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 UPDATE 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] "/Library/Frameworks/R.framework/Versions/3.0/Resources/library/sequenza/data/abf.
```

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
```

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)
```

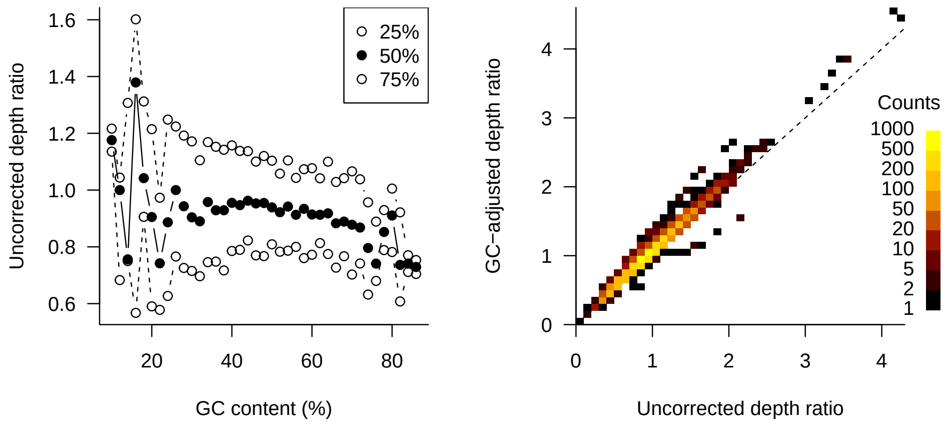
WHAT IS THIS? EXPLAIN... (not run)

```
> 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$raw[,2], gc.stats$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))
```

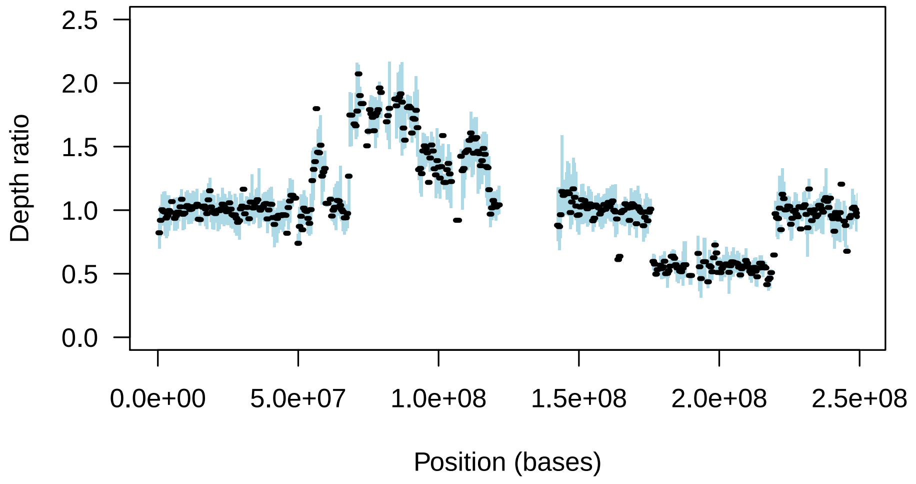


Figure 1: Depth ratio profile visualization over a single chromosome.

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)
```

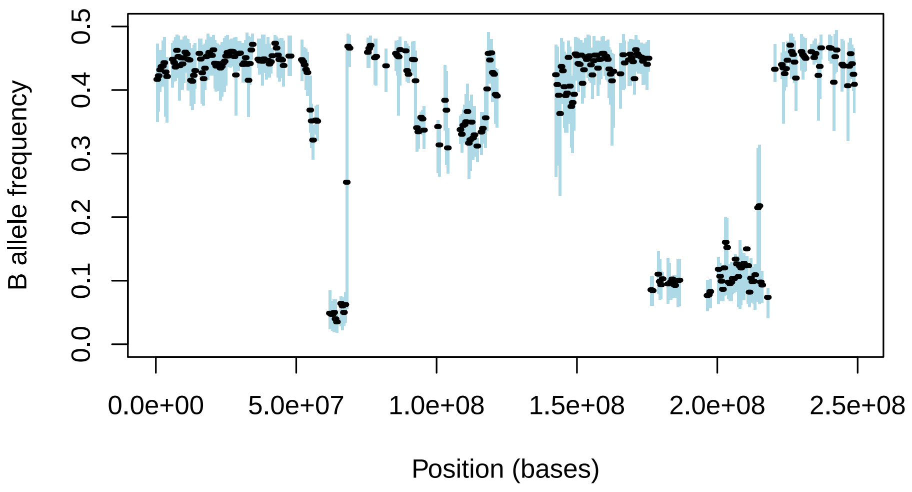


Figure 2: B-allele frequency profile visualization over a single chromosome.

