DMRforPairs vignette

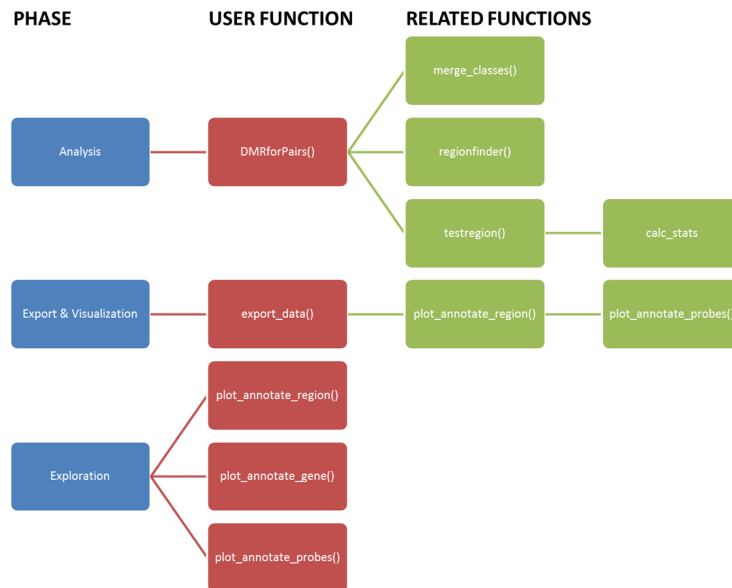Martin A. Rijlaarsdam

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1 Overview

Figure 1: Flowchart of the DMRforPairs algorithm. The user progresses vertically through the pipeline while the algorithm uses the subsequent functions as indicated by horizontal connectors. The tuning function for the DMRforPairs parameters (min_n and min_distance) is not depicted, but can be used to explore the number of regions identified/probes included for various pairs of settings.

This is a demo illustrating the usage of DMRforPairs (Figure 1). DMRforPairs is designed to identify Differentially Methylated Regions between unique samples using array based methylation profiles. Regions are identified as genomic ranges with sufficient probes located in close proximity to each other and which are optionally annotated to the same functional class (see reference manual, merge_classes() function). Differential methylation is evaluated by comparing the methylation values within each region between individual samples and

(if the difference is sufficiently large), testing this difference formally for statistical significance.

The following remarks apply to the vignette:

1. the "annotate.significant" and "annotate" parameters have been set to FALSE to facilitate the speed of building the package/vignette and to allow running the vignette on computers that are not connected to the internet. The power of DMRforPairs is however greatly enhanced by the Gviz based visualizations that include annotation information from Ensembl. Therefore, it is advised to change these parameters to TRUE if internet is avaliable. We recommend annotate.relevant to be set to FALSE al all times unless very small sections of the genome are analyzed (see documentation)

2. the vignette requires writing permissions in the working directory.

3. parallelization has been disabled in all examples as well as in this vignette. This is done to provide polite code for people sharing compute cycles.

2 Setting up the data and settings

2.1 The Data

Load the data. This dataset provides the average methylation values on chromosome 7 of two commercially available EBV transfected lymphoblastoid cell lines from healthy individuals (NA17105 (African American male) and NA17018 (Chinese female)). The dataset also contains this data for the breast cancer cell line MCF7 (Soule et al. 1973) and the HPV negative squamous-cell vulva carcinoma cell line A431 (Giard et al. 1973 and Hietanen et al. 1995). For a full description of the dataset (+references) and its format, please see the reference manual.

```
> library(DMRforPairs)
> data(DMRforPairs_data)
```

Columns 1 - 6 of the data indicate information about individual probes (n=29,974) and their annotation. Columns 7 - 10 indicate M-values for all samples and columns 11 - 14 represent the associated beta values.

```
> head(CL.methy,2)

            targetID chromosome position class.gene.related
cg00081087 cg00081087          7 34873912       Body;Body;Body
cg00087298 cg00087298          7 33149137             TSS200
           class.island.related      gene.symbol    A431.M       MCF7.M
cg00081087                        AAA1;NPSR1;NPSR1  -4.027028  -0.4932408
cg00087298               Island                RP9 -13.872031 -12.2916392
           NA17105.M  NA17018.M    A431.beta    MCF7.beta NA17105.beta
cg00081087 -6.300535  -2.475343 5.782836e-02 0.4153514568  0.012827340
cg00087298 -8.549864 -12.007505 6.669415e-05 0.0003185493  0.003003211
           NA17018.beta
cg00081087 0.1524629099
cg00087298 0.0004419503
```

