Functional normalization: reproducible report

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July 8, 2014

1 Introduction

All the code that was used to produce the results and plots of the paper can be found at https://github.com/Jfortin1/funnorm_repro. In the following sections, we provide instructions about how to use the different scripts to recreate the results and figures.

Once the GitHub repository is cloned into a personal laptop, this report can be reproduced by setting the `funnormDir` below to the path of your repository.

```
funnormDir <- "/Users/Jean-Philippe/funnorm_repro" # My path
setwd(funnormDir)
```

In the main folder, the shell script `jobs.sh` contains all the ordered jobs that were submitted to the cluster in order to get the different results.

Extraction of the data

The names of the samples for each dataset can be found in the folder `/designs`. The IDAT files for the samples from TCGA (KIRC and AML) can be downloaded using the TCGA Data Portal and read into R using the command `read.450k` from the *minfi* in R.

Metadata

The folder `/metadata` contains the mappings file from TCGA for the AML and KIRC datasets, for both 27k and 450k platforms. These `.csv` files contain the names of the samples, the plate information, sample tissue and histology of the samples (used as the phenotype for the subsequent analysis).

The file `clinical_patient_laml-1.txt` contains the clinical leukemia tumor subtype defined by *fixme: FAB*.

The file `link27kto450k.Rda` pairs the AML 27k samples to their corresponding 450k samples.

Experimental designs

The folder `/designs` contain all the design information to construct the discovery and validation datasets used throughout the analysis. For instance, for the KIRC dataset, the design file is called `design_kirc.Rda` and contains the sample names, the phenotypic group and the discovery/validation set identification as follows:

```
setwd(file.path(funnormDir, "designs"))
load("design_kirc.Rda")
head(design_kirc)
```

```
##                           sampleName group        set
## 6042316009_R02C02 6042316009_R02C02 Tumor Validation
## 6042316009_R03C01 6042316009_R03C01 Tumor Validation
## 6042316009_R04C01 6042316009_R04C01 Tumor Validation
## 6042316009_R04C02 6042316009_R04C02 Tumor Validation
## 6042316009_R06C01 6042316009_R06C01 Tumor Validation
## 6042316009_R06C02 6042316009_R06C02 Tumor Validation
```

The file `create.design.aml.R` extracts the AML tumor subtype from the file `clinical_patient_laml-1.txt` for the AML samples.

Plate information

The directory `/plate_info` contains the physical plate information of the samples for each dataset.

```
setwd(file.path(funnormDir,"plate_info"))
load("kirc_plate_450k.Rda")
head(kirc_plate_450k)
```

```
##                           sampleName plate
## 6042324009_R01C01 6042324009_R01C01    90
## 6042324009_R02C01 6042324009_R02C01    90
## 6042324009_R04C01 6042324009_R04C01    90
## 6042324009_R05C01 6042324009_R05C01    90
## 6042324009_R02C02 6042324009_R02C02    90
## 6042324009_R03C02 6042324009_R03C02    90
```

The companion script `create.plate.info.R` was used to extract the physical plate information of the samples.

Loading the data in R

For each dataset, we read the data into *R* using the packages *minfi* and *methylumi*. The first package produces an `RGChannelSet` for each dataset, while the latter package produces a `MethyLumiSet`. The

reason why we use both packages to read the data is that the *noob* normalization method is implemented in the *methylumi* package, while all other normalization methods are compatible with *minfi*. For each dataset, the files are saved in the `/raw_datasets` directory. For instance, `rgset_kirc.Rda` and `methylumi_kirc.Rda` correspond to the `RGChannelSet` and `methyLumiSet` for the KIRC dataset, respectively.

Production of the discovery and validation datasets

The folder `dis_val_datasets` contains the code to create the discovery and validation datasets objects. For instance, the script `create.dis.val.methylumi.R` will produce the `MethyLumiSet` object for each discovery and validation subsets of each datasets, and these objects will be saved in this directory under the name `methylumi_dis_XXX` and `methylumi_val_XXX` where XXX is the name of the dataset, for instance *kirc*.

2 Normalization

All the scripts to produce the normalized datasets can be found in the folder `/norm_datasets`.

Normalization for raw, funnorm, quantile, SWAN and dasen methods

The file `create.norm.R` is used to produce the normalized datasets for the raw, funnorm, quantile, SWAN and dasen methods. It will produce files of the form `norm_dis_XXX.Rda` and `norm_val_XXX.Rda` in the same directory that contains the normalized data.

For example, the object stored in `norm_dis_kirc.Rda` is a list of 5 matrices corresponding to the normalized Beta values for each aforementioned method, for the KIRC discovery dataset.

Normalization for noob method

Since the *noob* method requires a `MethyLumiObject`, we use a different script to produce the normalized datasets. The script `create.norm.noob.R` is used to produce these datasets, and the normalized datasets are saved under the name `noob_dis_XXX.Rda` and `noob_val_XXX.Rda`.

Normalization for funnorm + noob method

The script `create.funnorm.noob.R` generates the normalized data for the funnorm method that includes the background correction as implemented in the *methylumi* (noob). The normalized data can be found in the files `funnorm_noob_dis_XXX.Rda` and `funnorm_noob_val_XXX.Rda`.

Normalization for BMIQ

Because BMIQ is extremely slow and no parallel method is implemented yet, the normalization of the data with BMIQ requires a special treatment.

The script `split.bmiq.R` will read in it `RGChannelSet`, and will split the data into individual samples, and resave the samples separately in the folder `/funnorm_repro/raw_datasets/all_samples`. This folder contains the 1460 individual samples saved in `.Rda files` (for instance, `6042308157_R06C01.Rda`).

Since BMIQ does not perform between-array normalization, we can normalize all these samples in an embarrassingly parallel way; that's what the script `create.norm.bmiq.R` does. It uses the version 1.3 of BMIQ that can be found in the `/scripts` folder.

The normalized samples will be saved in the folder `/funnorm_dir/norm_datasets/all_samples_bmiq`.

The script `merge.norm.bmiq.R` will merge back the normalized samples and will create the files `bmiq_dis_XXX.Rda` and `bmiq_val_XXX.Rda` for each dataset XXX.

Merging all the normalized data

For each dataset, the script `merge.all.norm.R` will merge all the normalized data above in files called `all_norm_dis_XXX.Rda` and `all_norm_val_XXX.Rda`.

3 Creation of the DMPs

For each dataset, we create a list of differentially methylated positions (DMPs) with the corresponding dataset phenotype. The script `create.dmps.R` located in the directory `/dmps` is used to create those lists. For each dataset, the results are stored in files `dmps_dis_XXX.Rda` and `dmps_val_XXX.Rda`. Each file is a list (which each entry corresponding to a normalization method) of data frames containing the results. For instance, the top DMPs from the funnorm method for the KIRC discovery dataset can be accessed by

```
setwd(file.path(funnormDir,"dmps"))
load("dmps_dis_kirc.Rda")
head(dmps$funnorm)
```

The row names correspond to the 450k probes. The DMPs are produced with the function `dmpFinder` from the *minfi* package.

4 SVA analysis

The folder `/sva_results` contain the code to run SVA *fixme: citation* on each dataset. The script `create.sva.results.R` (which calls the script `/scripts/returnDmpsFromSVA.R`) will output the

results from SVA in files called sva_results_dis_XXX.Rda and sva_results_val_XXX.Rda for each dataset XXX.

The script create.sva.dmps.R will format the SVA results in a similar manner to the DMPs previously produced by the normalization methods so that subsequent code can be applied generally. The DMPs are saved in files called sva_dmps_dis_XXX.Rda and sva_dmps_val_XXX.Rda for each dataset XXX.

For instance, the SVA DMPs for the KIRC discovery dataset can be obtained by

```
setwd(file.path(funnormDir,"sva_results"))
load("sva_dmps_dis_kirc.Rda")
head(dmps)
```

The file number_pcs.txt described the number of surrogate variables found by SVA for each dataset.

5 RUV analysis

The scripts to run RUV-2 can be found at /scripts/RUV.functions. The folder ruv_results contains the different scripts to produce the results from the RUV analysis. In a first time, the script create.ruv.results.tuning.R is used to find the optimal number k of factors to remove. The two scripts create.ruv.results.plots.data.R and create.ruv.results.plots.data.2.R are used to visualize the optimal number k of factors to remove. The script create.optimal.ruv.R is used to create the normalized RUV datasets with the optimal k chose by the user. Finally, the script create.ruv.dmps.R is used to extract the DMPs from the RUV corrected datasets. The file optimal$_k$.$txtcontainsthevaluesofkforeachdatasetthatwereusedinthepaper$.

6 ComBat analysis

Because ComBat cannot be applied when there is perfect confounding between batch and phenotype, we only applied the ComBat method to the following datasets: KIRC Discovery, KIRC validation and AML.

The folder /combat_results contain the code to produce the results from ComBat. Specifical the script create.combat.results.R will output the results of ComBat in the files combat_re combat_results_dis_val.Rda and combat_results_aml.Rda.

Similar to the SVA script, the script create.combat.dmps.R will produce the DMPs list for each dataset.

7 Creation of the DMPs data for the 27K data

KIRC data

In the folder /kirc_27k, the script create.dmps.R will produce the DMPs for the 27K samples for the KIRC dataset with a plate adjustment using the package *limma*.

The results will be stored in the file dmps_27k_kirc_plate_adjusted.Rda.

AML data

In the folder /aml_27k, the script create.dmps.R will produce the DMPs for the 27K samples for the AML dataset with a plate adjustment using the package *limma*.

The results will be stored in the file dmps_27k_aml_plate_adjusted.Rda.

8 Creation of the Discovery/Validation ROC data

In the folder /roc_data, the scripts create.roc.data.XXX.Rda, where XXX is either blank, *sva*, *sva.funnorm*, *rub* or *ruv.funnorm* will produce the discovery/validation ROC data for the EBV, Blood and KIRC datasets for the normalized data, the SVA results, the funnorm + SVA results, the RUV results and the funnorm + RUV results respectively.

The results will be stored in files called *XXX_rocData_YYY_ZZZ.Rda* where XXX depends on the method, and where YYY and ZZZ depends on the dataset. For instance, ruv_funnorm_roc stores the ROC data from the funnorm + RUV method, with 100K loci used as the truth, for the KIRC dataset.

9 Creation of the concordance data

In the folder /roc_data, the script create.overlap.data.R will create the concordance curves data between the discovery and validation subjects for the EBV, Blood and KIRC datasets, for each method.

The results will be stored in the files overlapData_XXX_YYY.Rda where XXX is either 100 or 1000 (step to calculate the concordance) and YYY is the name of the dataset. For instance, the file overlapData_100_kirc.Rda contains the concordance curves data for the KIRC dataset, where the concordance was calculated at every 100 loci.

10 External validation with 27k data

The directory /external_validations contains the script to create the overlap and ROC data of the KIRC and AML datasets when the 27K data DMPs results are used as the truth.

The scripts external.validation.kirc.R and external.validation.aml.R create the concordance curves and ROC curves data for the KIRC and AML datasets, respectively.

For the concordance curves, the results are stored in the files overlapData1K_XXX.Rda where XXX is either kirc_dis, kirc_val or aml.

For the ROC curves data, the results are stored in the files rocdata_27k_XXX where XXX is either kirc_dis, kirc_val or aml.

11 External validation with the WGBS data

To assess the quality of the top DMPs for the Ontario-EBV dataset, we use an external dataset from whole-genome bisulfite sequencing as described in the manuscript. The blocks and DMPs data from the WGBS can be found at /external_validations under the names blocks_ebvs_null.rda and dmrs_ebv_null.rda respectively. The script external.validati will produce the overlap/replication percentages for each dataset and will save the results in the file data.wgbs.replication.Rda.

12 Sex analysis

Creation of the probes X-inactivation status

The file nature03479-s9.xls, which was downloaded from the supplemental material at http://www.nature.com/nature/journal/v434/n7031/full/nature03479.html, contains the X-inactivation status that was used throughout the present paper. The script create.sex.pr extracts the information of the file nature03479-s9.xls to assign an X-inactivation status for each of the X-chromosome probes of the 450K array. The status is saved in the file x.probes.status.Rda; the intermediate file x.inactivation.data.Rda is a representation of nature03479-s9.xls in a format readable by the R software. The script create.x.y.probes.R extracts the probe names for the probes mapping to the X and Y chromosome and save them in the files chrY.Rda and chrX.Rda.

Creation of the ROC data for the Ontario-Gender dataset

The directory /roc_data_gender contains the script create.roc.data.gender.R which generates the file rocDataGender.Rda that contains the ROC curves data for the sex prediction using the X-inactivation status described above.

13 Sensitivity analysis

We have run Funnorm for $k = 1, \ldots, 10$ number of principal components. The directory /sensitivity_analysis contains the different scripts to produce the sensitivity analysis. The script sensitiviy.norm.R creates the normalized dataset for each k; the normalized data will be saved in /sensitivity_analysis/norm_datasets. The scripts sensitivity.dmps.R and sensitivity.roc.R create the DMPs and ROC data for each value of k, for each dataset.

14 Sample size simulation

To assess the performance of Funnorm for small sample sizes, we devised the following simulation scheme for the Ontario-EBV dataset. First, we kept the discovery dataset intact to make sure to have a reasonable gold standard in our discovery-validation ROC curves; we only simulated different sample sizes for the validation subset. For different sample sizes $n \in \{10, 20, 30, 50, 80\}$, we randomly chose half of the samples from the EBV-transformed samples, and the other half from the lymphocyte samples. For instance, for $n = 10$, we randomly picked 5 samples from each of the treatment groups; we repeated this subsampling $B = 100$ times, which generated 100 ROC discovery-validation ROC curves for each n, for a total of 500 ROC curves.

For a fixed n, we took the mean of the $B = 100$ ROC curves as well as the 0.025 and 0.975 quantiles to mimic a 95% confidence interval.

The different scripts are located in the directory simulation_samplesize.

The script create.subsamples.matrices.R creates a matrix of subsampling ids for each of $n \in \{10, 20, 30, 50, 80\}$, and saved the results in the file subsamples.matrices.Rda.

The file create.norm.R creates the 500 simulated validation datasets for the data normalize with Funnorm. The file create.raw.R creates the 500 simulated validation datasets for the raw data. The file create.noob.R creates the 500 simulated validation datasets for the data normalized with noob. The file create.bmiq.R creates the 500 simulated validation datasets for the data normalized with BMIQ. The file create.norm.other.R creates the 500 simulated validation datasets for the remaining normalization methods.

The subfolders *fixme: names of the folders* contain the simulated datasets for the corresponding datasets.

15 Technical Replicates

The folder /technical_replicates contains the code to normalize the 19 trios of technical replicates analyzed in the paper. In particular, the script analysis.R outputs the

file technicalVariances.Rda. The files tri.Rda and dataLink.csv allows the user to map sample names to their trio id.

16 Effect sizes

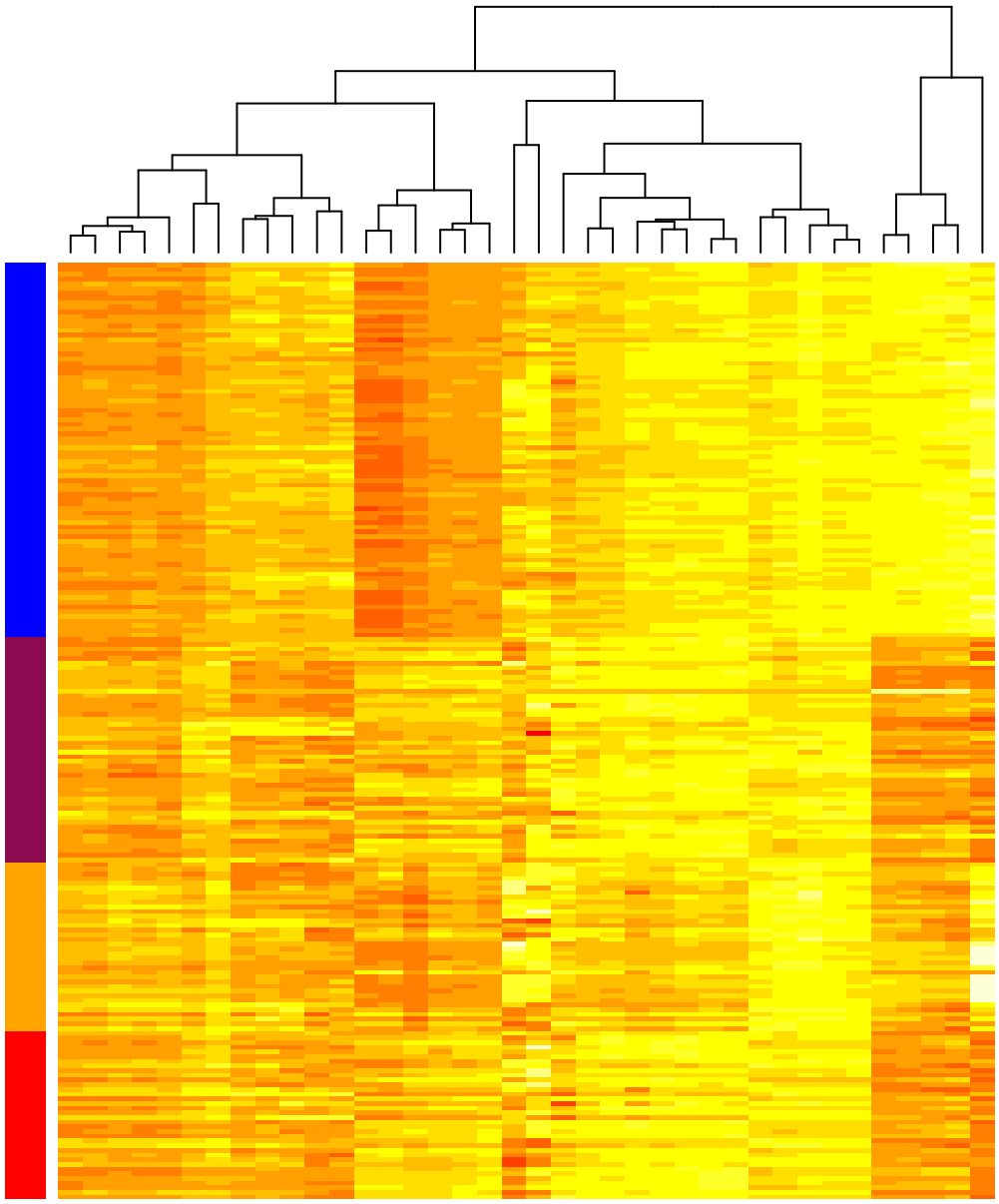
The script create.effect.size.R in the folder /effect_size_analysis computes the effect size for each dataset.

17 Reproducible plots

Please find below the scripts that generated all the figures in the paper.

Heatmap and PCA

```
source(file.path(funnormDir, "paper_figures/generate.heatmap.R"));
create.heatmap(print=TRUE)
```



