GENE315 CNV lab - week 1

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Background: copy number variation

Deviation from the diploid copy number in genomic segments (>50 bp) is known as copy number variation (CNV). Copy number variants often encompass genes and are an important but poorly understood source of variation in genomes. In humans copy number variation has been associated with phenotypes such as autoimmune disease and weight. IRGM is a relatively simple 20kb insertion-deletion upstream of the immunity-related GTPase family M gene and is associated with Crohn's disease (Prescott et al. 2010). FCGR3B (Fc gamma receptor 3B) is within a more complex locus (refer lectures) with copy number typically varying from 0 to 4 within a population (McKinney and Merriman, 2012). It is associated with autoimmune diseases such as rheumatoid arthritis and systemic lupus erythematosus.

Generation of copy number calls from 1000 Genomes data

High-throughput DNA sequencing technologies provide the ability to examine copy number variation on a whole genome scale. When a sample is sequenced it is assigned an average coverage level, which reflects the number of bases sequenced relative to the size of the genome. For example, if 300 million 100bp reads are generated for a 3Gbp genome the average coverage will be 10x (number of reads x read length / genome size = 300,000,000 x 100 / 3,000,000). Analysis approaches based on read depth use this idea to identify regions that exhibit large deviations away from the expected level of coverage to identify changes in copy number. For example, a region exhibiting 30x coverage in a diploid genome that has been sequenced to a depth of 20x could reflect a gain of one additional copy of that region of DNA.

In order to generate copy number calls based on read depth, aligned sequence data are required. The 1000 Genomes Project provides such data for the genomes of thousands of individuals from populations around the world. In this part of the lab we will use data from the 1000 Genomes Project to investigate variation in gene copy number at the FCGR3B and IRGM loci.

Aligned DNA sequence read data will be provided for a subset of the 1000 Genomes sample data for 1Mbp regions around the two loci of interest. At each locus you will use the R software to perform:

- Generation of read depth information
- Visualisation of read depth across the region of interest
- Segmentation analysis to identify regions of altered copy number

Below is an example of the R code required to perform an analysis of CNV at the FCGR locus - we will work through this code in the lab. You will then need to alter the provided code to perform a similar analysis for IRGM so you can answer the questions on the final page of this document.

Overview: 1000 Genomes Project

In this lab you will be looking at copy number variation in samples from the 1000 Genomes Project, using publicly available high-throughput sequencing data. The link for the 1000 Genomes Project is:

http://www.internationalgenome.org/ (http://www.internationalgenome.org/)

The first couple of questions for your assignment relate to the 1000 Genomes Project - you should be able to find the answers to those questions by having a look around their website.

Aligned sequence data from the 1000 Genomes Project was obtained from their ftp download site. For this lab we are using a relatively small subset of the full data (310 samples) out of 2535, from three of the 26 populations included in the study.