2.2 The Settings

First, a number of possible settings for the min_distance (the maximal distance accepted between probes in a region) and min_n (the minimal number of probes in a region) are evaluated.

```
> parameters=expand.grid(min_distance = c(200,300), min_n = c(4,5))
> recode=1
> results.parameters =  tune_parameters(parameters,
+  classes_gene=CL.methy$class.gene.related,
+  classes_island=CL.methy$class.island.related,
+  targetID=CL.methy$targetID,
+  chr=CL.methy$chromosome,
+  position=CL.methy$position,
+  m.v=CL.methy[,c(7:8)],
+  beta.v=CL.methy[,c(11:12)],
+  recode=recode,
+  gs=CL.methy$gene.symbol,
+  do.parallel=0)
> results.parameters

     min_distance min_n n.regions n.valid.probes n.probes.included
[1,]          200     4        40            589               178
[2,]          300     4        50            589               228
[3,]          200     5        32            589               154
[4,]          300     5        43            589               216
```

In the rest of this vignette, the default setting of minimally 4 probes per region is used. These have to be in < 200 bp distance of each other. The threshold for a relevant median difference in M value between the samples is set to 1.4. Benjamini Hochberg corrected p-values <0.05 are deemed significant. The parameter experiment sets name of the experiment which is reflected in the name

3

of the folder with results that will be created in the working directory.

```
> min_n=4
> d=200
> dM=1.4
> pval_th=0.05
> experiment="results_DMRforPairs_vignette"
> method="fdr"
> clr=c("red","blue","green")
```

3   Run DMRforPairs

The algorithm is most conveniently executed by calling the wrapper for the analysis part (DMRforPairs()) which returns the results of all the separate steps. DMRforPairs runs automatically, showing regular status updates. Analysis can take quite long, especially on a genome wide scale (several hours). The demo data should generally finish within a few minutes. The wrapper subsequently performs:

1. Recoding of the probe classes according to a custom or build in scheme.

2. Identification of regions with sufficient probe density (i.e. number of probes and proximity) over all genomic regions at which probes are annotated in the dataset .

3. Calculation of relevant statistics (e.g. median difference in M and beta values) and performing of formal tests to see if the difference is significant.

These steps are extensively described in the reference manual.

```
> output=DMRforPairs(
+   classes_gene=CL.methy$class.gene.related,
+   classes_island=CL.methy$class.island.related,
+   targetID=CL.methy$targetID,
+   chr=CL.methy$chromosome,
+   position=CL.methy$position,
+   m.v=CL.methy[,c(8:10)],
+   beta.v=CL.methy[,c(12:14)],
+   min_n=min_n,min_distance=d,min_dM=dM,
+   recode=recode,
+   sep=";",
+   method=method,
+   debug.v=FALSE,gs=CL.methy$gene.symbol,
+   do.parallel=0)
```

4

3.1 Examining the primary output of DMRforPairs

3.1.1 Recode probe classes (merge_classes())

Orginal probe classes...

```
> head(output$classes$pclass,3)

           [,1]
cg00081087 "Body;Body;Body;"
cg00087298 "TSS200;Island"
cg00139681 ";"
```

Recoded probe classes...

```
> head(output$classes$pclass_recoded,3)

           [,1]
cg00081087 "gene;NA;NA"
cg00087298 "NA;tss;island"
cg00139681 "NA;NA;NA"
```

Row numbers of probes without a recoded class...

```
> head(output$classes$no.pclass,10)

 [1]   3   6 13 15 17 33 38 41 46 50
```

Classes used for recoding...

```
> output$classes$u_pclass

[1] gene    tss     island
Levels: gene island tss
```

Merge classes returns a reduced set of probe data (annotation, M and beta
values) including only probes associated with at least one recoded class. In
case of recode=2 this implicates all probes in the dataset. This reduced set of
probes is designated "valid" in the remainder of this vignette and in the reference
manual.