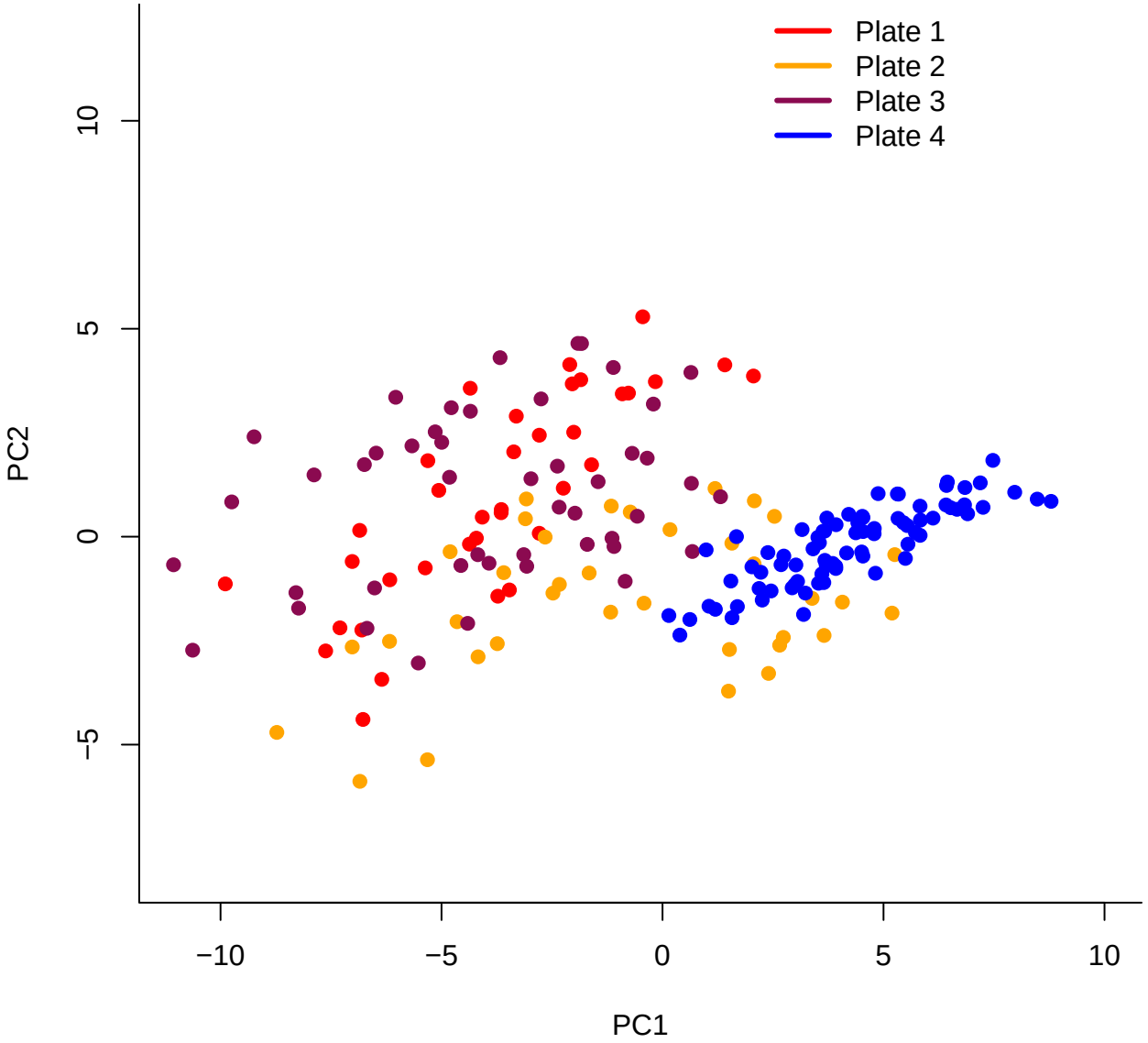
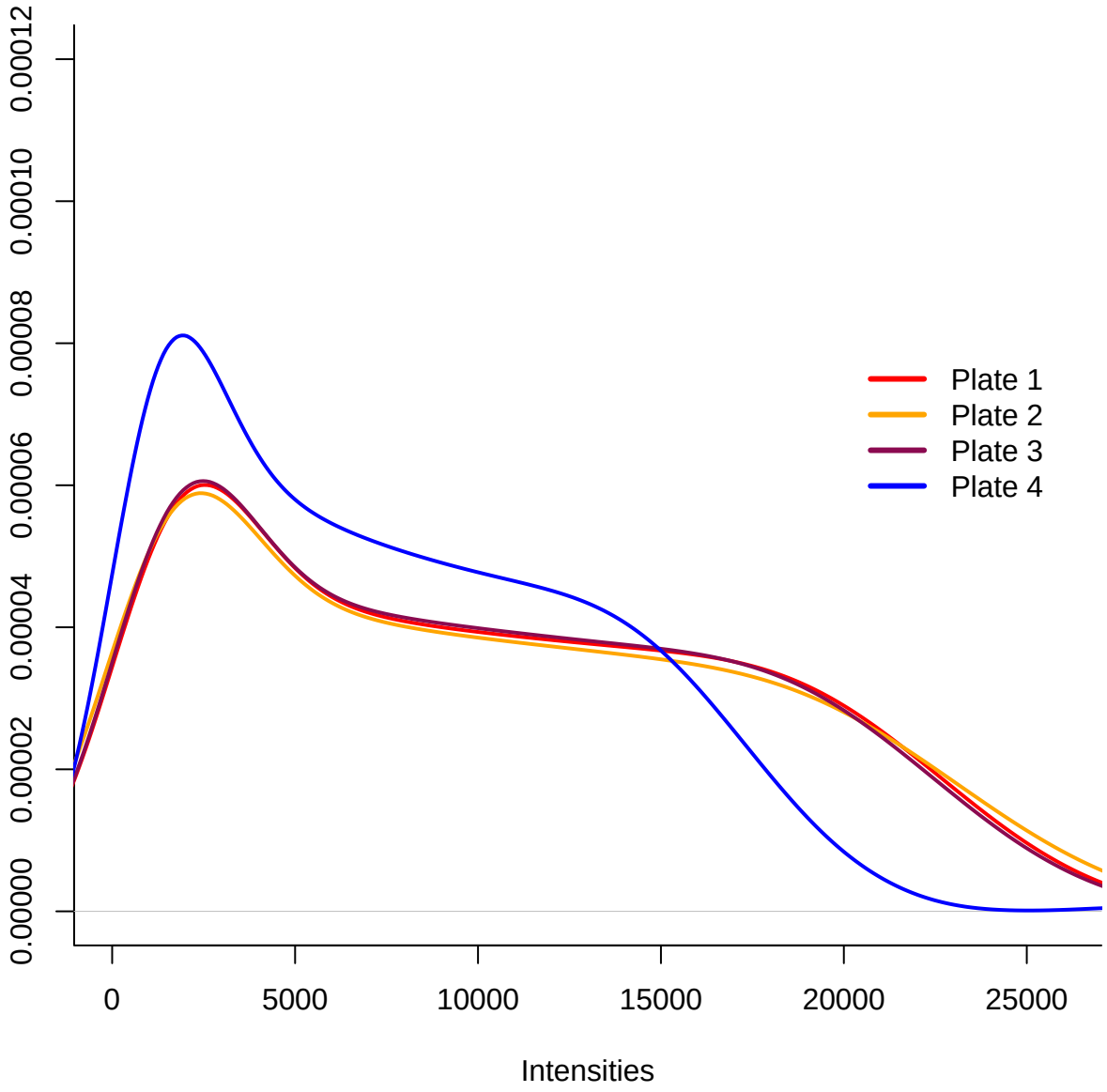


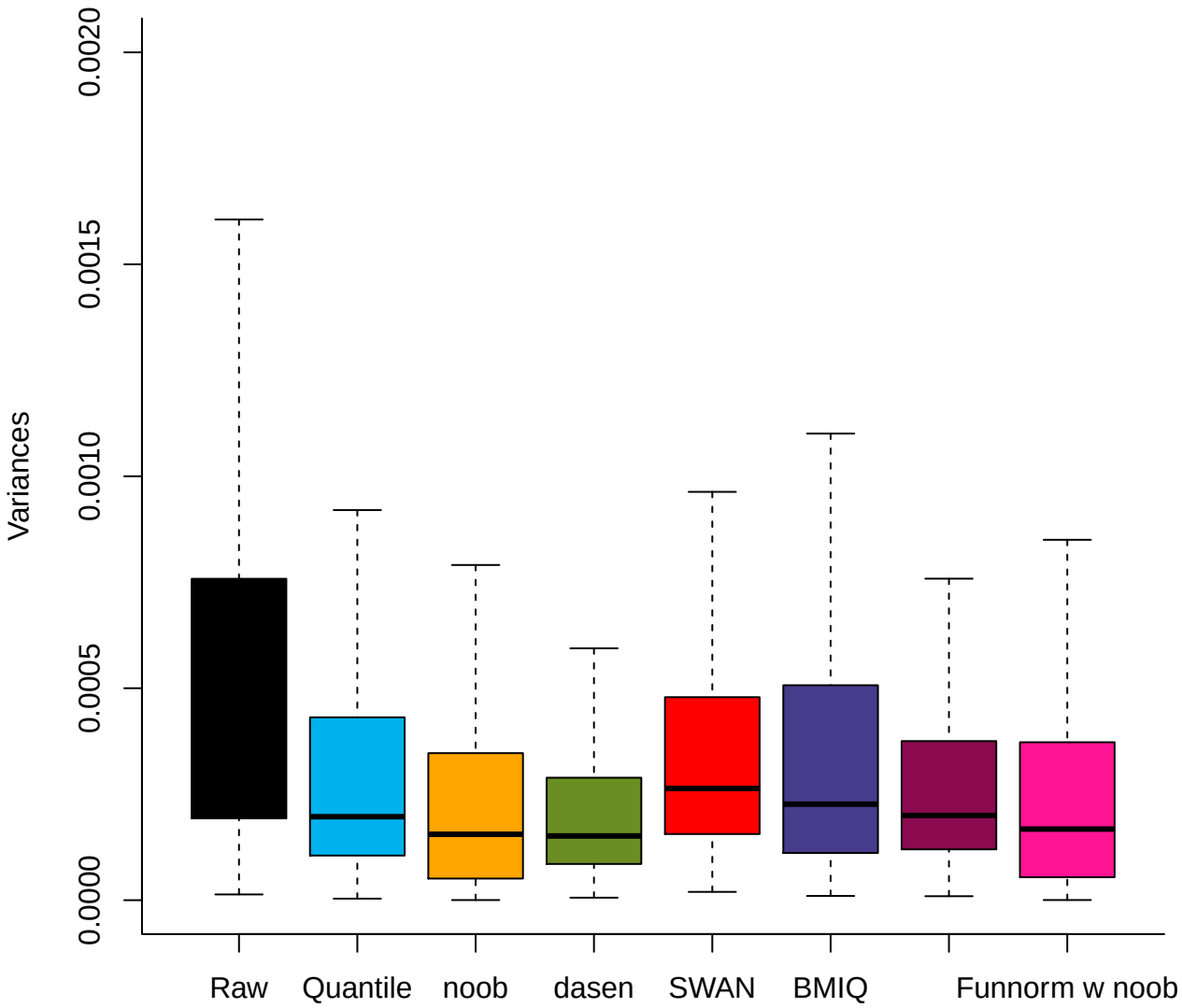
Plate 1
Plate 2
Plate 3
Plate 4
PC2
PC1
10
5
0
−5
−10
−5
0
5
10




```
## pdf
##   2
```

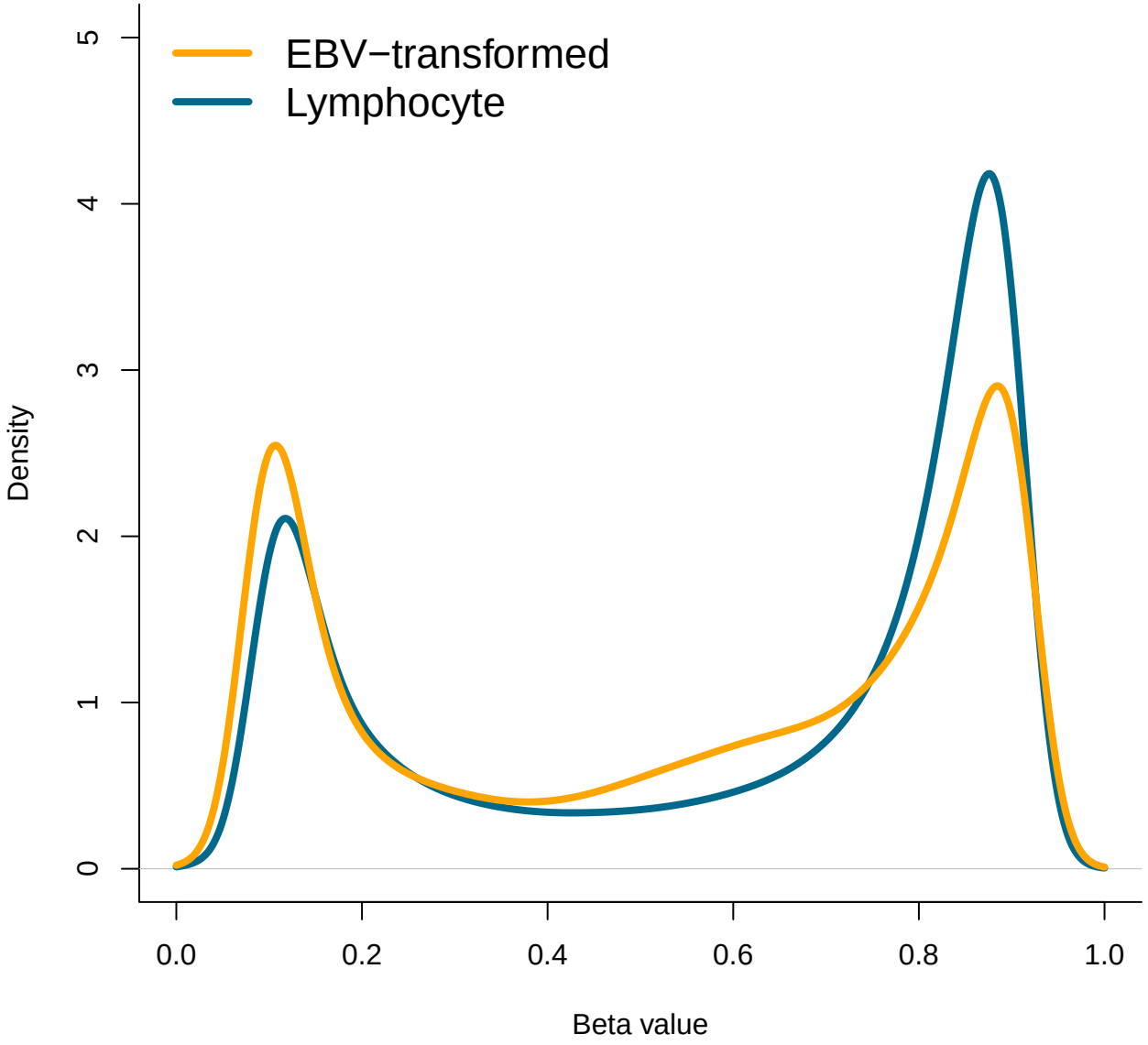
Technical replicates

```
source(file.path(funnormDir, "paper_figures/generate.replicates.plot.R"));
create.replicates(print=TRUE)
```



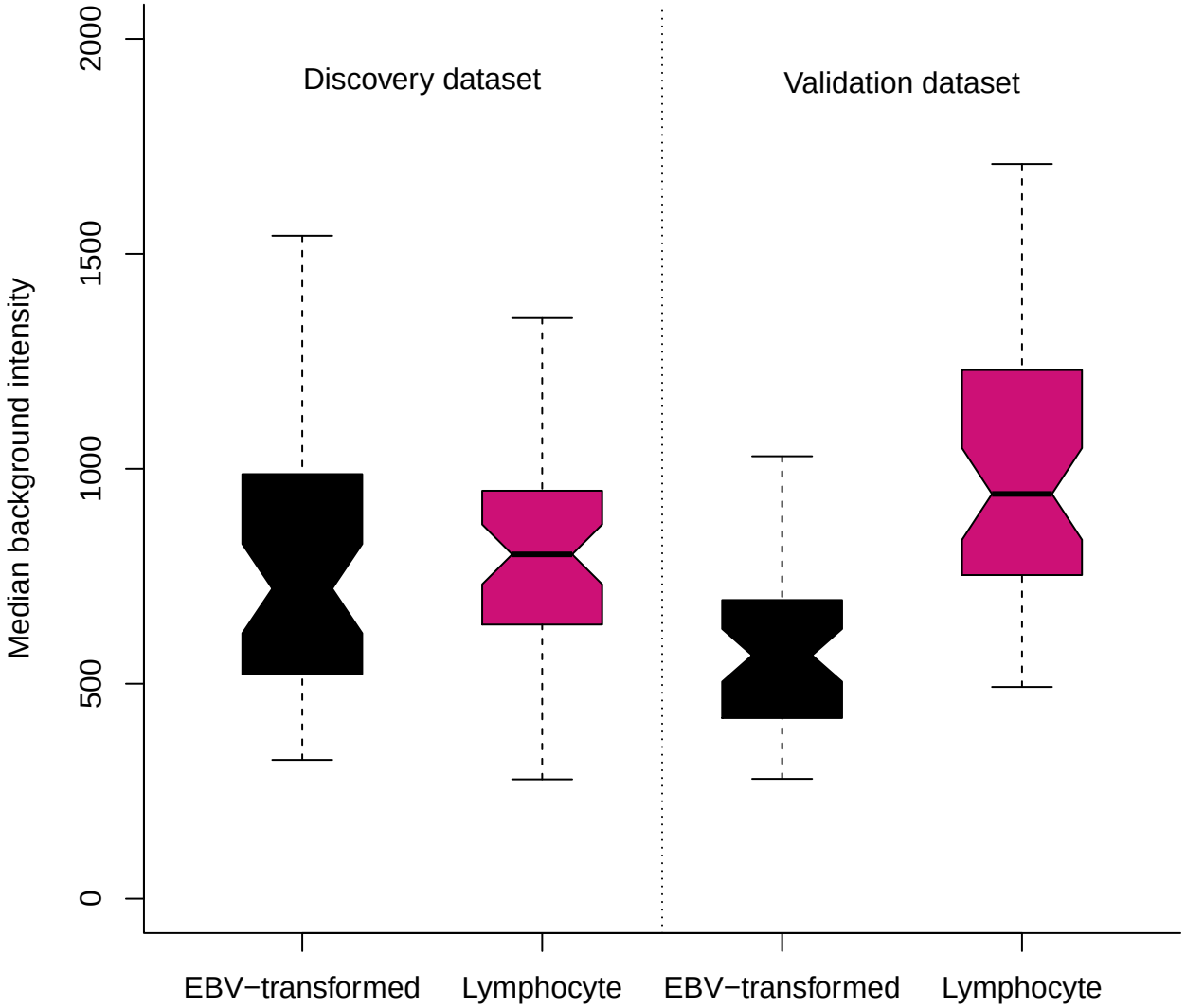
Marginal distributions

```
source(file.path(funnormDir, "paper_figures/generate.lympho.marginal.R"));
create.marginal(print=TRUE)
```

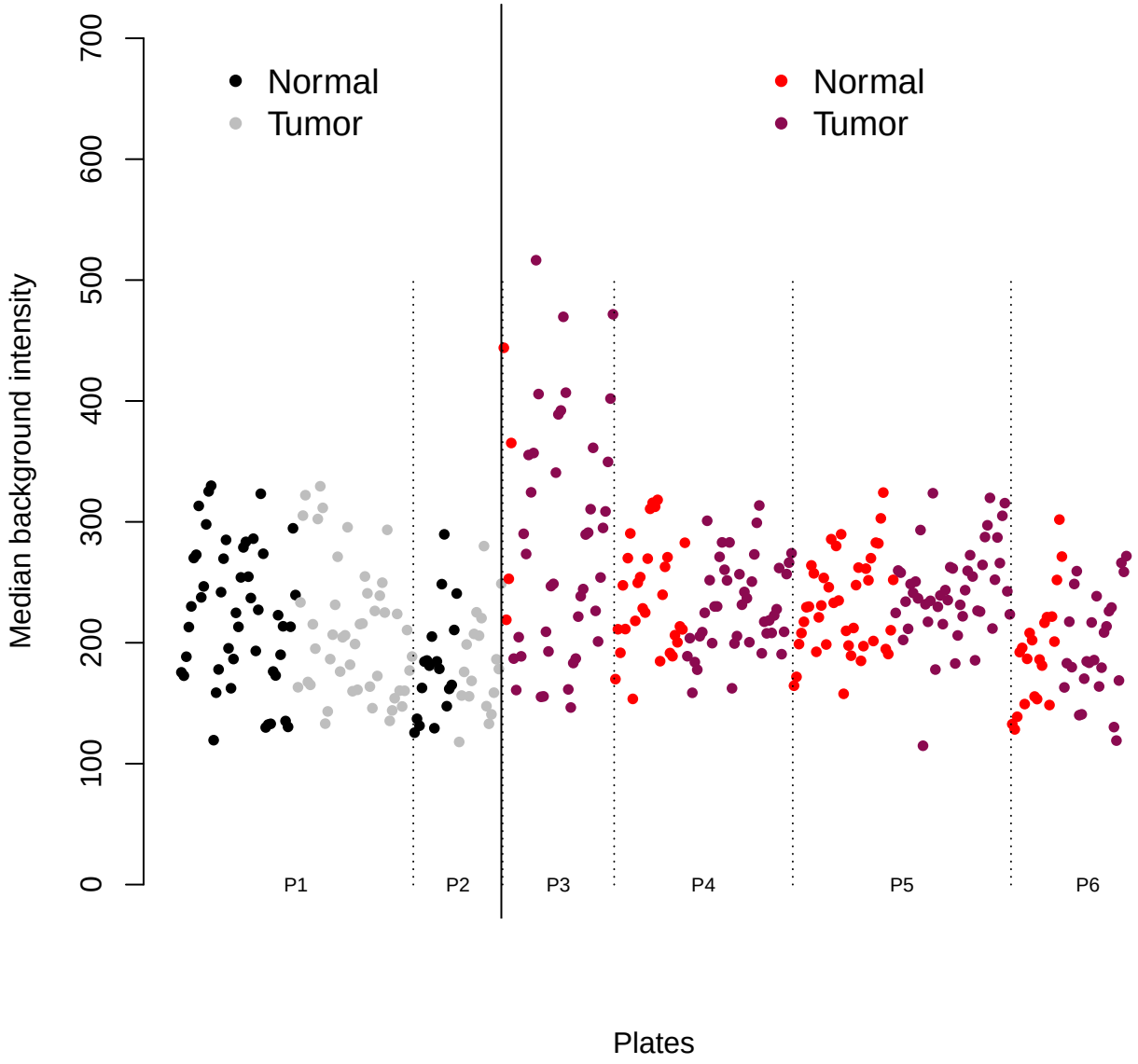


In silica batch effects

```
source(file.path(funnormDir, "paper_figures/generate.design.plot.R"));
create.design.plot.ebv(print=TRUE)
```



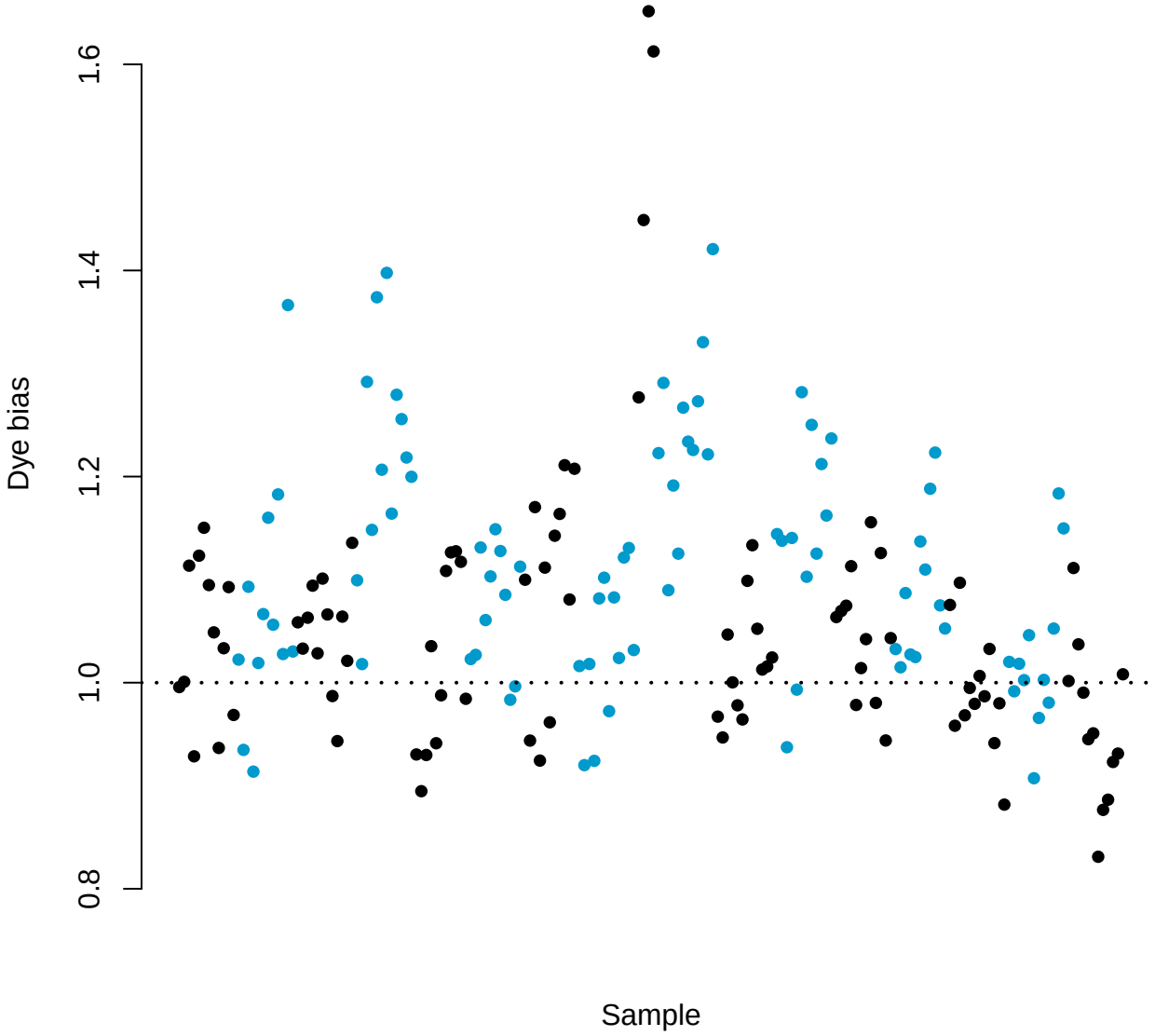

```
create.design.plot.kirc(print=TRUE)
```