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       1.028167 0.382      C>T
496           1 13111750         44        48.02       1.028167 0.417      A>C
637           1 15821826         54       497.97       1.028167 0.398      G>T
1077          1 19983391         72        77.08       1.012928 0.558      G>C
1364          1 26878353         60        50.00       1.012928 0.520      C>A
1504          1 32627966         54       120.78       1.012928 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      1.1118211 0.382      C>T
496           1 13111750         44        48.02      1.2727273 0.417      A>C
637           1 15821826         54       497.97      0.8927813 0.398      G>T
1077          1 19983391         72        77.08      0.9354839 0.558      G>C
1364          1 26878353         60        50.00      1.3019694 0.520      C>A
1504          1 32627966         54       120.78      1.0796178 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")
```

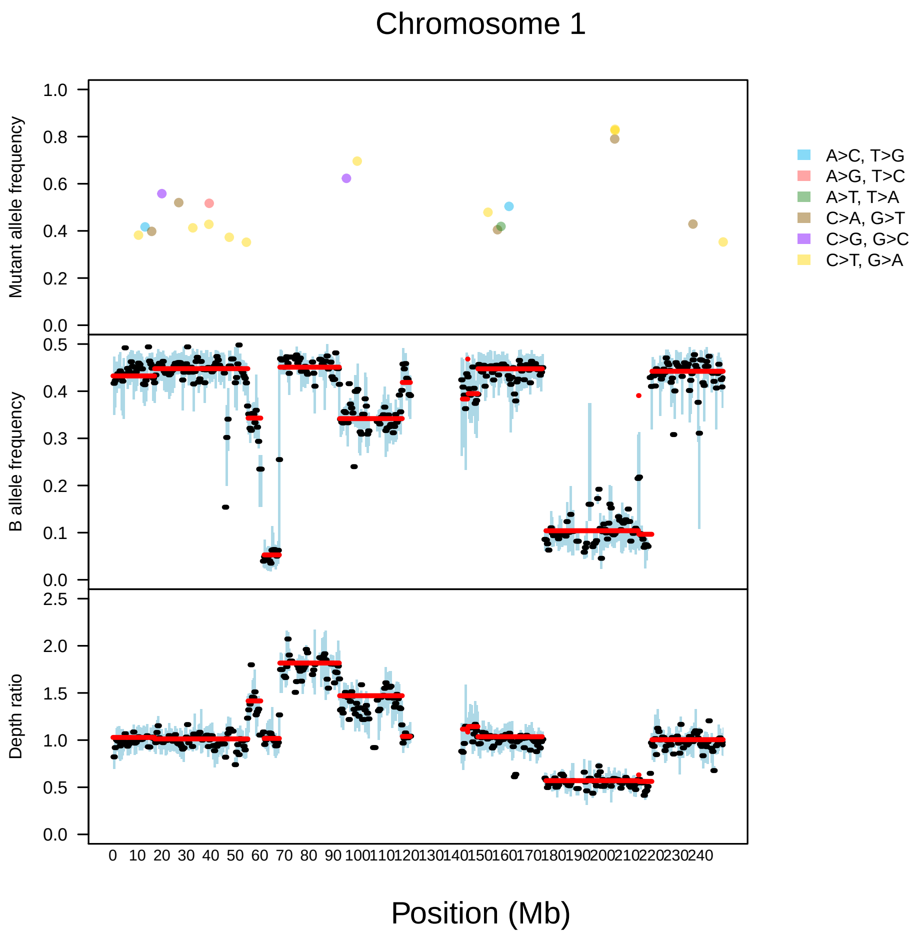


Figure 3: Plots of Mutation (top), B-allele frequencies (middle) and depth ratio (bottom) for chromosome position.

11 Inference of cellularity and DNA-content

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.content = seq(0.5,3,0.05), mc.cores = 4)
```

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

NEED EXPLANATIONS HERE:

```
> head(CP)
```

```
  dna.content cellularity         L
1        0.50         0.1 -476.9822
2        0.55         0.1 -440.3015
3        0.60         0.1 -410.5284
4        0.65         0.1 -386.2940
5        0.70         0.1 -366.7464
6        0.75         0.1 -351.1103
```

```
> dna.c.cint <- get.ci(CP[,c(1,3)])
> dna.c.cint$confint
```

```
[1] 1.0 1.8
```

```
> dna.c.cint$max.l
```

```
1
```

```
> cellu.cint <- get.ci(CP[,c(2,3)])
> cellu.cint$confint
```

```
[1] 0.79 0.89
```

```
> cellu.cint$max.l
```

```
0.89
```

```
> cp.plot(CP)
```

