DNA-Modification Detection with SMRT-Sequencing Using R

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Contents

Abstract

This documents describes a programing interface to Pacific Biosciences compare H5 files. These files provide additional data beyond basecalls and quality values obtained during a sequencing run. The data contained in compare H5 files can be used to discover base modifications, e.g., DNA methylation events. In addition to demonstrating the detection of DNA methylation in a two-sample statistical testing context, we present an `R` API for extracting data from PacBio HDF5 files.

1 Introduction

Base modifications are important in understanding a variety of biological processes such as gene expression, host-pathogen interactions, DNA damage and DNA repair. Single-Molecule Real-Time (SMRT) sequencing has the potential to revolutionize the study of base modifications through direct detection on unamplified source material. Traditionally, it has been a challenge to study the wide variety of modifications that are seen in nature. Most high throughput techniques focus on 5-methylcytosine – made accessible through bisulfite treatment and subsequent amplification techniques

to detect this methylation at single-base resolution. In contrast, SMRT-sequencing does not require genetic alterations to the source material in order to view base modifications. Instead, measurements of the kinetics of base additions are made during the normal course of sequencing. These kinetic measurements present characteristic patterns in response to a wide variety of base modifications. As a result of this relatively simple mechanism to detect base modifications, it is now possible to study more than just 5-methylcytosine in a high-throughput fashion. Bacterial modifications such as 6-methyladenine, 4-methylcytosine, or 5-hydroxymethylcytosine are accessible to study using a single sequencing method on the PacBio RS. As our understanding of kinetic information grows, the analysis of base modifications using SMRT technology will continue to become easier and faster, making accessible a rich new frontier of scientific study.

In this document we demonstrate how to perform DNA modification detection using the suite of R packages developed and used at Pacific Biosciences. These APIs provide the developer with low-level access to all information collected during a sequencing run. This document serves two purposes (1) to demonstrate the use of the pbh5 R package to access low-level data produced during a SMRT-sequencing run and (2) to provide a starting point for users to conduct their own kinetic analysis.

1.1 R Packages/System Requirements

In this analysis we will make heavy use of the pbh5 and pbutils R packages. In addition, the pbh5 package depends on the h5r package. Finally, we will also make use of the ggplot2 and xtable packages available on CRAN[1]. All of the analysis conducted here can be performed using the pbh5 package exclusively, however, the code to execute this document depends on the aforementioned packages.

```
> require(pbh5)
> require(pbutils)
> require(xtable)
> require(ggplot2)
> source("utils.R")
```

In addition to R package requirements, this document requires a system with approximately 3-4 Gigabytes of memory and a recent version of R, i.e., ≥R-2-11. Finally, this document is a "vignette", i.e., the code and text is all contained in analysis.Rnw, the code extracted can be found in analysis.R. To "run" this document, the user can perform the following from the top-level directory:

```
make analysis-build
```

This will download the data (cmp.h5 files) into the Data directory and then run the analysis.Rnw document.

1.2 Experimental Setup

Example sequencing data from the PacBio RS, subjected to the kinetic analysis described below, comprise two different sources of input DNA (1) Synthetically methylated DNA with a few site-specific modifications per template and (2) DNA library data from lambda phage. For the synthetically modified data, we have a 5 identical (from a nucleotide sequence perspective) templates, of which four templates have DNA base modifications at particular sites. One is a control template which will be used in comparison to each treatment template. For the lambda data set, both DAM+ and DAM-

[1] http://www.r-project.org

	nAlignments	nMolecules	nMovies	nReferences
2x_4mC	100649	24288	1	1
2x_5hmC	31746	8583	1	1
2x_5mC	36755	9393	1	1
2x_6mA	74165	20120	1	1
control	36177	9163	1	1

Table 1: Summary of synthetically methylated datasets used in this document.

preparations as well as whole-genome amplifications for both the DAM+ and DAM- preparations (either containing or lacking DAM methylase, respectively) are sequenced. The DAM or DNA methyl-transferase specifically methylates the adenine base of the GATC motif in DNA. Additionally, the lambda DNA sample contains methyl-transferases for other motifs.

	nAlignments	nMolecules	nMovies	nReferences
6mA_dam-_native	149435	35849	2	1
6mA_dam+_native	146323	32149	2	1
6mA_dam-_WGA	160201	37604	2	1
6mA_dam+_WGA	97859	24474	2	1

Table 2: Summary of lambda datasets used in this document.

2 Exploring the Data

As described above, SMRT-sequencing provides a rich set of information beyond that of traditional sequencing platforms. Specifically, here we focus on information about the kinetic behavior of the polymerase at specific positions in the reference sequence. We first examine high-level summaries of the data, such as yield, read length, and accuracy. We will focus on the Lambda data for some of the major exploratory work because it provides a larger number of sequencing contexts to investigate. First, we describe some of the major components of the `R` API which we will use throughout this document to analyze the two different modification datasets.

2.1 Working with the Compare H5 File

The *cmp.h5* file (pronounced comp H5 or compare H5) provides a rich set of data resulting from the alignment of PacBio data to a reference sequence. The cmp.h5 file may contain one or more movies (sequencing runs) and contains all of the alignments for that movie's reads to a reference fasta file.

```
> cmpH5 <- PacBioCmpH5("../Data/Lambda/6mA_dam+_native/data/aligned_reads.cmp.h5")
> cmpH5

class of: PacBioCmpH5
file: aligned_reads.cmp.h5
Version: 1.2.0.SF
```

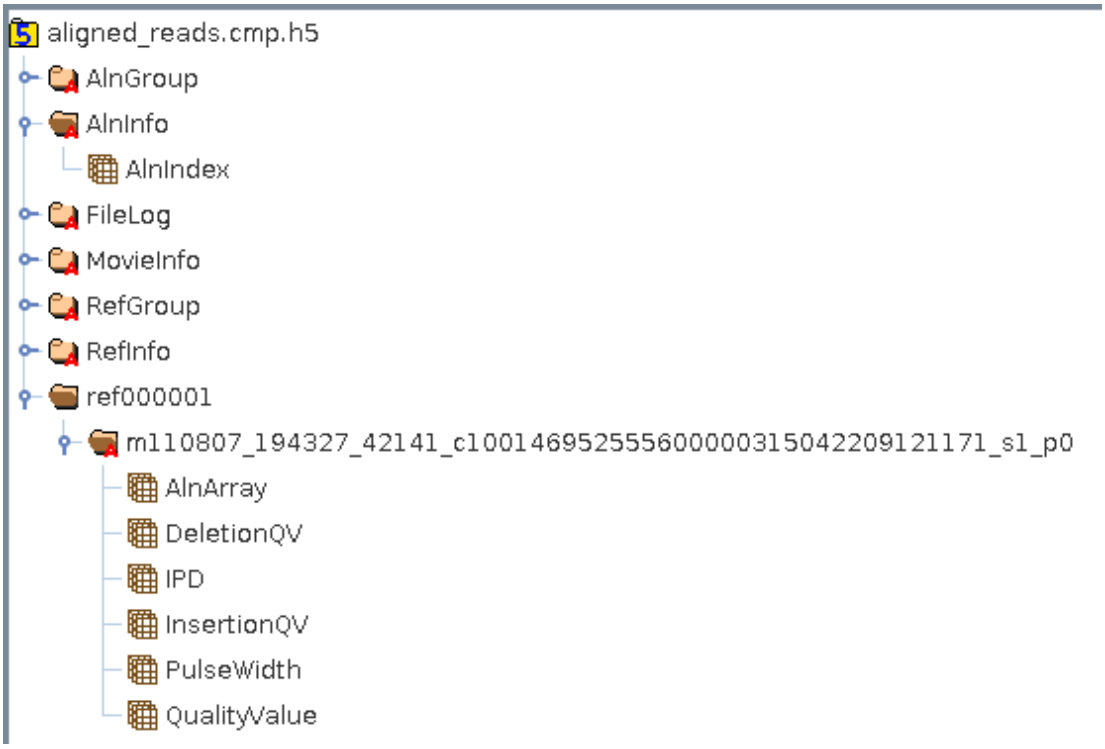


Figure 1: *cmp.h5 Structure* - A screenshot of the cmp.h5 file structure as seen through the "hdfview" tool provided by the hdfgroup.org. PacBio cmp.h5 files provide a wealth of additional information about the sequencing run. Broadly speaking, HDF5 files can be thought of as a file system for your data – allowing one to organize both metada and experimental results in consistent structures.

```
N Alignments: 146323
N ReadGroups: 2
N RefSeqs: 1
```

The core of the file is represented by the "AlnIndex" and the corresponding "AlnArray" datasets. Alignments, QualityValues, and kinetic data are stored at the "refGroup/alnGroup" level of the hierarchy, e.g.,

```
> group <- "/ref000001/m110818_122604_42141_c100129202555500000315043109121114_s1_p0"
> g <- getH5Group(cmpH5, group)
> ls(g)

[1] "."            "AlnArray"     "DeletionQV"   "IPD"          "InsertionQV"
[6] "PulseWidth"   "QualityValue"
```

Each of these datasets contain all of the alignment related data for a given "alignment group" which tends to be a movie. The alignments are packed together in a compact format and the "AlnIndex" contains the relevant information on how to extract a particular alignment. The long "basename" portion of the path represents the movie name, whereas the "dirname" portion represents the reference sequence. The mapping between the canonical "ref000001" and the name in the fasta file can be found using the refGroup function. For our work, the most pertinent datasets are: AlnArray, IPD (inter-pulse duration), and PulseWidth.

As mentioned above, all of the alignments in the file are stored in a global alignment index. In addition to providing summary statisitics about alignments, e.g., the number of mismatches, deletions, etc., the index provides the offsets into the alignment datasets for fast random access to alignments.

```
> head(alnIndex(cmpH5), 2)
```

```
      ID
1 109944
2   8671
                                                                  alnGroupPath
1 /ref000001/m110818_122604_42141_c100129202555500000315043109121114_s1_p0
2 /ref000001/m110818_122604_42141_c100129202555500000315043109121114_s2_p0
                                                        movieName   refName
1 m110818_122604_42141_c100129202555500000315043109121114_s1_p0 ref000001
2 m110818_122604_42141_c100129202555500000315043109121114_s2_p0 ref000001
     fullRefName tStart tEnd alignedStrand holeNumber setNumber strobeNumber
1 lambda_NEB3011      1   98             1      47143         1            0
2 lambda_NEB3011      1  108             1      77027         2            0
  moleculeID rStart rEnd mapQV nMatches nMisMatches nInsertions nDeletions
1     367143     10  104     0       88           0           7         10
2     717027    320  435   254      104           0          12          4
  offsetBegin offsetEnd nBackRead nOverlap
1    11153264  11153368         0        0
2     2966605   2966724         1        1
```

In general, these details can be ignored and users can interact with the file via the accessor API functions. For instance, to access an alignment:

```
> alns <- getAlignments(cmpH5, idx = c(1, 200, 3))
> lapply(alns, head, n = 2)
```

```
[[1]]
     read reference
[1,] "T"  "T"
[2,] "A"  "A"

[[2]]
     read reference
[1,] "A"  "A"
[2,] "C"  "C"

[[3]]
     read reference
[1,] "G"  "G"
[2,] "G"  "G"
```

The API provides a large set of functions with the above signature, i.e., `cmpH5` and `idx`, where `idx` is an index vector which must contain values between 1 and `nrow(cmpH5)` inclusive. These naturally

refer to the rows in the `alnIndex(cmpH5)`. In addition to `getAlignments`, other useful functions include: `getIPD`, `getPulseWidth`, and `getQualityValue`.