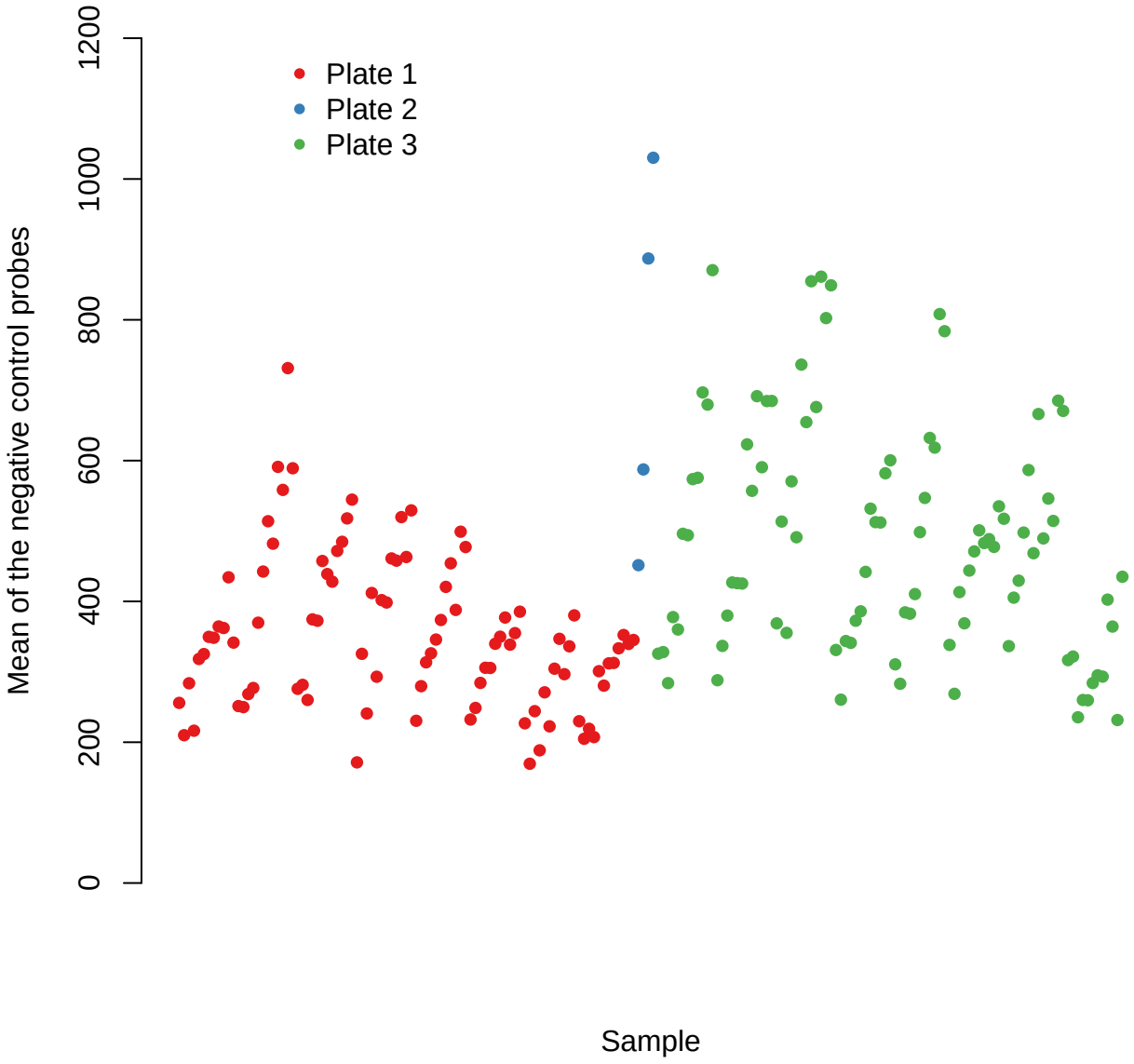



```
## pdf
##   2
```

Plate and dye bias effects

```
source(file.path(funnormDir, "paper_figures/generate.dyebias.plot.R"));
create.dyebias(print=TRUE)
```

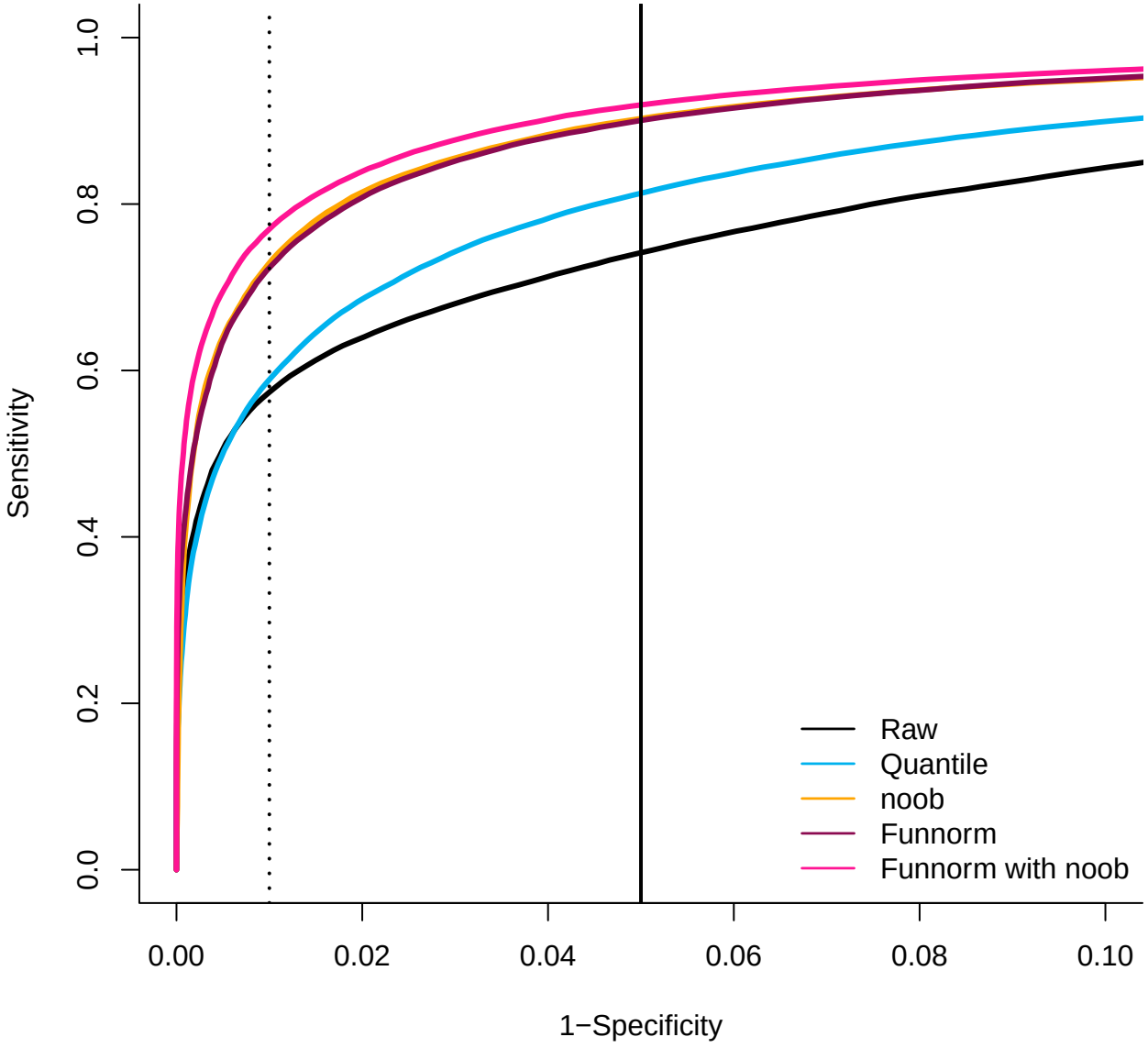

Dye bias
1.6
1.4
1.2
1.0
0.8
Sample

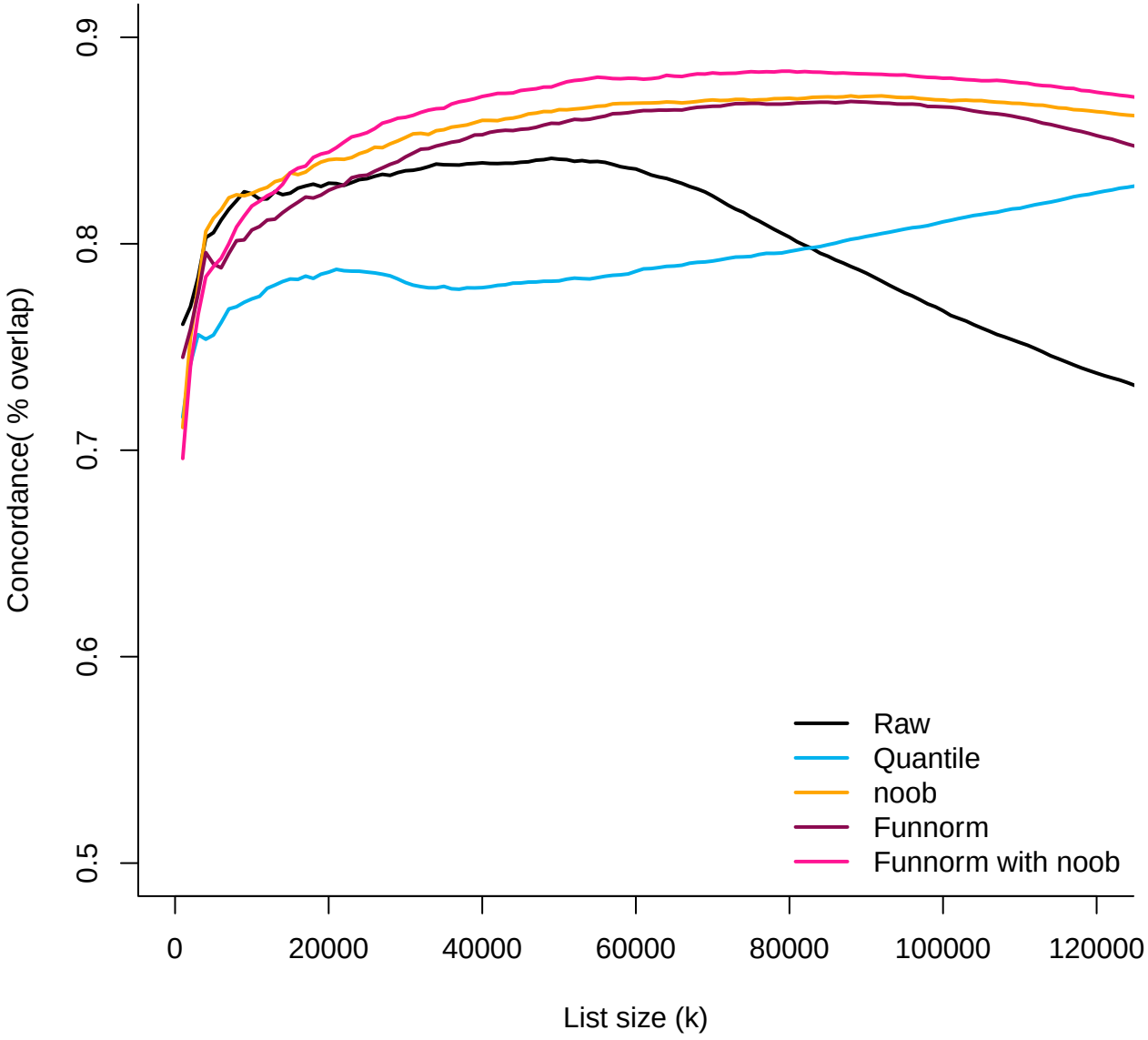



```
## pdf
##   2
```

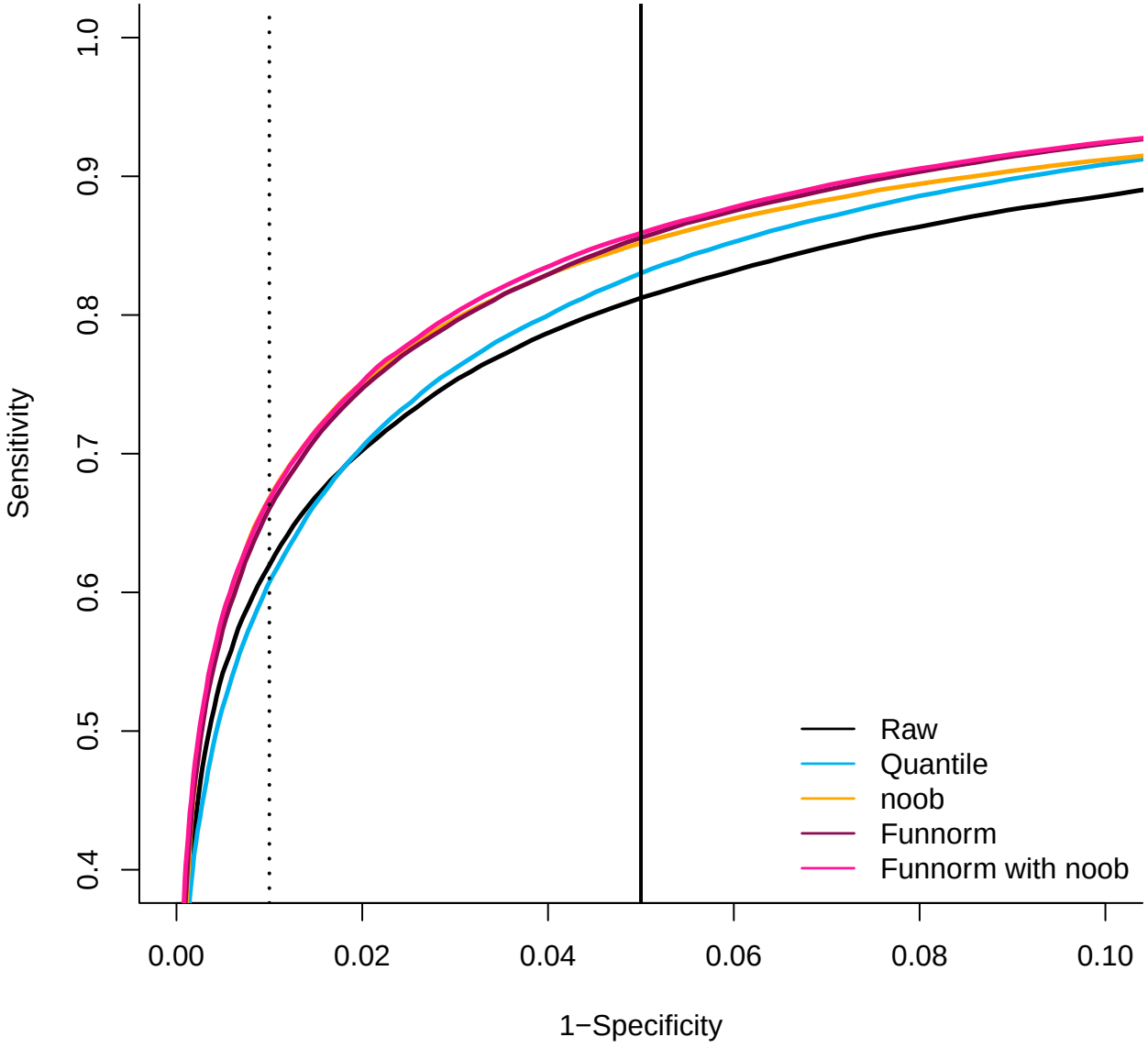
Main results

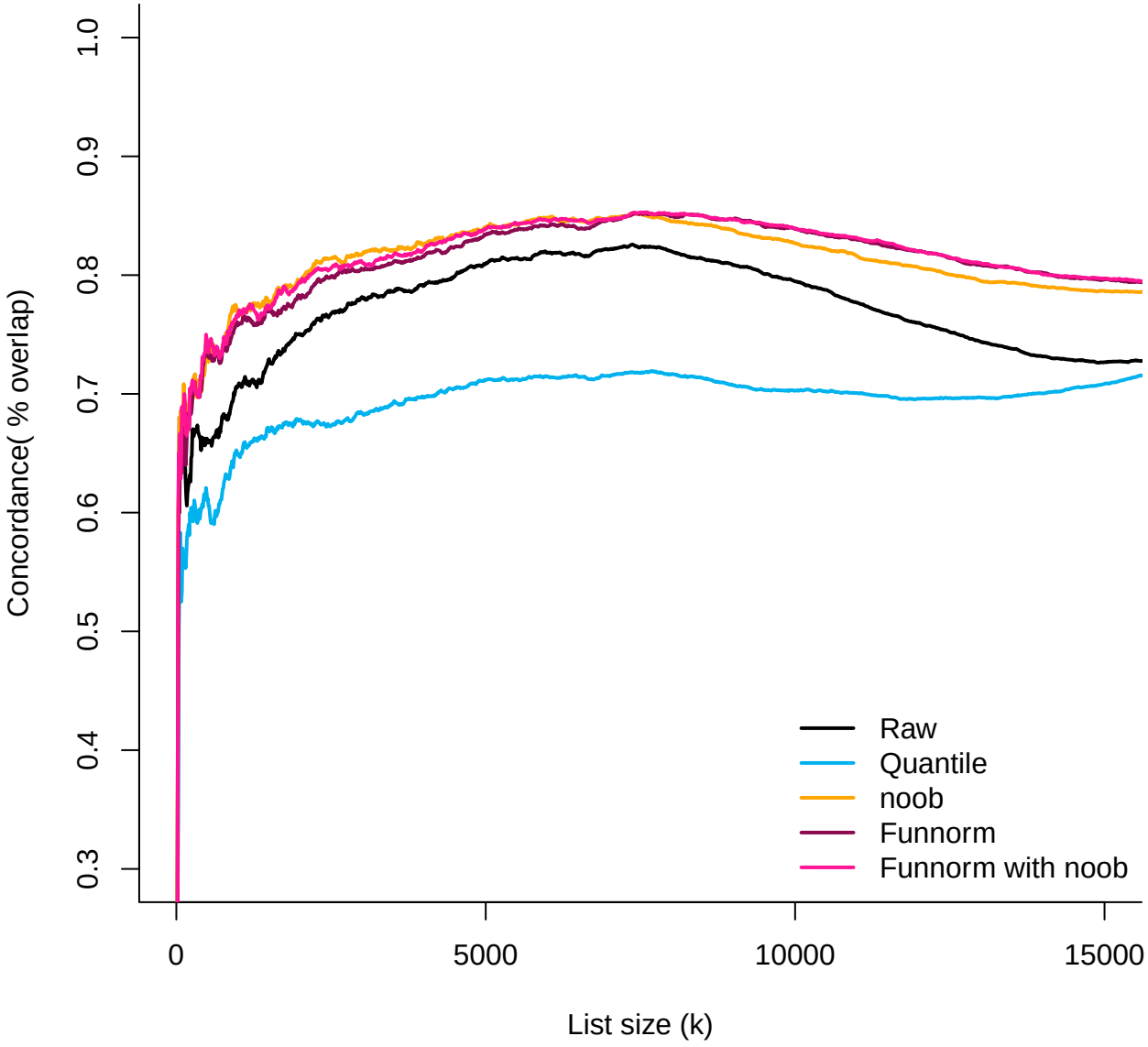
```
source(file.path(funnormDir, "paper_figures/generate.main.results.plots.R"));
create.main.ebv(print=TRUE)
```


Sensitivity
1−Specificity
0.0
0.2
0.4
0.6
0.8
1.0
0.00
0.02
0.04
0.06
0.08
0.10
Raw
Quantile
noob
Funnorm
Funnorm with noob

