sequenza usage example

Francesco Favero[*], Aron C. Eklund

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[*]favero@cbs.dtu.dk

1 Abstract

Deep sequence of tumor DNA along with corresponding normal DNA can provide a rich picture of the mutations and aberrations that characterize the tumor. However, analysis of this data can be impeded by of tumor cellularity and heterogeneity and by unwieldy data. Here we describe the *sequenza* software system, which comprises a fast python-based pre-processor and an R-based analysis package. Sequenza enables the efficient estimation of tumor cellularity and ploidy, and generation of copy number, loss-of-heterozygosity, and mutation frequency profiles.

This document details a typical analysis of matched tumor-normal exome sequence data using *sequenza*.

2 Minimum requirements

Software: R Operating system: Linux, OSX, Windows, ... (any that runs R) Memory: Minimum 1GB of RAM. Recommended >2Gb. Disk space: ? times the size of total data

R version Bioconductor version ? Python version and modules

3 Getting ready with Sequenza package/Installing R/Setting up Sequenza

—– download from bitbucket/cbs.dtu.dk —– how to install it. R CMD INSTALL sequenza_version.tar.gz

—– Copy sequenza utils to some location ? —– Setting PATH ?

A typical workflow by Sequenza is as follow : 1. Convert pileup to abfreq 2. GC normalization 3. Obtain depth ratio and B allele frequencies 4. Allele-specific segmentation 5. Infer cellularity and DNA-index by model fitting 6. Call CNAs(CNVs ?) and mutations

4 Preparing inputs for Sequenza

In order to obtain precise mutational and aberration patterns in a tumor sample, Sequenza requires a matched normal sample from the same patient. In short, the following files are needed to get started with Sequenza.

1. A pileup file from the tumor specimen 2. A pileup file from the normal specimen 3. A FASTA reference genomic sequence file (optional, for GC-content correction)

We recommend using preprocessed and quality filtered BAM files to obtain mpileup calls for both samples.

Pileup files can be generated using `samtools` (ref). The genome sequence file can be obtained from (url). samtools mpileup -f hg19.fasta -Q 20 normal.bam samtools mpileup -f hg19.fasta -Q 20 tumor.bam

5 First the non-R part: preprocessing data

For convenience and efficiency we have implemented preprocessing algorithms in an external (not called from R) Python program. The program is provided with the package; it's exact location can be found like this:

```
> system.file("exec", "sequenza-utils.py", package="sequenza")
```

```
[1] ""
```

You may wish to copy this program to a location on your path. NOTE: this script requires several UNIX tools and thus probably not work on Windows (HOW ABOUT CYGWIN ?).

Extract average GC content in 50-base genomic windows:

```
    #  sequenza-utils.py GC-windows -w 50 hg19.fa | gzip > hg19.gc50Base.txt.gz
```

Process the two pileup files to obtain an "abfreq" file containing alleles and mutation frequency.

```
    #  sequenza-utils.py pileup2tab -gc hg19.gc50Base.txt.gz -r 0001-normal_blood.pileup.gz
-s 0001-met2.pileup.gz -q 20 -n 10 -o 0001-met2.abfreq.txt.gz
```

—– UPDATE ME UPDATE ME UPDATE ME UPDATE ME UPDATE ME UPDATE ME —–

6 Read the preprocessed data (*abfreq* file) into R

The remainder of this example takes place in R.

Load the sequenza package:

```
> library("sequenza")
```

Find the example data file:

```
> data.file <-  system.file("data", "abf.data.abfreq.txt.gz", package = "sequenza")
> data.file
```

```
[1] "/usr/local/Cellar/r/3.0.1/R.framework/Versions/3.0/Resources/library/sequenza/dat
```

The abfreq file can be read all at once, but processing one chromosome at a time is less demanding on computational resources and might be preferable. (Note that the demo data included with sequenza is only chromosome 1)

Read only the data corresponding to chromosome 1:

```
> abf.data <- read.abfreq(data.file, chr.name = "1")
```