3.1.2 Identify probe-dense regions (regionfinder())

Potential regions of interest...

```
> head(output$regions$boundaries,4)

  chr   start_bp      end_bp length_bp n_probes regionID regionIDall    ClassAll
1   7 33080496   33080615       120        4        1           1        gene
2   7 34118464   34118935       472        5        2        2;38 gene;island
3   7 34873912   34874196       285        4        3           3        gene
4   7 105172664 105173132       469        5        4           4        gene
```

Probes with associated class after recoding (valid probes)...

```
> head(output$regions$valid.probes,2)

          rowID    probeID chr position       pClass
cg00081087    1 cg00081087   7 34873912    gene;NA;NA
cg00087298    2 cg00087298   7 33149137 NA;tss;island
```

Associated m and beta values for all samples for each valid probe...

```
> head(output$regions$valid.m,2)

               MCF7.M NA17105.M  NA17018.M
cg00081087  -0.4932408 -6.300535  -2.475343
cg00087298 -12.2916392 -8.549864 -12.007505

> head(output$regions$valid.beta,2)

           MCF7.beta NA17105.beta NA17018.beta
cg00081087 0.4153514568  0.012827340 0.1524629099
cg00087298 0.0003185493  0.003003211 0.0004419503
```

Region to probe map: matrix of valid probes (rows) and recoded probe classes
(columns) with either NA if not included in any potential region of interest
or the ID of the region the probe is assigned to. By definition each probe
can only be associated to one region per class. Region IDs are specific to a
dataset and a set of DMRforPairs parameters. Region IDs are therefore not
interchangable between datasets/experiments and primarily serve as identifiers
during exploration of the dataset.

```
> head(output$regions$perprobe,4)

           gene tss island
cg00081087    3  NA     NA
cg00087298   NA  14     35
cg00156506   NA  NA     NA
cg00280235   NA  NA     NA
```

3.1.3 Calculation of relevant statistics and testing (testregion())

This output is structured like output$regions$boundaries but is supplemented with descriptive statistics and formal test results per region.

```
> head(output$tested,1)

  chr start_bp   end_bp length_bp n_probes regionID regionIDall ClassAll
1   7 33080496 33080615      120        4        1           1     gene
  beta.median.MCF7.beta beta.median.NA17105.beta beta.median.NA17018.beta
1             0.9848456              0.003448186              0.003629177
  m.median.MCF7.M m.median.NA17105.M m.median.NA17018.M
1        6.094547          -9.110979          -8.158476
  median.delta.beta.MCF7.M.minus.NA17105.M
1                                0.9693837
  median.delta.beta.MCF7.M.minus.NA17018.M
1                                0.9685009
  median.delta.beta.NA17105.M.minus.NA17018.M
1                                 -0.0001267329
  median.delta.m.MCF7.M.minus.NA17105.M median.delta.m.MCF7.M.minus.NA17018.M
1                               14.6789                              13.85861
  median.delta.m.NA17105.M.minus.NA17018.M pairwise.p.MCF7.M.vs.NA17105.M
1                                -0.5443935                    0.02857143
  pairwise.p.MCF7.M.vs.NA17018.M pairwise.p.NA17105.M.vs.NA17018.M
1                     0.02857143                        0.1142857
  max.abs.median.delta    p.value p.value.adjusted
1              14.6789 0.01246768       0.03562195
```

4 Export and visualization

The export_data() function performs a complete export of all results to TSV, pdf and png files for all (relevant) regions. These overviews are generated in increasing detail for:

1. all regions

2. regions with a relevant difference ($> dM$) and

3. regions with a significant difference.

HTML tables are used to access the results and describe the analysis (Figure 2). Thumbnails of the methation pattern of a region are presented in the tables (2 and 3) as well as general statistics. Links to detailed statistics (tsv) and (pairwise) visualizations (pdf) are provided. Regions with a relevant difference can be looked up in the Ensembl database resulting in annotated figures of the methylation pattern. Also, direct links to the regions in the Ensembl and UCSC