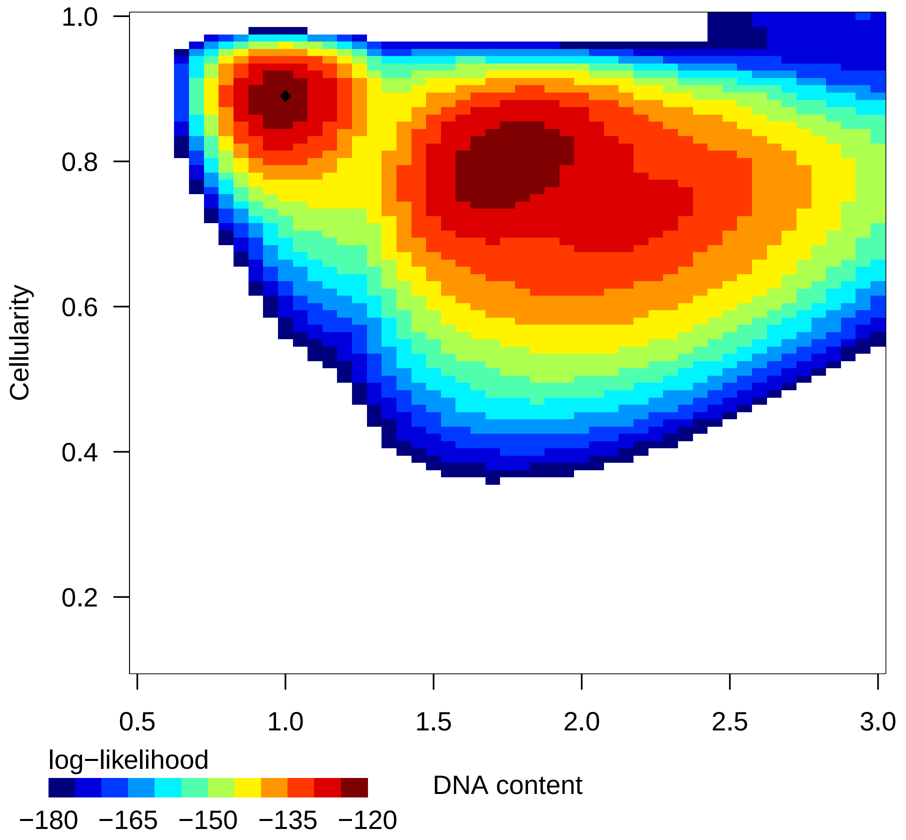


Figure 4: Result from the Bayesian inference over the defined range of cellularity and DNA content. The color indicates the log-likelihood of the corresponding cellularity/DNA-content values.

```
> par(mfrow = c(2,2))
>    cp.plot(CP)
>    plot(cellu.cint$values[,c(2,1)], ylab = "cellularity",
+          xlab = "likelihood", type = "l")
>    abline(h = cellu.cint$confint, lty = 2, lwd = 0.5, col = "red")
>    plot(dna.c.cint$values[,c(1,2)], xlab = "DNA-content",
+          ylab = "likelihood", type = "l")
>    abline(v = dna.c.cint$confint, lty = 2, lwd = 0.5, col = "red")
```

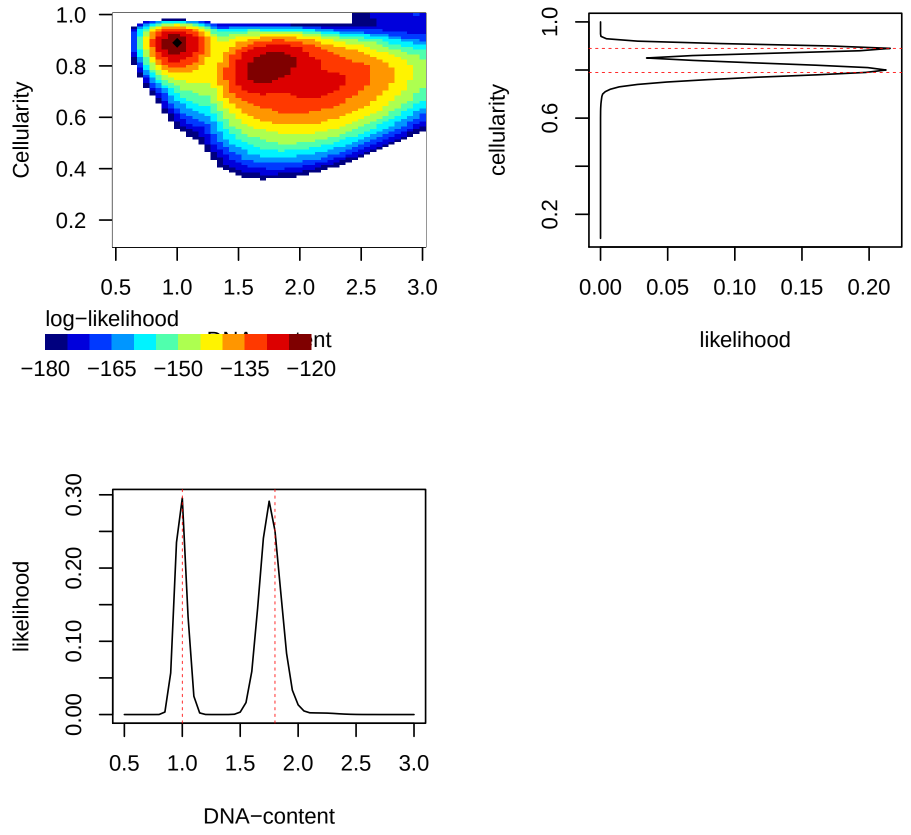


Figure 5: Plot of the log likelihood with respective cellularity and DNA-content probability distribution and confidence intervals.