Alternatively, read all data at once (not run):

```
> abf.data <- read.abfreq(data.file)
```

```
> str(abf.data)
```

```
'data.frame':        5349 obs. of  13 variables:
 $ chromosome               : Factor w/ 1 level "1": 1 1 1 1 1 1 1 1 1 1 ...
 $ n.base                   : int  13116 13118 13327 881918 884091 884101 900298 9005
 $ base.ref                 : Factor w/ 5 levels "A","C","G","N",..: 5 1 3 3 2 1 2 1
 $ depth.normal             : int  53 51 48 55 85 76 108 106 31 41 ...
 $ depth.sample             : int  33 33 27 37 65 59 78 72 14 16 ...
 $ depth.ratio              : num  0.623 0.647 0.563 0.673 0.765 0.776 0.722 0.679 0.
 $ Af                       : num  0.645 0.606 0.577 0.514 0.597 0.558 0.5 0.564 0.38
 $ Bf                       : num  0.355 0.394 0 0.486 0.387 0.442 0.5 0.436 0 0.4 ..
 $ ref.zygosity             : Factor w/ 2 levels "het","hom": 1 1 2 1 1 1 1 1 2 1 ...
 $ GC.percent               : num  58 58 60 64 70 58 70 66 82 64 ...
 $ sample.reads.above.quality: num  0.94 1 0.96 1 0.95 0.88 0.92 0.54 0.93 0.94 ...
 $ AB.germline              : Factor w/ 10 levels "A","AC","AG",..: 9 3 8 3 6 2 6 2 8
 $ AB.sample                : Factor w/ 64 levels ".","A0.004:C0.623",..: 1 1 23 1 1
```

7 Quality control step? (EXPLAIN)

Each nucleotide aligned in the sequencing is associate with a quality score. The *sequenza-utils* software is capable of filtering the base with the quality lower then a specified value (default is 20), and returns the rate of reads that have passed the filter in the column *sample.reads.above.quality*, while the *depth.sample* column contains the raw depth calculated in the pileup (from samtools). The product of the rate of bases that have passed the quality check and the total amount or reads aligned at the same nucleotide return the number of reads that have passed the quality check.

```
> abf.data$good.s.reads <- abf.data$depth.sample *
+                          abf.data$sample.reads.above.quality
```

8 GC-normalization

The number of reads at a given genomic position can be affected by the local GC content. We attempt to remove this bias as in (ref).

It is possible to gather gc-content information from the entire file (normally this would be the entire genome, but in our example it contains only chromosome 1):

```
> gc.stats <- gc.sample.stats(data.file)
```

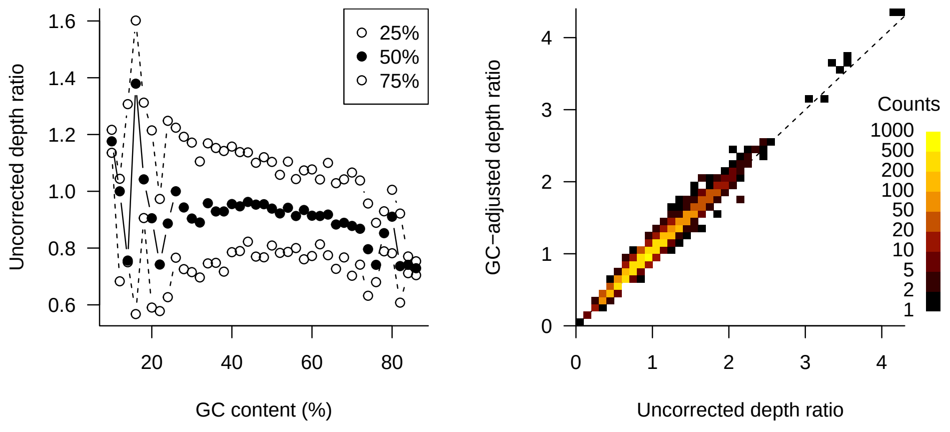
Or alternatively, it is possible to collect the GC-contents information from an object loaded in the environment.

```
> gc.stats <- gc.norm(ratio = abf.data$depth.ratio,
+                     gc = abf.data$GC.percent)
```

