

# sequenza usage example

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## 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 Starting data

Sequenza requires: 1. A pileup file from the tumor specimen 2. A pileup file from the normal specimen 3. A FASTA genome sequence file (optional, for GC-content correction)

Pileup files can be generated using `samtools` (ref). The genome sequence file can be obtained from (url).

## 3 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.

Extract average GC content in 50-base genomic windows:

```
# abfreqtools.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.

```
# abfreqtools.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 UP-  
DATE ME —

## 4 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.zygotity    : 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
```

## 5 Quality control step? (EXPLAIN)

NEED TO EXPLAIN THIS:

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

## 6 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).

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

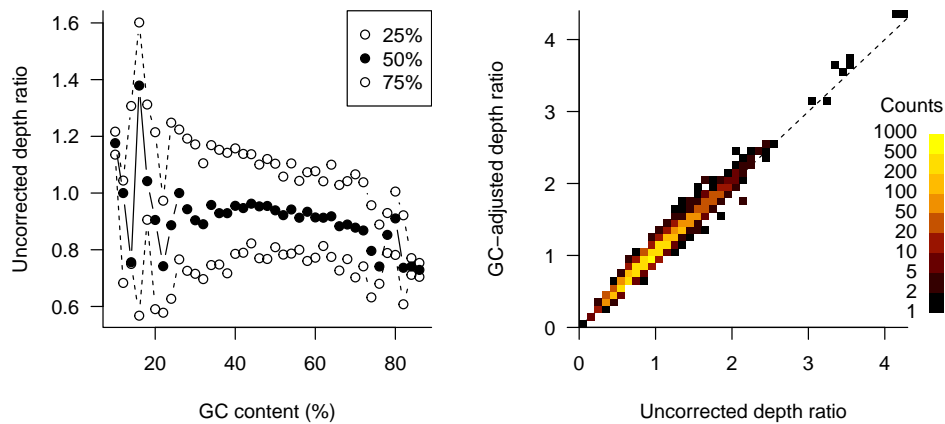
```
> gc.stats <- gc.sample.stats(data.file)
```

WHAT IS THIS? EXPLAIN... (not run)

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

Calculate the GC-adjusted 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')
```



## 7 Create genomic profiles

### 7.1 First, the depth ratio

Summarize the depth ratio 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))
```

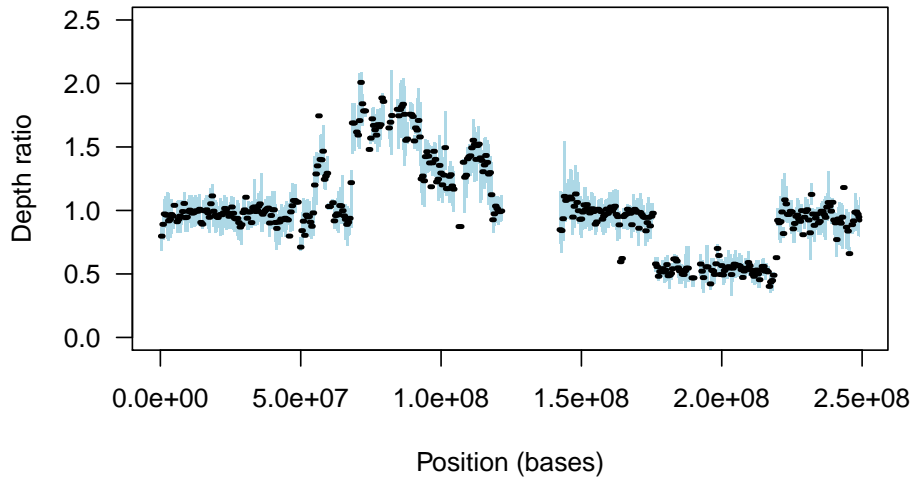


Figure 1: Depth ratio profile visualization over a single chromosome.

## 7.2 Next, the B-allele frequencies

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

Summarize the BAF 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)
```