12 Call CNVs and mutations using the estimated parameters

```
> cellularity <- cellu.cint$max.l
> cellularity

0.89

> dna.content <- dna.c.cint$max.l
> dna.content

1
```

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.content = dna.content,
+                             avg.depth.ratio = 1)
> head(mut.alleles)

   CNr CNt Mt         L
4    2   2  1 -13.66768
41   2   2  1 -12.62514
42   2   2  1 -13.02744
43   2   2  1 -16.25295
44   2   2  1 -14.06167
45   2   2  1 -12.76822

> 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       1.028167      C>T   2   2  1 -13.66768
496           1 13111750 0.417       1.028167      A>C   2   2  1 -12.62514
637           1 15821826 0.398       1.028167      G>T   2   2  1 -13.02744
1077          1 19983391 0.558       1.012928      G>C   2   2  1 -16.25295
1364          1 26878353 0.520       1.012928      C>A   2   2  1 -14.06167
1504          1 32627966 0.413       1.012928      C>T   2   2  1 -12.76822
```

12.2 Detect Copy number variation

```
> cn.alleles <- baf.bayes(Bf = seg.s1$Bf, depth.ratio = seg.s1$depth.ratio,
+                         cellularity = cellularity, dna.content = dna.content,
+                            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    1.028167     866   2 1 1
2          1  17013750  55100328 0.44782729  1056    1.012928    1072   2 1 1
3          1  55183366  60381491 0.34292696    68    1.415367      68   3 2 1
4          1  61743160  67960720 0.05276295   120    1.017667     122   2 2 0
5          1  68151685  92445257 0.45096790   262    1.817312     265   4 2 2
6          1  92568263 118165328 0.34186189   348    1.470577     355   3 2 1
          L
1 -12.06399
2 -11.71369
3 -11.53540
4 -10.48273
5 -11.65117
6 -11.55367
```

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.content = dna.content,
+                 avg.depth.ratio = 1)
```

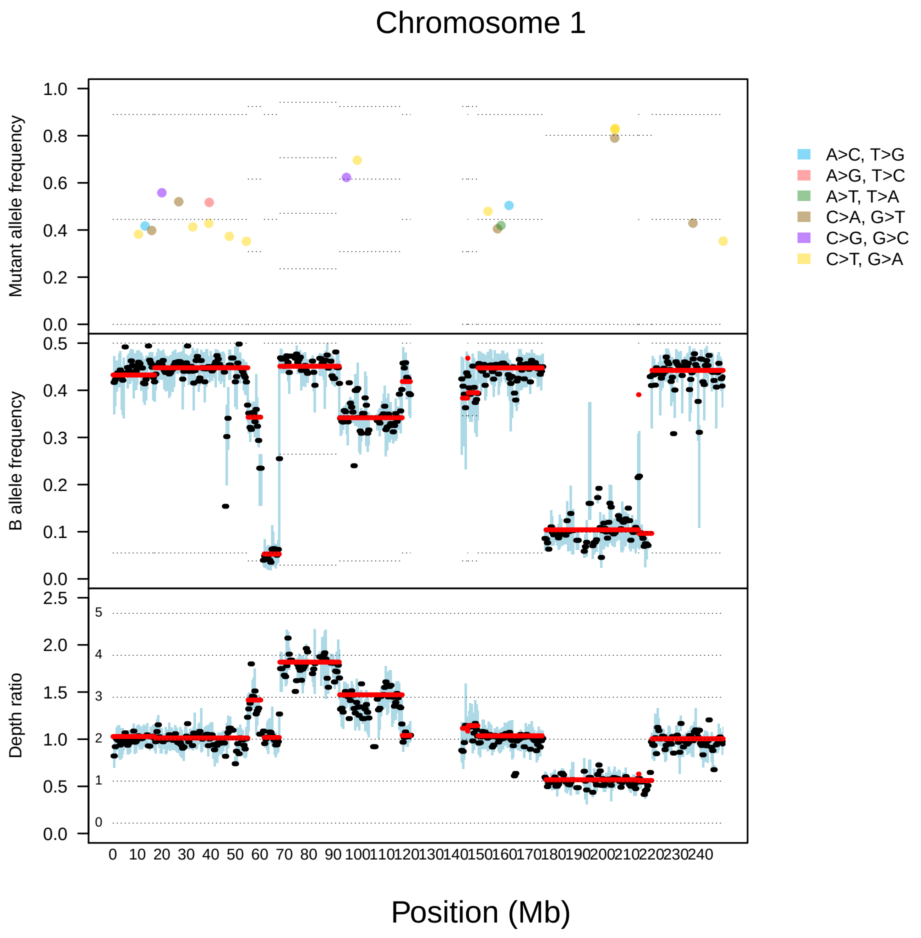


Figure 6: Plots of Mutation (top), B-allele frequencies (middle) and depth ratio (bottom) for chromosome position. Horizontal dotted line indicate different copy number/allelic state.