To get more information on the cmp.h5 file format refer to: PacBio DevNet. Also, to get help on the pbh5 package, try `?pbh5`. In the remainder of the document, we will typically hide the code to not disrupt the document flow. As mentioned above, all of the code can be found in either analysis.Rnw or analysis.R

2.2 Visualizing Kinetic Properties of the System

In this section, we visualize pulse width and IPD (inter-pulse duration) distributions. These two, especially IPD, represent the primary source of data informing about possible base modifications. In Figures 2, a schematic of the trace signal is plotted. In this figure all pulses have the same magnitude for simplicity, and indeed we will focus on the distributions of durations of incorporation (pulse width) and between incorporation events (IPD). In the aforementioned figures, we have drawn a red arrow to indicate the IPD at a position of interest. Each read covering a region gives us information about the incorporation events. We can compare that to a control sample where no modifications are present.

We want to examine the various sources of variation in the IPD and pulse width distributions. In our case, we will compare a function of the IPD distribution in a treatment sample (where we believe there to be modifications) to that of a control sample (where we have removed them).

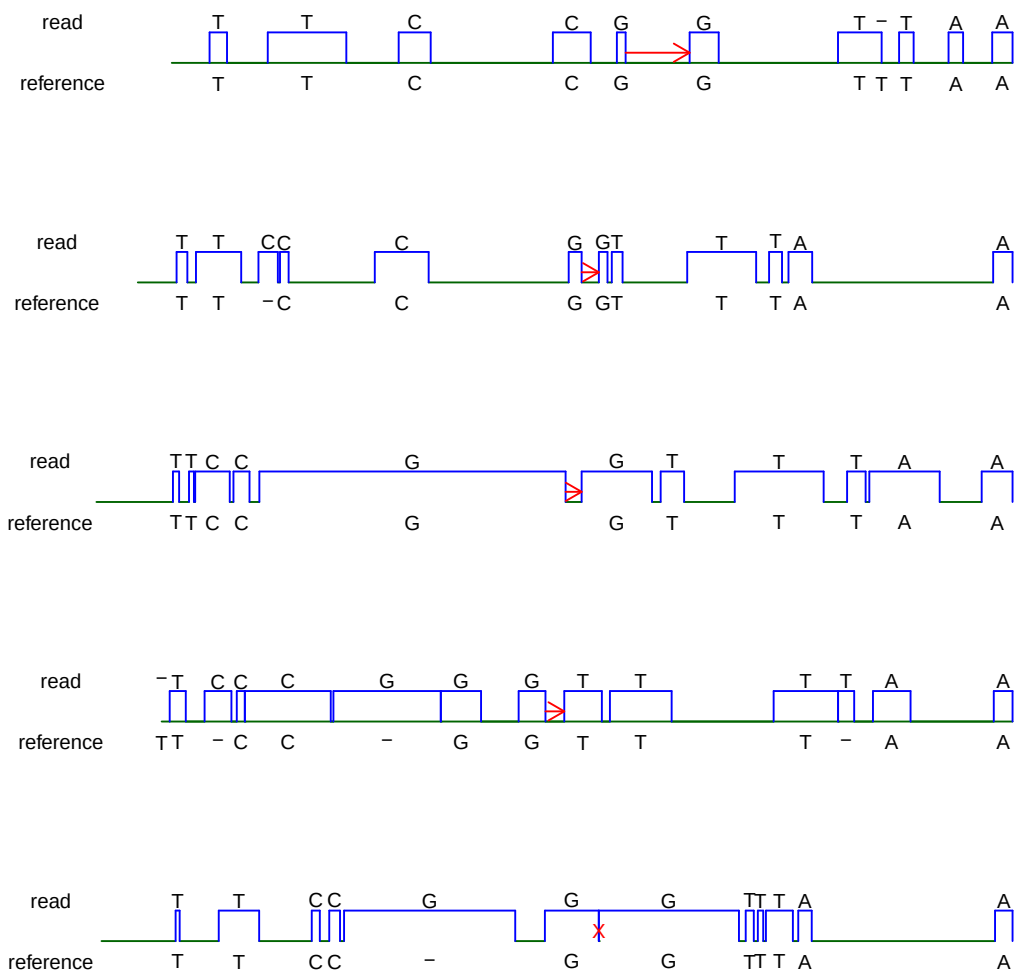
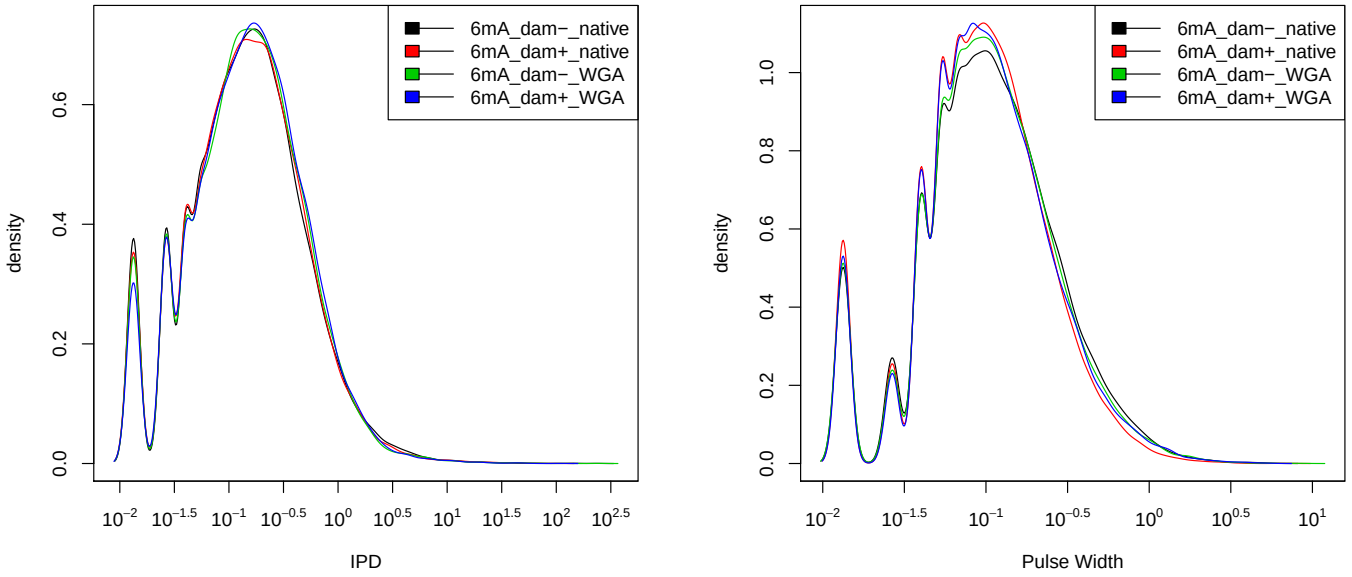
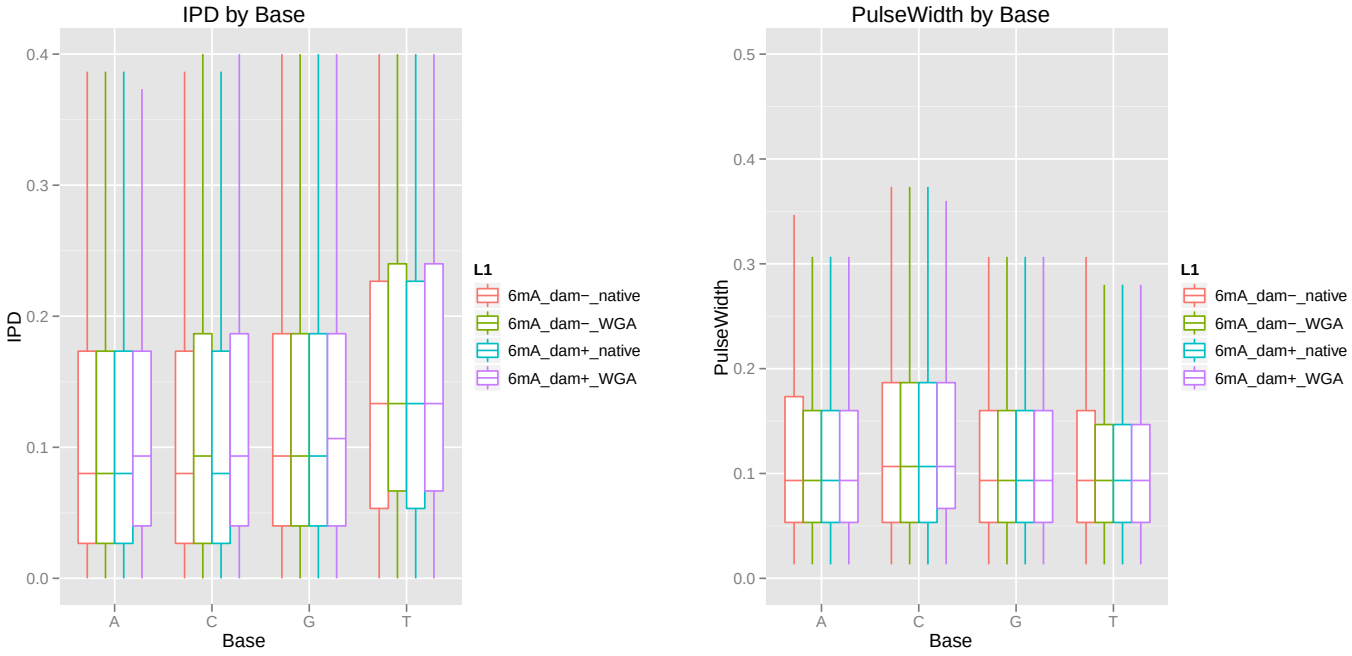


Figure 2: *Trace Schematic* - Here we plot "trace" views directly from the cmp.h5 file. The presence of random insertion/deletion/mismatch errors adds additional complexity to correctly assign kinetic parameters to a particular incorporation event.



(a) (b)

Figure 3: *Global IPD and PulseWidth Densities* - Here we plot the global IPD and PulseWidth distributions. This distribution is a mix of incorporations of the 4 nucleotides. Both IPD and PulseWidth are currently stored in the file in seconds rather than frames. The spikes occur because of the finite frame rate of the detector.



(a) (b)

Figure 4: *IPD and PulseWidth By Base* - Here we plot the IPD and PulseWidth distributions stratified by the base being incorporated. There is a base effect on IPD, i.e., which base is incorporated changes the kinetic behavior of the enzyme.