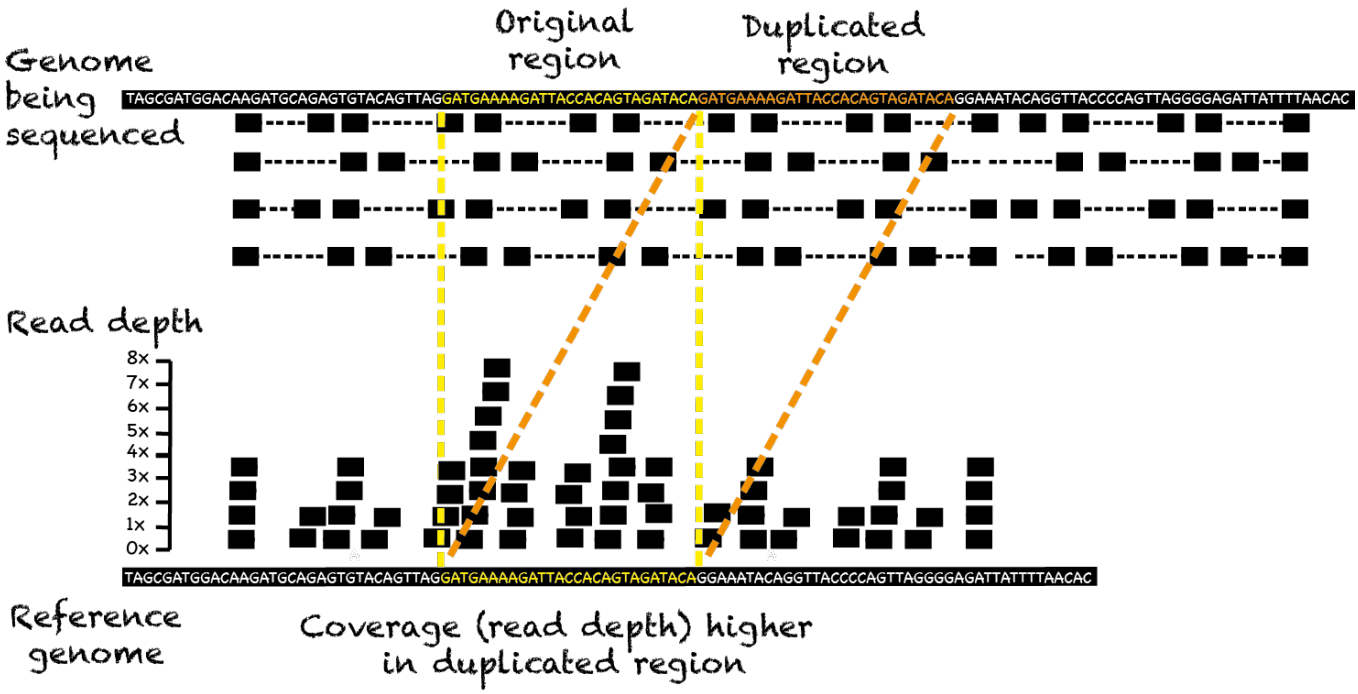
As mentioned in class, the aligned data for each sample is stored in the BAM format on the ftp server. One of the nice things about BAM is that you can *index* the data files, which makes it possible to retrieve a subset of the data (e.g., from a particular region of the genome) without having to download the entire data file.

This is an important point, as the data files for a single sample are fairly large (e.g., around 30GB). For this lab I have downloaded data from regions around the two genes of interest (FCGR3B and IRGM). For each of these regions, the data extracted was only between 3MB & 20MB, making it relatively quick and (somewhat) easy to retrieve data for large numbers of samples.