14 Working with SNP array data

MAKE THIS A TOPIC A SEPARATE DOCUMENT

14.1 Preparing the data

```
> data(BAF)
> data(logR)

> sample.i <- data.frame(chromosome = BAF$chrs, n.base = BAF$pos,
+                        Bf = BAF$S1, adjusted.ratio = logR$S1,
+                        depth.sample = 1, good.s.reads = 1,
+                        ref.zygosity = 'hom', stringsAsFactors = FALSE)
```

14.1.1 Correcting logR with a normal sample, or with the mean logR value

Without a reference sample (normal germline sample) we can try to divide for the mean value. It would be correct to use the germline logR.

```
> sample.i$adjusted.ratio <- 2^(sample.i$adjusted.ratio)
> sample.i$adjusted.ratio <- sample.i$adjusted.ratio / mean(sample.i$adjusted.ratio)
```

14.1.2 Retrive the homozygous position

It should be available a germline sample to get the heterozygours SNP, doing in the same sample it's a risk if the sample is pure. A treshold around 0.25 or 0.35 can be picked to subset the heterozygous position on the germline. In the example we are lowering the treshold while taking the SNP from the same aberrant sample.

```
> het.lim <- 0.2
> is.het <- sample.i$Bf >= het.lim & sample.i$Bf <= 1 - het.lim
> sample.i$ref.zygosity[is.het] <- 'het'
> sample.i$Bf[sample.i$Bf >= 0.5] <- 1 - sample.i$Bf[sample.i$Bf >= 0.5]
> sample.het.i <- sample.i[is.het, ]
```

14.2 Windowing logR values.

```
> snp.r.win <- windowValues(x = sample.i$adjusted.ratio,
+                           positions = sample.i$n.base,
+                           chromosomes = sample.i$chromosome,
+                           window = 1e6, overlap = 1)
```

14.3 Windowing B-allele frequencies values.

```
> snp.b.win <- windowValues(x = sample.het.i$Bf,
+                           positions = sample.het.i$n.base,
+                           chromosomes = sample.het.i$chromosome,
+                           window = 1e6, overlap = 1)
```

14.4 Chromosome view without mutation

```
> chromosome.view(baf.windows = snp.b.win[[1]],
+                 ratio.windows = snp.r.win[[1]],
+                  min.N.ratio = 1)
```

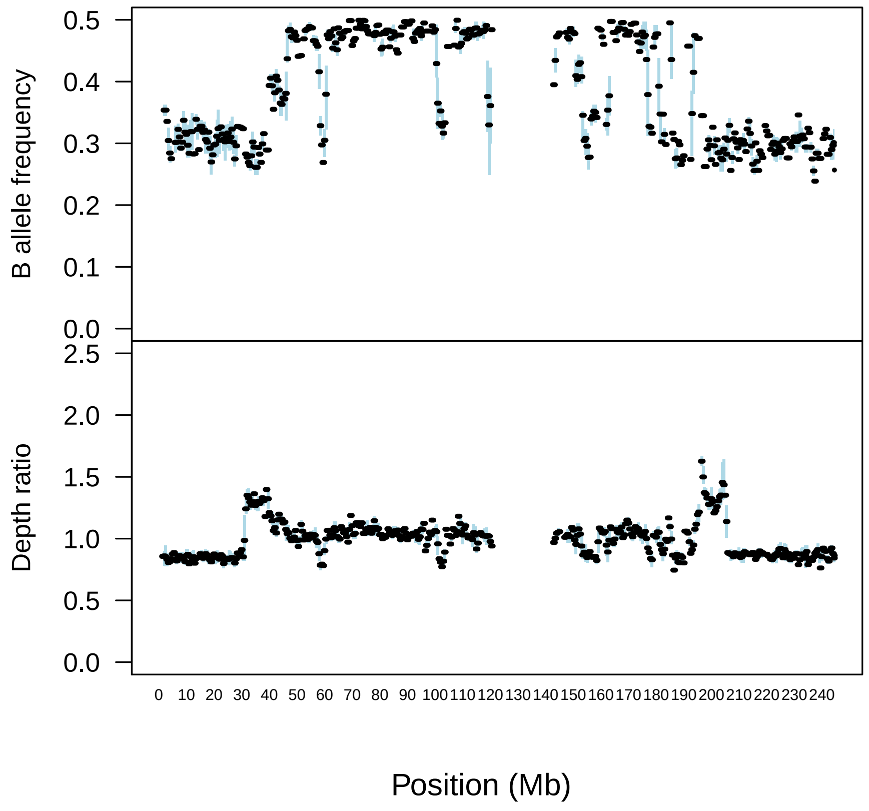


Figure 7: Plots B-allele frequencies (top) and un-logged-logR (bottom) with SNP array data.