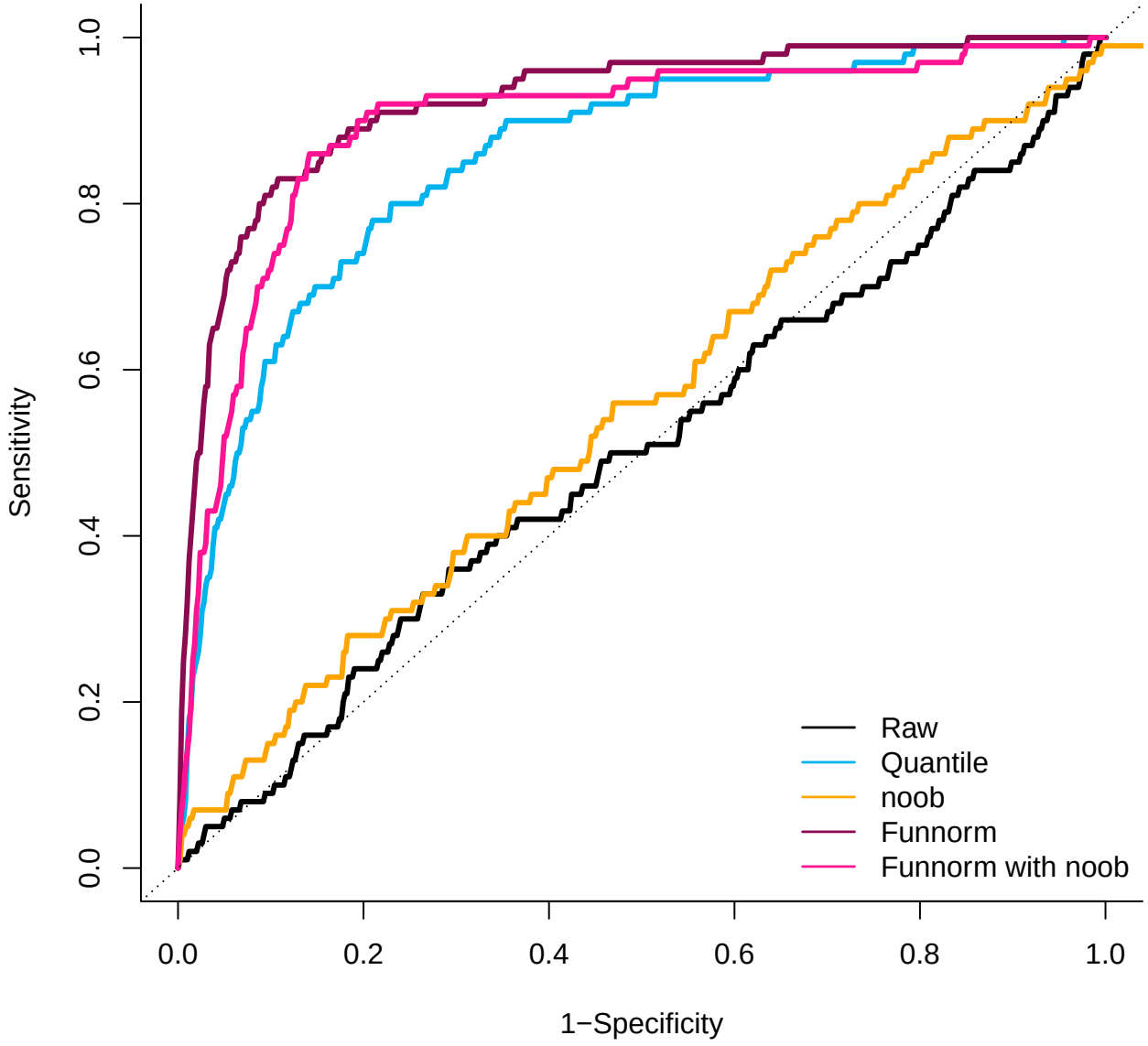



```
create.main.kirc(print=TRUE)
```

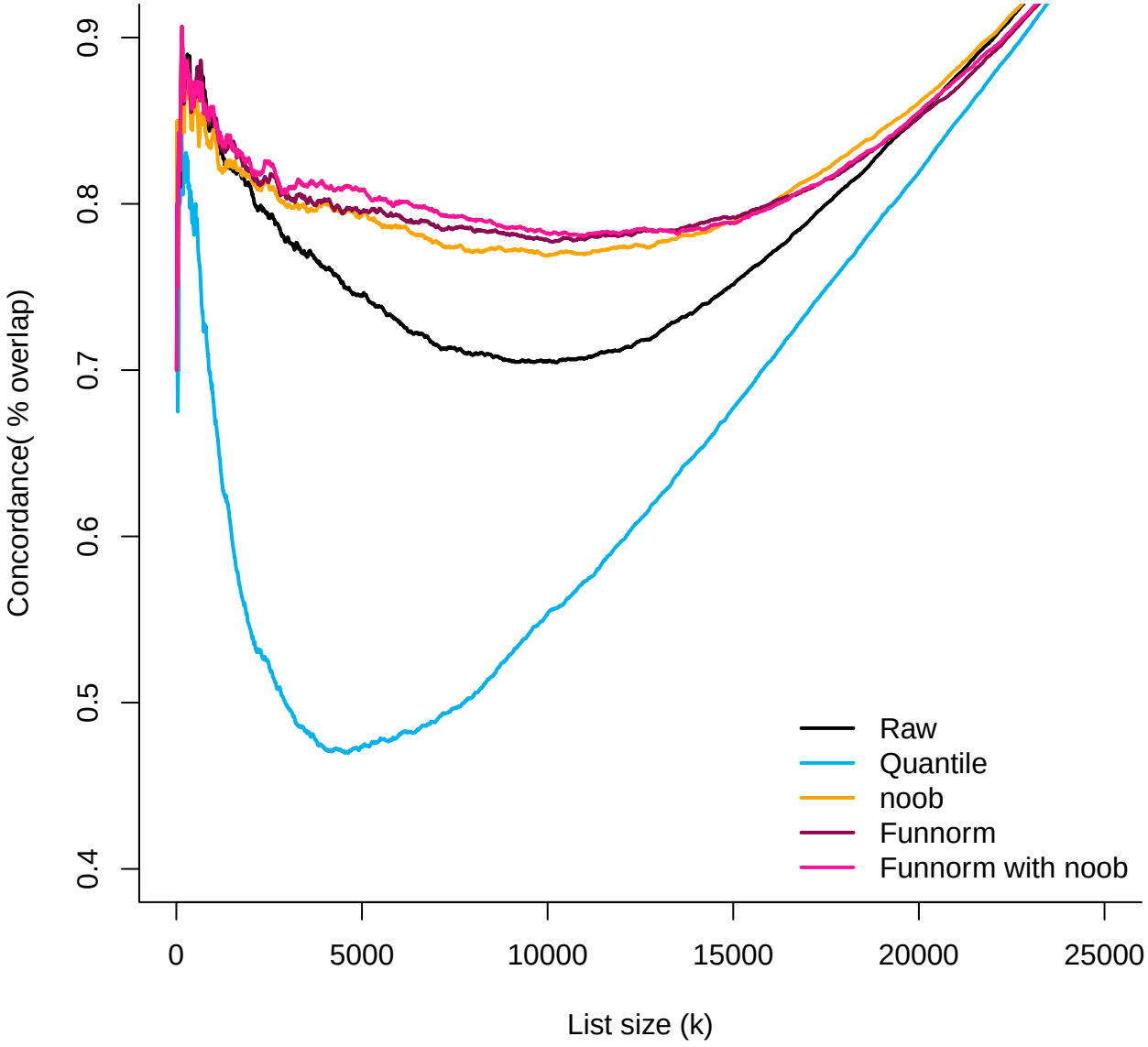

Sensitivity
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.00
0.02
0.04
0.06
0.08
0.10
1−Specificity
Raw
Quantile
noob
Funnorm
Funnorm with noob

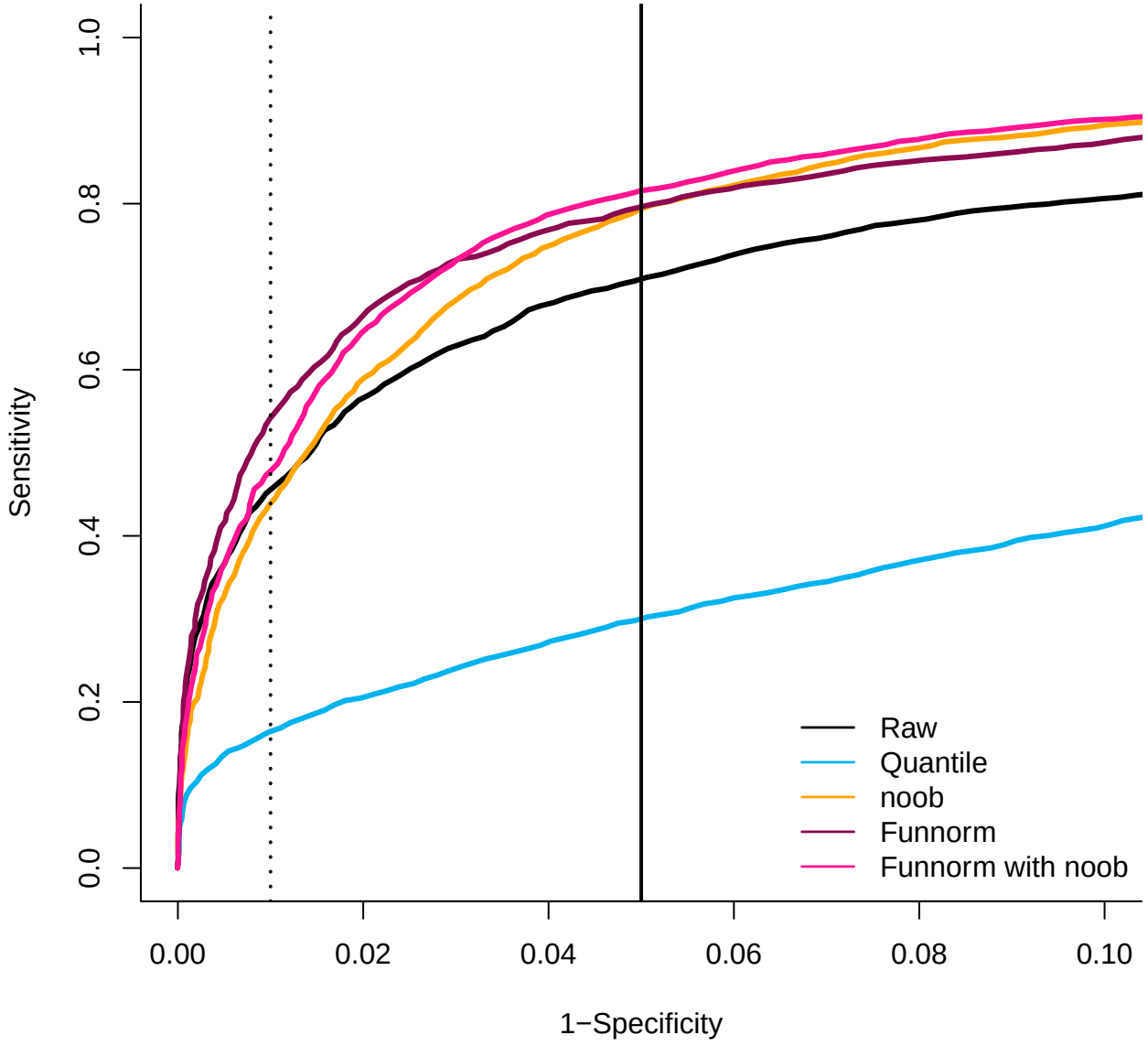



```
create.main.blood(print=TRUE)
```



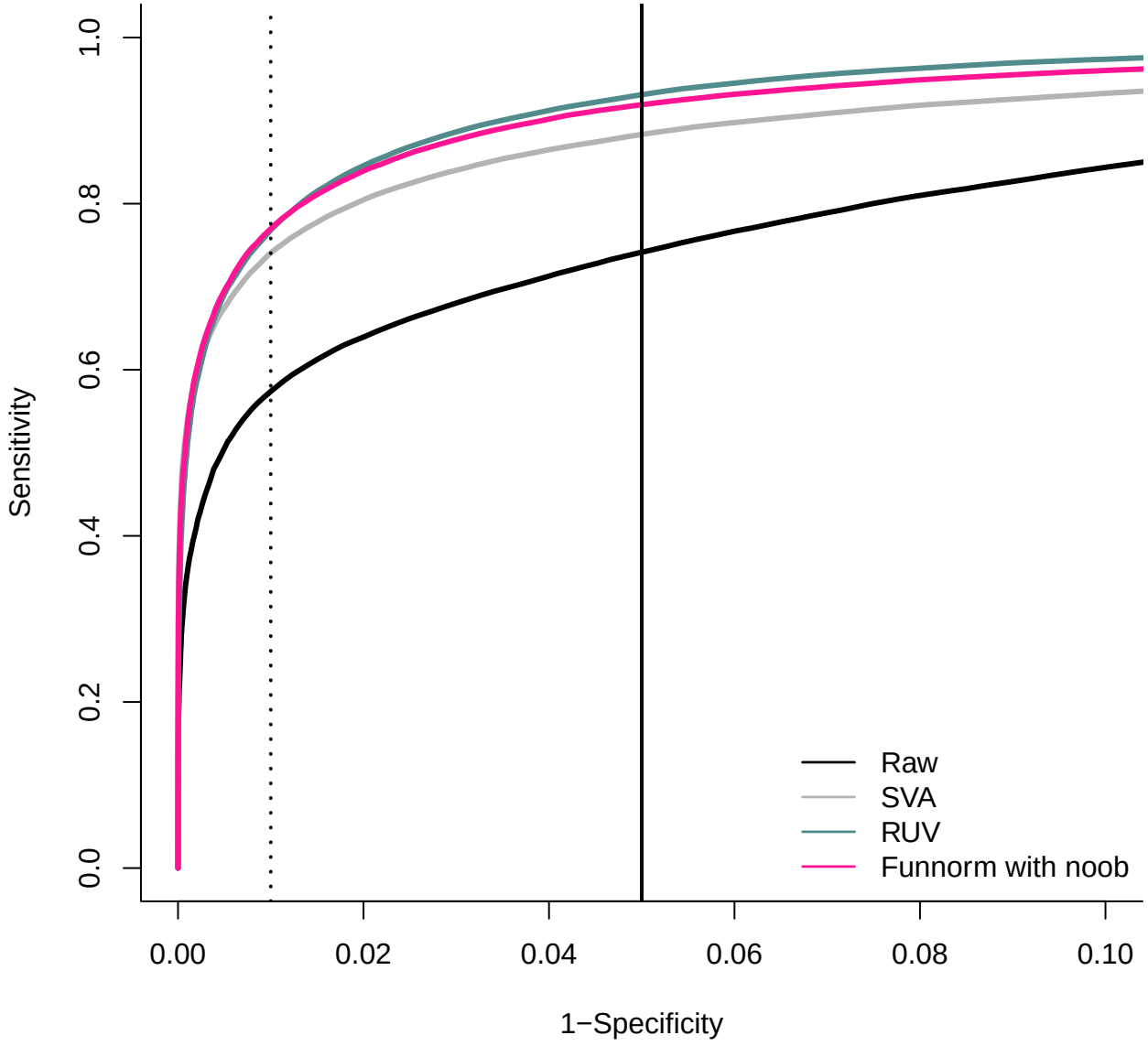

```
create.main.aml(print=TRUE)
```


Concordance(% overlap)
0.4
0.5
0.6
0.7
0.8
0.9
0
5000
10000
15000
20000
25000
List size (k)
Raw
Quantile
noob
Funnorm
Funnorm with noob

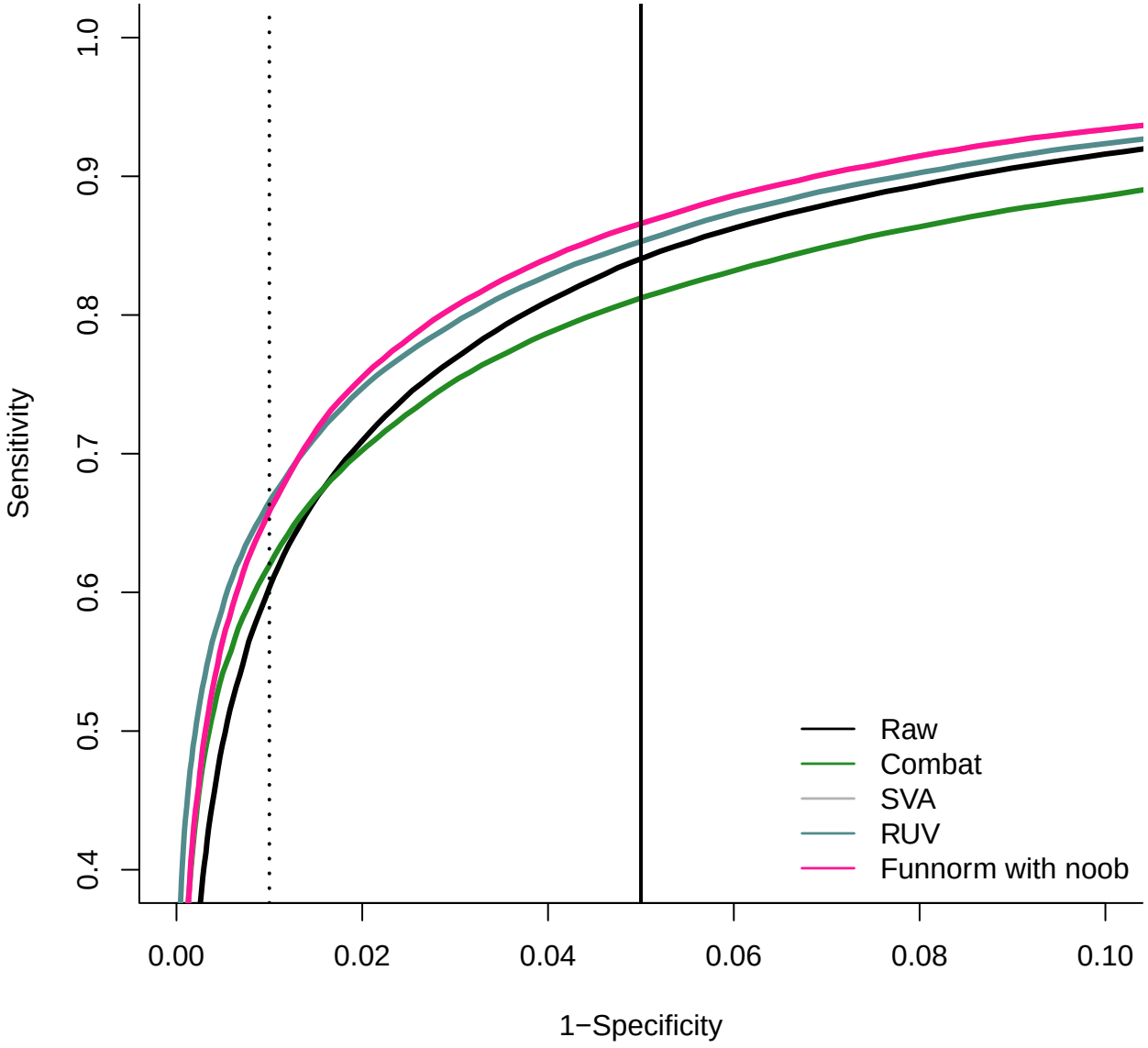


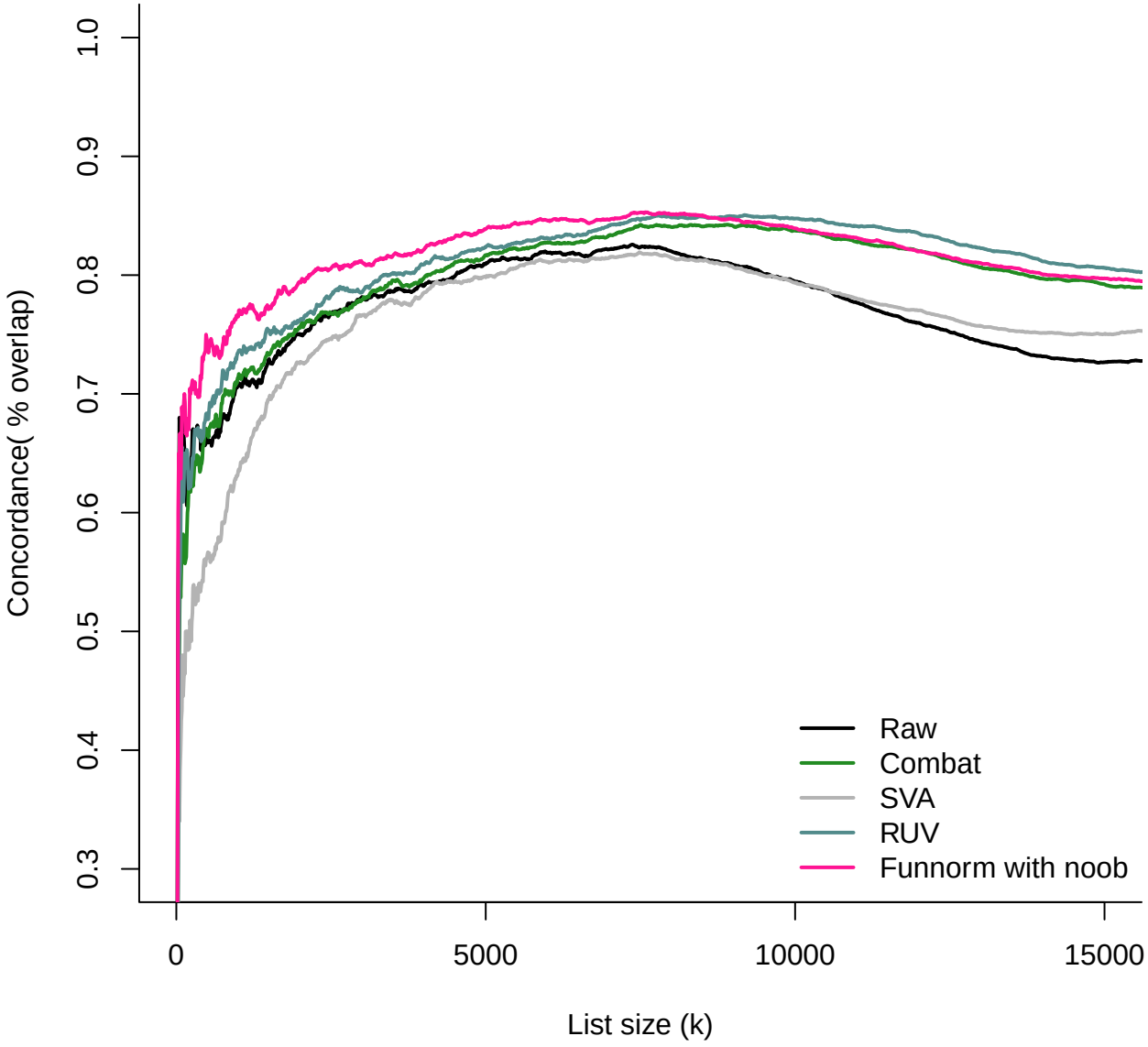
SVA,RUV and ComBat results

```
source(file.path(funnormDir, "paper_figures/generate.sva.ruv.results.plots.R"));
create.sva.ruv.ebv(print=TRUE)
```