Calculate the GC-normalized depth ratio:

```
> gc.vect  <- setNames(gc.stats$raw.mean, gc.stats$gc.values)
> abf.data$adjusted.ratio <- abf.data$depth.ratio /
+                            gc.vect[as.character(abf.data$GC.percent)]

> par(mfrow = c(1,2), cex = 1, las = 1, bty = 'l')
> matplot(gc.stats$gc.values, gc.stats$raw,
+         type = 'b', col = 1, pch = c(1, 19, 1), lty = c(2, 1, 2),
+         xlab = 'GC content (%)', ylab = 'Uncorrected depth ratio')
> legend('topright', legend = colnames(gc.stats$raw), pch = c(1, 19, 1))
> hist2(abf.data$depth.ratio, abf.data$adjusted.ratio,
+       breaks = prettyLog, key = vkey, panel.first = abline(0, 1, lty = 2),
+       xlab = 'Uncorrected depth ratio', ylab = 'GC-adjusted depth ratio')
```



9 Create genomic profiles

9.1 First, the depth ratio

Summarize the depth ratio by binning the data in overlapping genomic windows:

```
> abf.r.win <- windowValues(x = abf.data$adjusted.ratio,
+                positions = abf.data$n.base,
+                chromosomes = abf.data$chromosome,
+                window = 1e6, overlap = 1,
+                weight = abf.data$depth.normal)
```

```
> plotWindows(abf.r.win[[1]], log2.plot = FALSE,
+               ylab = "Depth ratio", xlab = "Position (bases)",
+               main = names(abf.r.win)[1], las = 1, n.min = 1,
+               ylim = c(0, 2.5))
```

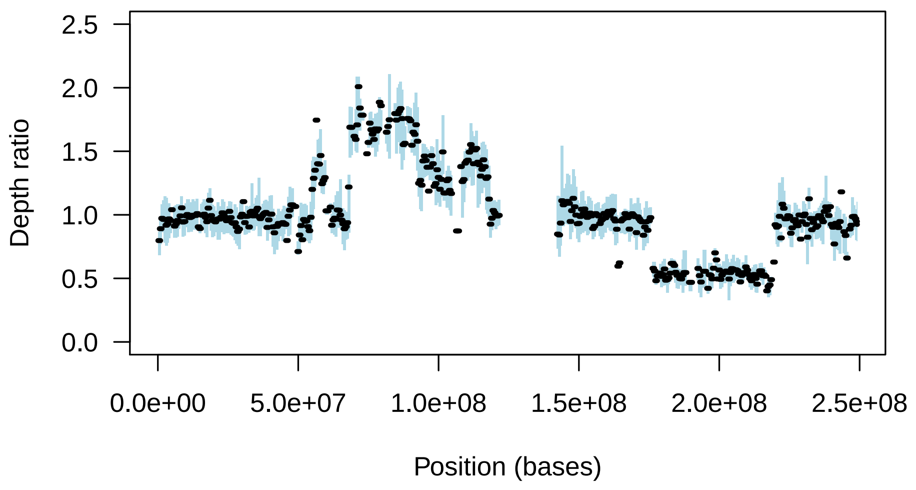


Figure 1: Depth ratio profile visualization over a single chromosome.

9.2 Next, the B-allele frequencies

The column *ref.zygosity* contains the zygosity derived from the germline sample. the possible values are *het* for heterozygous positions and *hom* for homozygous positions.

```
> abf.hom  <- abf.data$ref.zygosity == 'hom'
> abf.het  <- abf.data[!abf.hom, ]
```

Summarize the BAF by binning the data in overlapping genomic windows (including only those positions called heterozygous in the normal sample):

```
> abf.b.win <- windowValues(x = abf.het$Bf,
+                 positions = abf.het$n.base,
+                 chromosomes = abf.het$chromosome,
+                 window = 1e6, overlap = 1,
+                 weight = round(x = abf.het$good.s.reads, digits = 0))
```

```
> plotWindows(abf.b.win[[1]], ylim = c(0, 0.5),
+               main = names(abf.r.win)[1], xlab = "Position (bases)",
+               ylab = "B allele frequency", n.min = 10)
```