14.5 Segmenting with the *copynumber* package

```
> breaks <- find.breaks(sample.het.i, gamma = 40, kmin = 20, baf.thres = c(0, 0.5))
> seg.i  <- segment.breaks(sample.i, breaks = breaks)
```

14.6 Using the Bayesian inference on segmented SNP arrays

```
> weights.snp   <- 150 + round((seg.i$end.pos - seg.i$start.pos)/1e6 , 0)
> filter.size   <- (seg.i$end.pos - seg.i$start.pos) >= 3e6

> avg.unlogR <- mean(sample.i$adjusted.ratio, na.rm = TRUE)
> CP.snp <- baf.model.fit(Bf = seg.i$Bf[filter.size],
+                          depth.ratio = seg.i$depth.ratio[filter.size],
+                          weight.ratio = weights.snp[filter.size],
+                          weight.Bf = weights.snp[filter.size],
+                          avg.depth.ratio = avg.unlogR,
+                          cellularity = seq(0.1,1,0.01),
+                          dna.content = seq(0.5,3,0.05), mc.cores = 4,
+                          priors.labels = 2, priors.values = 1)

> dna.c.cint <- get.ci(CP.snp[,c(1,3)])
> dna.c.cint$confint

[1] 1.4 1.5

> dna.c.cint$max.l

1.45

> cellu.cint <- get.ci(CP.snp[,c(2,3)])
> cellu.cint$confint

[1] 0.23 0.27

> cellu.cint$max.l

0.25

> cellularity <- cellu.cint$max.l
> dna.content <- dna.c.cint$max.l
```

14.7 Cellularity and DNA-content plot for SNP array

```
> cp.plot(CP.snp)
```

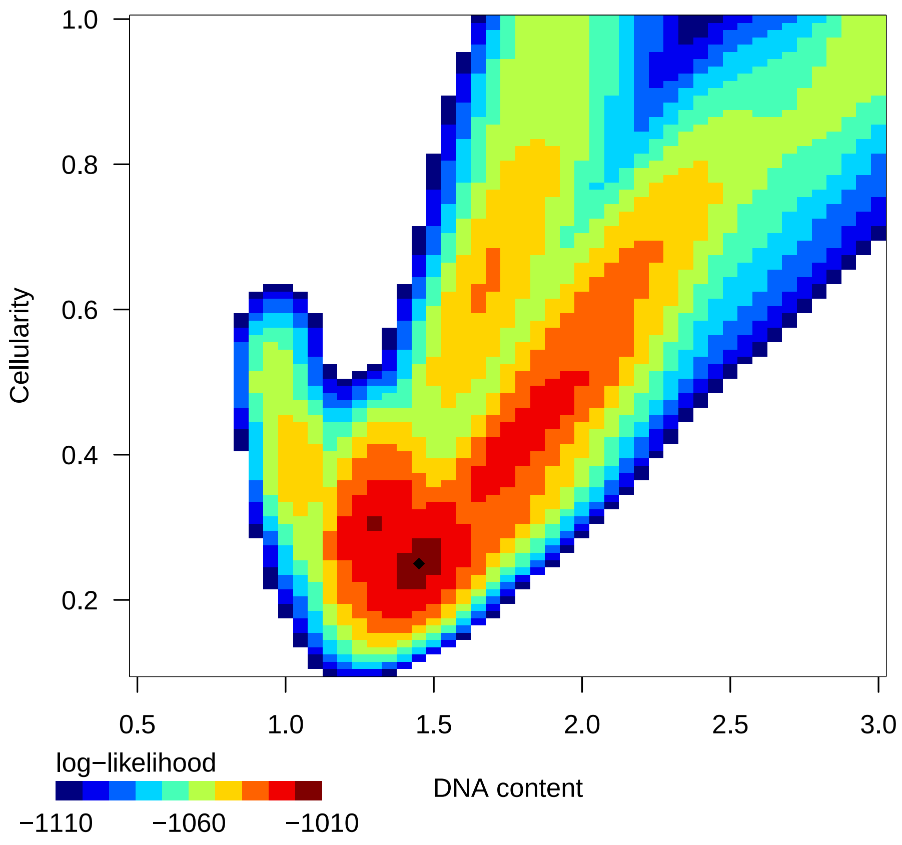


Figure 8: Result from the Bayesian inference over the defined range of cellularity and DNA-content from artificial SNP array data. The color indicate the log-likelihood of the corresponding cellularity/DNA-content values.

```
> par(mfrow = c(2,2))
>    cp.plot(CP.snp)
>    plot(cellu.cint$values[,c(2,1)], ylab = "cellularity",
+         xlab = "likelihood", type = "l")
>    abline(h = cellu.cint$confint, lty = 2, lwd = 0.5, col = "red")
>    plot(dna.c.cint$values[,c(1,2)], xlab = "DNA-content",
+         ylab = "likelihood", type = "l")
>    abline(v = dna.c.cint$confint, lty = 2, lwd = 0.5, col = "red")
```