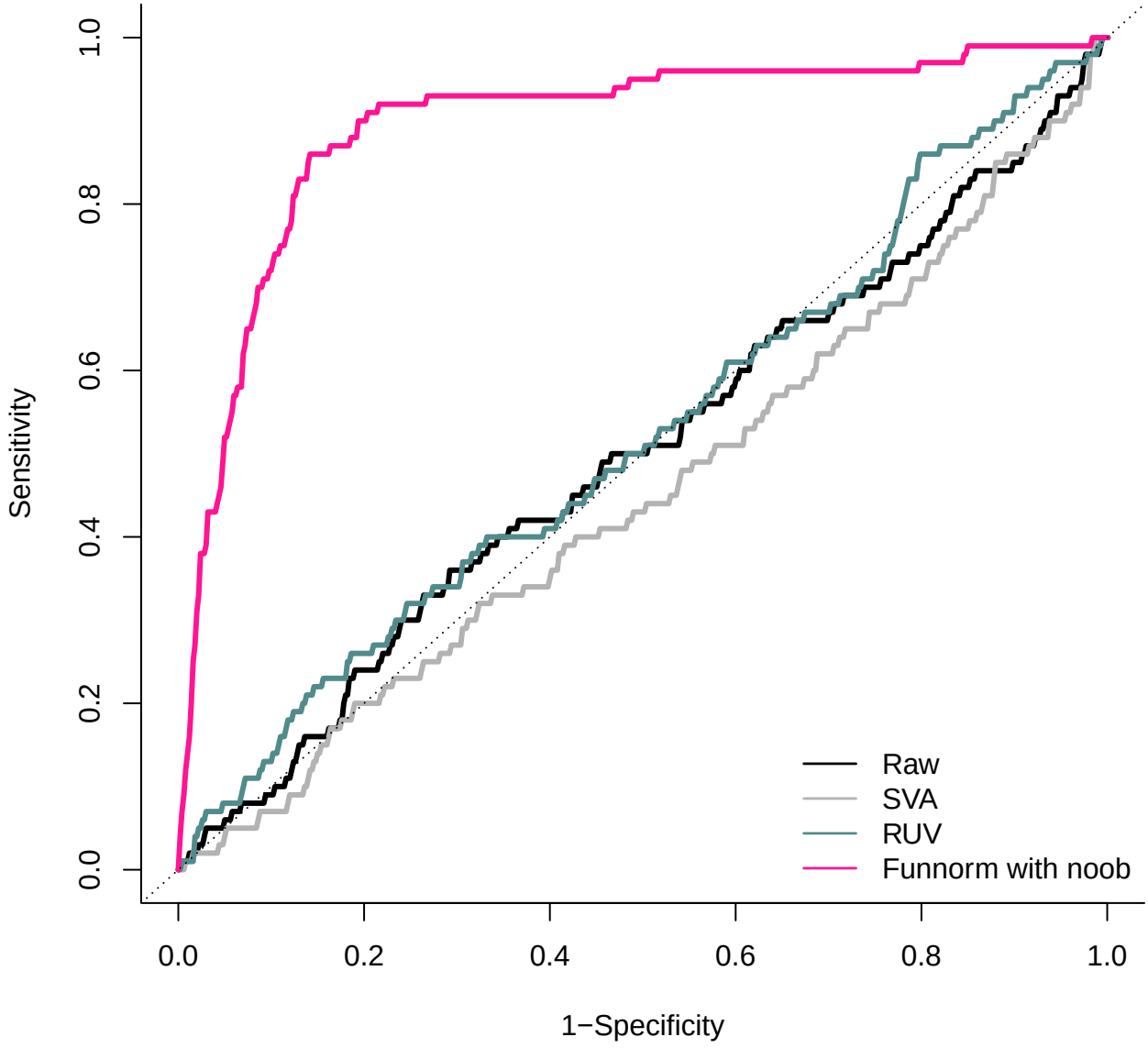



```
create.sva.ruv.kirc(print=TRUE)
```

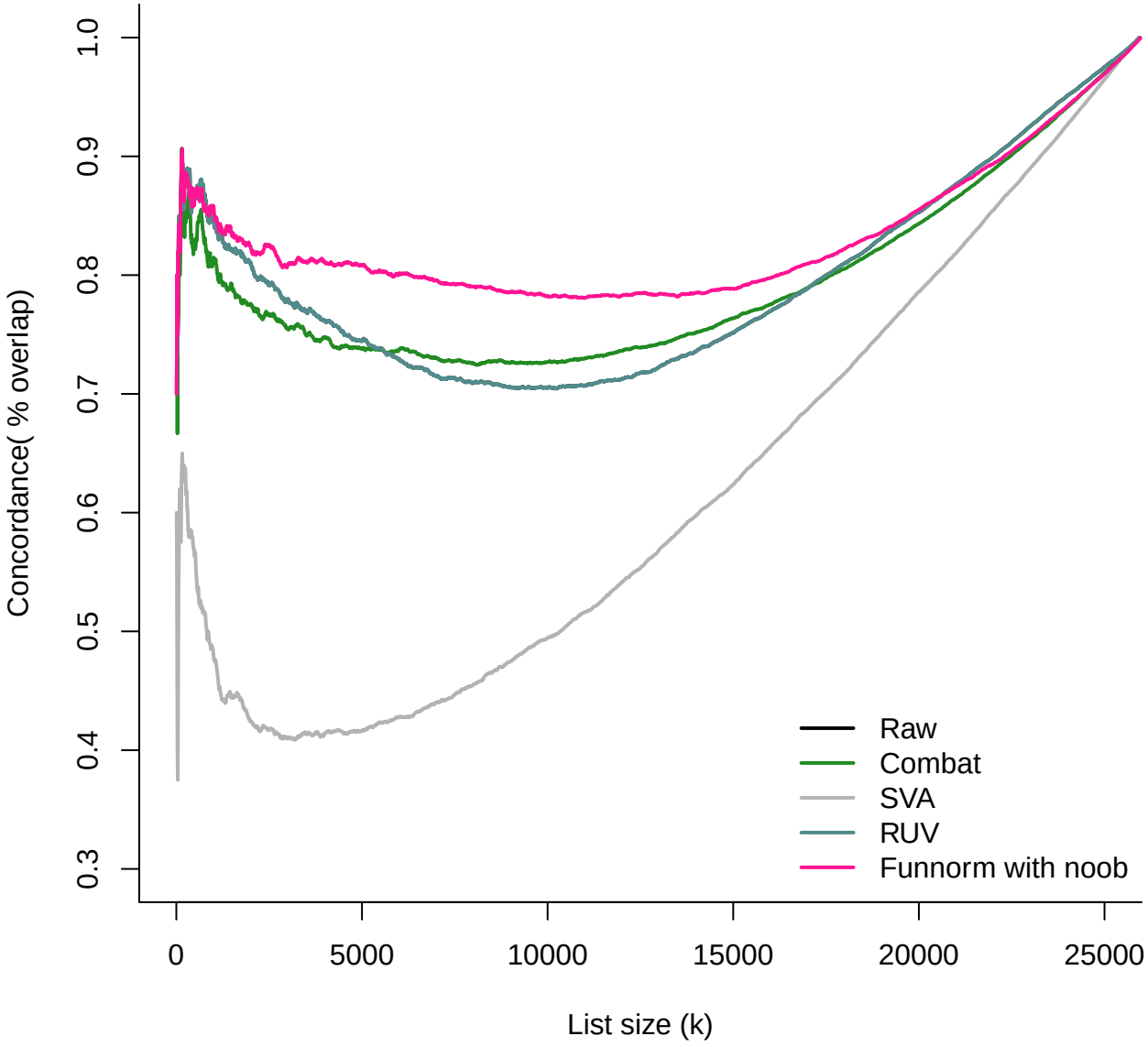

Sensitivity
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.00
0.02
0.04
0.06
0.08
0.10
1−Specificity
Raw
Combat
SVA
RUV
Funnorm with noob

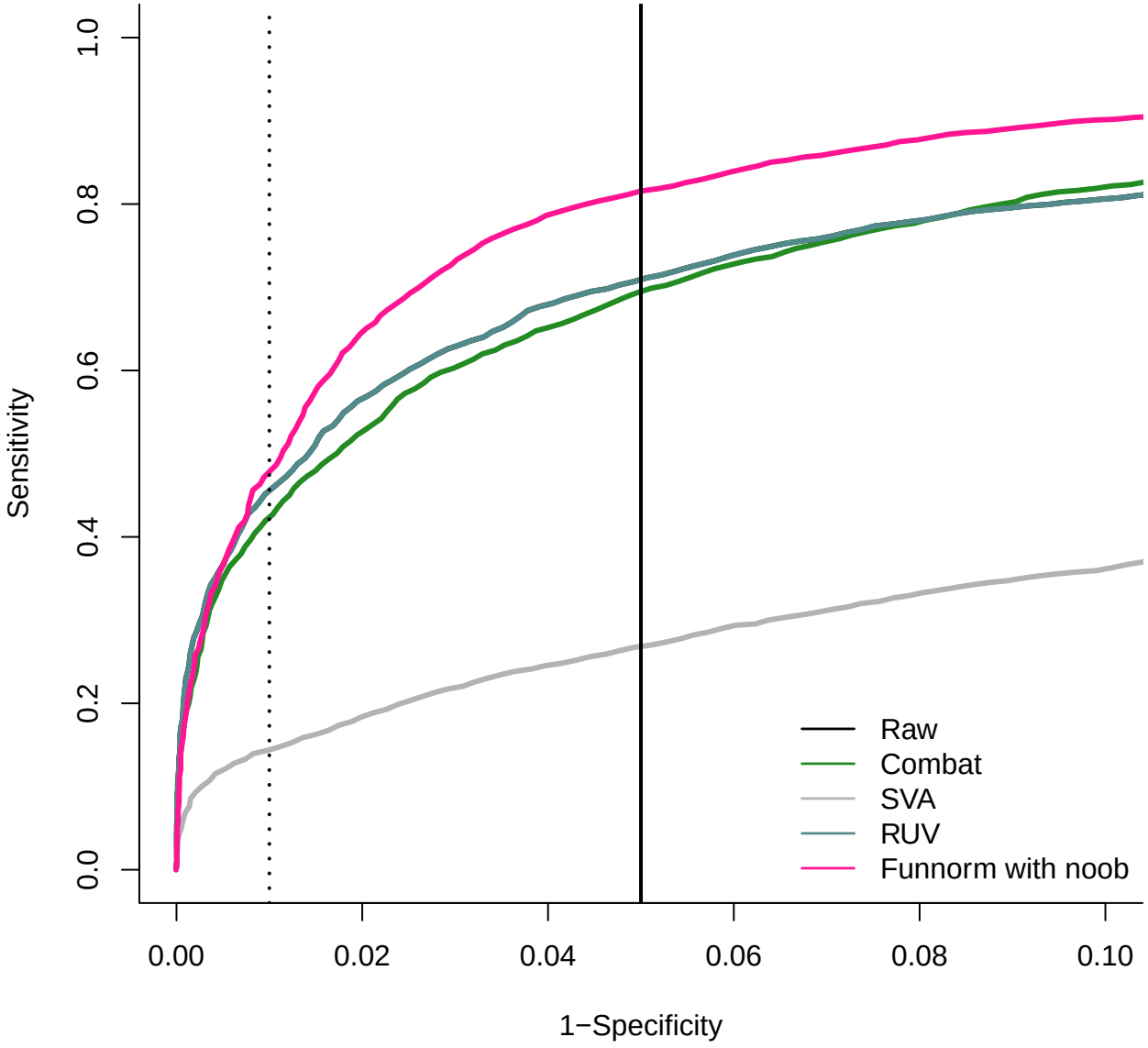


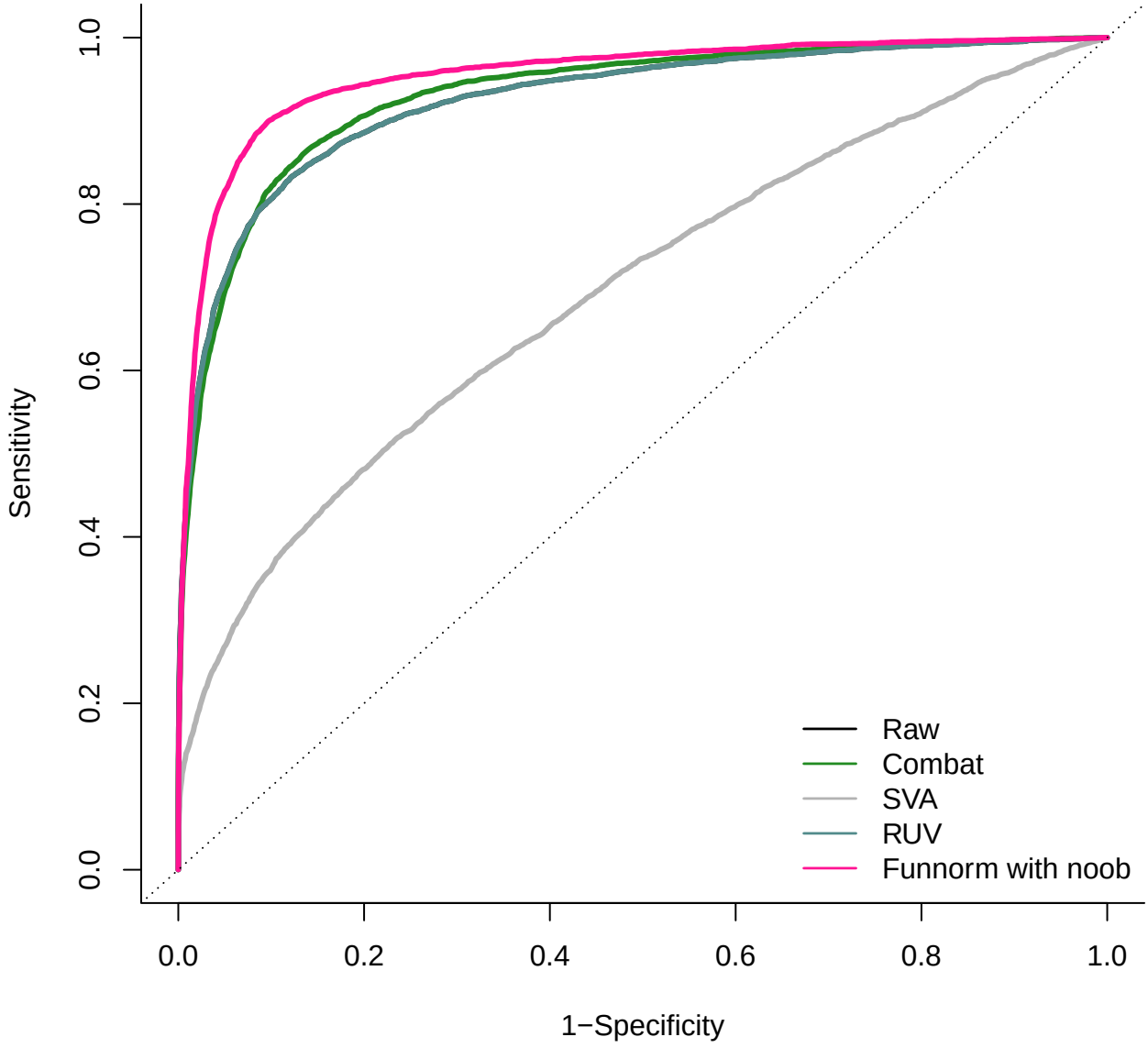

```
create.sva.ruv.blood(print=TRUE)
```




```
create.sva.ruv.aml(print=TRUE)
```

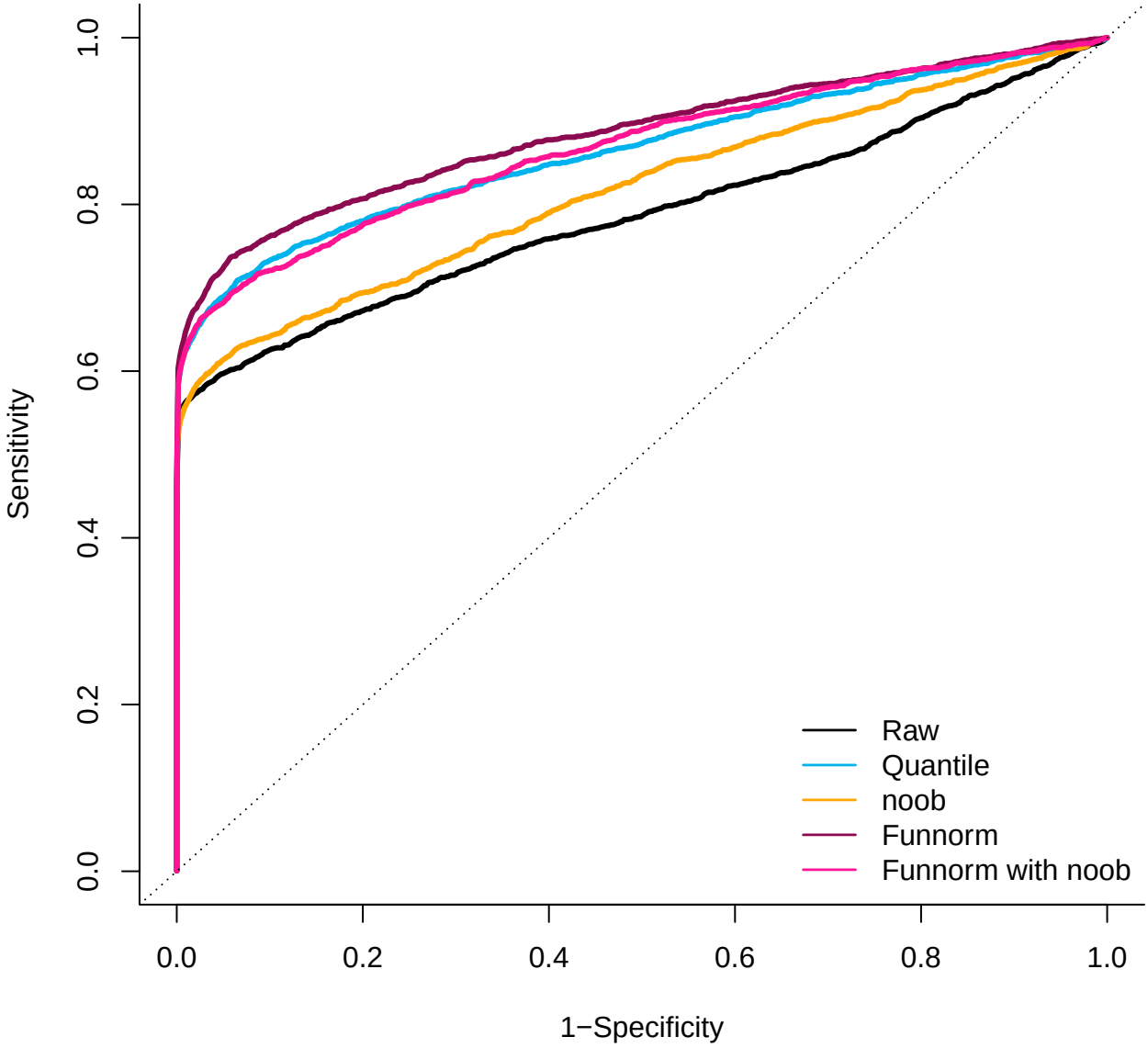

Concordance(% overlap)
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1.0
0
5000
10000
15000
20000
25000
List size (k)
Raw
Combat
SVA
RUV
Funnorm with noob