Thumbnail	Chr	Start	End	Links	Length	n	ID	Class	MCF7.beta	NA17105.beta	NA17018.beta	Gene.Symbol	dM	p	p.adj
	7	106505688	106505961	PDF STATS ENSEMBL UCSC	274	5	46	island	0.966	0.01175	0.00440	PIK3CG (margin: PIK3CG)	15.99	0.00815	0.0272
	7	33080496	33080615	PDF STATS ENSEMBL UCSC	120	4	1	gene	0.985	0.00345	0.00363	AVL9; NT5C3A (margin: AVL9; NT5C3A; AC074338.4; RN7SL505P)	14.68	0.01247	0.0356
	7	107300939	107301048	PDF STATS ENSEMBL UCSC	110	5	29	tss	0.899	0.01680	0.02933	SLC26A4; SLC26A4-AS1 (margin: SLC26A4; SLC26A4-AS1)	8.96	0.00752	0.0272
	7	107300939	107301288	PDF STATS ENSEMBL UCSC	350	7	10	gene;island	0.899	0.01680	0.02933	SLC26A4; SLC26A4-AS1 (margin: SLC26A4; SLC26A4-AS1)	8.79	0.00120	0.0120

Figure 2: Example of the HTML output of DMRforPairs

genome browsers are presented.By default, DMRforPairs creates a folder (experiment.name) within the current working directory for the output (export_data() function). This is done because a complete export generates a large number of files. Visualization and export can take quite long depending on the status of biomart (Ensembl).