The getByTemplatePosition function retrieves data for `idx` reads. It takes a function, f, which returns a list of vectors or matrices where the length or number of rows is equal to the alignment length for alignment i. Typically, one just passes in an existing function, such as getIPD or getPulseWidth.

```
> head(getByTemplatePosition(cmpH5, idx = 1:2, f = getIPD))
```

```
  position read ref idx strand        elt
1        1    C   C   1      1 0.17333291
2        2    C   C   1      1 0.06666651
3        3    C   C   1      1 0.17333291
4        4    G   G   1      1 0.22666611
5        5    C   C   1      1 0.13333301
6        6    C   C   1      1 0.39999902
```

Additionally, there are a number of high-level data access functions related to retrieving the information in the cmp.h5 file by position and context. Again, these functions take a vector of indices which refer to the reads in the alignment index to be used, e.g.,

```
> head(makeContextDataTable(cmpH5, idx = 1:2, up = 2, down = 2))
```

```
    elt.ipd    elt.pw elt.tpos context.P01 context.P02 context.P03 context.P04
1 0.3999990 0.0533332       96           T           A           T           T
2 0.4266656 0.1066664       95           A           T           T           T
```

```
3 0.1066664 0.0799998       94          T          T          T          T
4 0.7866647 0.0799998       93          T          T          T          A
5 0.0399999 0.0533332       92          T          T          A          A
6 0.0399999 0.1466663       91          T          A          A          A
  context.P05
1           T
2           T
3           A
4           A
5           A
6           T
```

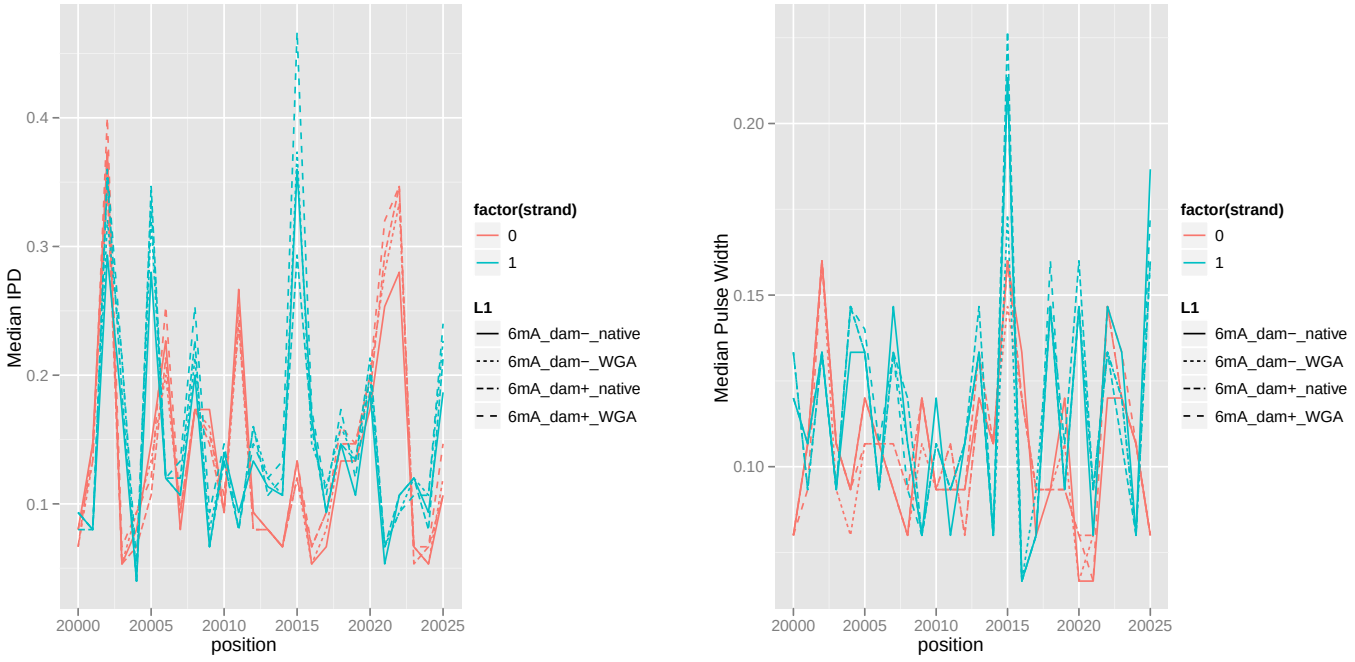
Another useful function for summarizing data by context is:

```
> s <- summarizeByContext(cmpH5, idx = 1:100, up = 1, down = 1,
+     statF = getPulseWidth)
> head(s)

    count     value
AAA  1481 0.1066664
AAC   517 0.0933331
AAG   466 0.0799998
AAT   356 0.0933331
ACA   279 0.1333330
ACC   265 0.1333330
```

Throughout this document we will be using these two or three functions for data access. Below we define a convenience function which takes a range along the genome and then retrieves the results of f for those reads. An important point to notice is that the getReadsInRange function returns any read that overlaps either the start or the end of the range. Therefore, portions of reads will not be within $[\text{start}, \text{end}]$, hence the subset below.

```
> getByPositionAndStrand <- function(f = getIPD, s = 20000, e = 20025) {
+     pbutils::collapse(lapply(cmpH5s, function(cmpH5) {
+         x <- getByTemplatePosition(cmpH5, idx = getReadsInRange(cmpH5,
+             1, s, e), f = f)
+         x <- subset(x, position >= s & position <= e)
+         ddply(x, c("strand", "position"), function(a) {
+             median(a$elt, na.rm = T)
+         })
+     }))
+ }
> byPositionAndStrandIPD <- getByPositionAndStrand()
> byPositionAndStrandPW <- getByPositionAndStrand(f = getPulseWidth)
```



(a) (b)

Figure 5: *IPD and PulseWidth by Strand and Position* - Here we plot the median of the IPD distribution conditioned on both strand and position. We can see the presence of strong position and strand effects.

2.3 Context-specific Effects

Finally, we investigate the effect of sequence context on IPD and pulse width distributions. Alignment-level data from a cmp.h5 file are always stored with respect to the bases being incorporated. Therefore, when one retrieves an alignment from the cmp.h5 file, if that alignment is labeled as a reverse strand alignment: `getTemplateStrand(cmpH5) == 1`, then the reference sequence is reverse complemented rather than the read. The importance of this representation is that we always store the data (e.g., alignments, IPDs, pulse widths, etc.) in the direction in which the bases are incorporated.

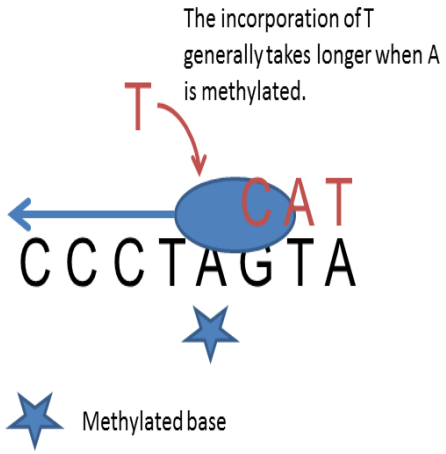


Figure 6: *GATC Cartoon* - A cartoon depicting the incorporation of "T" which will be "delayed" when the complement A base is methylated. The "T" base is what is stored in the "AlnArray" data structures.

```
> getTemplateStrand(cmpH5)[1:10]

 [1] 1 1 0 0 1 1 0 1 0 1
```

```
> tmp <- getByTemplatePosition(cmpH5, idx = 1:2)
> head(tmp[order(tmp$position, tmp$strand), ])
```

```
    position read ref idx strand       elt
1          1    C   C   1      1 0.17333291
106        1    C   C   2      1 0.22666611
2          2    C   C   1      1 0.06666651
107        2    C   C   2      1 0.29333261
3          3    C   C   1      1 0.17333291
108        3    C   C   2      1 0.17333291
```

We can use the associateWithContext to get a data element by context. There are a couple of relevant options to consider. First, context can either be determined by the read bases or by the reference bases. In either case, gaps are removed from either the read or the reference and then context is computed.

```
> tmp <- associateWithContext(cmpH5, idx = 1:2, f = getTemplatePosition,
+     collapse = T, useReference = T)
> head(tmp[order(tmp$elt), ])
```

```
    elt context
94    3   CGCCC
198   3   CGCCC
93    4   CCGCC
197   4   CCGCC
92    5   GCCGC
196   5   GCCGC
```

Here we used the reference context to group the results of the function call f and you can see that there are two different contexts for the same position (the results of f is stored in the column with name "elt") – this occurs because we still maintain the orientation of the alignments in terms of read space, so for the reference context of 'GGGCG' there are the set of reverse strand reads with the context 'CGCCC'.

```
> contextTable <- associateWithContext(cmpH5, idx = sample(1:nrow(cmpH5),
+     size = 1000), f = getIPD, collapse = T, useReference = T,
+     up = 1, down = 1)
> par(cex.axis = 0.65)
> boxplot(split(contextTable$elt, contextTable$context), ylim = c(0,
+     0.5), las = 2, main = "Context-specific IPD distributions",
+     ylab = "IPD", outline = FALSE, col = rep(1:4, each = 4))
> legend("topleft", c("A", "C", "G", "T"), fill = 1:4, bg = "white")
```

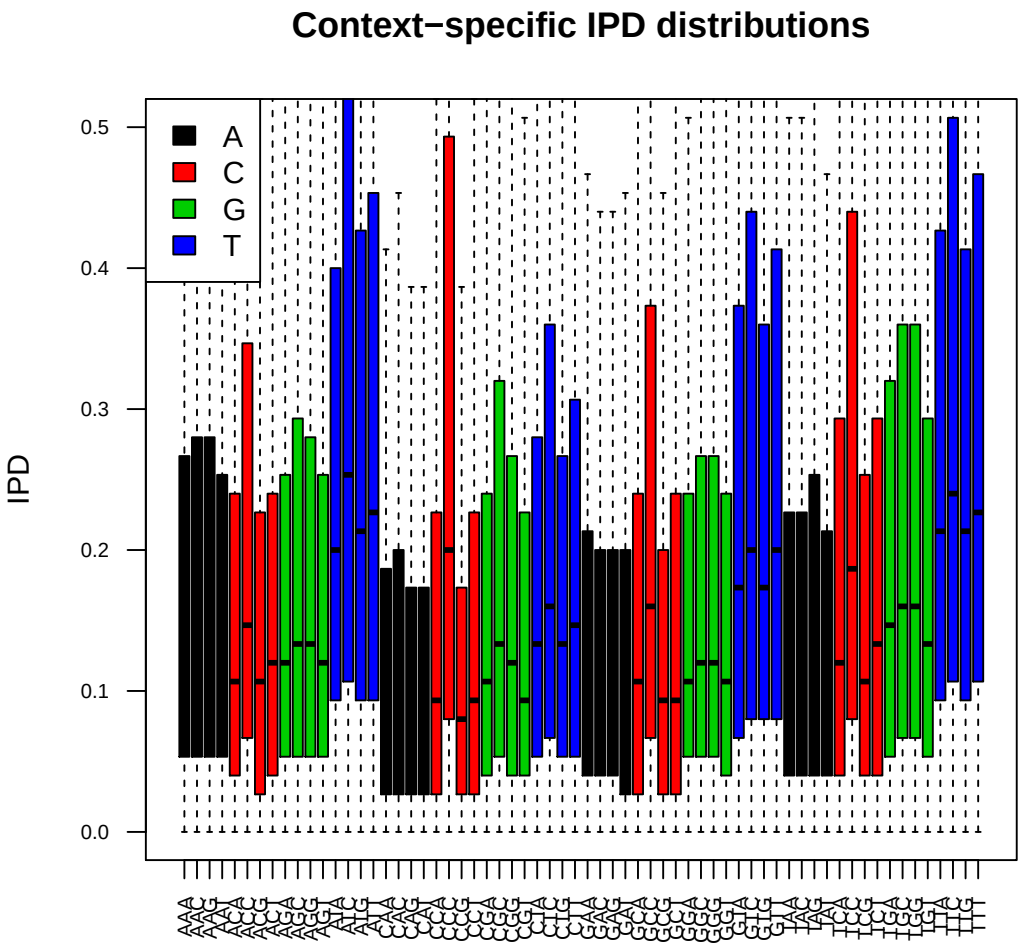


Figure 7: *IPD by Context* - Plots of IPD by context. We can see that the IPD distribution depends on context. Here the boxplots have been colored by the base being incorporated.