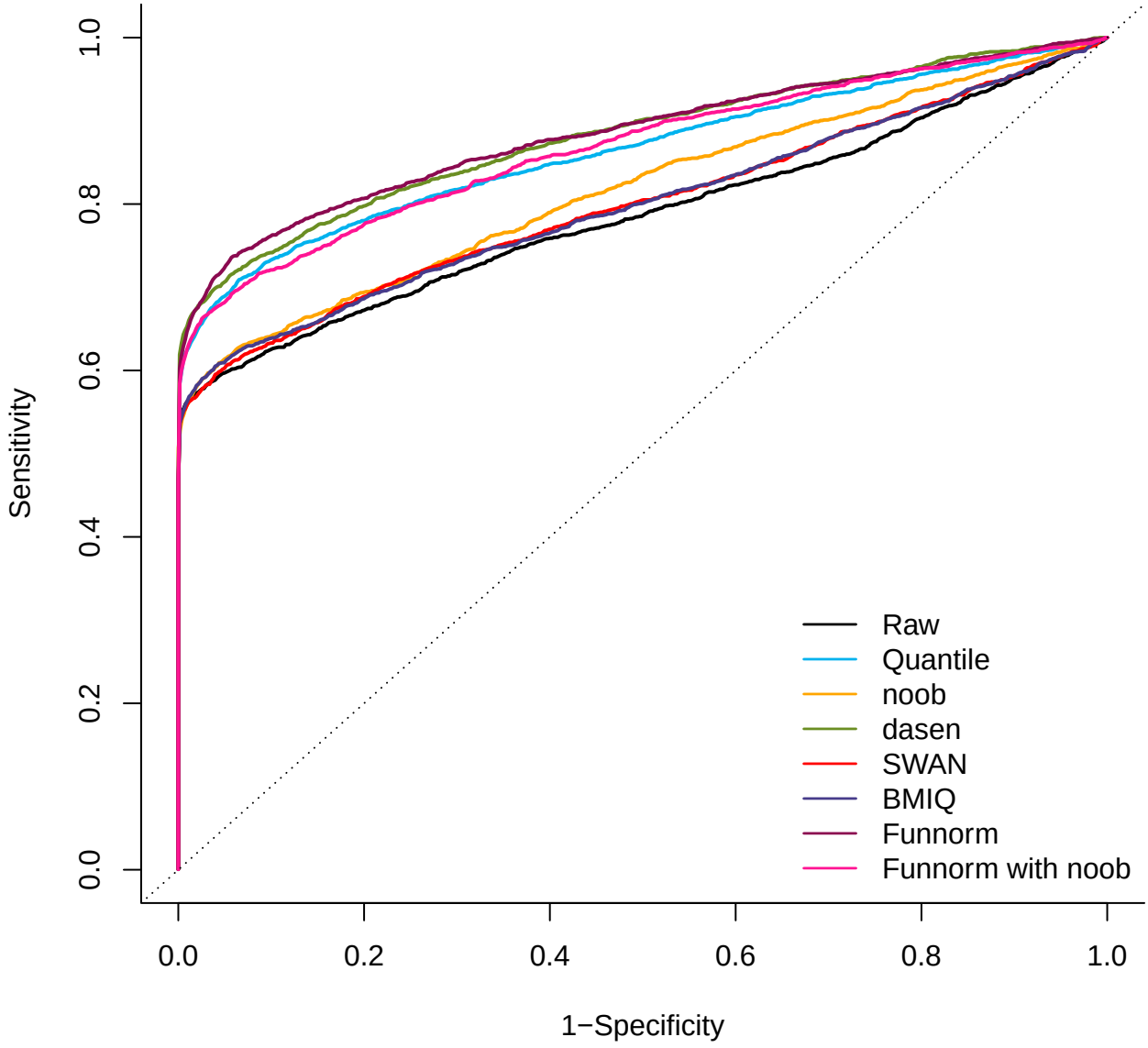

Sensitivity
0.0
0.2
0.4
0.6
0.8
1.0
0.00
0.02
0.04
0.06
0.08
0.10
1−Specificity
Raw
Combat
SVA
RUV
Funnorm with noob



Sex results

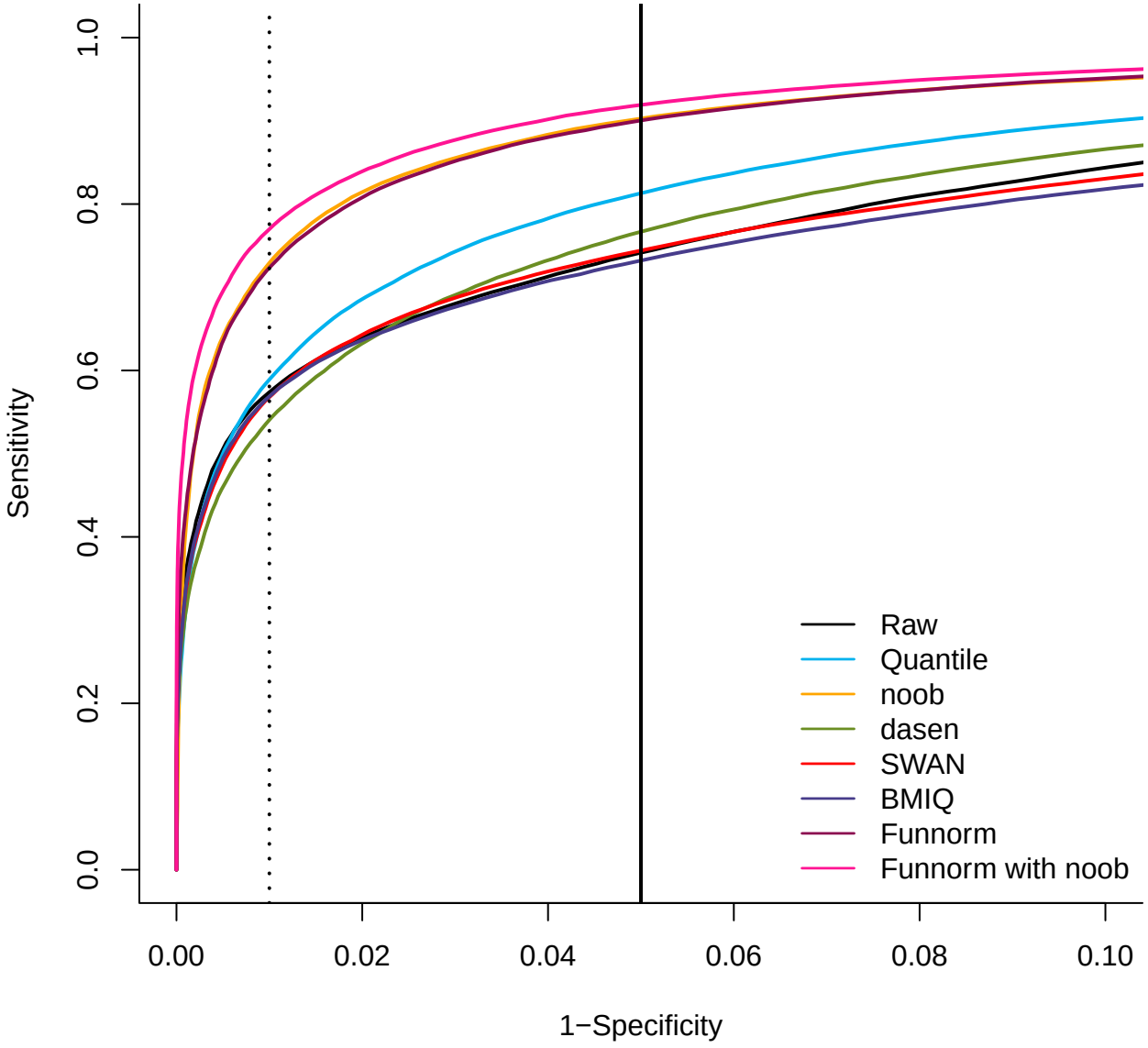
```
source(file.path(funnormDir, "paper_figures/generate.roc.gender.plots.R"));
create.roc.gender(print=TRUE)
```

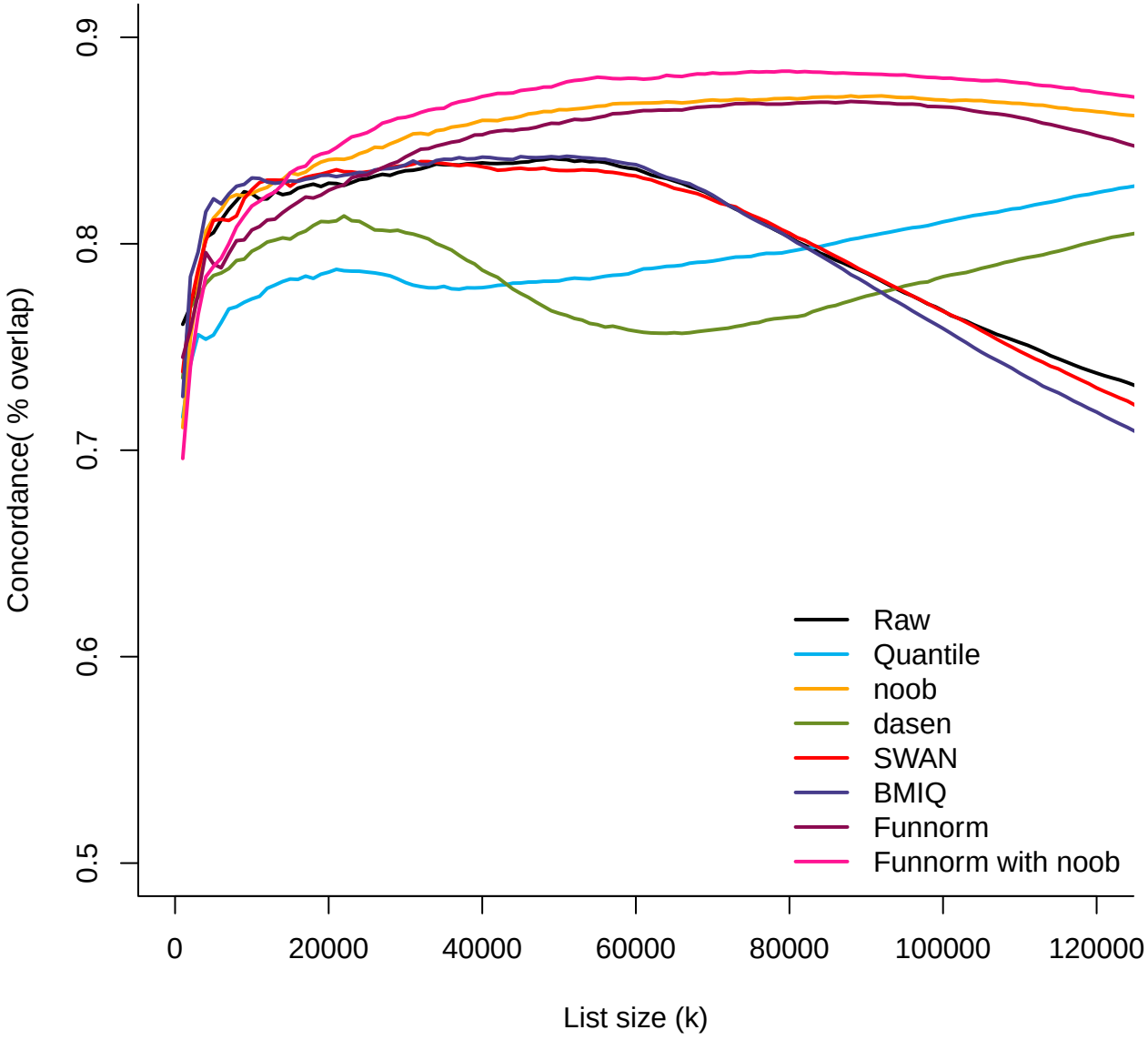

Sensitivity
1−Specificity
0.0
0.2
0.4
0.6
0.8
1.0
Raw
Quantile
noob
Funnorm
Funnorm with noob



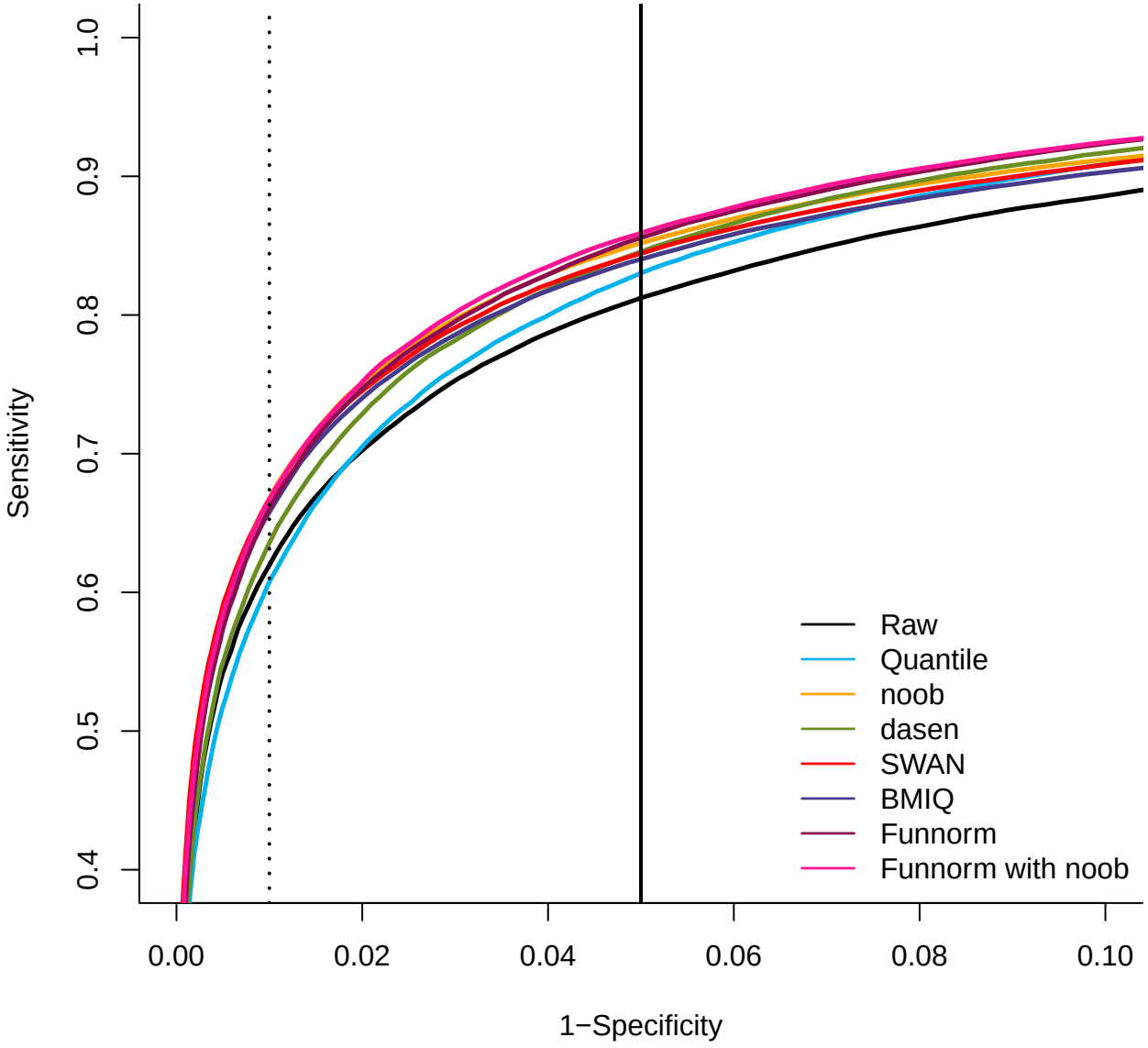
Additional Results

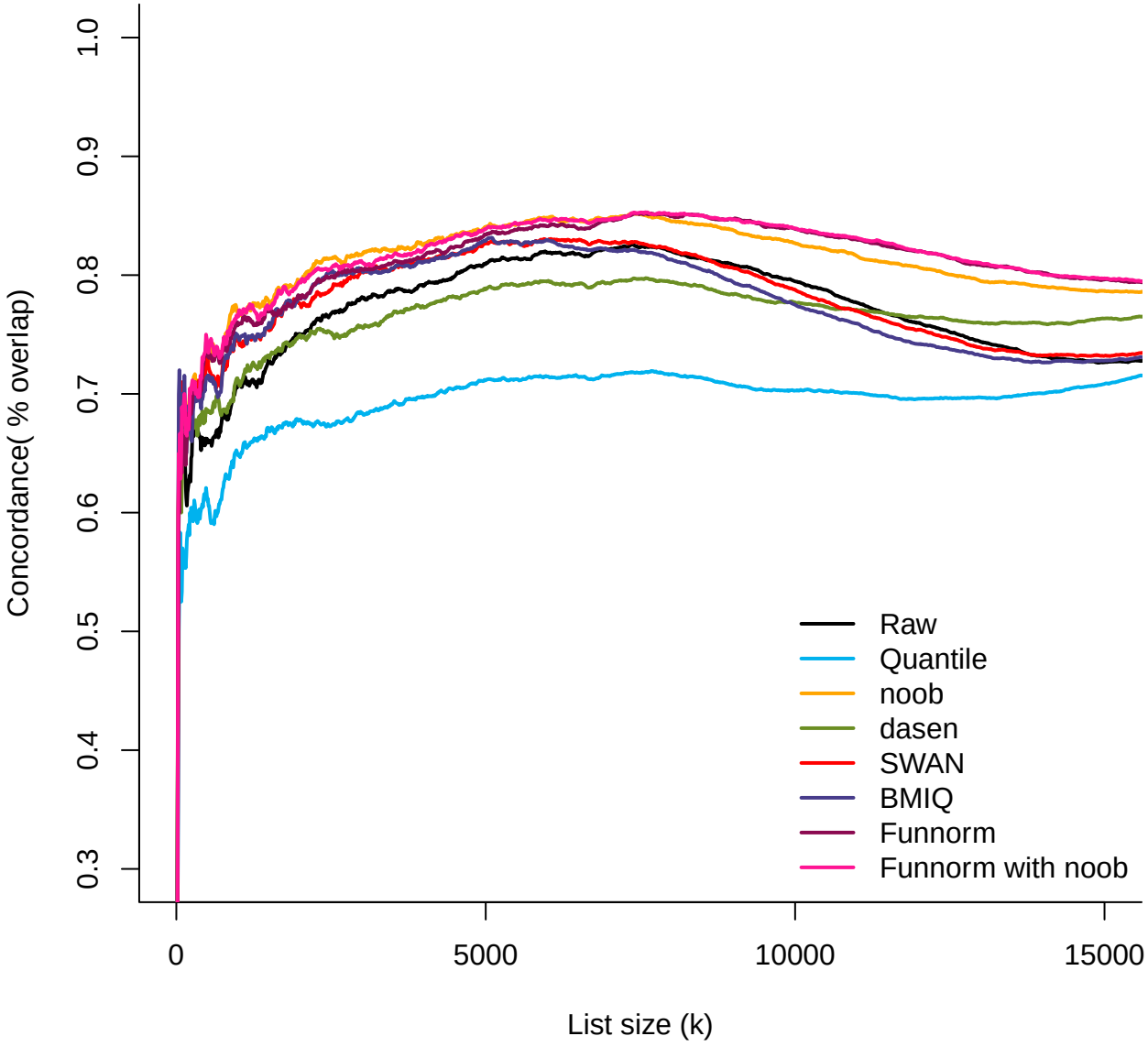
```
source(file.path(funnormDir, "paper_figures/generate.add.results.plots.R"));
create.add.ebv(print=TRUE)
```


Sensitivity
1−Specificity
0.0
0.2
0.4
0.6
0.8
1.0
0.00
0.02
0.04
0.06
0.08
0.10
Raw
Quantile
noob
dasen
SWAN
BMIQ
Funnorm
Funnorm with noob