```
> tested_inclannot=export_data(
+   tested=output$tested,
+   regions=output$regions,
+   th=pval_th,min_n=min_n,min_dM=dM,min_distance=d,
+   margin=10000,clr=clr,method=method,experiment.name=experiment,
+   annotate.relevant=FALSE,annotate.significant=FALSE,
+   FigsNotRelevant=FALSE,debug=FALSE)
```

Please see the "results_DMRforPairs_vignette" folder in your working directory for the output of the vignette.

PIK3CG was one of the genes strongly differentially methylated in 1 of the samples relative to the other two. By clicking on the PDF link, the output region can be further studied (Figure 3). Additional statistics are accessible via the STATS link in the HTML table (significant.html).
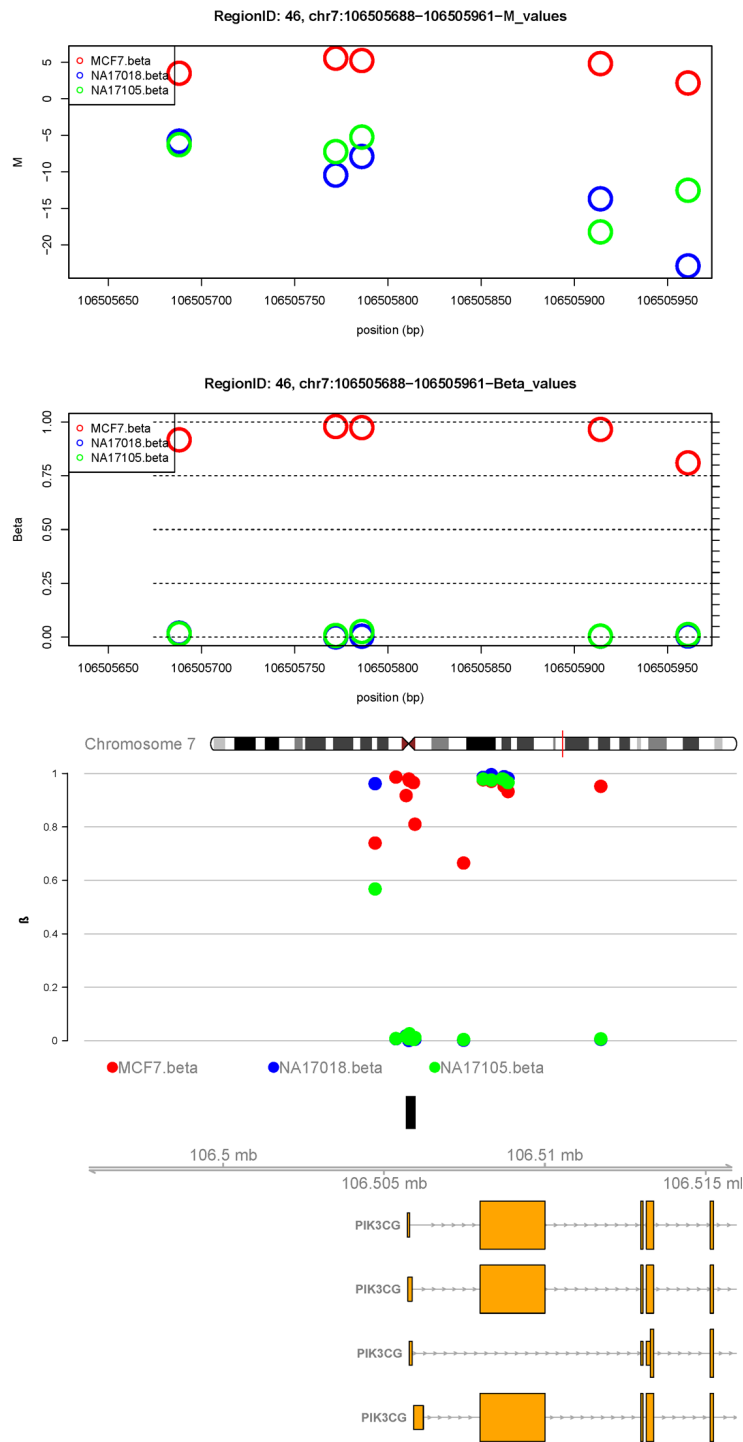
Figure 3: Differentially methylated region around the TSS of PIK3CG.