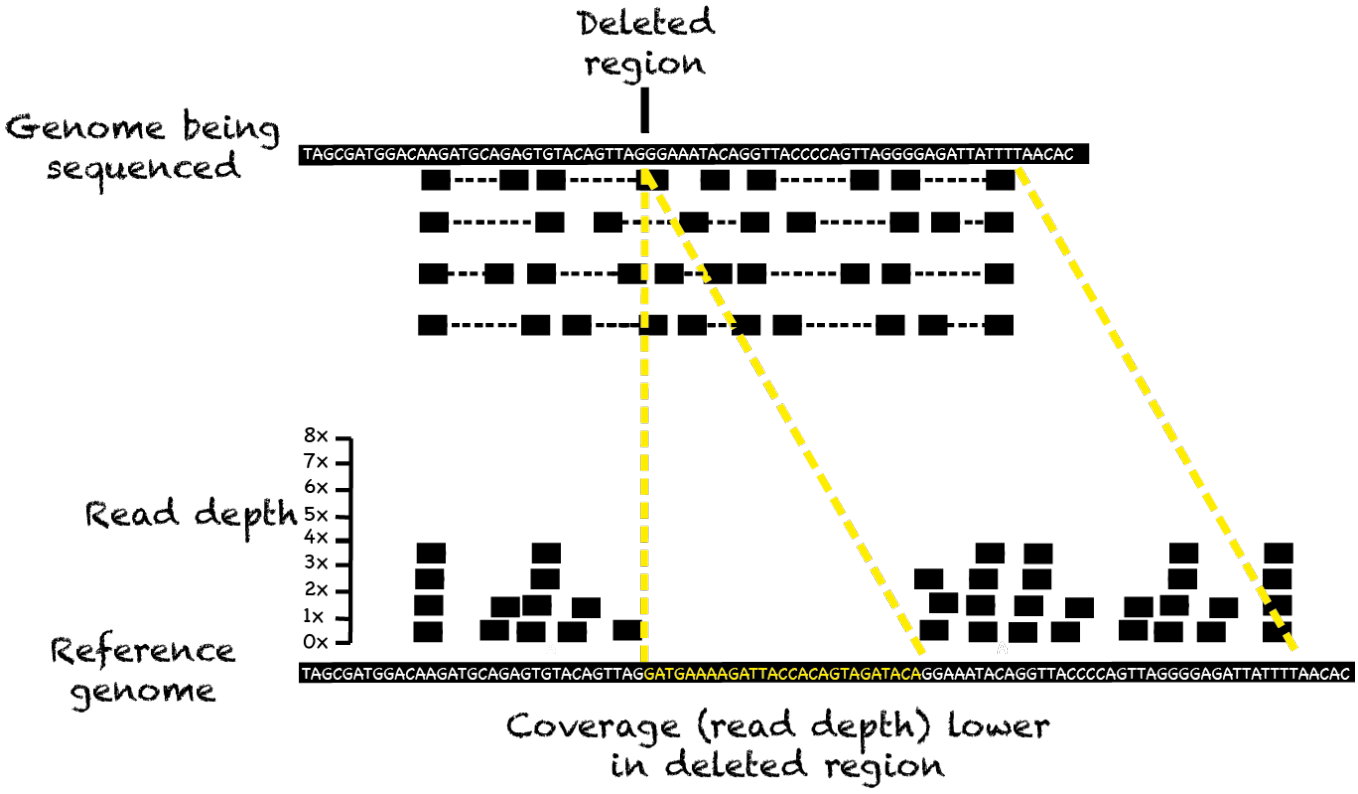
Recap: read depth and copy number

In class we talked about read depth (number of times a base of DNA was read by the sequencer) could be used to investigate changes in copy number. The plot below shows an example of a duplication, with a corresponding increase in read depth across the duplicated

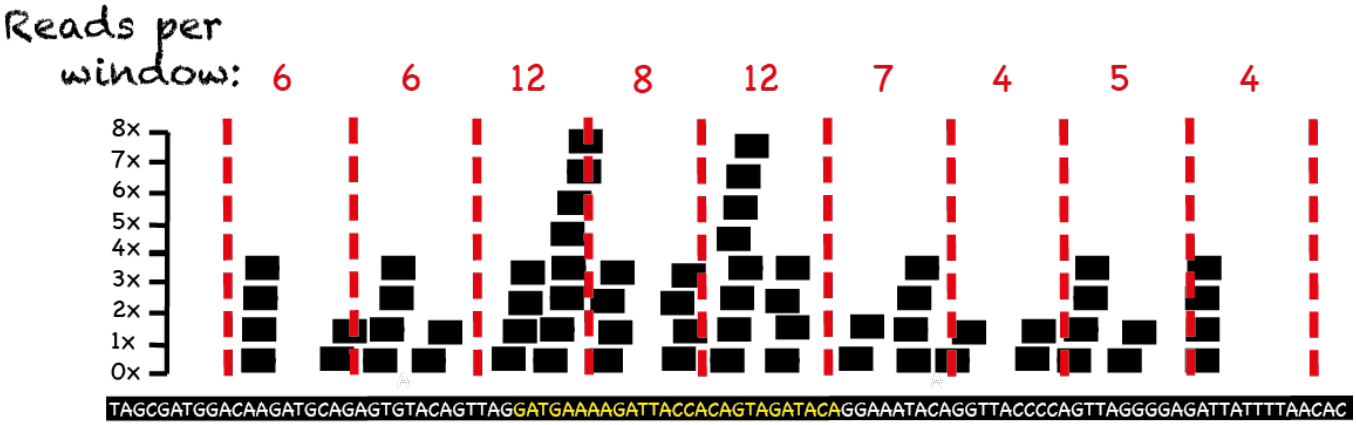
region:



The opposite occurs with deletions - decreased read depth across the deleted region, as shown in the following plot:



For low coverage samples (i.e., samples for which the average read depth is relatively low, e.g., less than 10x) simply calculating read depth at every base can make for very noisy data. An alternative is to split the region of interest into fixed-width *windows* (e.g., 500 or 1000bp), and calculate the total number of reads whose alignments start in each window (just keeping track of the start positions avoids double-counting, as read alignments can only start in one place). The plot below illustrates this idea, with the red lines indicating the windows, and the counts denoting the number of aligned reads starting in each window.