We can use the associateWithContext to see modification patterns which might follow motifs, rather than specific positions. In this case, we focus on the DAM+ condition of the Lambda dataset as GATC motif is mostly modified.

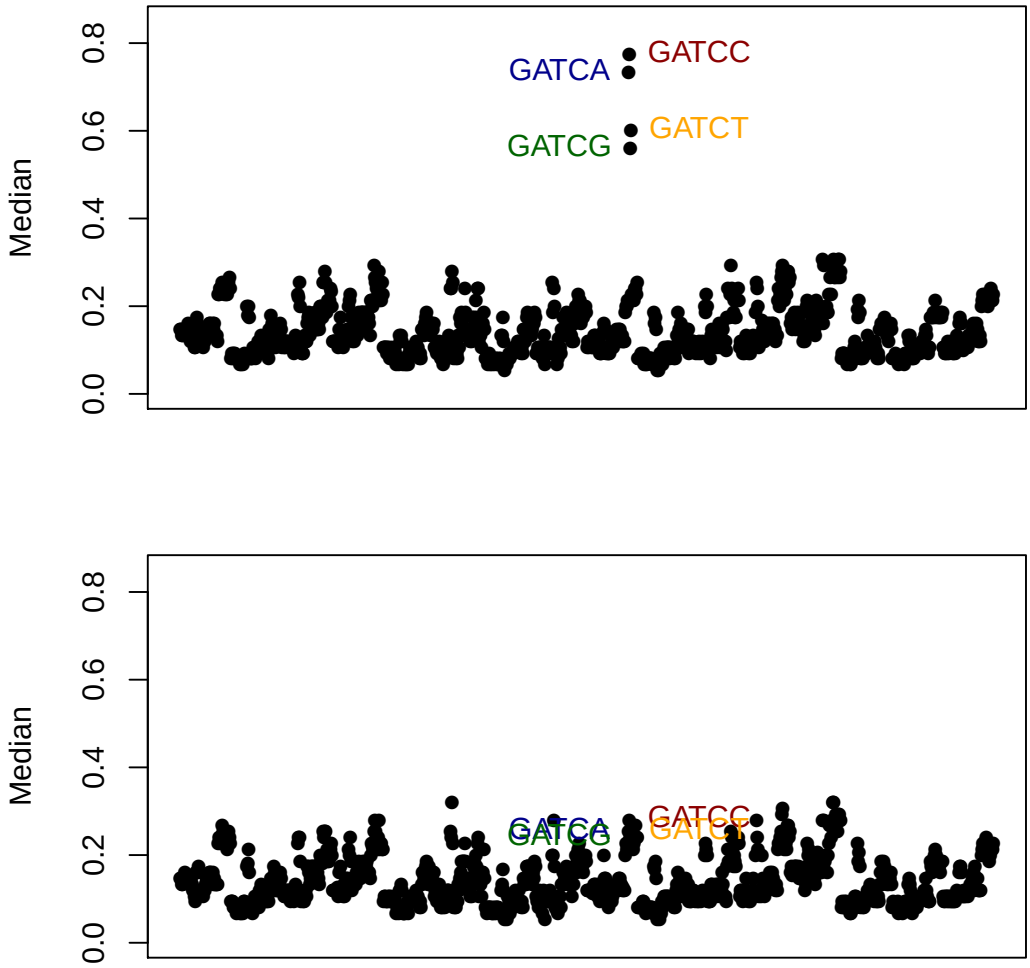


Figure 8: *Context-specific Modifications* – Here we plot the median IPD for 5 based contexts for both the DAM+ and DAM- Lambda strains. First, the range of IPDs is quite similar for all non-modified motifs, i.e., the motif effect is larger than the the strain effect - this will be clearer when we directly compare the IPD measurements across samples.

3 Statistical Testing

In this section we focus on two-sample statistical tests comparing the IPD distribution in a sample containing DNA base modifications to a control sample. Each particular DNA modification has a different kinetic signature at and around the modified base, and more sophisticated methods will take that into account in the future. In this section, we will first focus on the synthetic data sets where the modified positions are known. Here, we will look at detection as a function of coverage. In general, with sufficient coverage the difference between IPD distributions can be detected, however, certain

modifications do not have a large effect on the kinetics of the polymerase and therefore to detect these smaller effects we need to observe the incorporation event more times, i.e., more sequencing fold coverage is required. Additionally, the effects of a modified base might occur around the actual modifications as opposed to the exact methylated site, due to the contact the DNA polymerase makes with the DNA template over an extended region surrounding the modified base position.

We can view this as a simple two-sample statistical testing problem where IPD measurements obtained from the native sample are compared to IPD measurements obtained from a control sample. As in many high-throughput sequencing experiments, some of the canonical assumptions, e.g., independence, normality, etc. might not be satisfied. In addition, one necessarily cares about multiple testing as there are many sites to test. At the end of this section we will discuss natural enhancements to the simple procedures demonstrated here.

Before we begin to analyze the modification signal for the synthetic template data, it is important to understand the topology of the synthetic molecule. The sequence which we align to is a reverse complement of itself with a hairpin at one end and a SMRTbell template at the other end. In the default pipeline, the SMRTbell template is found and the reads are partitioned into individual "subreads" based on that adapter location.

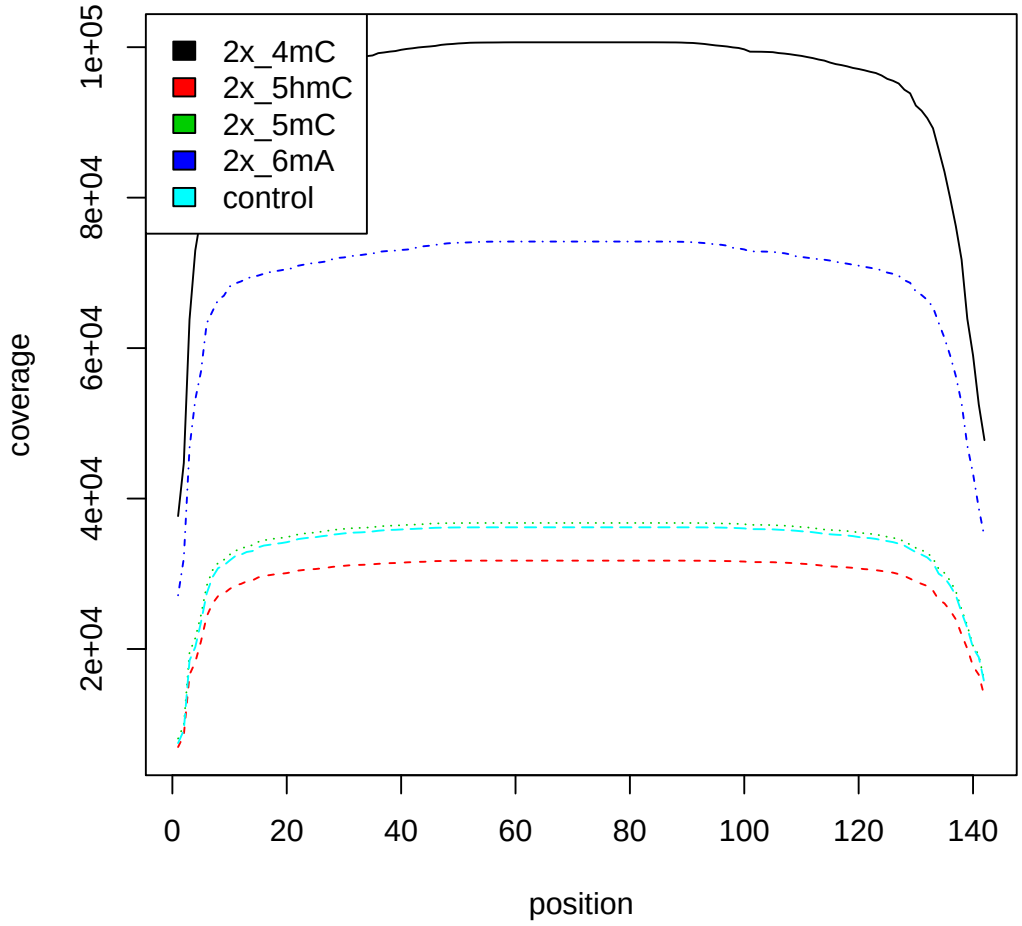


Figure 9: *Coverage Across Synthetic Reference* - Here we plot the "pileup" coverage across the synthetic template. We can see the extreme dropoff of coverage towards the ends of the sequence.