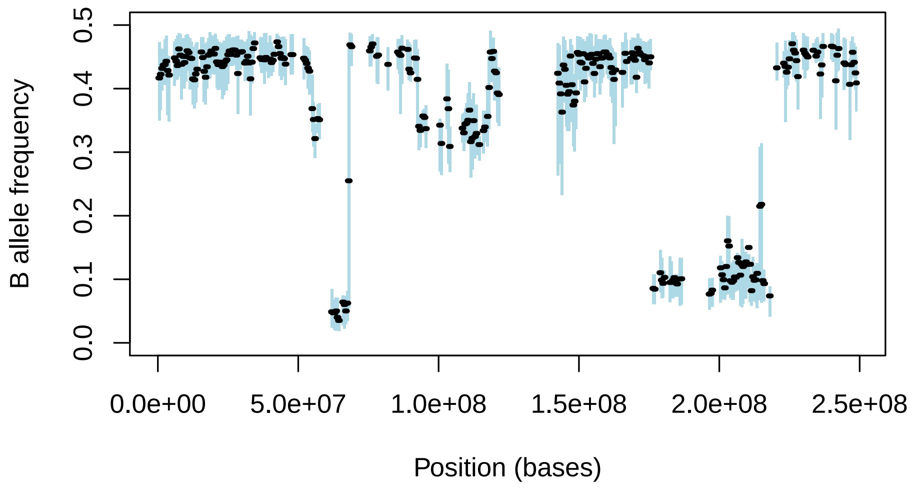


Figure 2: B-allele frequency profile visualization over a single chromosome.

10 Allele-specific segmentation

10.1 Find genomic breakpoints

To find breakpoints we use the allele-specific segmentation algorithm from the *copynumber* package [1].

```
> breaks <- find.breaks(abf.het, gamma = 80, kmin = 10, baf.thres = c(0, 0.5))
> head(breaks)
```

```
  chrom start.pos   end.pos
1     1     13116  17013363
2     1  17013750  55100328
3     1  55183366  60381491
4     1  61743160  67960720
5     1  68151685  92445257
6     1  92568263 118165328
```

Now obtain the segment values:

```
> seg.s1 <- segment.breaks(abf.data, breaks = breaks)
```

11 Select mutations by mutation frequency

I the genotype file (the *abfreq* file) the mutation are detected as homozygous position with a decreased frequency of the germline nucleotide. A set of nucleotide not present in the germline is present with the relative frequency in the column *AB.sample*. Being a frequency derived by the number of reads covering the position, the accuracy of the measurement is depending on the depth in the considered position. In order to filter the mutations the function *mutation.table* allow to filter the present mutation to a define level of frequency, a desired number of reads depth, and a desired number of mutated nucleotide per position. Additionally it is possible to swap the *adjusted.ratio* column with the corresponding value after segmentation.

```
> mut.tab <- mutation.table(abf.data, mufreq.treshold = 0.15,
+                           min.reads = 40, max.mut.types = 1,
+                           min.type.freq = 0.9, segments = seg.s1)
```

However it is optional, without providing the segmented data the *adjusted.ratio* would remains unchanged.

```
> mut.tab.no.seg <- mutation.table(abf.data, mufreq.treshold = 0.15,
+                           min.reads = 40, max.mut.types = 1,
+                           min.type.freq = 0.9)
```

```
> dim(mut.tab)
```

```
[1] 22  7
```

```
> head(mut.tab)
```

```
    chromosome   n.base GC.percent good.s.reads adjusted.ratio     F mutation
286          1 10436585         50       207.58      0.9982462 0.382      C>T
496          1 13111750         44        48.02      0.9982462 0.417      A>C
```

```
637           1 15821826          54       497.97      0.9982462 0.398      G>T
1077          1 19983391          72        77.08      0.9813842 0.558      G>C
1364          1 26878353          60        50.00      0.9813842 0.520      C>A
1504          1 32627966          54       120.78      0.9813842 0.413      C>T
```

```
> head(mut.tab.no.seg)
```

```
     chromosome   n.base GC.percent good.s.reads adjusted.ratio     F mutation
286           1 10436585          50       207.58      1.0785423 0.382      C>T
496           1 13111750          44        48.02      1.2353979 0.417      A>C
637           1 15821826          54       497.97      0.8704422 0.398      G>T
1077          1 19983391          72        77.08      0.9201950 0.558      G>C
1364          1 26878353          60        50.00      1.2760504 0.520      C>A
1504          1 32627966          54       120.78      1.0526037 0.413      C>T
```

12 Plot chromosome view with mutations, BAF, depth ratio and segments

```
> chromosome.view(mut.tab = mut.tab, baf.windows = abf.b.win[[1]],
+                 ratio.windows = abf.r.win[[1]], min.N.ratio = 1,
+                 segments = seg.s1, main = "Chromosome 1")
```

