaks intensities.

```
mireclip_inten <-
  mireclip_data %>%
  dplyr::select(contains("CH010"))
```

```
N_counts <-
  mireclip_data %>% dplyr::select(contains("CH010"),
                                  MIRNA=Symbol_miRNA,ENSG=ensg)
peak_cols <- grep(colnames(N_counts),
                  pattern="CH010")

N_counts[,peak_cols] <- ceiling(N_counts[,peak_cols]) + 1
```

```r
# Get total chimeric counts.
n <- N_counts[, peak_cols] %>% colSums() %>%
    as.vector() %>%
    as.numeric()

ind_test0 <- function(x,dist="nbinom") {

  f <-
    list('binom'=function(nij,pi,pj,n) pbinom(q = nij,
                                               size=n,
                                               prob = pi*pj,
                                               lower.tail = T),

         'nbinom'=function(nij,pi,pj,n) {
             pnbinom(q = nij,size= 1,
                     prob = pi*pj,
                                               lower.tail = T)

         }
  )


  res <- future_lapply(seq_len(nrow(x)), function(k) {
    gene_i <- x$ENSG[k]
    mirna_j <- x$MIRNA[k]

  # Get chimeric counts.
  nij <- x[k, peak_cols] %>%
    as.vector() %>%
    as.numeric()

  # Get marginal gene and mirna counts.
  ni <- x[x$ENSG == gene_i ,
                      peak_cols] %>%
    colSums() %>%
    as.vector() %>%
    as.numeric()

  nj <- x[x$MIRNA == mirna_j,
                      peak_cols] %>%
    colSums()%>%
    as.vector() %>%
    as.numeric()

    # Get marginal probabilities.
    pij <- nij / n
    pi <- ni / n
    pj <- nj / n

  pvalue <-
   f[[dist]](nij,pi,pj,n)

  return(pvalue)
}) %>% do.call(what = rbind)
```

```
  return(res)
}
future::plan(multisession, workers = 4)
chim_test <- ind_test0(N_counts)
# Close me buddies.
future::plan(sequential)

mireclip_inten <-
  idr2d::preprocess(x = mireclip_inten %>%
              mutate_all(~ . + 1.1) %>%
               as.matrix(),
             value_transformation = "identity",
            jitter_factor = 0) %>%
  log()
```

Represent peaks based on most enriched miRNA.

```
sig_enriched_mirnas <-
  lapply(unique_peaks$genomic_info, function (peak) {
  cat(".")
  tmp <- which(mireclip_data$genomic_info == peak)
  if (length(tmp) == 1) {
    mu <- mean(mireclip_inten[tmp,])
    sd <- sd(mireclip_inten[tmp,])
    mirna_pval <- pt(2 * mu/sd, lower.tail = FALSE, df = 3)
    mirna_padj <- mirna_pval * n_ind_peaks
    mirna_padj <-
      ifelse(mirna_padj>= 1, 0.99, mirna_padj)

    if (mirna_padj < 0.1)
        return(mireclip_data$Symbol_miRNA[tmp])
   else  NA
  }
  else {
    mu <- rowMeans(mireclip_inten[tmp,])
    sd <- MatrixGenerics::rowSds(mireclip_inten[tmp,])
    mirna_pval <- pt(2 * mu/sd, lower.tail = FALSE, df = 3)
    mirna_padj <- mirna_pval * n_ind_peaks
    mirna_padj <-
      ifelse(mirna_padj>= 1, 0.99, mirna_padj)

    if (any(mirna_padj < 0.1))
        return(mireclip_data$Symbol_miRNA[tmp][mirna_padj < 0.05])
    else NA
  }
})

with_sig_mirna <-
  !(lapply(sig_enriched_mirnas, is.na) %>%
      unlist())
names(with_sig_mirna) <-
  names(sig_enriched_mirnas) <-
  unique_peaks$genomic_info
```

```
mireclip_data$with_sig_mirna <- with_sig_mirna[mireclip_data$genomic_info]
```