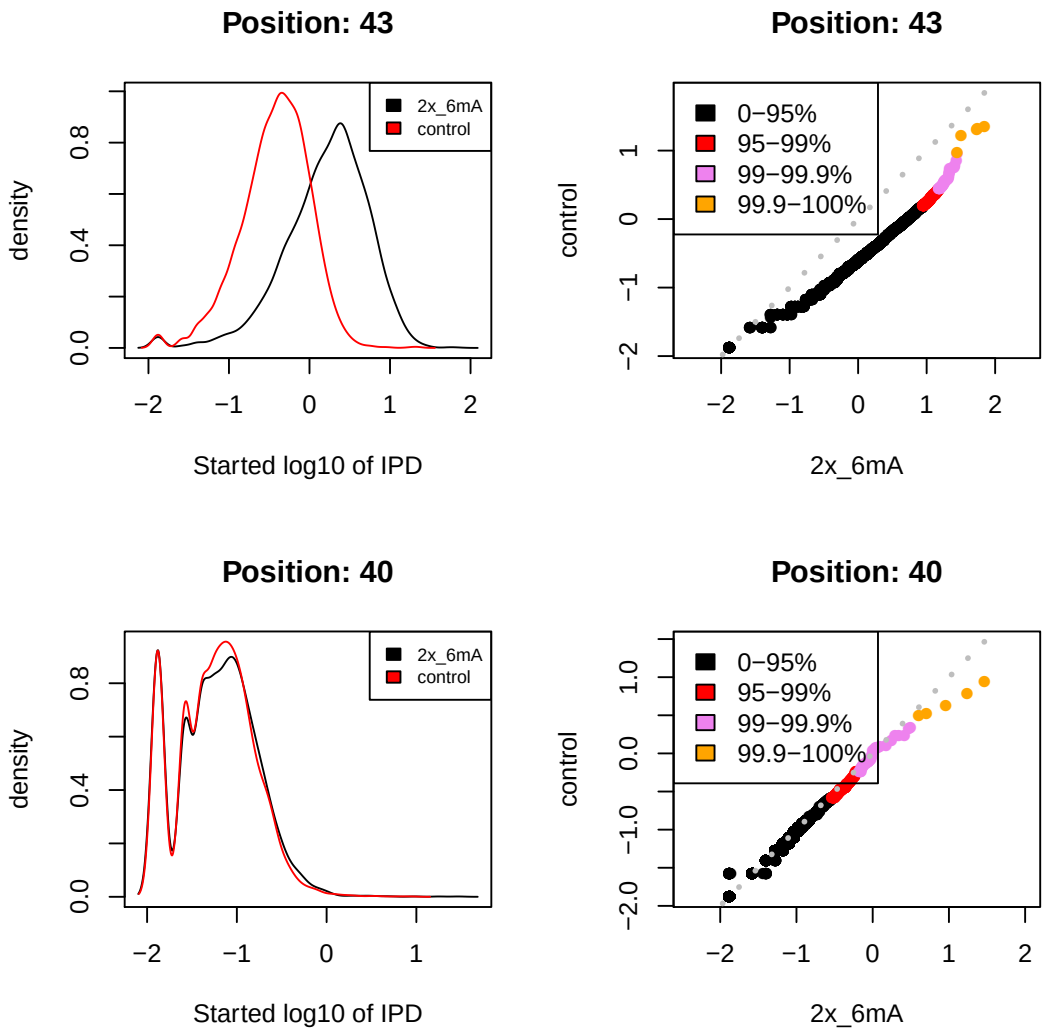


Figure 10: *Density at Position and Strand* - Here we plot the IPD distribution for both a truly modified site as well as a non-modified site. We can see that there is a very large effect on the distribution when the modification is a methyl-A.

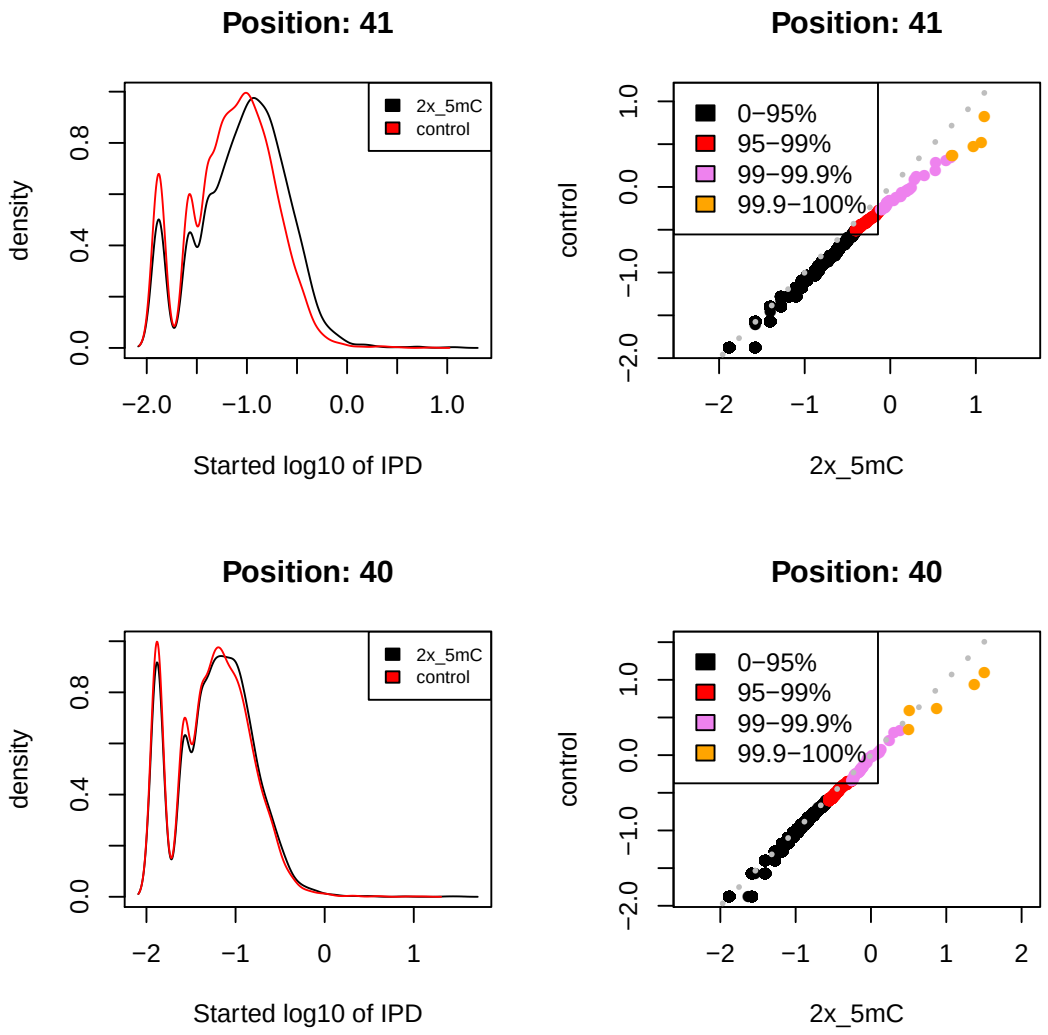


Figure 11: *Density at Position and Strand* - Here we plot the IPD distribution for both a truly modified site as well as a non-modified site. We can see that the effect on IPD is much smaller when the modification is a 5-methylcytosine indicating that we will need larger sample sizes to obtain the same precision as a methyl-A.

As Figures 8, 10, and 11 demonstrate, a natural statistic when comparing the IPDs of a control sample to a native or modified sample is the mean ratio, either logged or unlogged. We refer to the following statistic S as the IPD ratio:

$$S = \frac{1/N_{native} \sum_{i=1}^{N_{native}} IPD_{i,native}}{1/N_{control} \sum_{i=1}^{N_{control}} IPD_{i,control}} \tag{1}$$

Here S is specific to a particular reference position, and N_{native} corresponds to the number of IPD events at that position in the native sample. In Figure 12, we plot S as defined above for each of the four methylated synthetic templates vs. control.

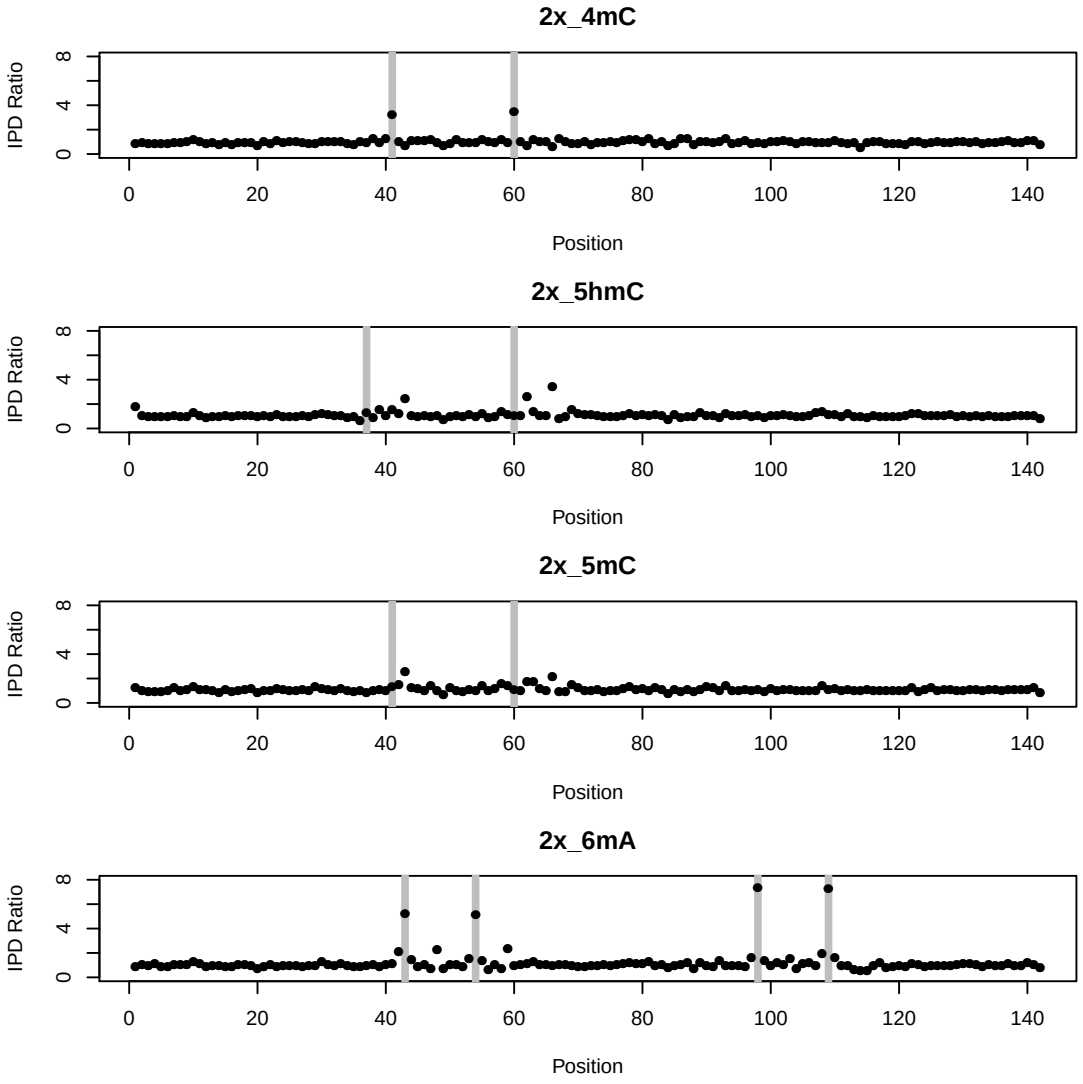


Figure 12: *IPD Ratios* - Here we plot the IPD ratio statistic. This statistic is a measure of the mean shift in IPD distributions. One pertinent aspect of the IPD data that this plot shows is the different signatures for a given modification, with 4-mC having characteristic signals at the modified position, 5-mC and 5-hmC at 2 and 6 bases downstream of the position, and 6-mA at the modified position and 5 bases downstream.