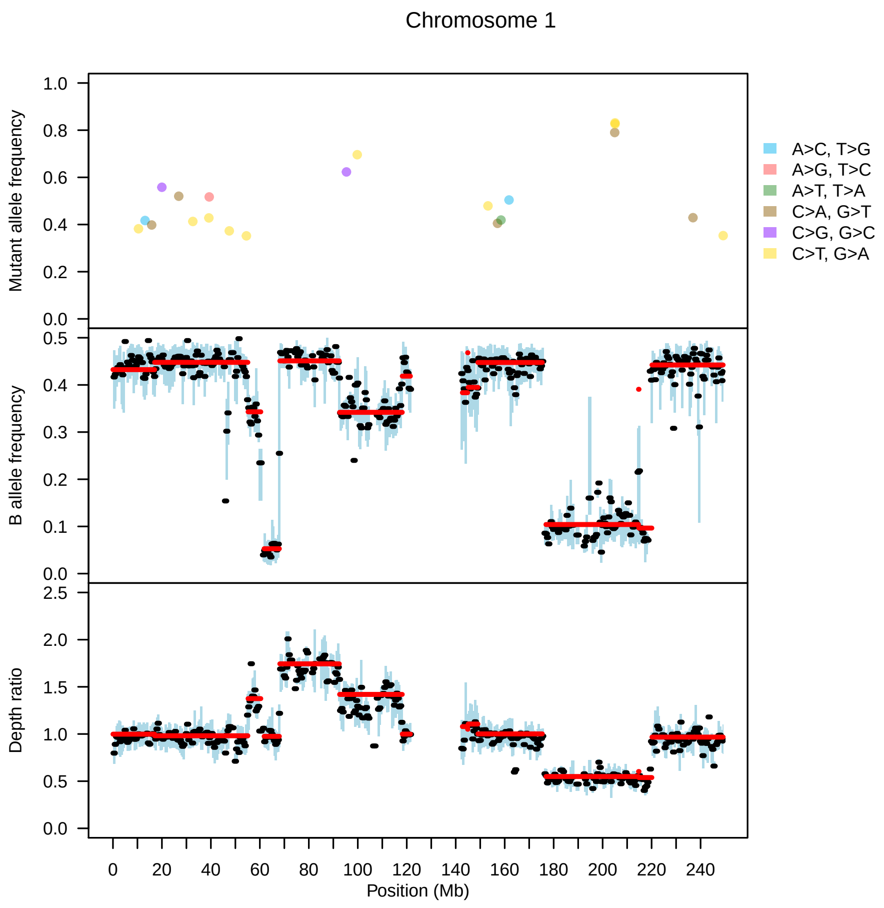


Figure 3: Plots of Mutation (top), B-allele frequencies (middle) and depth ratio (bottom) for chromosome position.

13 Inference of cellularity and DNA-index

NEEDS EXPLANATION:

```
> seg.filtered <- seg.s1[(seg.s1$end.pos - seg.s1$start.pos) > 5e6, ]
```

NEEDS EXPLANATION:

```
> weights.seg  <- 150 + round((seg.filtered$end.pos - seg.filtered$start.pos) / 1e6,
```

```
> avg.depth.ratio <- mean(gc.stats$adj[,3])
> avg.depth.ratio
```

```
[1] 1.202572
```

I DON'T UNDERSTAND WHY "avg.depth.ratio = 1" when we have calculated a different number above?:

```
> CP <- baf.model.fit(Bf = seg.filtered$Bf, depth.ratio = seg.filtered$depth.ratio,
+                     weight.ratio = weights.seg,
+                     weight.Bf = weights.seg,
+                     avg.depth.ratio = 1,
+                     cellularity = seq(0.1,1,0.01),
+                     dna.index = seq(0.5,3,0.05), mc.cores = 4)
```

WOULD IT MAKE MORE SENSE FOR THIS FUNCTION TO RETURN A MATRIX?