Rename features.

```
# Rename features.
region_rename <- c(
  "5utr" = "5' UTR",
  "CDS" = "CDS",
  "3utr" = "3' UTR",
  "noncoding_exon" = "Other",
  "miRNA" = "miRNA",
  "miRNA_proximal" = "miRNA",
  "5ss" = "Intron",
  "noncoding_5ss" = "Other",
  "3ss" = "Intron",
  "noncoding_3ss" = "Other",
  "proxintron" = "Intron",
  "noncoding_proxintron" = "Other",
  "distintron" = "Intron",
  "noncoding_distintron" = "Other",
  "tRNA" = "Other",
  "intergenic" = "Other"
)

mireclip_data$feature <-
  factor(x = mireclip_data$feature,
         levels = names(region_rename),
         labels = region_rename)
```

Get peaks with positive seed matching using scanMiR.

```
# Get peak strand.
strand <- as.character(strand(unique_peaks))
names(strand) <- unique_peaks$genomic_info

# Get affinity constants.
KSmodels <- getKdModels(species = "hsa")

seed_match_res <-
lapply(names(with_sig_mirna[with_sig_mirna]),
                function(peak) {
                  cat(".")
    # Get miRNA from enriched peaks.
    mirnas <- sig_enriched_mirnas[[peak]]

    peak_seqs_chr <- as.character(ifelse(strand[peak] == "+",
                            peak_seqs[peak],
                            reverseComplement(peak_seqs[peak])))

    # Does the peak sequence matches seed?
    seed_match <-
      try(aggregateMatches(findSeedMatches(seqs = peak_seqs_chr,
                seeds = KSmodels[mirnas],
                  p3.extra = TRUE,
                  onlyCanonical = FALSE,
                  minDist = -Inf,
                  verbose = FALSE,maxLogKd = Inf)), silent = TRUE)
   return(seed_match)
})

names(seed_match_res) <-
  names(with_sig_mirna[with_sig_mirna])

with_seed_match <- (lapply(seed_match_res, class) %>% unlist()) != "try-error"

mireclip_data$with_seed_match <- FALSE
mireclip_data$with_seed_match[mireclip_data$genomic_info %in%
                              names(with_seed_match)[with_seed_match]] <-
  with_seed_match
```

Create subset with different number of replicates.

```
# Subset replicates.
mireclip_inten_reps <-
  list("n_rep=2" = mireclip_inten[, 1:2],
       "n_rep=3" = mireclip_inten[, 1:3],
       "n_rep=4" = mireclip_inten[, 1:4])

# Check dimensions.
lapply(mireclip_inten_reps, dim)
$`n_rep=2`
[1] 3145    2

$`n_rep=3`
[1] 3145    3

$`n_rep=4`
[1] 3145    4
```

Set initial values for each method.

```
# Set parameters for each model.
methods_params <- list(
  eCV = list(max.ite = 1e4),
  gIDR = list(
     mu = 2,
     sigma = 1,
     rho = 0.5,
     p = 0.5,
    eps = 1e-3,
    max.ite = 100
  ),
  IDR = list(
     mu = 2,
     sigma = 1,
     rho = 0.5,
     p = 0.5,
    eps = 1e-3,
    max.ite = 100
  ),
  mIDR = list(
     mu = 2,
     sigma = 1,
     rho = 0.5,
     p = 0.5,
    eps = 1e-3,
    max.ite = 100
  )
)
```

Correlation analysis of impossed reproducible features.

```
tmp <-
     mireclip_data$with_seed_match

mireclip_data$Symbol_miRNA[tmp] %>% unique() %>% length()
r <- lapply(unique(mireclip_data$genomic_info[tmp]),function(x) {
  i <- which(mireclip_data$genomic_info[tmp] == x)
  if (length(i) > 1) {
    R<- cor(mireclip_inten[tmp, ][i,])
    r <- mean(R[upper.tri(R)])
    m <- mean(mireclip_inten[tmp, ][i,])
c(length(i),r,m)
      } else {c(NA,NA,NA)}})

d <- do.call(rbind,r) %>% as.data.frame() %>% drop_na()
```

Assess reproducibility with each method.