3.1 Testing by Coverage

Naturally, we generally want more than just an IPD ratio, or measure of the difference between two distributions, we want to know whether that difference would have been likely to be observed by chance. As is clear from Figure 12, the different modifications have different signal strengths and effect sizes. Below we investigate the performance of a Wilcox test when making the comparison for increasingly larger amounts of coverage. The Wilcox test is general and robust and does not depend on a particular form of the distribution. Where site-wise statistical testing becomes more subtle is the independence assumption between sites (i.e., position 10 and 11 contain a large number of common reads) as well as the independence assumption within molecule. Both of these assumptions need to be addressed when correcting for multiple testing.

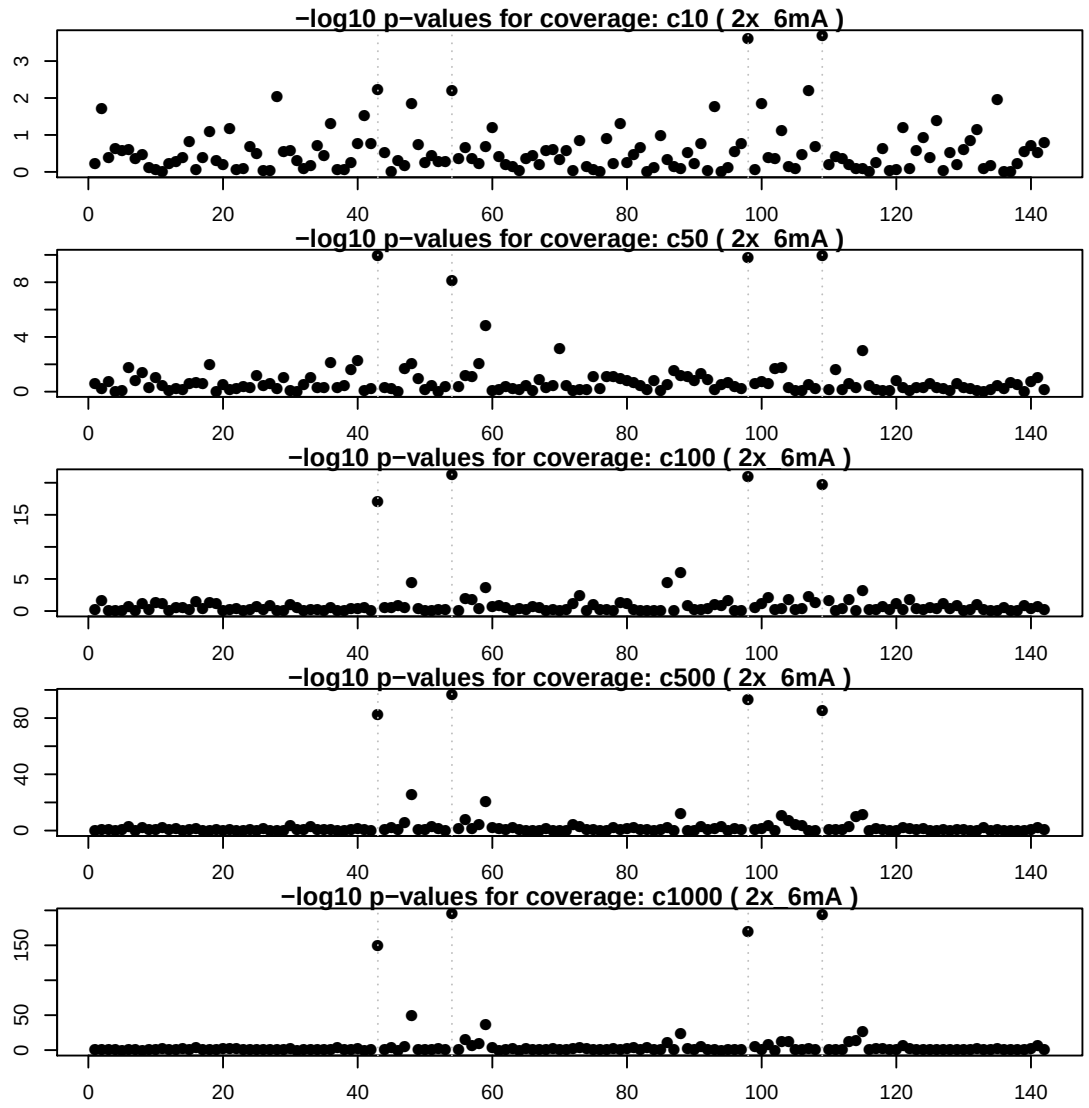


Figure 13: *Tests by Position* – Here we plot the $-\log_{10}$ p-values from the Wilcox test for increasing levels of coverage for the 2x_6mA modified template.

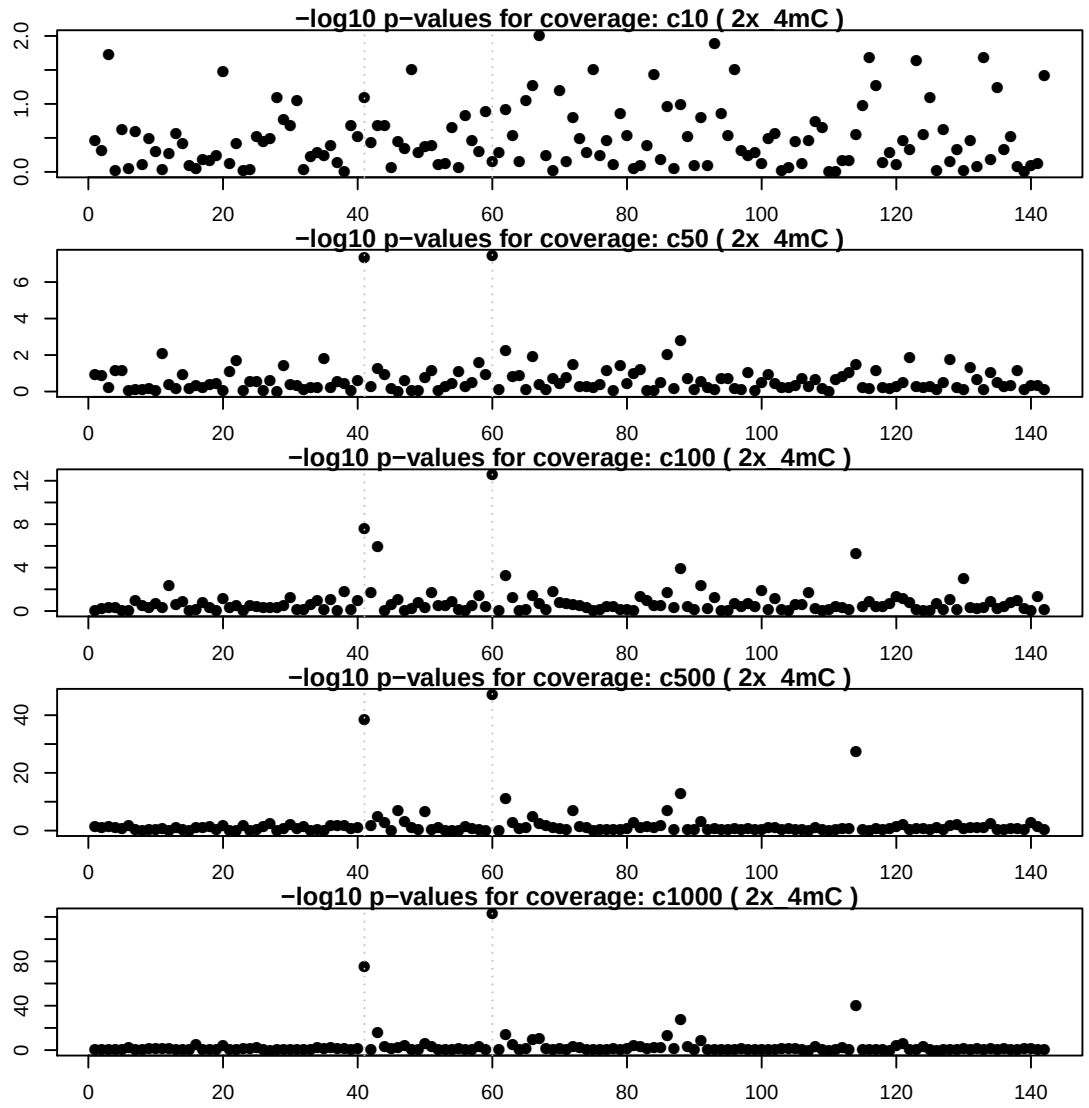


Figure 14: *Tests by Position* – Here we plot the $-\log_{10}$ p-values from the Wilcox test for increasing levels of coverage for the 2x_4mC modified template.