NEED EXPLANATIONS HERE:

```
> cint <- get.ci(CP)
> cp.plot(CP)
> cp.plot.contours(CP, add = TRUE, likThresh = c(0.5, 0.75, 0.95, 0.99))
```

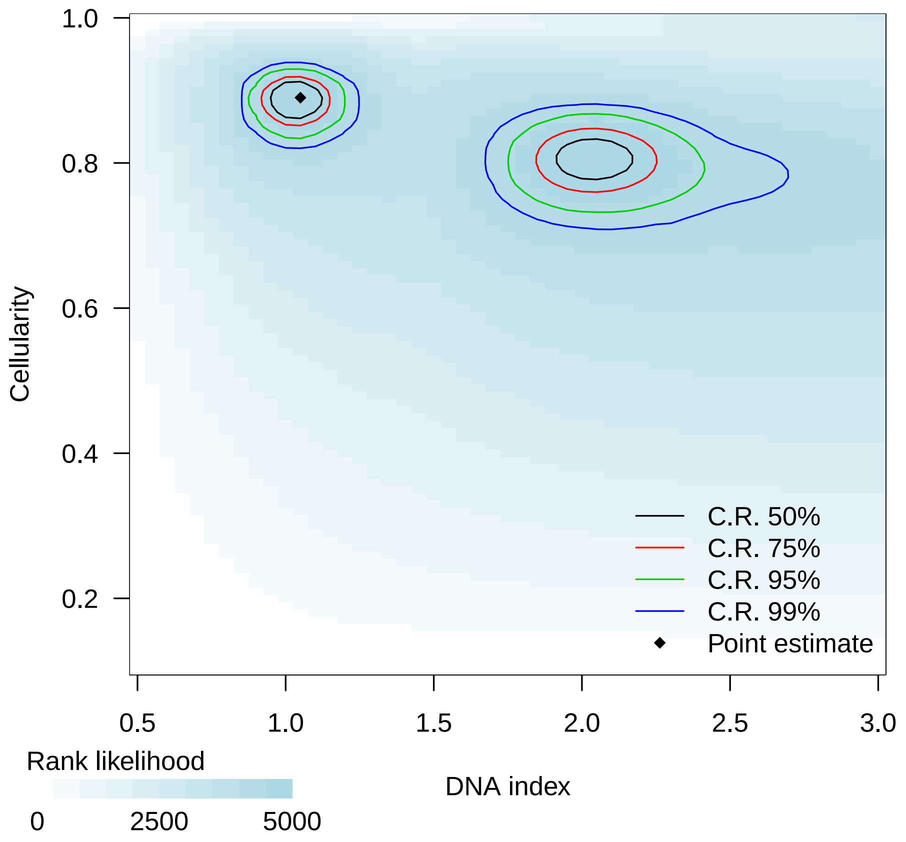


Figure 4: Result from the Bayesian inference over the defined range of cellularity and DNA-index. The color indicates the log-likelihood of the corresponding cellularity/DNA-index values.

```
> par(mfrow = c(2,2))
> cp.plot(CP)
> plot(cint$values.y, ylab = "Cellularity",
+      xlab = "likelihood", type = "n")
> select <- cint$confint.y[1] <= cint$values.y[,2] & cint$values.y[,2] <= cint$confi
> polygon(y = c(cint$confint.y[1], cint$values.y[select, 2], cint$confint.y[2]),
+         x = c(0, cint$values.y[select, 1], 0), col='red', border=NA)
> lines(cint$values.y)
> abline(h = cint$max.y, lty = 2, lwd = 0.5)
> plot(cint$values.x, xlab = "DNA index",
+      ylab = "likelihood", type = "n")
> select <- cint$confint.x[1] <= cint$values.x[,1] & cint$values.x[,1] <= cint$confi
> polygon(x = c(cint$confint.x[1], cint$values.x[select, 1], cint$confint.x[2]),
+         y = c(0, cint$values.x[select, 2], 0), col='red', border=NA)
> lines(cint$values.x)
> abline(v = cint$max.x, lty = 2, lwd = 0.5)
>
```