4.1 Examining the data further

There are also several functions to further explore the data based on the findings after export. These will be discussed in this section. For example, region 16 was highly relevant (median delta M=13.77). However, because of limited statistical power (n=4) the region did not survive correction for multiple testing. We might want to inquire this region further using the plot_annotate_region() function. By default relevant, but non-significant regions like 16 are not annotated. If we set annotate to TRUE in the example below we can appreciate that even though the number of probes is low (technical bias), the sudden consistent difference between MCF7 occurs right around the transcription start site of BMPER and the surrounding probes do not show this differential pattern (Figure 4). The plot_annotate_region() function also reports back the complete set of statistics and pairwise plots for the requested region.

```
> plot_annotate_region(output$tested,
+                      output$regions,
+                      margin=10000,
+                      regionID=16,
+                      clr=clr,
+                      annotate=FALSE,
+                      scores=TRUE,
+                      path=experiment)

$symbols_exact
[1] ""


$symbols_margin
[1] ""


$scores
                                                [,1]
beta.median.MCF7.beta                         0.9383
beta.median.NA17018.beta                      0.0088
beta.median.NA17105.beta                      0.0022
m.median.MCF7.M                               4.0696
m.median.NA17018.M                           -7.5344
m.median.NA17105.M                           -9.4626
median.delta.beta.MCF7.M.minus.NA17018.M      0.9244
median.delta.beta.MCF7.M.minus.NA17105.M      0.9272
median.delta.beta.NA17018.M.minus.NA17105.M   0.0016
median.delta.m.MCF7.M.minus.NA17018.M        11.8639
median.delta.m.MCF7.M.minus.NA17105.M        13.7677
median.delta.m.NA17018.M.minus.NA17105.M      1.3083
pairwise.p.MCF7.M.vs.NA17018.M                0.0286
pairwise.p.MCF7.M.vs.NA17105.M                0.0286
pairwise.p.NA17018.M.vs.NA17105.M             0.6857
max.abs.median.delta.m                       13.7677
p.value                                       0.0231
n.probes                                      4.0000
```
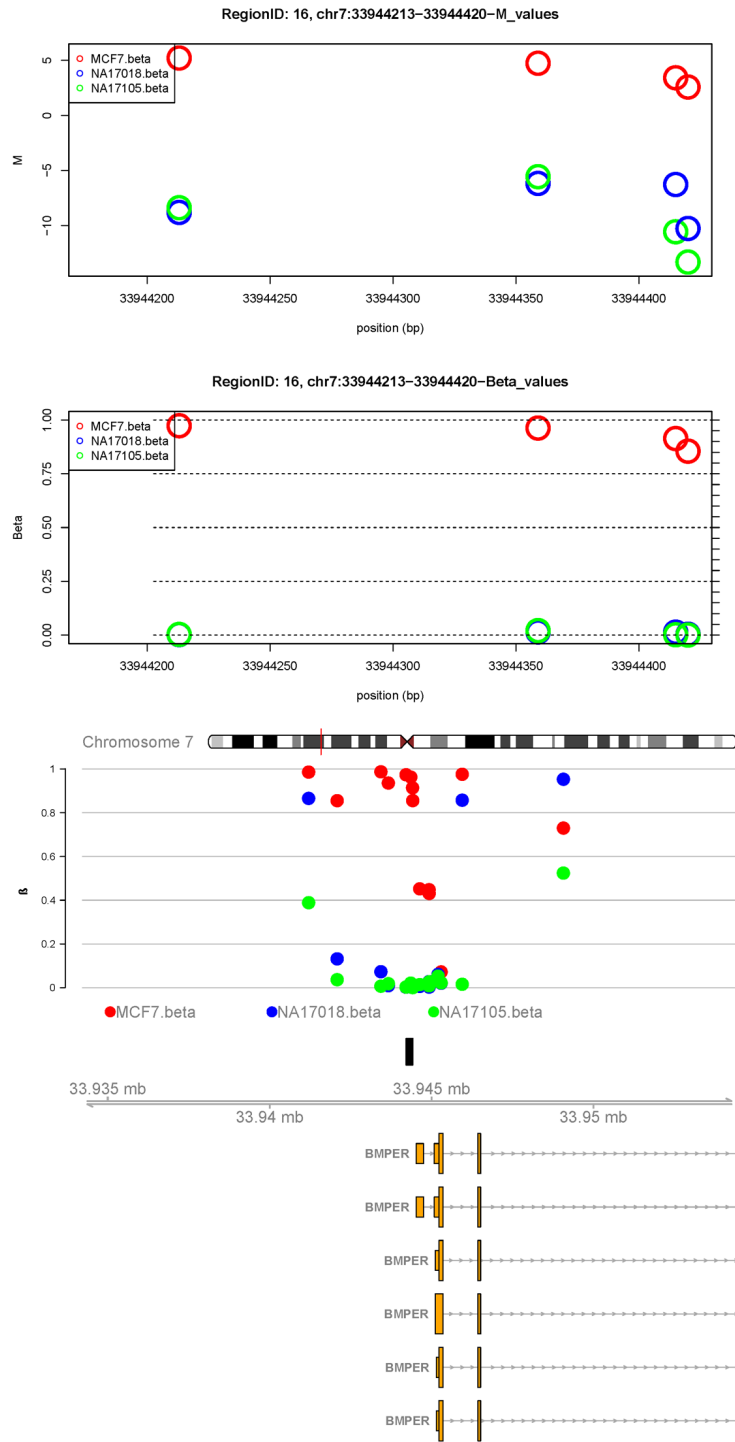
Figure 4: Differentially methylated region 16 - relevant, but not significant.