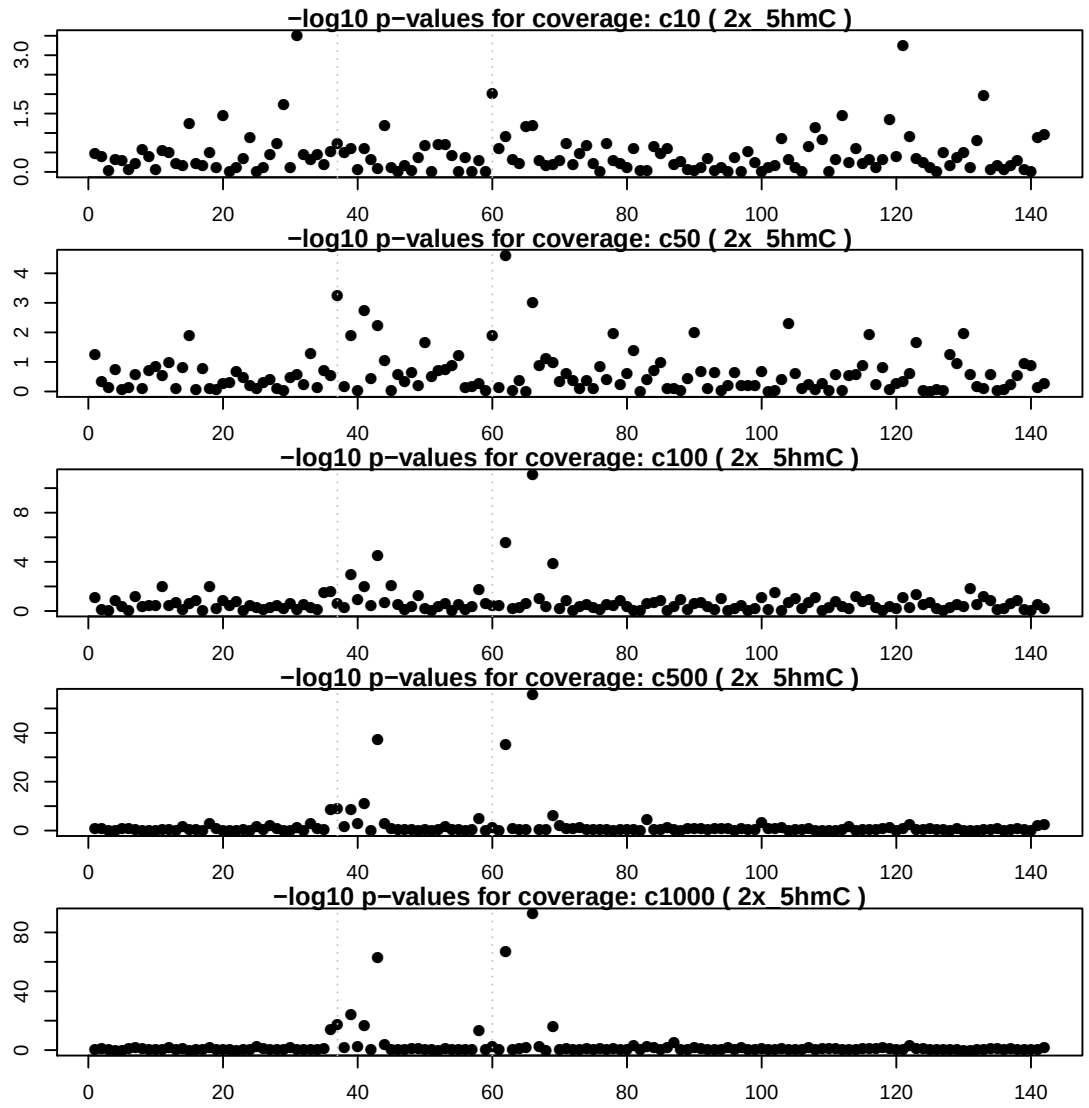


Figure 15: *Tests by Position* – Here we plot the $-\log_{10}$ p-values from the Wilcox test for increasing levels of coverage for the 2x_5hmC modified template.

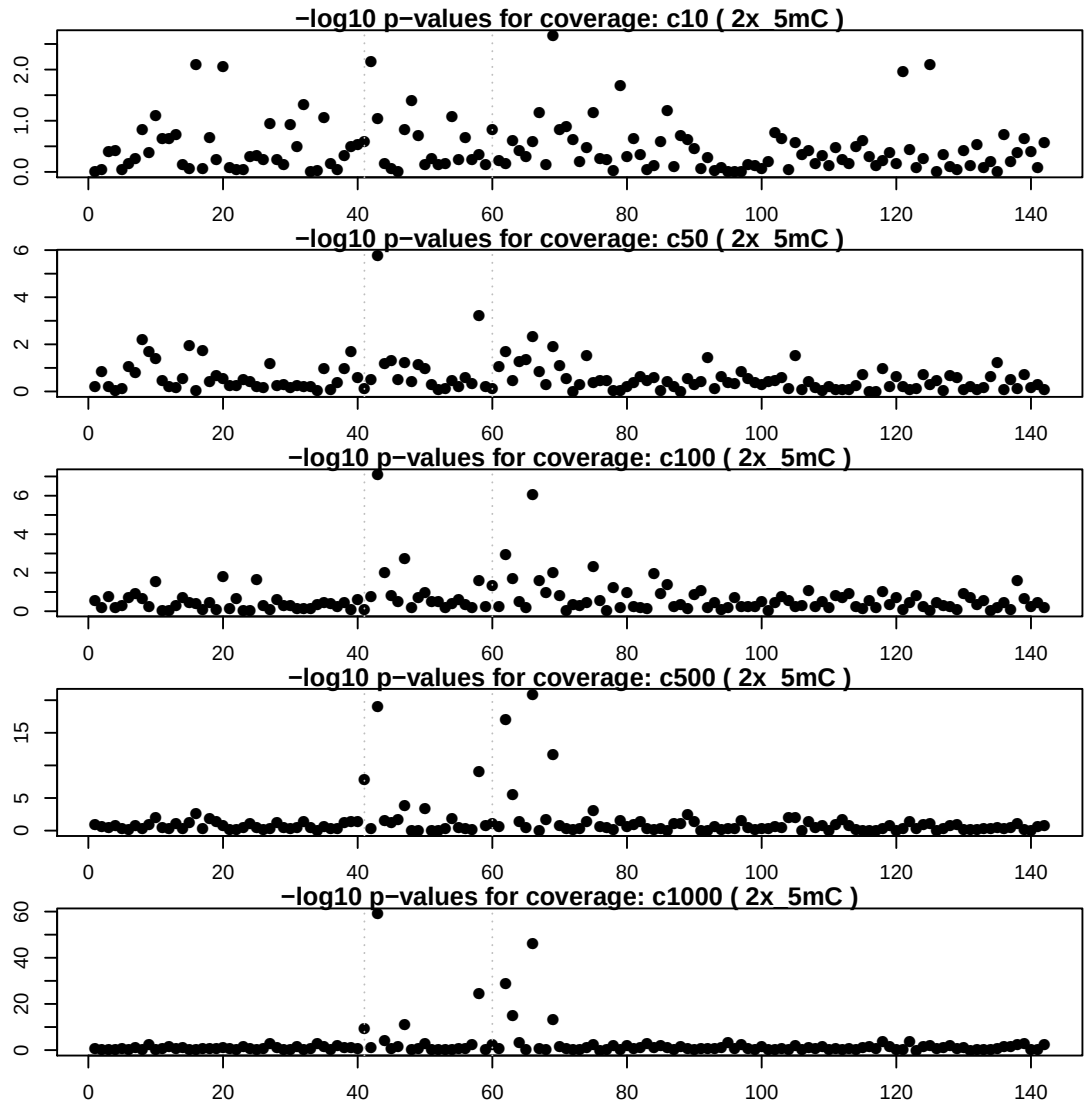


Figure 16: *Tests by Position* – Here we plot the $-\log_{10}$ p-values from the Wilcox test for increasing levels of coverage for the 2x_5mC modified template.

3.2 ROC Analysis

In this section we evaluate the performance of 3 different statistical tests via ROC analysis. We can determine if a particular testing procedure outperforms another in general. Additionally, we can get a sense of our true-positive and false-positive rates for a particular modification. The different tests that we employ are three related tests where each position is tested independently of the other positions. In general, as we can see from the $-\log_{10}$ p-values by position plots, the effect of a modification alters the IPD distribution in nearby bases. More sophisticated tests can take this into account in the future.

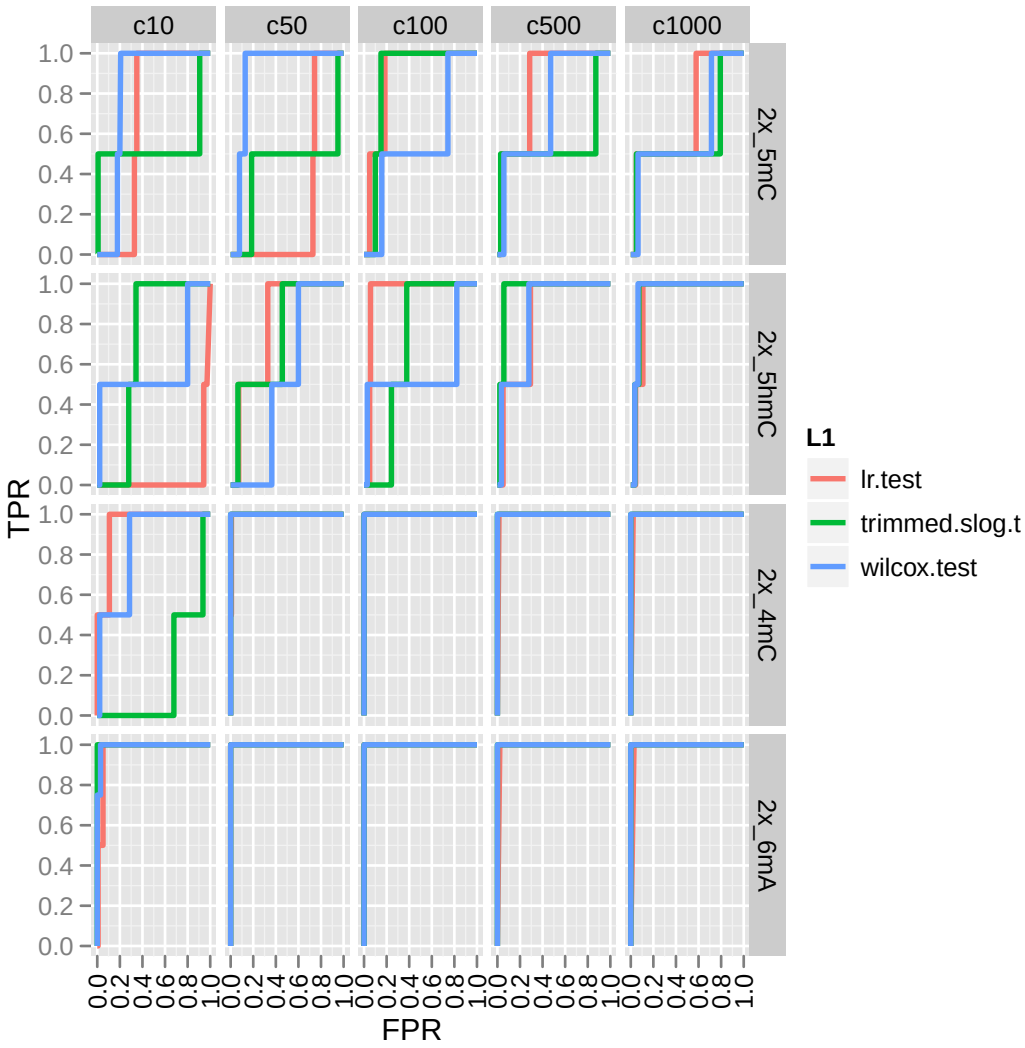


Figure 17: *ROC Curves* - Here we plot ROC curves for the three different testing procedures faceted by coverage and modification type. These curves demonstrate the differences in the magnitude of the modification effects. It is clear the 6mA is quite easy to detect, even at a relatively low level of coverage. However, it is equivalently clear that 5mC has a much smaller effect on the IPD distribution.

As we can see, the differing test procedures do not produce dramatically different results. The false positives in the case of the 5mC modification are to be expected with this simple analytical model as its effect on the IPD occurs three bases downstream. Future improvements to the analysis can take this effect into account. We can see that at sufficient coverage our false positive/true positive tradeoff is quite good for 5hmC, 4mC, and 6mA modifications.

3.3 Statistical Testing in Lambda DNA

A dataset from a biological sample is the lambda dataset where we have 4 distinct conditions (Table 2). In this context, we have both a DAM+ and a DAM- condition which we can compare as well as their corresponding native vs. WGA preparations. For simplicity, we can compare DAM+ and DAM- and focus on GATC methylation sites as our true positives.