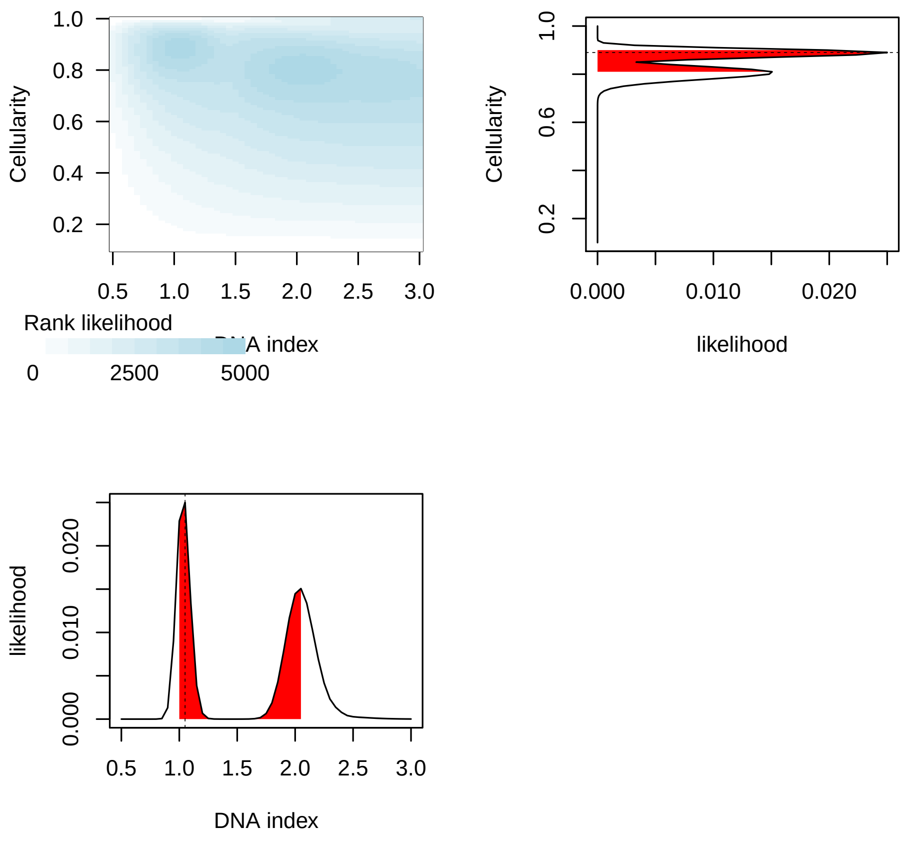


Figure 5: Plot of the log likelihood with respective cellularity and DNA-index probability distribution and confidence intervals.

14 Call CNVs and mutations using the estimated parameters

```
> cellularity <- cint$max.y
> cellularity

[1] 0.89

> dna.index <- cint$max.x
> dna.index

[1] 1.05
```

14.1 Detect mutated alleles

```
> mut.tab.clean <- na.exclude(mut.tab)
> mut.alleles <- mufreq.bayes(mufreq = mut.tab.clean$F, depth.ratio = mut.tab.clean$
+                              cellularity = cellularity, dna.index = dna.index,
+                              avg.depth.ratio = 1)
> head(mut.alleles)

   CNr CNt Mt         L
4    2   2  1 -13.67293
41   2   2  1 -12.63039
42   2   2  1 -13.03269
43   2   2  1 -16.28677
44   2   2  1 -14.09548
45   2   2  1 -12.80203

> head(cbind(mut.tab.clean[,c("chromosome","n.base","F","adjusted.ratio", "mutation"

     chromosome   n.base     F adjusted.ratio mutation CNr CNt Mt         L
286           1 10436585 0.382      0.9982462      C>T   2   2  1 -13.67293
496           1 13111750 0.417      0.9982462      A>C   2   2  1 -12.63039
637           1 15821826 0.398      0.9982462      G>T   2   2  1 -13.03269
1077          1 19983391 0.558      0.9813842      G>C   2   2  1 -16.28677
1364          1 26878353 0.520      0.9813842      C>A   2   2  1 -14.09548
1504          1 32627966 0.413      0.9813842      C>T   2   2  1 -12.80203
```