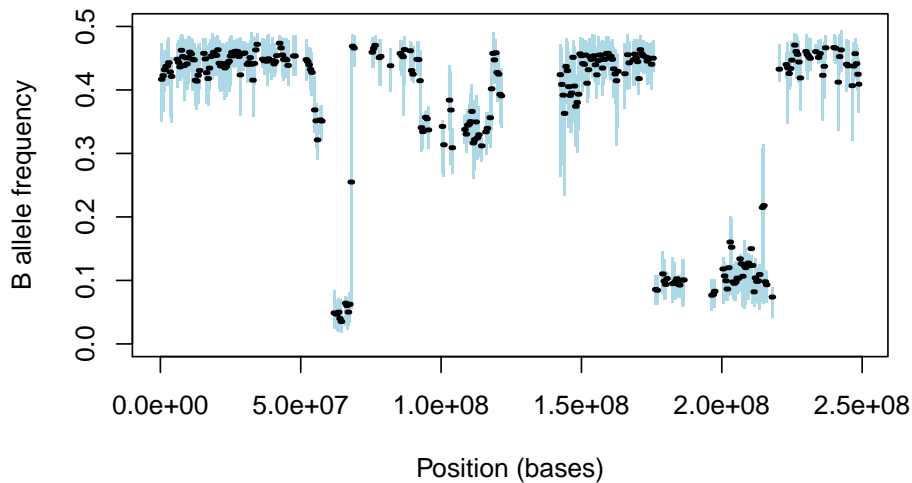


Figure 2: B-allele frequency profile visualization over a single chromosome.

## 8 Allele-specific segmentation

### 8.1 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)
```

## 9 Select mutations by mutation frequency

```
> 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)
```

NEEDS EXPLANATION HERE... what is the following object?

```
> 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



## 10 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")
```

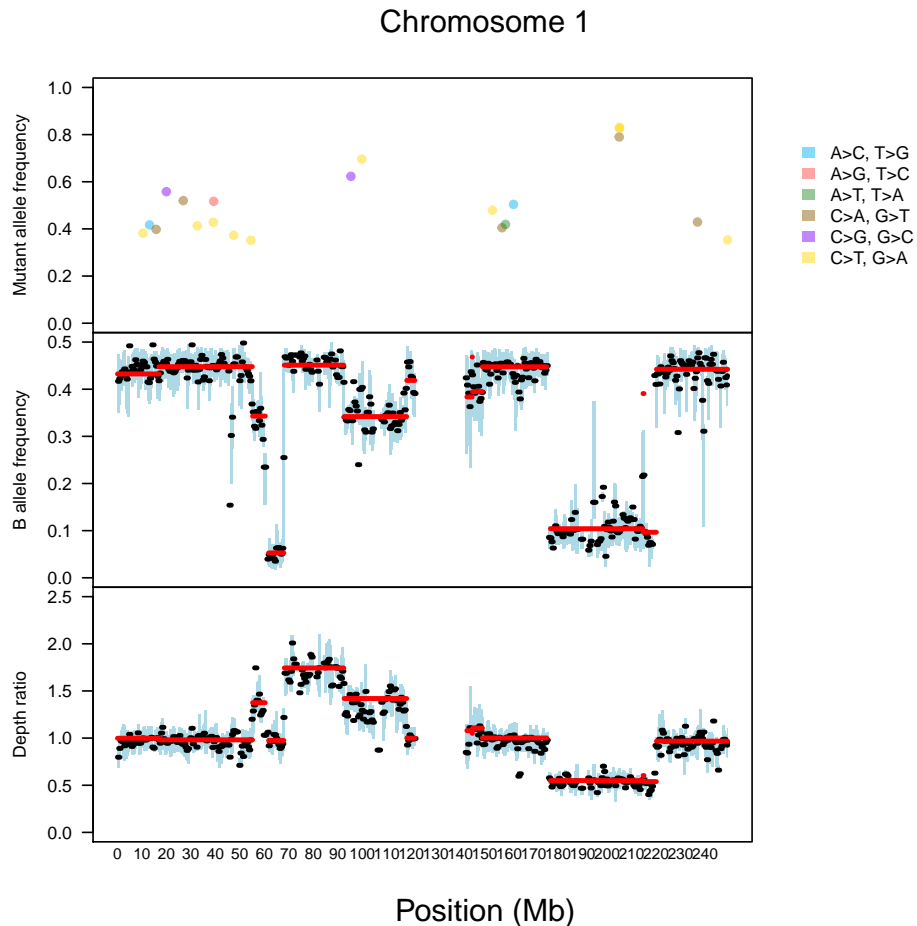


Figure 3: Plots of Mutation (top), B-allele frequencies (middle) and depth ratio (bottom) for chromosome position.

## 11 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))
```