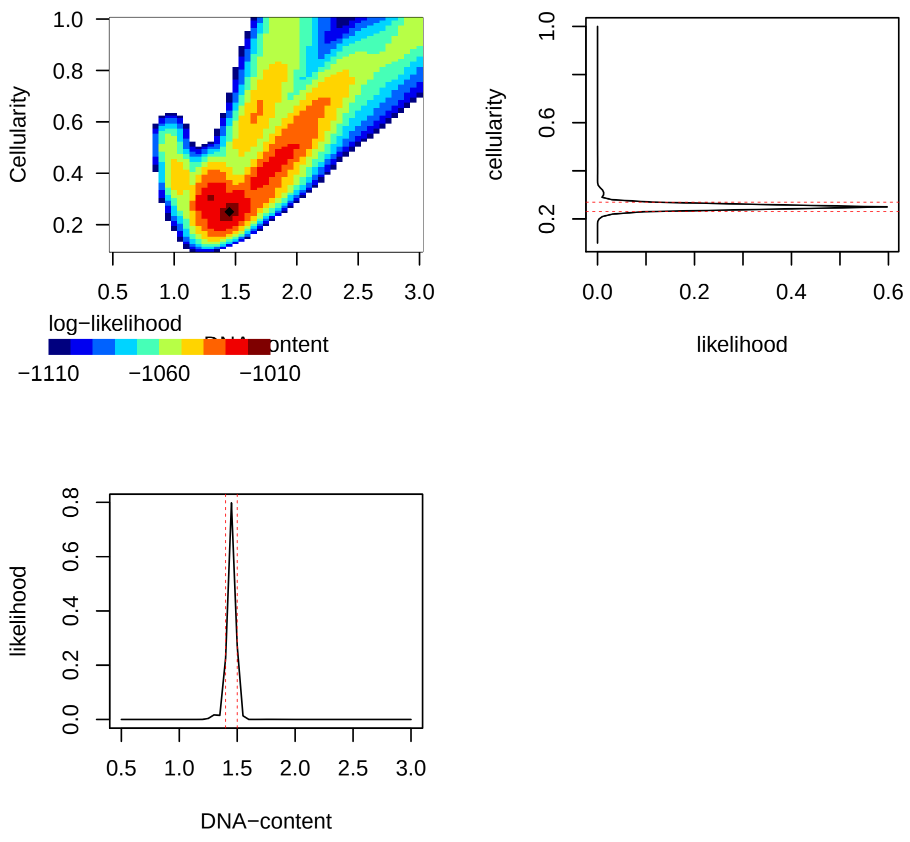


Figure 9: Plot of the log likelihood with respective cellularity and DNA-content probability distribution and confidence intervals.

14.8 Call for copy number variation using inferred parameters.

```
> snp.seg.cn <- baf.bayes(Bf = seg.i$Bf,
+                            depth.ratio = seg.i$depth.ratio,
+                            avg.depth.ratio = avg.unlogR,
+                             cellularity = cellularity,
+                             weight.ratio = 2 * 300,
+                             weight.Bf = 300, ratio.priority = FALSE,
+                             dna.content = dna.content, CNt.max = 10)
> segmented.snp <- cbind(seg.i, snp.seg.cn)
> head(segmented.snp[segmented.snp$chromosome == 1, ])

  chromosome start.pos   end.pos        Bf N.BAF depth.ratio N.ratio CNt A B
1          1   2189662  28792900 0.3076600    85   0.8445499     130   4 4 0
2          1  29582868  40285096 0.2899053    19   1.2582041      43   8 7 1
3          1  40630391  46296225 0.3774100    20   1.1287311      29   7 5 2
4          1  46437972  57009803 0.4780000    31   1.0157311      43   6 3 3
5          1  57301533  64307493 0.4303579    19   0.9750263      28   6 4 2
6          1  65068455 100351185 0.4793541    61   1.0493888     107   6 3 3
          L
1 -14.51342
2 -14.59017
3 -14.56633
4 -14.59775
5 -15.09243
6 -14.57161
```

14.9 Graphical representation of copy number with SNP arrays

```
> chromosome.view(baf.windows = snp.b.win[[1]],
+                    ratio.windows = snp.r.win[[1]],  min.N.ratio = 1,
+                    segments = segmented.snp[segmented.snp$chromosome == "1", ],
+                    cellularity = cellularity, dna.content = dna.content,
+                    avg.depth.ratio = avg.unlogR, main = "1")
```