The DMRforPairs script also contains a wrapper to visualize the methylation pattern in and around a specific gene (gene symbol) as long as the gene symbol is annotated in the Illumina manifest. The plot_annotate_gene() function also reports back the complete set of statistics and pairwise plots for that gene. We will follow up on BMPER here (Figure 5).

```
> plot_annotate_gene(gs="BMPER",
+                    regions=output$regions,
+                    margin=10000,
+                    ID="BMPER",
+                    clr=clr,
+                    annotate=FALSE,
+                    path=experiment)

$symbols_exact
[1] ""

$symbols_margin
[1] ""

$scores
                                                       [,1]
beta.median.MCF7.beta                             0.67506214
beta.median.NA17018.beta                          0.27153857
beta.median.NA17105.beta                          0.02429967
m.median.MCF7.M                                   1.05783234
m.median.NA17018.M                               -1.44791040
m.median.NA17105.M                               -5.35393171
median.delta.beta.MCF7.M.minus.NA17018.M          0.26227534
median.delta.beta.MCF7.M.minus.NA17105.M          0.36175373
median.delta.beta.NA17018.M.minus.NA17105.M       0.01144306
median.delta.m.MCF7.M.minus.NA17018.M             2.38364051
median.delta.m.MCF7.M.minus.NA17105.M             4.83587754
median.delta.m.NA17018.M.minus.NA17105.M          1.03137346
pairwise.p.MCF7.M.vs.NA17018.M                    0.00006834
pairwise.p.MCF7.M.vs.NA17105.M                    0.00000002
pairwise.p.NA17018.M.vs.NA17105.M                 0.06660043
max.abs.median.delta.m                            4.83587754
p.value                                           0.00000017
n.probes                                         40.00000000
```
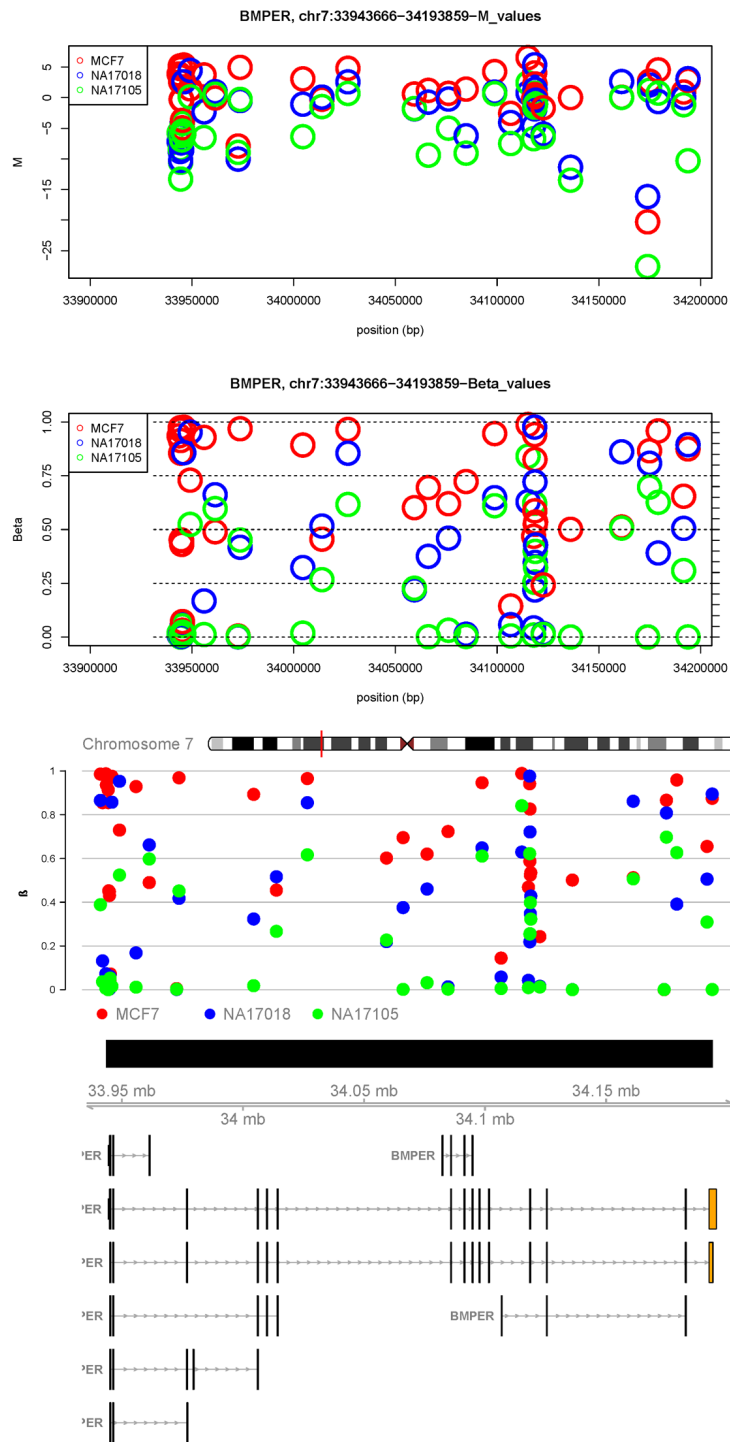
Figure 5: Methylation througout the BMPER gene.