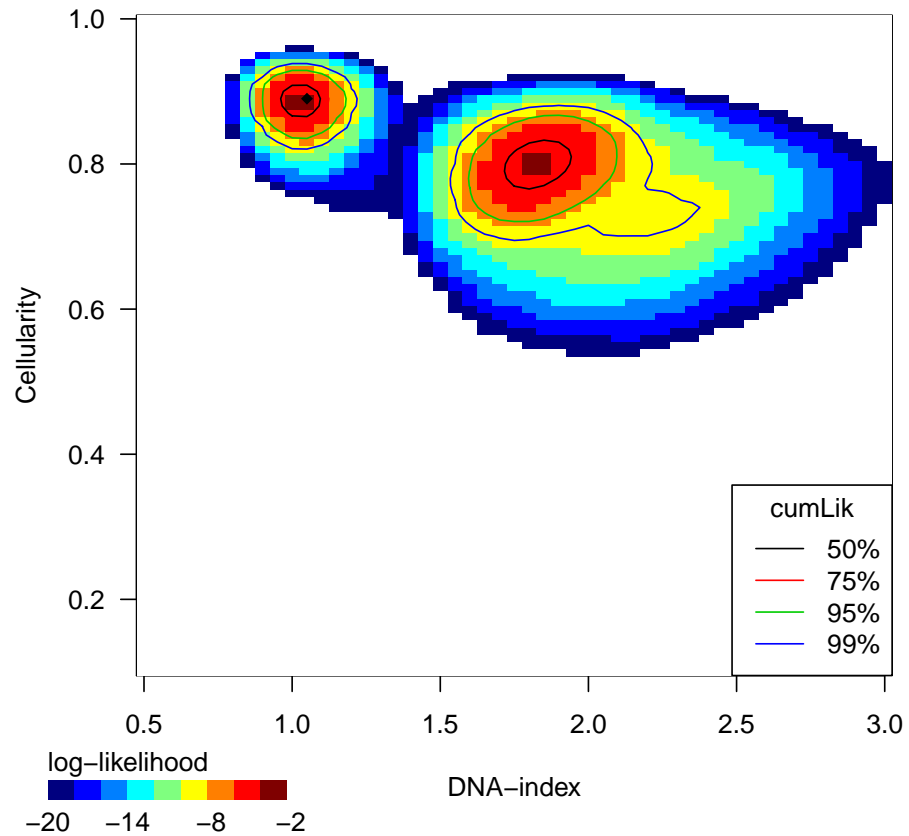


Figure 4: Result from the Bayesian inference over the defined range of cellularity and DNA-index. The color indicates the log-likelihood of the corresponding cellularity/DNA-index values.

```

> 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)
>

```

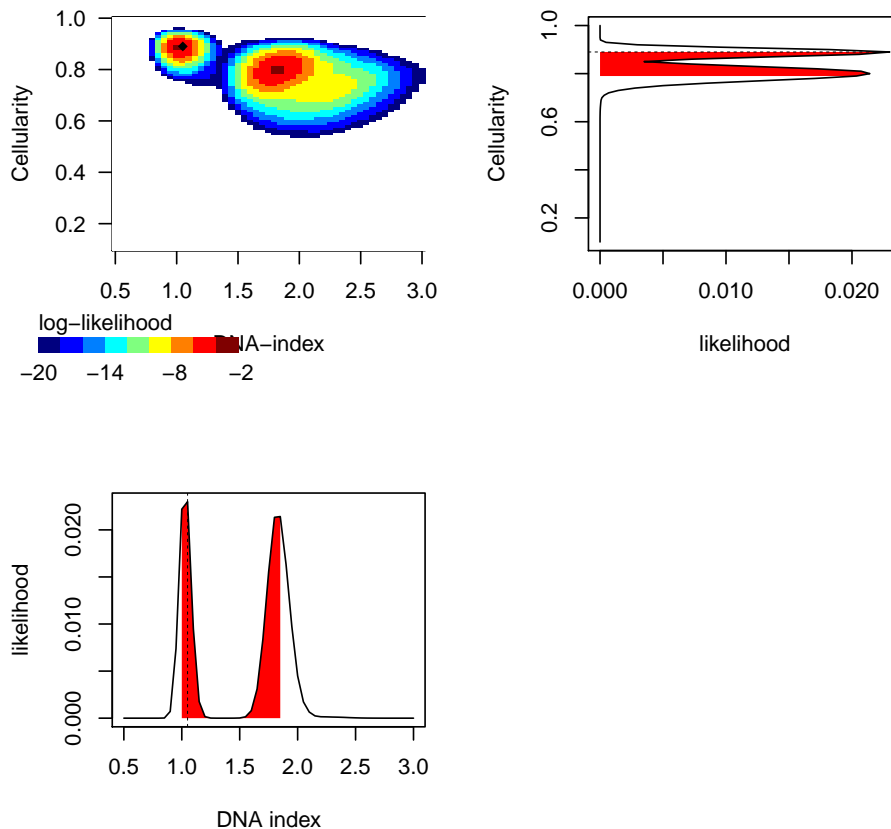


Figure 5: Plot of the log likelihood with respective cellularity and DNA-index probability distribution and confidence intervals.