Calculating read depth

In order to generate windows and calculate read depth for the regions and samples we are interested in for this lab, we need to split the region into fixed sized windows, and then take the aligned data and count how many reads begin in each window. To do this we can use the *samtools* application (or the *Rsamtools* package from within R). Unfortunately (or luckily, depending on how much you like writing code) the Rsamtools can be tricky to install, so I have carried out this process for you.

Getting started in R

We will be performing our R-based analysis using the RStudio application. There are a number of ways to use RStudio for this lab:

- on your own computer, with R and RStudio installed
- on the lab computers in ACAL

Downloading the data

The data and R files needed for this lab are located on Blackboard in a zip file in the “Module 2: Analysis of genetic variation in humans” folder. Download this file to the desktop, and unzip it.
In R, set your working directory to be the folder that is created when the file is unzipped.

The zip file contains three files that we will use this week:

- FCGR-counts.csv - data for the FCGR region
- IRGM-counts.csv - data for the IRGM region
- plotCNV.R - R function to generate plots of the window counts

We’ll use the other three files next week:

- CNcalls.csv
- IRGM_rs13361189.csv
- FCGR_rs117435514.csv

Each of the data files contains per window count data across the region of interest, for a collection of samples from the 1000 Genomes Project. If you want to see the data format, you can open the files in Microsoft Excel (genomic positions as rows, samples as columns).

Data generation parameters and samples

The following parameters were used to generate the data for the two regions of interest:

Gene region	Chr	Start	End	Length	Window
IRGM	5	150124000	150324000	200Kbp	500bp
FCGR	1	161300000	161800000	500Kbp	1000bp

The populations for which data were downloaded are:

Population	N (number of samples)	Columns of data set
CEU	99	1 - 99
CHB	103	100 - 202
YRI	108	203 - 310

IMPORTANT NOTE

The analyses below relate to the data from the FCGR locus. For your assignment, you need to perform a similar analysis for IRGM. The code below will be very helpful - you will just need to replace the FCGR data with the data for IRGM. **MAKE SURE YOU SAVE YOUR R COMMANDS IN A FILE. THIS WILL ALLOW YOU TO EASILY RERUN THEM (AND/OR ALTER THEM FOR THE IRGM DATA) LATER ON. ASK ME HOW TO DO THIS IF YOU ARE NOT SURE**.

Getting the data into R

In order to read the data into R, the `read.csv()` function can be used. The following code reads the data for the FCGR region into an object called `fcgrDat`. The `row.names = 1` setting tells the command that the row names for the data set can be found in the first column that is read in:

```
fcgrDat = read.csv('FCGR_counts.csv', row.names = 1)
```

We can get some information about the `fcgrDat` object as follows:

```
## Type of object
class(fcgrDat)
```

```
## [1] "data.frame"
```

```
## Size of object (rows, columns)
dim(fcgrDat)
```

```
## [1] 500 310
```

```
## First 10 row names
rownames(fcgrDat)[1:10]
```

```
##  [1] "161301000" "161302000" "161303000" "161304000" "161305000" "161306000"
##  [7] "161307000" "161308000" "161309000" "161310000"
```

```
## First 5 column names
colnames(fcgrDat)[1:5]
```

```
## [1] "NA06984" "NA06985" "NA06986" "NA06989" "NA06994"
```

```
## First 10 rows and first 5 columns of data
fcgrDat[1:10,1:5]
```

```
##           NA06984 NA06985 NA06986 NA06989 NA06994
## 161301000     108     186     155      74      67
## 161302000     105     192     152      79      50
## 161303000     117     186     178      81      63
## 161304000     134     192     201      58      55
## 161305000     118     188     174      69      62
## 161306000     101     153     144      69      62
## 161307000     101     158     179      60      75
## 161308000     128     160     188      75      56
## 161309000     105     173     151      74      80
## 161310000      76     190     150      50      56
```