14

DMRforPairs can also visualize custom genomic regions. The example below basically generates a zoomed in version of of the whole BMPER gene and shows that only the promoter region (before TSS) is differentially methylated (Figure 6). This overlaps with the region selected by DMRforPairs (region 16). The plot_annotate_custom_region() function also reports back the complete set of statistics for the requested custom genomic region.

```
> plot_annotate_custom_region(chr=7,
+                             st=33943000,
+                             ed=33945000,
+                             output$regions,
+                             margin=500,
+                             ID="BMPER_TSS",
+                             clr=clr,annotate=FALSE,
+                             path=experiment)

$symbols_exact
[1] ""

$symbols_margin
[1] ""

$scores
                                                       [,1]
beta.median.MCF7.beta                               0.913892
beta.median.NA17018.beta                            0.009059
beta.median.NA17105.beta                            0.008536
m.median.MCF7.M                                     3.409922
m.median.NA17018.M                                 -7.167840
m.median.NA17105.M                                 -6.908633
median.delta.beta.MCF7.M.minus.NA17018.M            0.900301
median.delta.beta.MCF7.M.minus.NA17105.M            0.912505
median.delta.beta.NA17018.M.minus.NA17105.M        -0.000737
median.delta.m.MCF7.M.minus.NA17018.M               9.973318
median.delta.m.MCF7.M.minus.NA17105.M              10.288598
median.delta.m.NA17018.M.minus.NA17105.M           -0.429142
pairwise.p.MCF7.M.vs.NA17018.M                      0.000041
pairwise.p.MCF7.M.vs.NA17105.M                      0.000041
pairwise.p.NA17018.M.vs.NA17105.M                   0.931427
max.abs.median.delta.m                             10.288598
p.value                                             0.000170
n.probes                                            9.000000
```
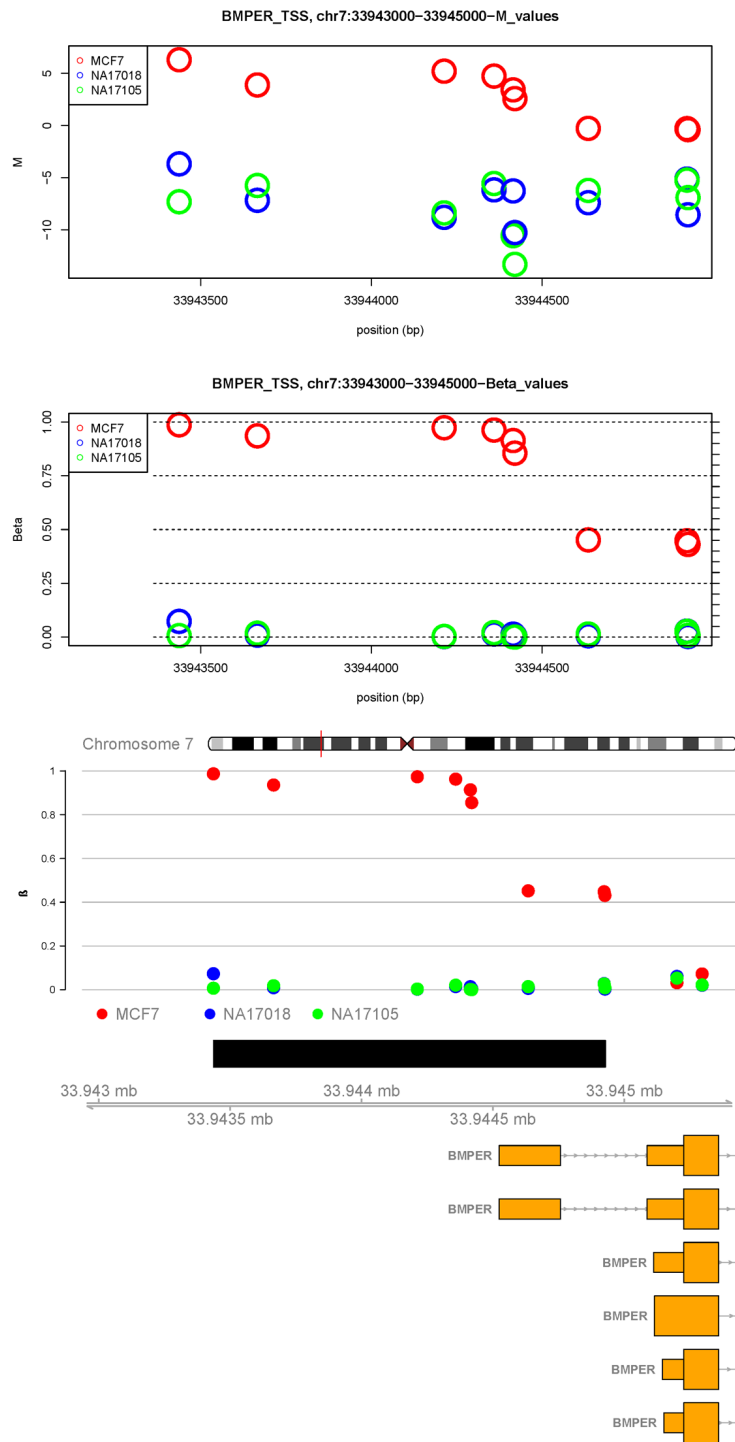
Figure 6: Methylation around the TSS of BMPER.