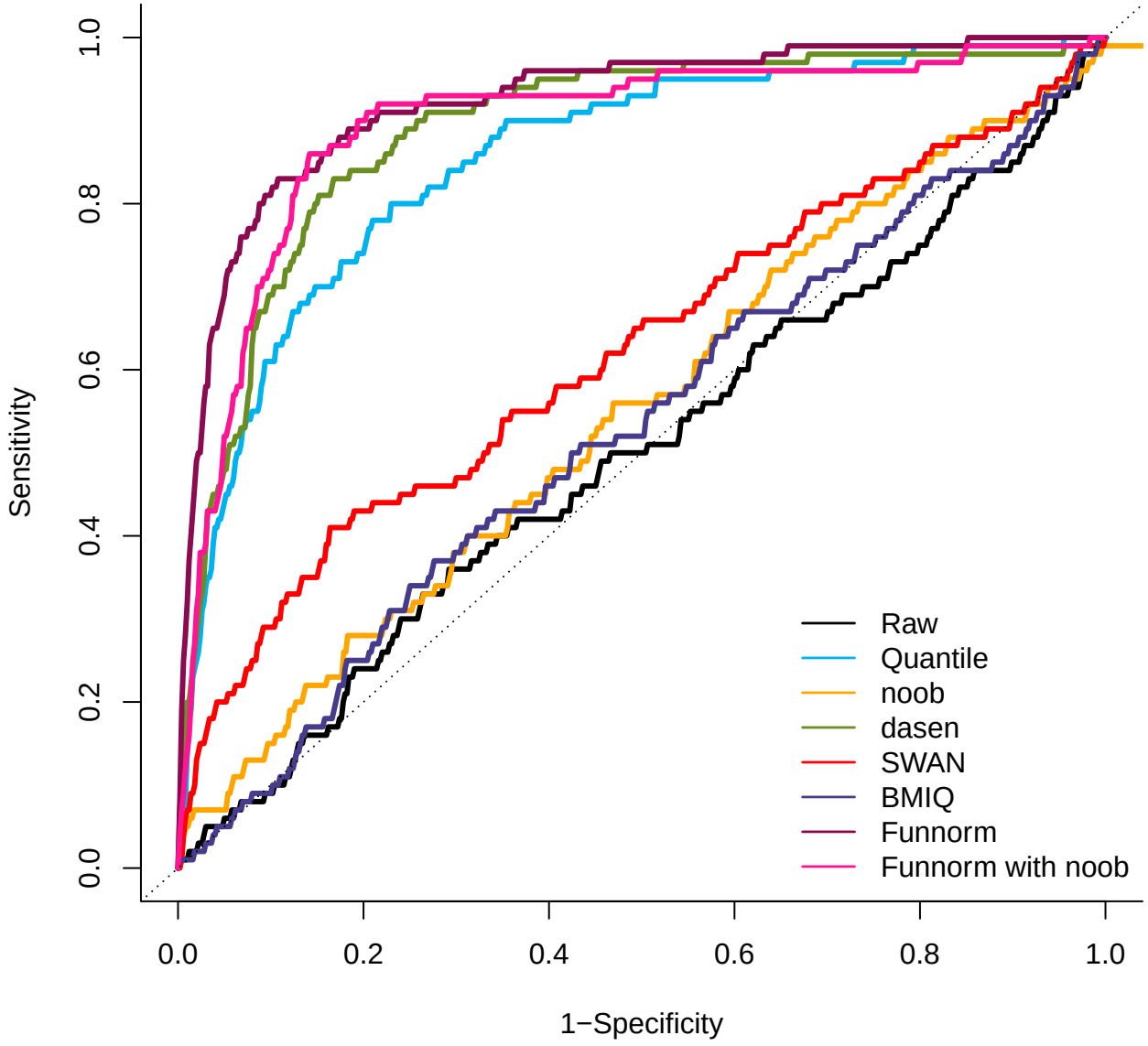



```
create.add.kirc(print=TRUE)
```

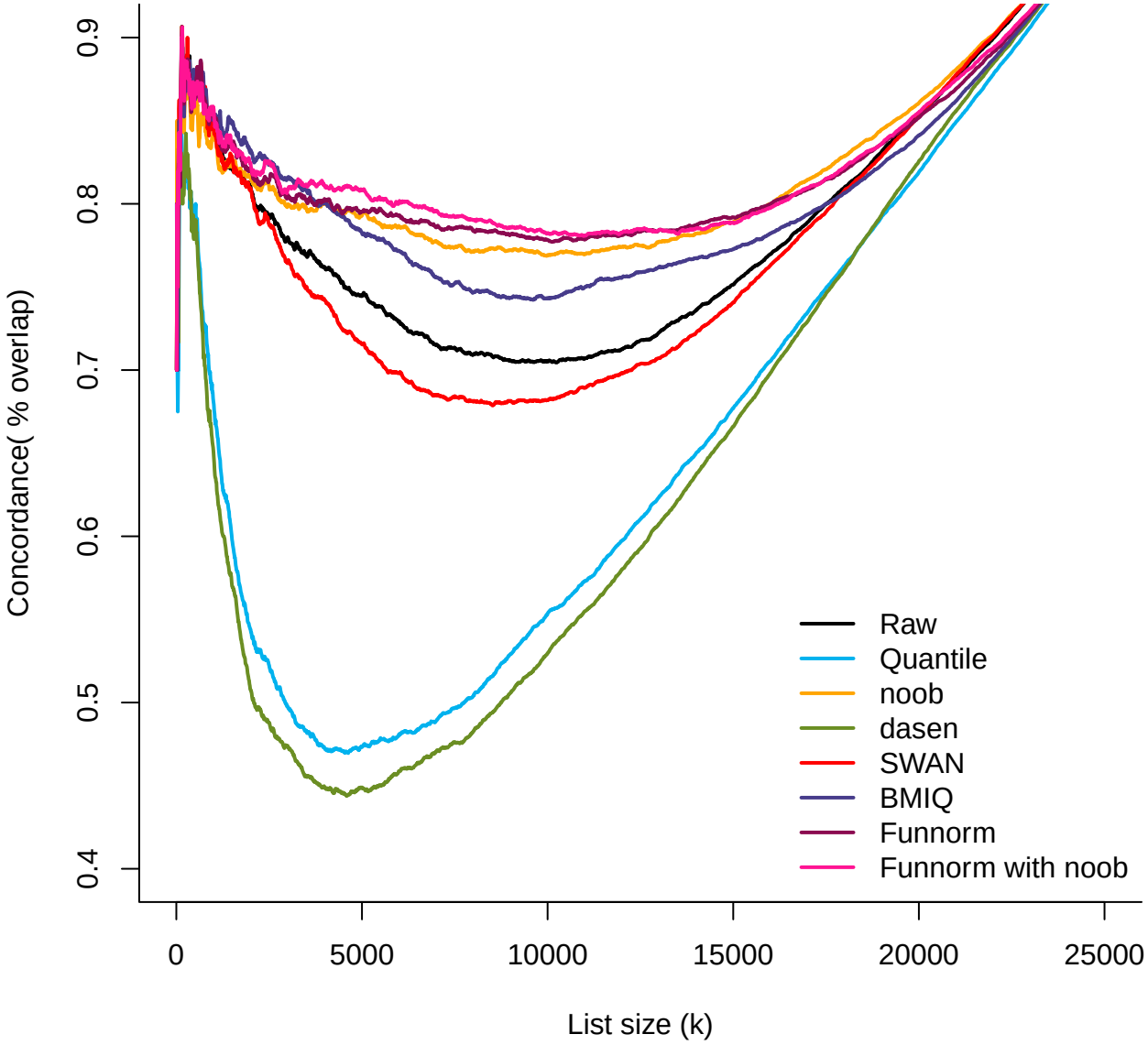

Sensitivity
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.00
0.02
0.04
0.06
0.08
0.10
1−Specificity
Raw
Quantile
noob
dasen
SWAN
BMIQ
Funnorm
Funnorm with noob

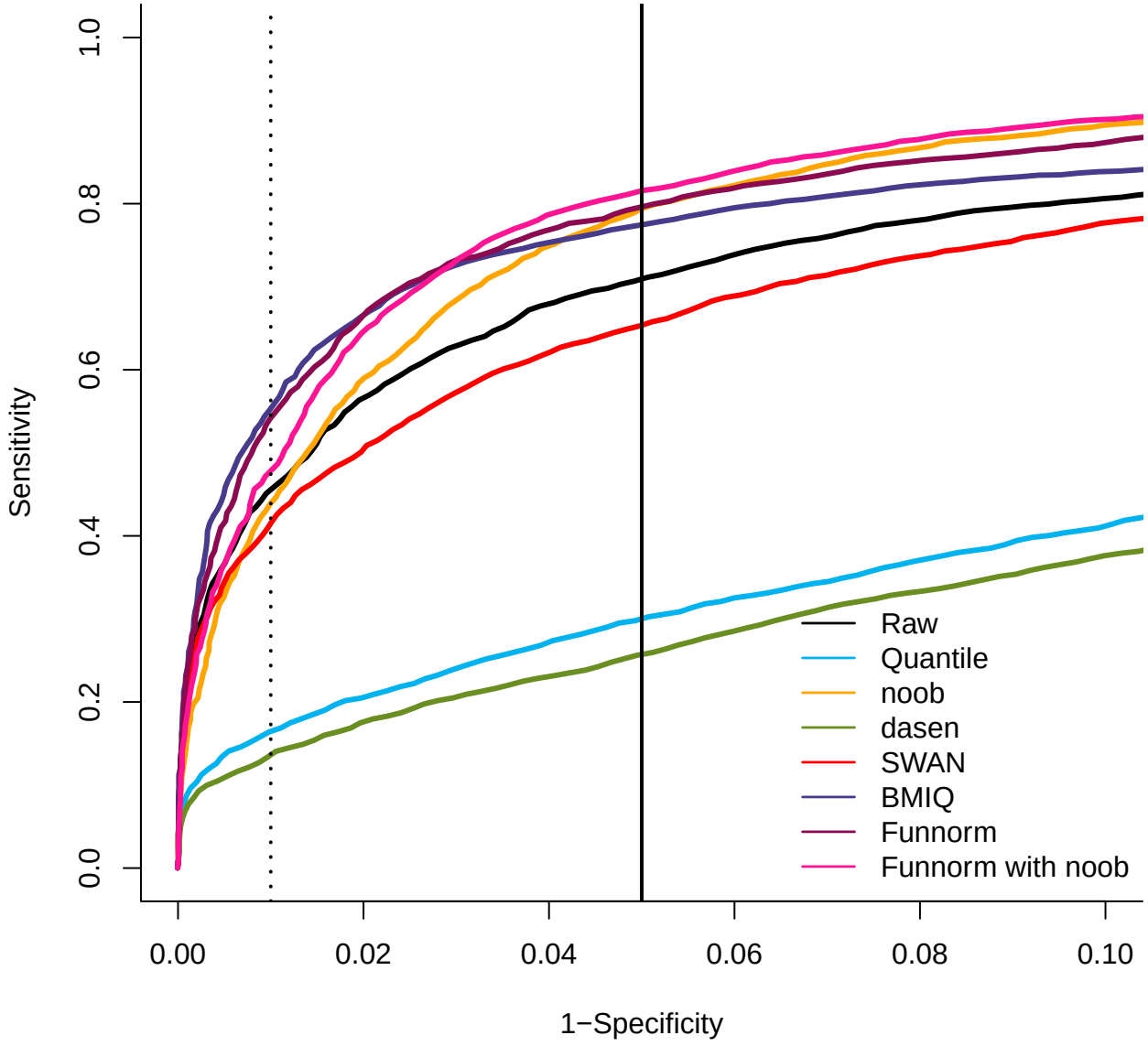



```
create.add.blood(print=TRUE)
```



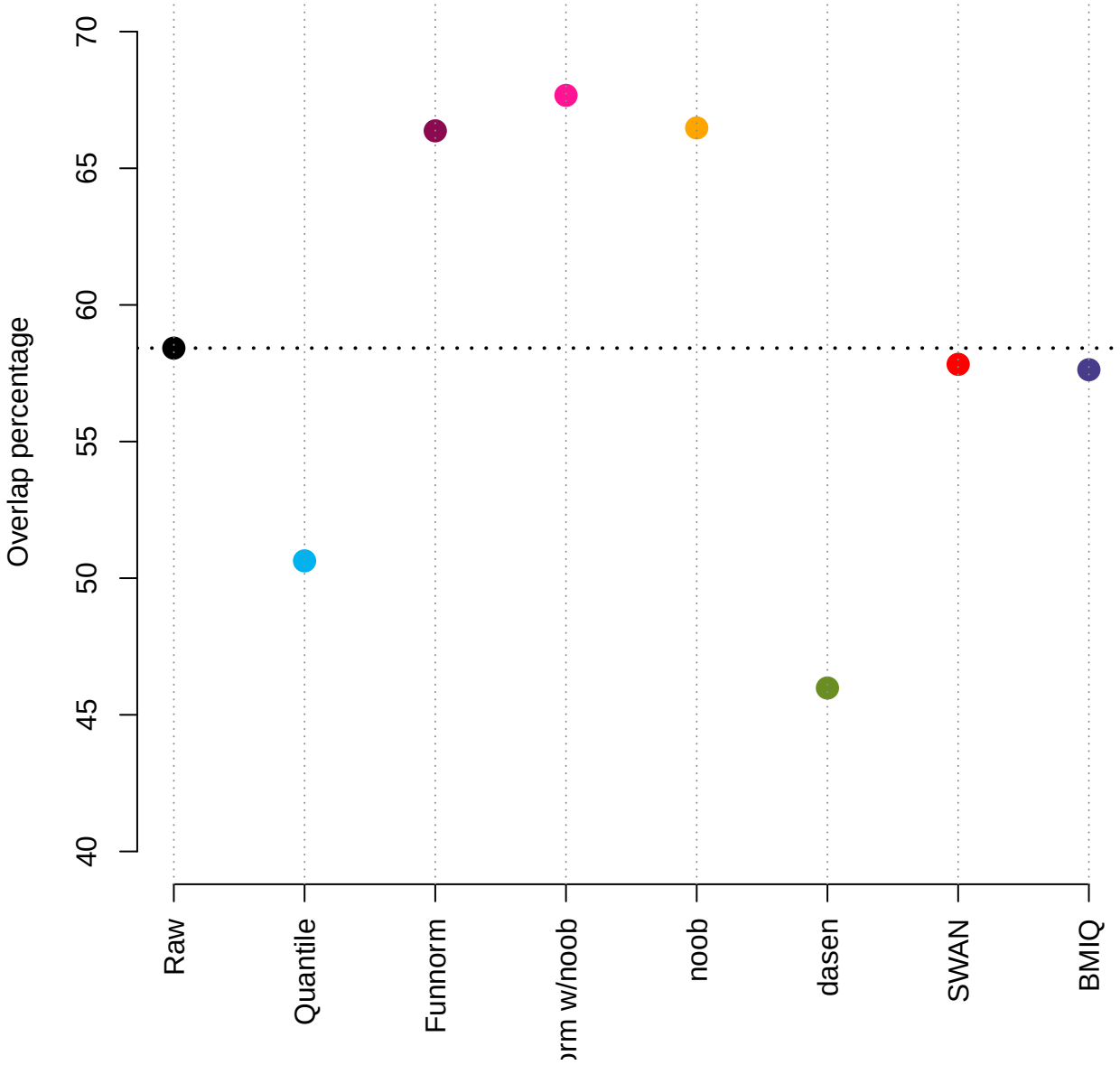

```
create.add.aml(print=TRUE)
```


Concordance(% overlap)
0.4
0.5
0.6
0.7
0.8
0.9
0
5000
10000
15000
20000
25000
List size (k)
Raw
Quantile
noob
dasen
SWAN
BMIQ
Funnorm
Funnorm with noob



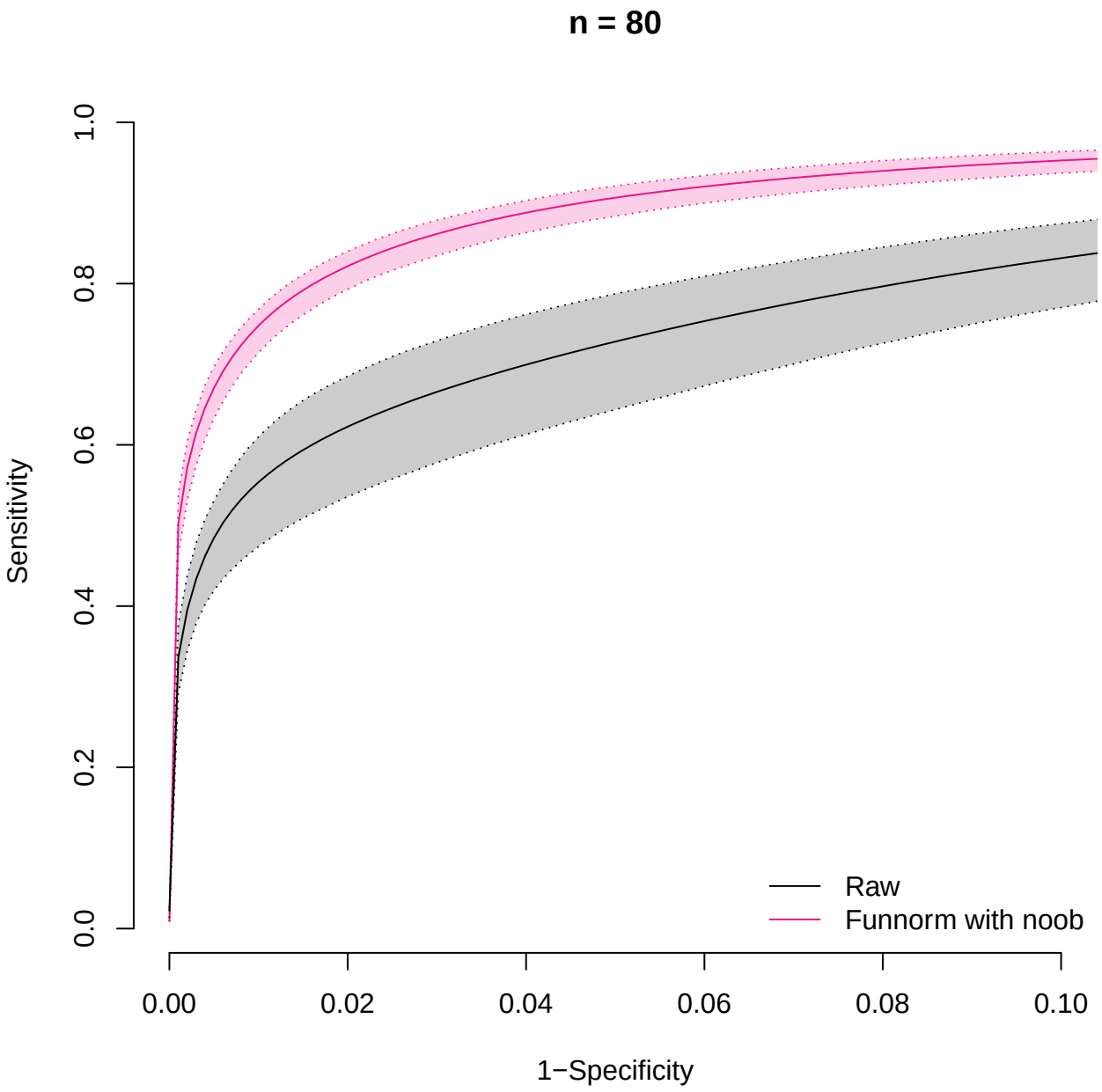
Dotplot for WGBS validation

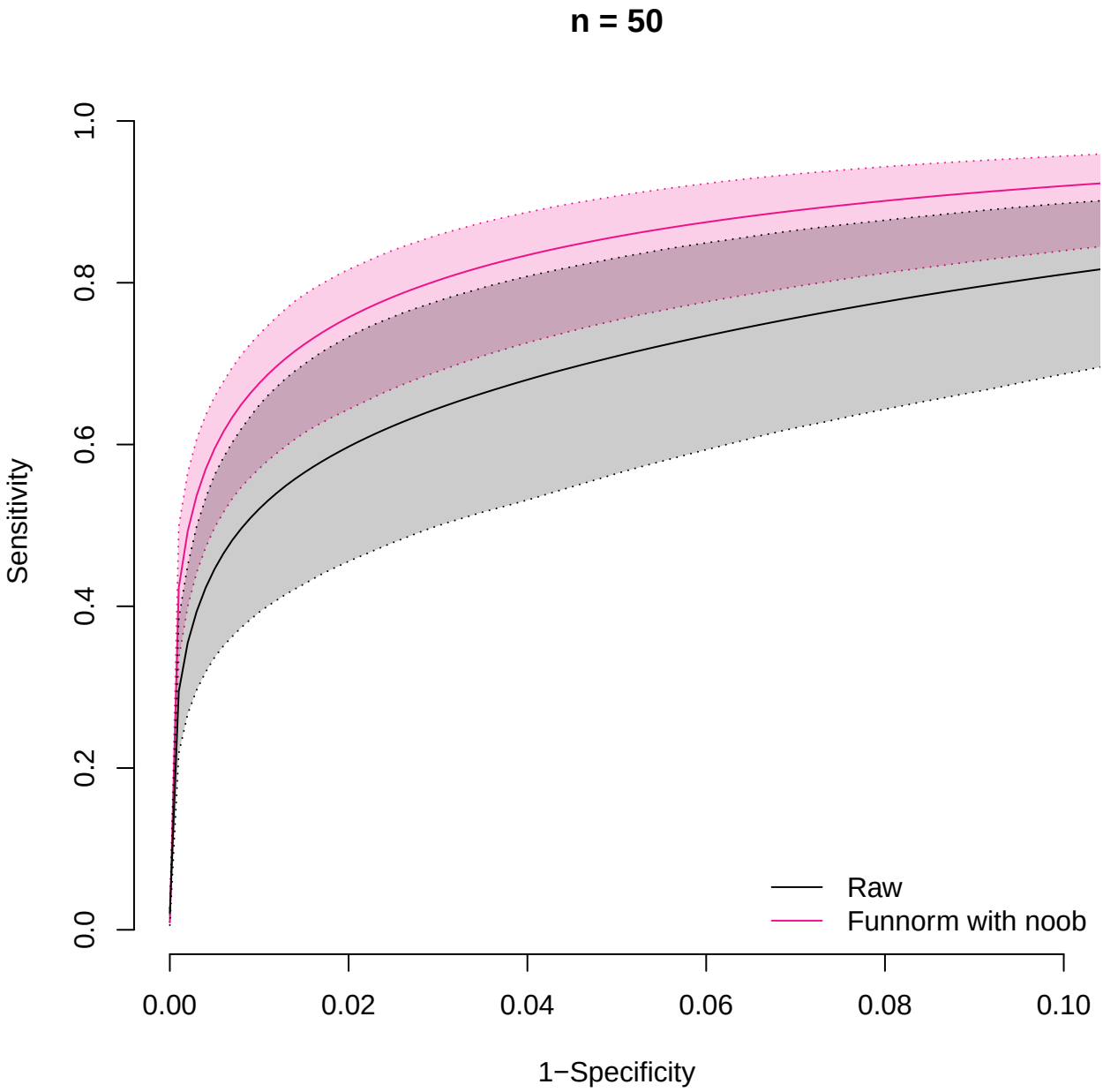
```
source(file.path(funnormDir, "paper_figures/generate.dot.plot.R"));
create.dot.plot(print=TRUE)
```