Create a table with all combinations of parameters.

```
(par_settings <- expand.grid(n_rep =  paste0("n_rep=",4:2),
                            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
```

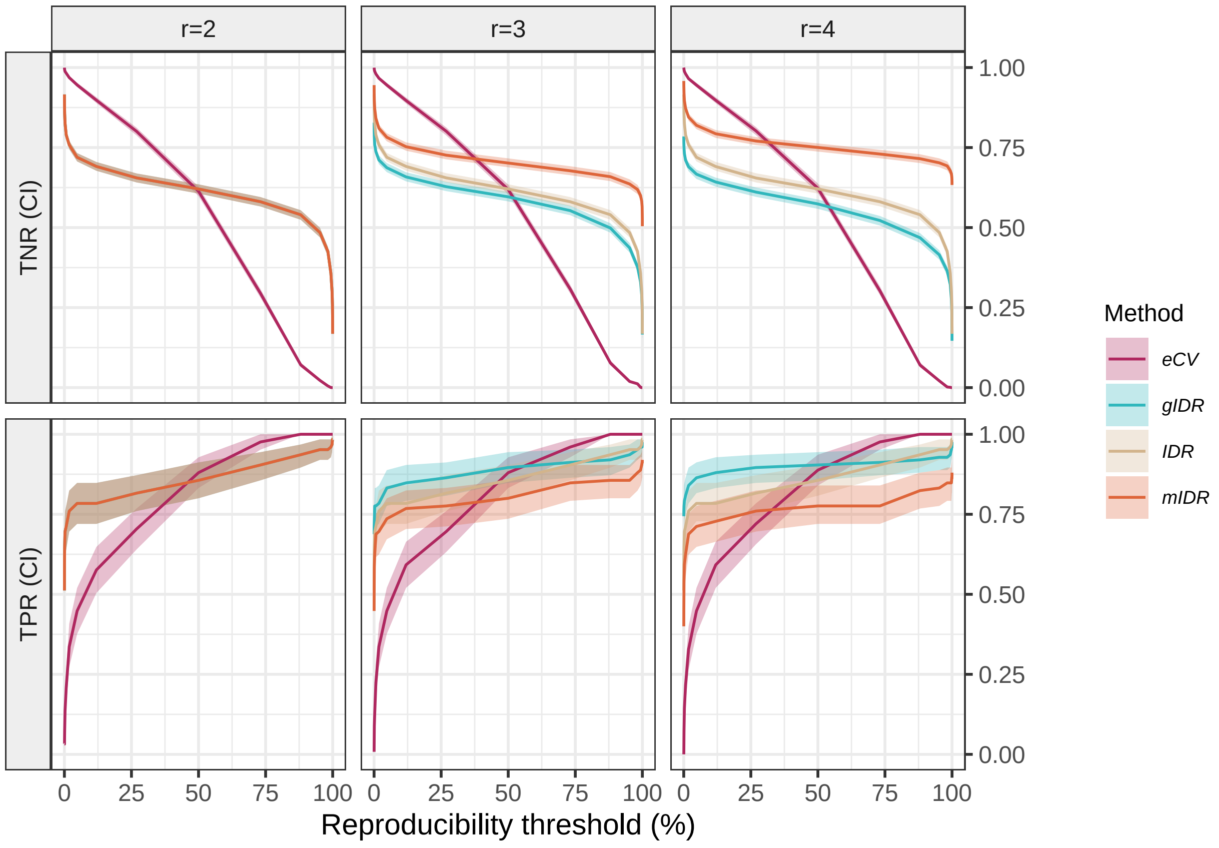
```
11 n_rep=3    eCV       11
12 n_rep=2    eCV       12
```

```r
# 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(mireclip_inten_reps[[n_rep]],
                         value_transformation = "identity",
                         jitter = 1e-4)
  } else {
    X <- mireclip_inten_reps[[n_rep]]
  }

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

   tmp <- exp(rowMeans(log(chim_test))) > 0.01 &
     mireclip_data$with_seed_match | mireclip_data$feature == "3'UTR"

   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_resRealmiReCLIP.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 = "Figure5.tiff",plot = p,device = "tiff",
       dpi=300,units = "in",width = 7,height = 5,scale = 0.85)
```