For this analysis, we will need to use the Biostrings package to determine where the GATC sites are in the genome. As an example we can look at one position.

```
> if (!require(Biostrings)) {
+     stop("Unable to execute Lambda testing examples without Biostrings package.")
+ }
> lambda <- read.DNAStringSet("../ReferenceRepository/lambdaNEB/lambdaNEB.fa")[[1]]
> matches <- matchPattern("GATC", lambda)
> gatcExample <- pbutils::collapse(lapply(cmpH5s[c("6mA_dam+_native",
+     "6mA_dam-_native")], function(cmp) {
+     s <- start(matches)[1]
+     e <- end(matches)[1]
+     subset(getByTemplatePosition(cmp, idx = getReadsInRange(cmp,
+         1, s, e), f = getIPD), position >= s & position <= e &
+         read == ref)
+ }))
```

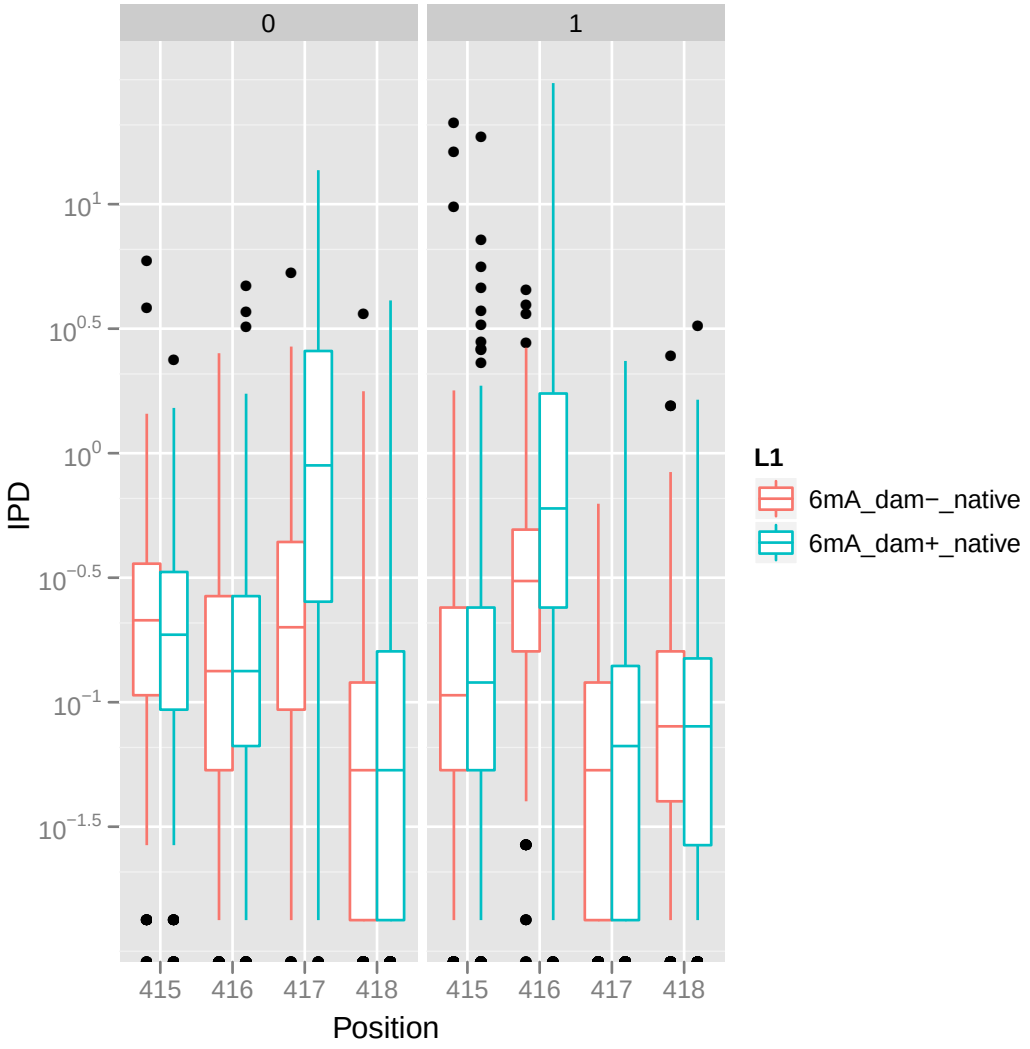


Figure 18: *GATC modification* - At this particular GATC modification, we can see that there is a strong signal of the modification and the reference position depends on the strand. This indeed makes sense as the strand we report means that we had to reverse complement the reference sequence to match the incorporated bases.

Finally, we wrap all this up into one function that users can use to generate a "top table" similar to that generated by the other packages that are common in the arena of differential expression. This table provides useful summary statistics and can be analyzed directly to determine which bases are most probably modified in a given sample.

```
> topTable <- makeTopTable(cmpH5s[["6mA_dam+_native"]], cmpH5s[["6mA_dam-_native"]],
+     start = 1, end = 5000)
> head(topTable)
```

```
     position reference read      p.value statistic n.control n.treatment
2533     2533         T    T 4.061199e-58  150313.0       436         422
551       551         T    T 8.576932e-52  135679.5       412         409
3071     3071         T    T 6.792062e-45   89537.0       368         298
```

```
417       417         T    T 3.263057e-40  143401.0       422         447
2169     2169         T    T 1.154287e-29  114931.0       364         431
2368     2368         T    T 7.818656e-27  154689.5       481         458
     ipd.ratio          fdr
2533  5.243457 2.030600e-54
551   9.717223 2.144233e-48
3071  7.271965 1.132010e-41
417   5.396483 4.078822e-37
2169  2.566337 1.154287e-26
2368  4.574853 6.515546e-24
```

This utility produces a table ordered by p-value, where each row represents a position in the reference. Users might very well like to modify this utility function, please refer to "utils.R" in the "Src" directory of this project.

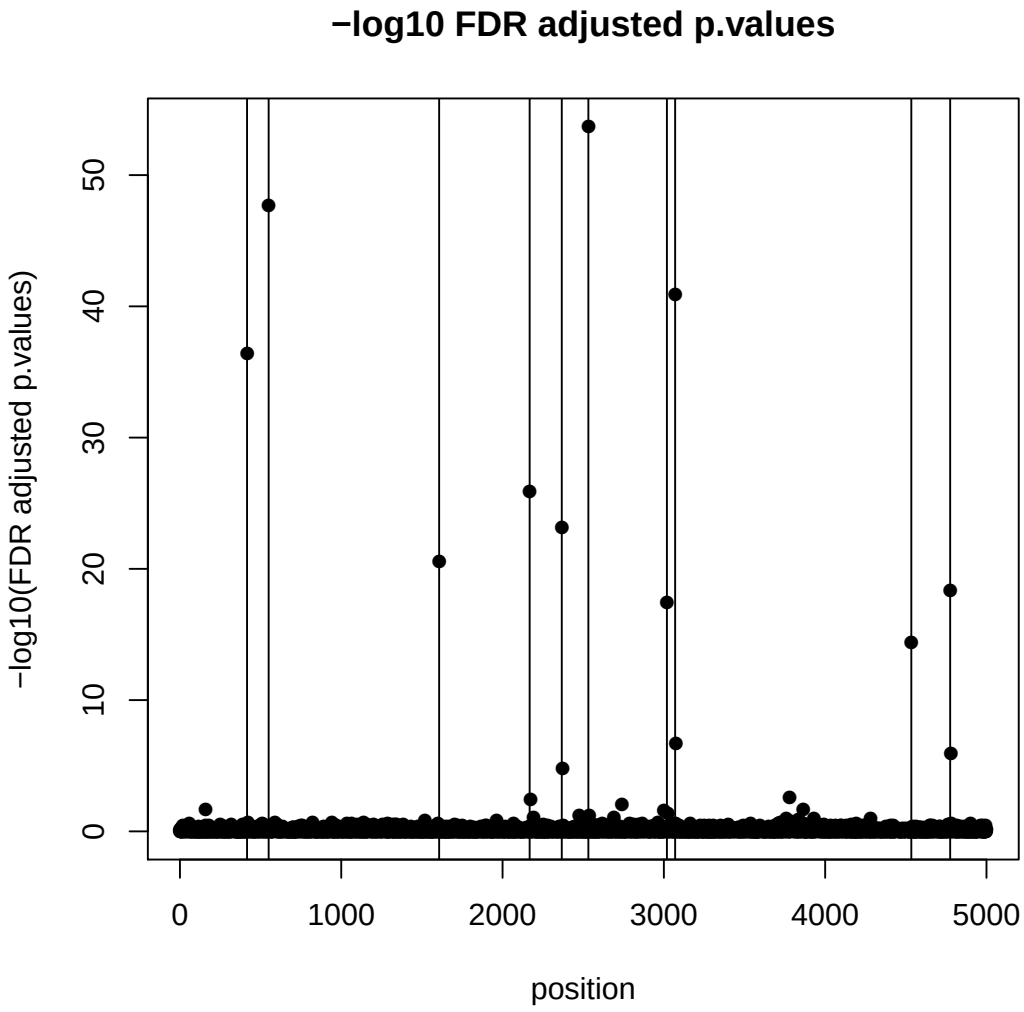


Figure 19: *Lambda FDR Adjusted P-values by Position* - For approximately 5000 bases across the Lambda genome we plot $-\log_{10} p$-values by position. Vertical lines indicate GATC positions in the genome.

4 Conclusion

This document attempts to provide a user with an overview of PacBio data and APIs which support performing Kinetics analyses. This document is an attempt to present the analyst with a starting point for their kinetics analyses using Pacific Biosciences data. This document is a "live" document in the sense that we will be updating the tools presented here for more complicated analyses.

There are a number of enhancements that can be conceived of in the two-sample comparison case. First, one might prefer a permutation test when comparing the native vs. WGA samples. In the "utils.R" file there exists an example of such a procedure. One might prefer an empirical Bayes approach when the number of reads is low. Finally, one might wish to investigate whether taking into account the modification signature rather than just the individual IPD provides a substantially more powerful test than just the individual position. We look forward to work with the bioinformatics community to develop implementations of these and other improvements.

A Session Info

Here we give information about the version of R and installed packages used to generate this document.

```
> sessionInfo()

R version 2.13.1 Patched (2011-09-13 r57007)
Platform: x86_64-unknown-linux-gnu (64-bit)

locale:
 [1] LC_CTYPE=en_US.UTF-8       LC_NUMERIC=C
 [3] LC_TIME=en_US.UTF-8        LC_COLLATE=en_US.UTF-8
 [5] LC_MONETARY=C              LC_MESSAGES=en_US.UTF-8
 [7] LC_PAPER=en_US.UTF-8       LC_NAME=C
 [9] LC_ADDRESS=C               LC_TELEPHONE=C
[11] LC_MEASUREMENT=en_US.UTF-8 LC_IDENTIFICATION=C

attached base packages:
[1] grid      stats     graphics  grDevices utils     datasets  methods
[8] base

other attached packages:
 [1] Biostrings_2.20.3 IRanges_1.10.6    ggplot2_0.8.9     proto_0.3-9.2
 [5] reshape_0.8.4     plyr_1.6          xtable_1.5-6      pbutils_1.0
 [9] pbh5_1.1          h5r_1.2

loaded via a namespace (and not attached):
[1] digest_0.5.0 tools_2.13.1
```