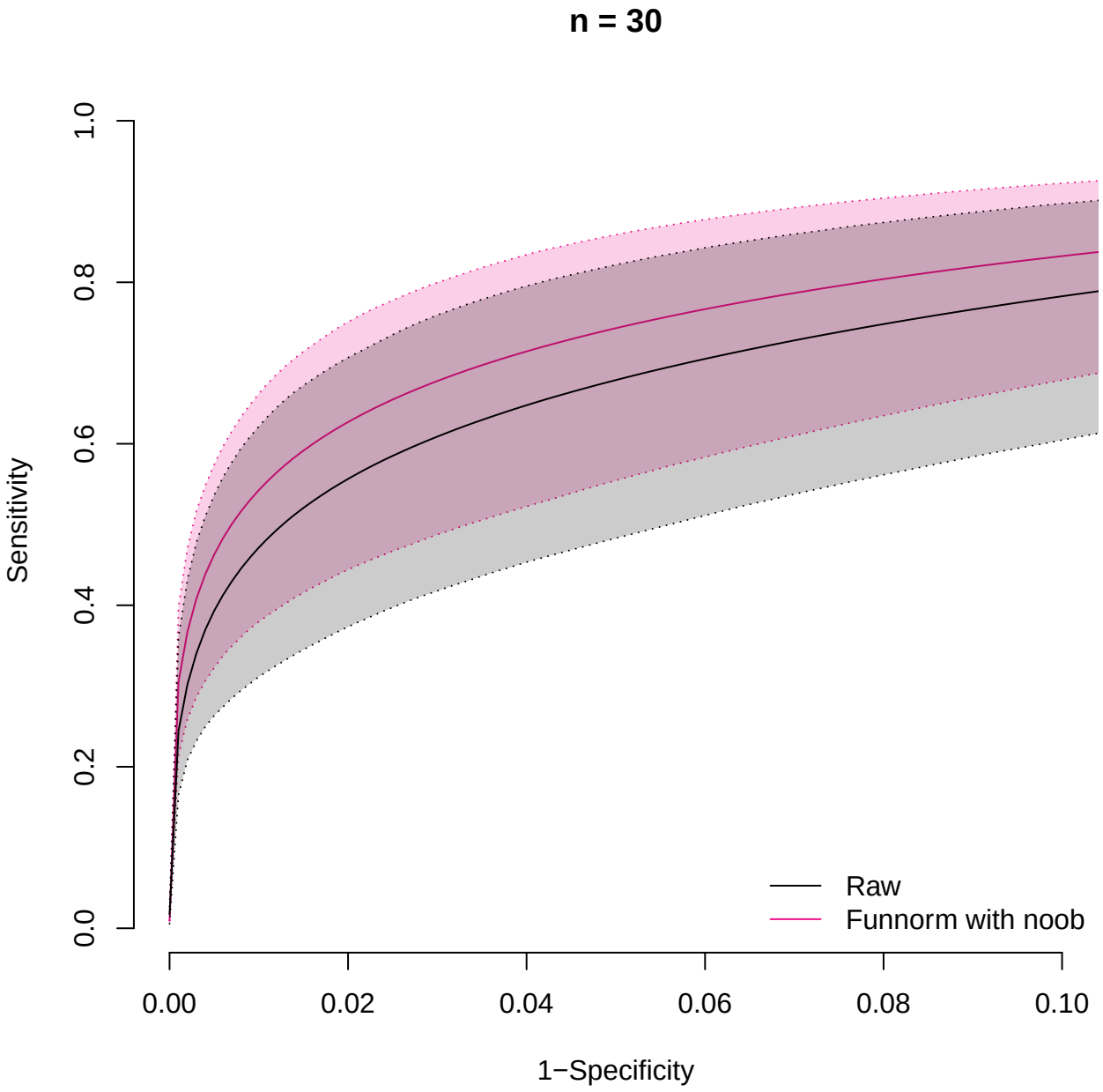


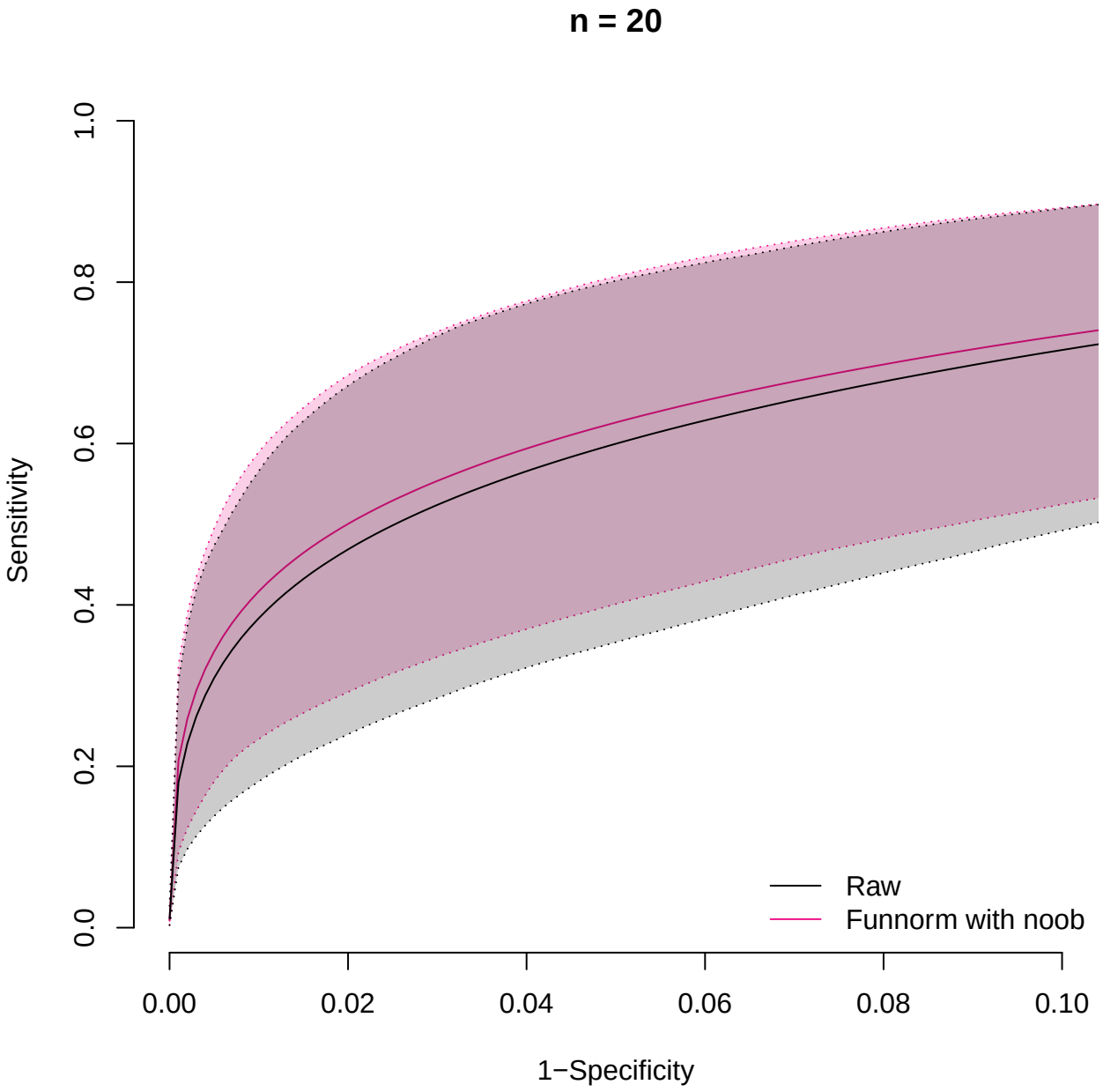
ROC curves for sample size simulation

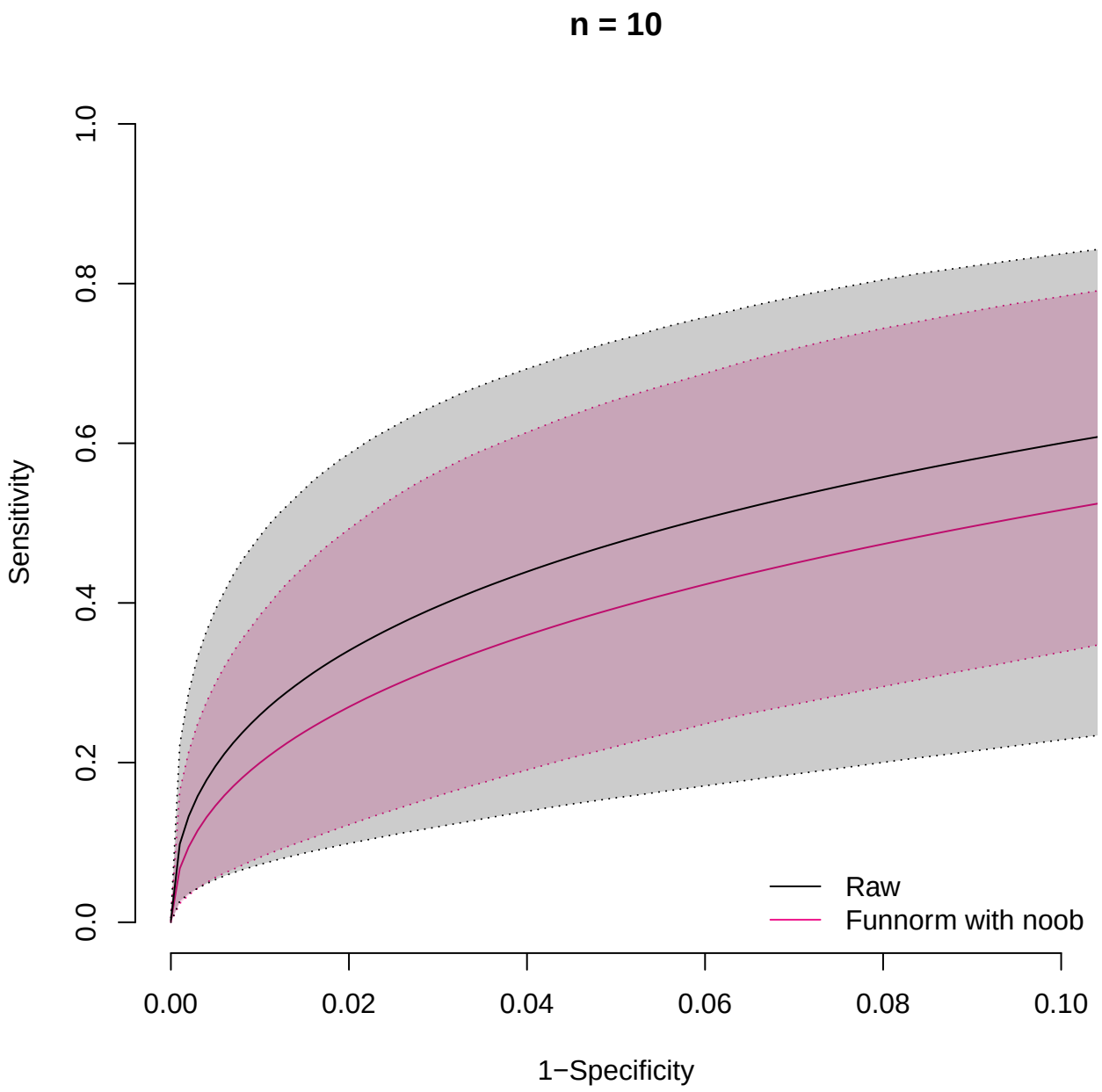
```
source(file.path(funnormDir, "paper_figures/generate.sample.size.simulation.plots.R"));
create.sample.size.sim.figures(print=TRUE)
```


n = 80
Sensitivity
1−Specificity
0.0
0.2
0.4
0.6
0.8
1.0
0.00
0.02
0.04
0.06
0.08
0.10
Raw
Funnorm with noob


n = 50
Sensitivity
1−Specificity
0.0
0.2
0.4
0.6
0.8
1.0
0.00
0.02
0.04
0.06
0.08
0.10
Raw
Funnorm with noob

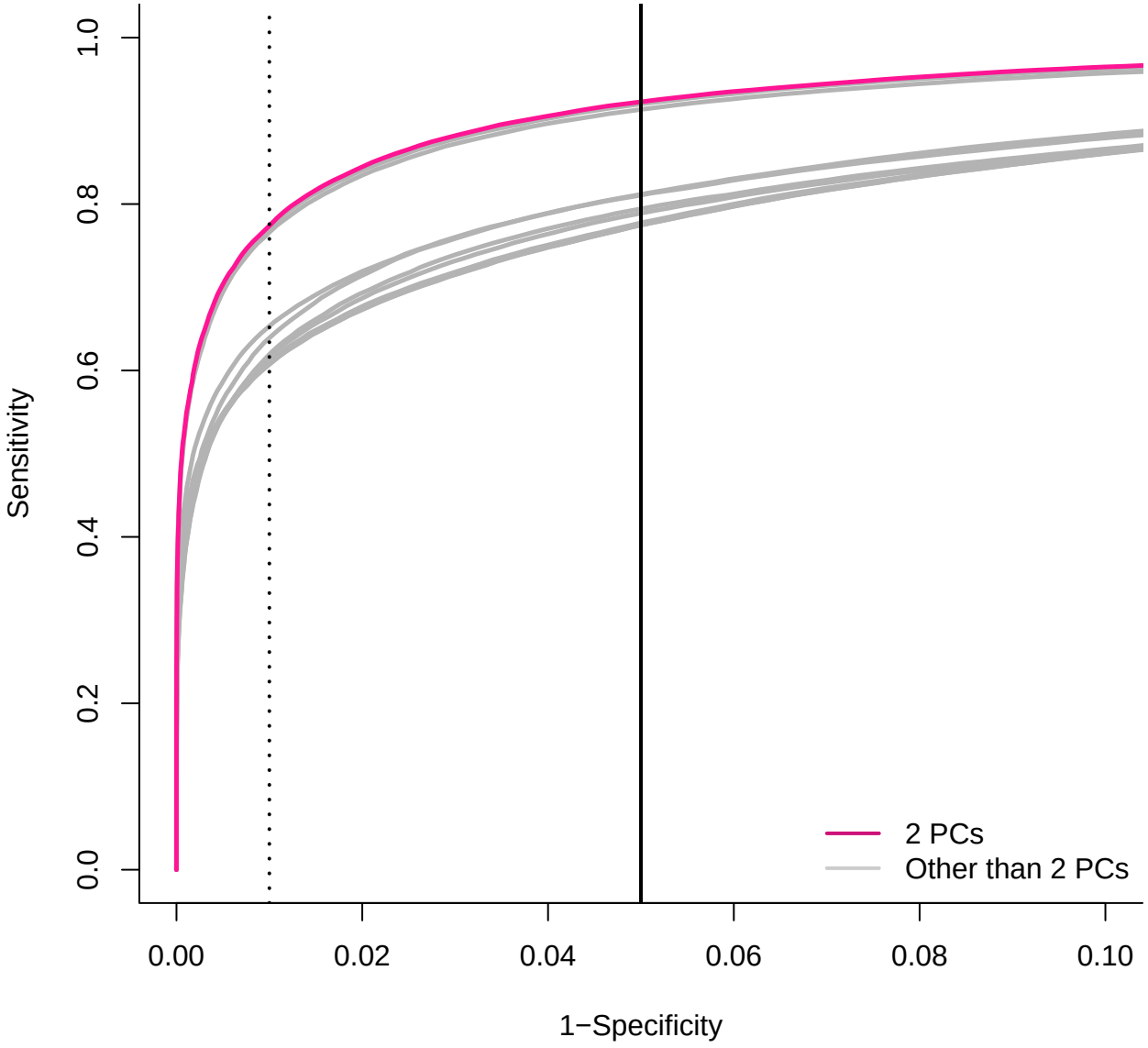

n = 30
Sensitivity
1−Specificity
0.0
0.2
0.4
0.6
0.8
1.0
0.00
0.02
0.04
0.06
0.08
0.10
Raw
Funnorm with noob

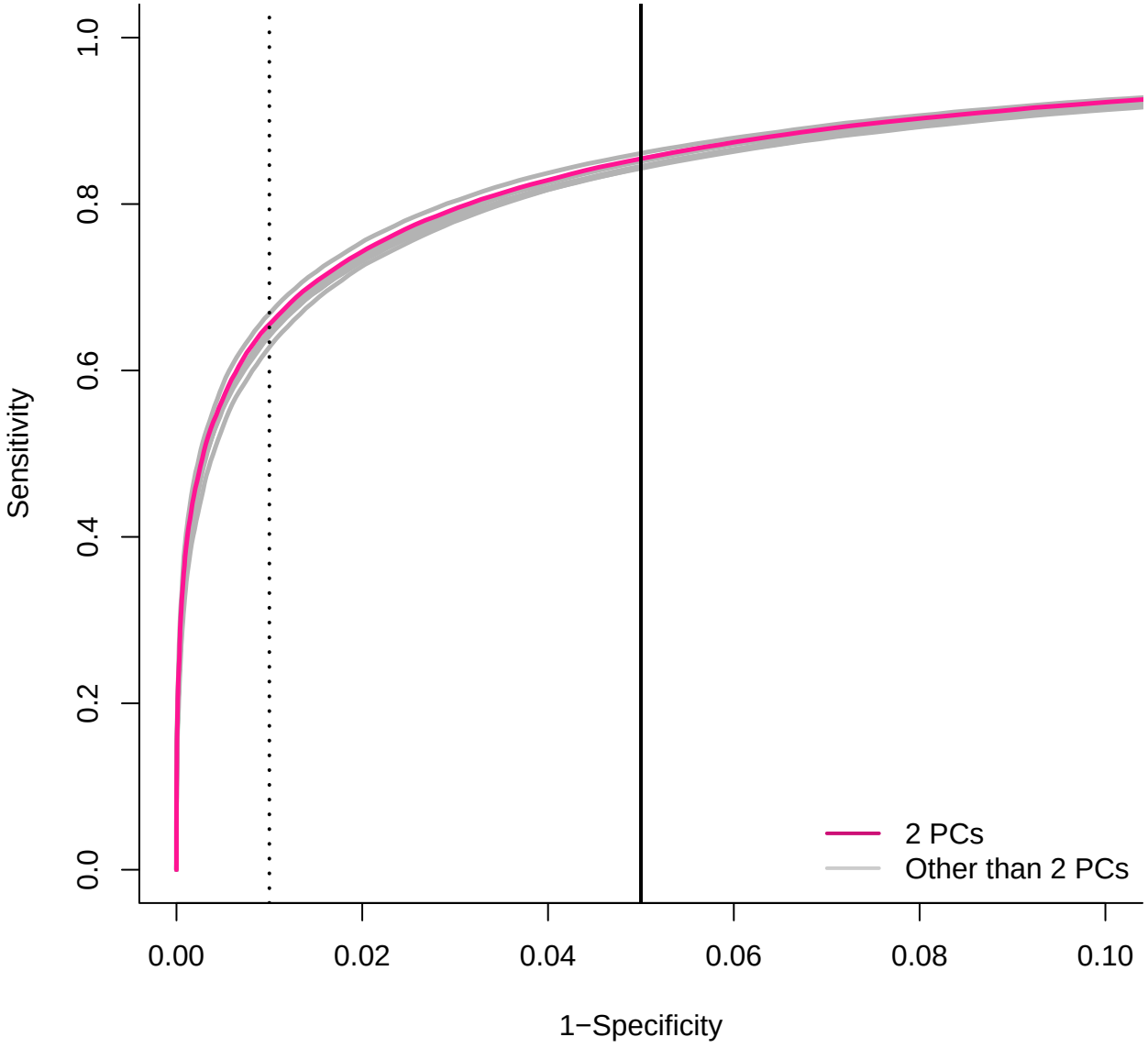

n = 20
Sensitivity
1−Specificity
0.0
0.2
0.4
0.6
0.8
1.0
0.00
0.02
0.04
0.06
0.08
0.10
Raw
Funnorm with noob

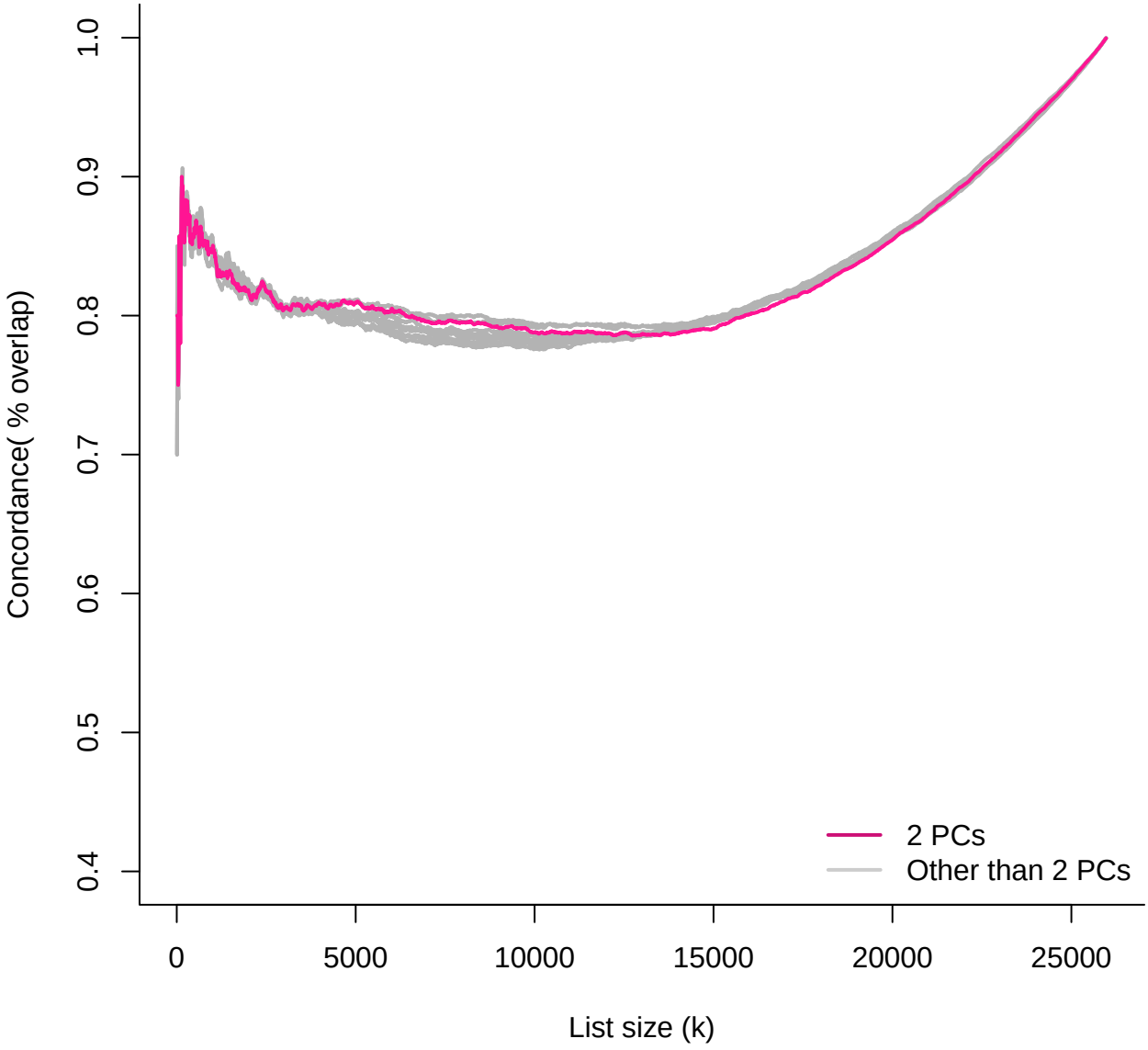


Curves for sensitivity analysis

```
source(file.path(funnormDir, "paper_figures/generate.sensitivity.analysis.plots.R"));
create.sens.analysis(print=TRUE)
```


Sensitivity
0.0
0.2
0.4
0.6
0.8
1.0
0.00
0.02
0.04
0.06
0.08
0.10
1−Specificity
2 PCs
Other than 2 PCs


Sensitivity
0.0
0.2
0.4
0.6
0.8
1.0
0.00
0.02
0.04
0.06
0.08
0.10
1−Specificity
2 PCs
Other than 2 PCs


Concordance(% overlap)
0.4
0.5
0.6
0.7
0.8
0.9
1.0
0
5000
10000
15000
20000
25000
List size (k)
2 PCs
Other than 2 PCs