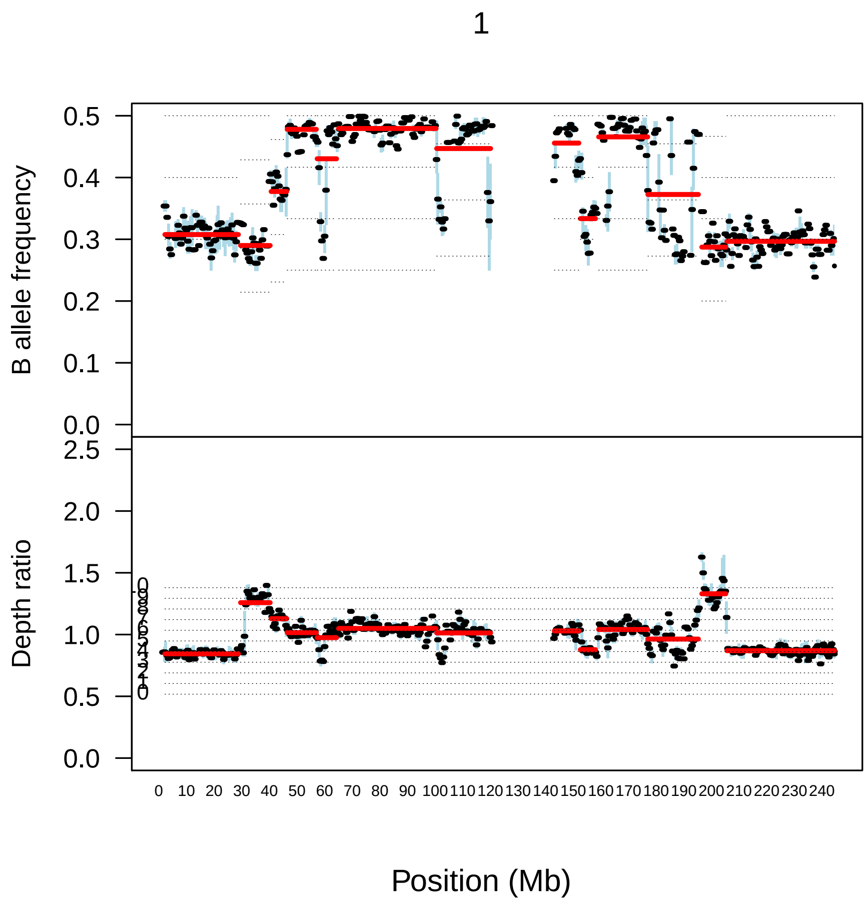


Figure 10: Plots B-allele frequencies (top) and un-logged-logR (bottom) with SNP array data. Chromosome 16. Horizontal dotted line indicate different copy number/ allelic state.

```
> chromosome.view(baf.windows = snp.b.win[[16]],
+                 ratio.windows = snp.r.win[[16]],  min.N.ratio = 1,
+                 segments = segmented.snp[segmented.snp$chromosome == "16", ],
+                 cellularity = cellularity, dna.content = dna.content,
+                 avg.depth.ratio = avg.unlogR, main = "16")
```

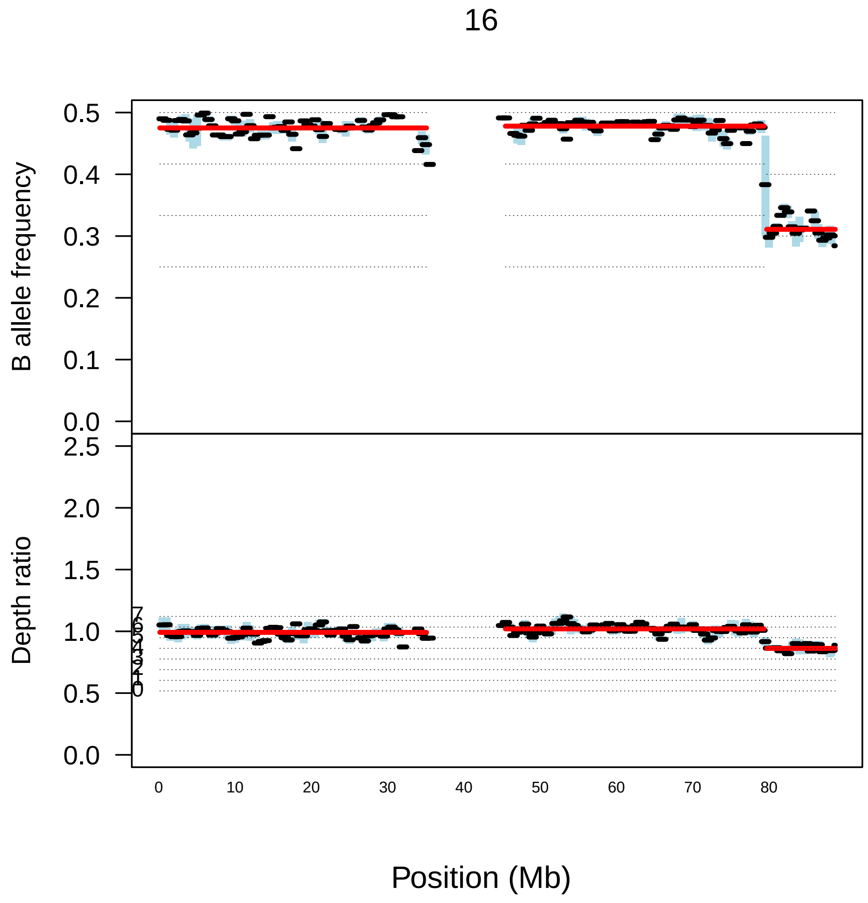


Figure 11: Plots B-allele frequencies (top) and un-logged-logR (bottom) with SNP array data. Chromosome 16. Horizontal dotted line indicate different copy number/ allelic state.

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.