14.2 Detect Copy number variation

```
> cn.alleles <- baf.bayes(Bf = seg.s1$Bf, depth.ratio = seg.s1$depth.ratio,
+                          cellularity = cellularity, dna.index = dna.index,
+                          avg.depth.ratio = 1)
> seg.s1.cn <- cbind(seg.s1, cn.alleles)
> head(seg.s1.cn)

  chromosome start.pos   end.pos         Bf N.BAF depth.ratio N.ratio CNt A B
1          1     13116  17013363 0.43238002   854   0.9982462     866   2 1 1
2          1  17013750  55100328 0.44782729  1056   0.9813842    1072   2 1 1
3          1  55183366  60381491 0.34292696    68   1.3751212      68   3 2 1
4          1  61743160  67960720 0.05276295   120   0.9751264     122   2 2 0
5          1  68151685  92445257 0.45096790   262   1.7436966     265   4 2 2
6          1  92568263 118165328 0.34186189   348   1.4197246     355   3 2 1
          L
1 -12.06924
2 -11.74751
3 -11.54012
4 -10.48264
5 -11.65910
6 -11.56429
```

15 Visualize detected copy number

```
> chromosome.view(mut.tab = mut.tab, baf.windows = abf.b.win[[1]],
+                 ratio.windows = abf.r.win[[1]],  min.N.ratio = 1,
+                 segments = seg.s1.cn, main = "Chromosome 1",
+                 cellularity = cellularity, dna.index = dna.index,
+                 avg.depth.ratio = 1)
```

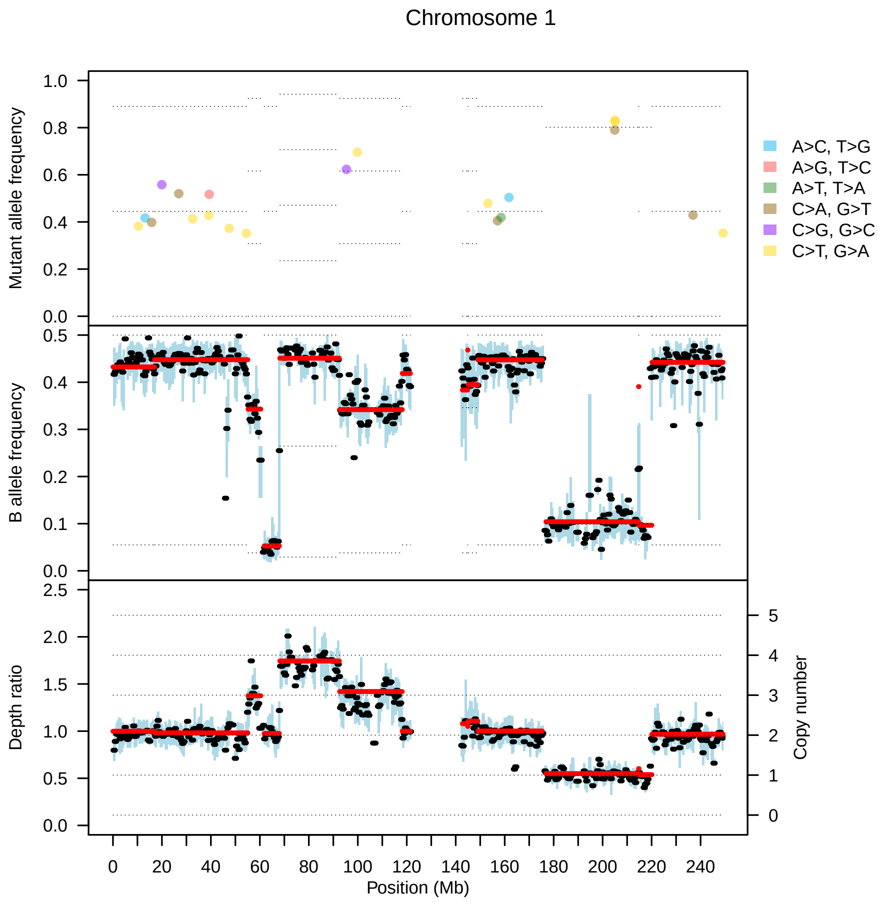


Figure 6: Plots of Mutation (top), B-allele frequencies (middle) and depth ratio (bottom) for chromosome position. Horizontal dotted line indicate different copy number/allelic state.

References

[1] Gro Nilsen, Knut Liestø l, Peter Van Loo, Hans Kristian Moen Vollan, Marianne B Eide, Oscar M Rueda, Suet-Feung Chin, Roslin Russell, Lars O Baumbusch, Carlos

Caldas, Anne-Lise Bø rresen Dale, and Ole Christian Lingjaerde. Copynumber: Efficient algorithms for single- and multi-track copy number segmentation. *BMC genomics*, 13:591, January 2012.