## 12 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
```

### 12.1 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	-13.68204
41	2	2	1	-12.63950
42	2	2	1	-13.04180
43	2	2	1	-16.29588
44	2	2	1	-14.10459
45	2	2	1	-12.81114

```
> 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	-13.68204
496	1	13111750	0.417	0.9982462	A>C	2	2	1	-12.63950
637	1	15821826	0.398	0.9982462	G>T	2	2	1	-13.04180
1077	1	19983391	0.558	0.9813842	G>C	2	2	1	-16.29588
1364	1	26878353	0.520	0.9813842	C>A	2	2	1	-14.10459
1504	1	32627966	0.413	0.9813842	C>T	2	2	1	-12.81114

### 12.2 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 -12.07834
2 -11.75661
3 -11.54033
4 -10.48384
5 -11.65777
6 -11.56998

```

## 13 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)
```

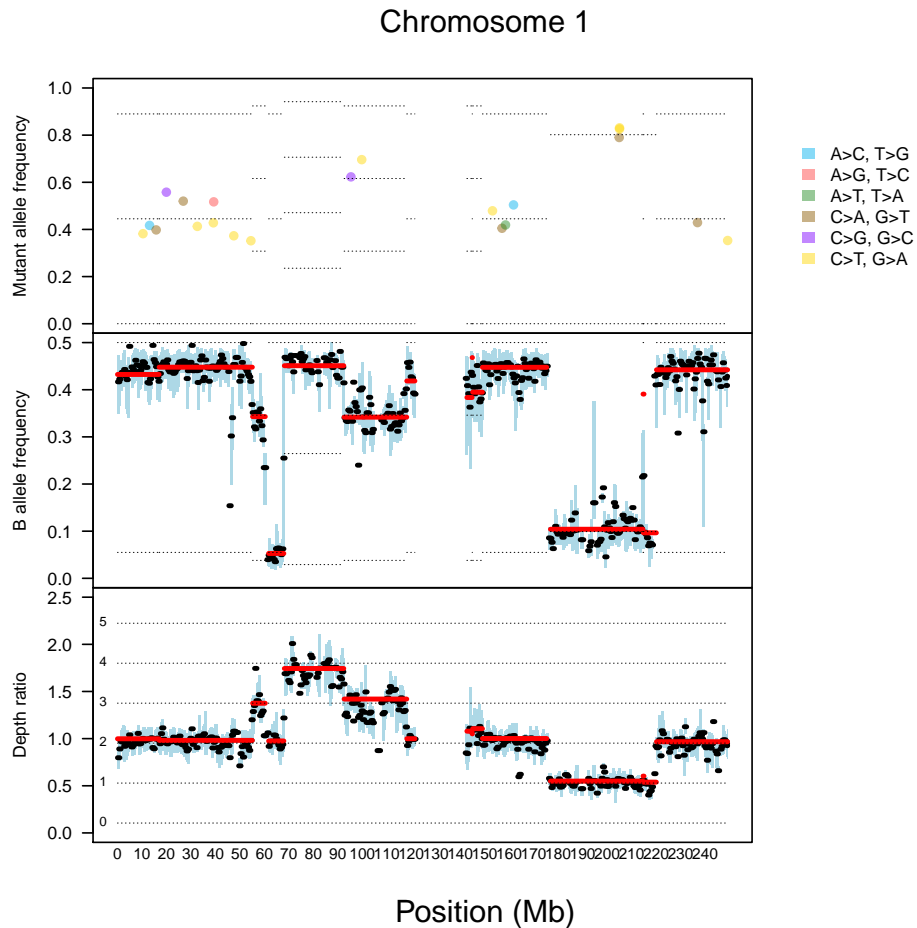


Figure 6: Plots of Mutation (top), B-allele frequencies (middle) and depth ratio (bottom) for chromosome position. Horizontal dotted line indicate different copy number/allelic state.

## References

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

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