We can access the data for a single sample in a few ways:

```
## First 10 observations for the 1st sample (NA06984)...
fcgrDat[1:10,1]
```

```
##  [1] 108 105 117 134 118 101 101 128 105  76
```

```
## ...which is the same as:
fcgrDat[1:10,"NA06984"]
```

```
##  [1] 108 105 117 134 118 101 101 128 105  76
```

```
## All of the data for the first sample (NA06984):
fcgrDat[,"NA06984"]
```

```
##   [1] 108 105 117 134 118 101 101 128 105  76 107 148 146 115 137 101 113 104
##  [19]  96 130 114 143 121 144 110 126 119 122 111 140  94  98 117 149 142 127
##  [37] 109  94 124 137 100 106  93 126 139 120 121 125 119 131  95 150 155 127
##  [55] 118  91 138 119 106  81  93 105 144 135 113 105  83 134 127 128 124 135
##  [73] 103 111 124 102 109  97 109 117 146 125  94 137 117  73  47 114  95 130
##  [91] 101 103 104  41 159  87 107 119 111  97 121  64 108 112 131 121 105 108
## [109] 161  68  46 116 170 238 351 393 291  41  57 147 189 337 327 373 167  44
## [127] 127 139 324 251 362 224  53  96 140 197 313 408 317 135  44 203 113 108
## [145] 108 161 112 145  83 124 113 137 148 168 123 128 110 134  99 156 143 142
## [163] 129 136 116 125 136 116 145 131 165 126 141 118 113 123 119 128 126  94
## [181] 113 110 145 129 152 137 115 152 148 137 115 149 115  85  44  91 110 126
## [199] 127 132 107 109 121 132 124 120 178 154 117 120 111 147 123 147 110 130
## [217] 141 125 152 133 145 125 138  88 157 137  92 156 128 127 129 117 119 139
## [235] 135  85 131 136  95 140 165 127 142 106 163 132 123 114 137 102 121 127
## [253] 121 126 106 108 132 141  98  83  95 140 139 119 125  91  80 119 104  70
## [271] 100 119 129  92 120  68  79  85  99 106 125 118 103 111 144 117  80  74
## [289] 139 102 100 107 110 105  99 120 125 129  77  89 115  81  66 104  98  88
## [307] 106 121 102 114 106 105 135 114 118 117 108 140 114 154 100  90  52  99
## [325] 102 101 106 168 151 122 137 156 110 127 103 124 117  97  98  99 115 142
## [343]  97 119  65  91 113 133 154 105 143 141 140 115 117 116 143  94 122 103
## [361] 112 121 115  90 175 130 152 113 101 126 158 116 148 118 141 131 111 125
## [379] 140 118 124 112 110 132 154 134 137 165 118 117 100 128 146 102 110 108
## [397]  38  93 110 128 117 133 100 106 124 114 116 118 127 137 124 100 122 141
## [415] 120 111 115 119 111 122 106 128 112 118 118 117 103 141 110 100 118 130
## [433] 127 137 115 125 129 108 117 105 142 138 116 133 108 118 111 118 103 136
## [451] 108 103 120 120 111 111 114 117 130 116 143 138 125 127 120 121 114 134
## [469]  75 138 136 109 141  99 128 145 148 144 126 107 112 145 123 122 110 111
## [487] 124 142 145 120  86 108 120 153 110 111 129 116 152 104
```

We can use this approach to calculate some basic statistics for the data from this sample:

```
## Calculate the median number of counts per window for sample NA06984:
median( fcgrDat[,"NA06984"] )
```

```
## [1] 119
```

```
## Calculate the total number of reads that aligned across this region for
## sample NA06984:
sum( fcgrDat[,"NA06984"] )
```

```
## [1] 62151
```

```
## Calculate the range of the counts in this region for sample NA06984:
range( fcgrDat[,"NA06984"] )
```

```
## [1]  38 408
```

Exploratory analysis

Since we know the length of the reads (100bp) and the size of the region around the FCGR genes (500Kbp), we can caculate the average read depth for this region. We use the median to reduce the impact of outlying observations (such as changes in copy number):

```
## Average read depth is given by:
## (Read length) x (Number of Reads) / (Length of Region)
100 * sum( fcgrDat[,"NA06984"] ) / 500000
```

```
## [1] 12.4302
```

So the average read depth across this region for sample NA6984 is 12.43 (i.e., on average, each base in this region was read 12.43 times in this sample).

What about all of the other samples? We can use some other functions to perform similar calculations on all of the samples simultaneously. The `colMeans()` function calculates the mean of every column in an R data.frame:

```
## I've suppressed the output here - it's just a page of numbers...
colMeans( fcgrDat )
```

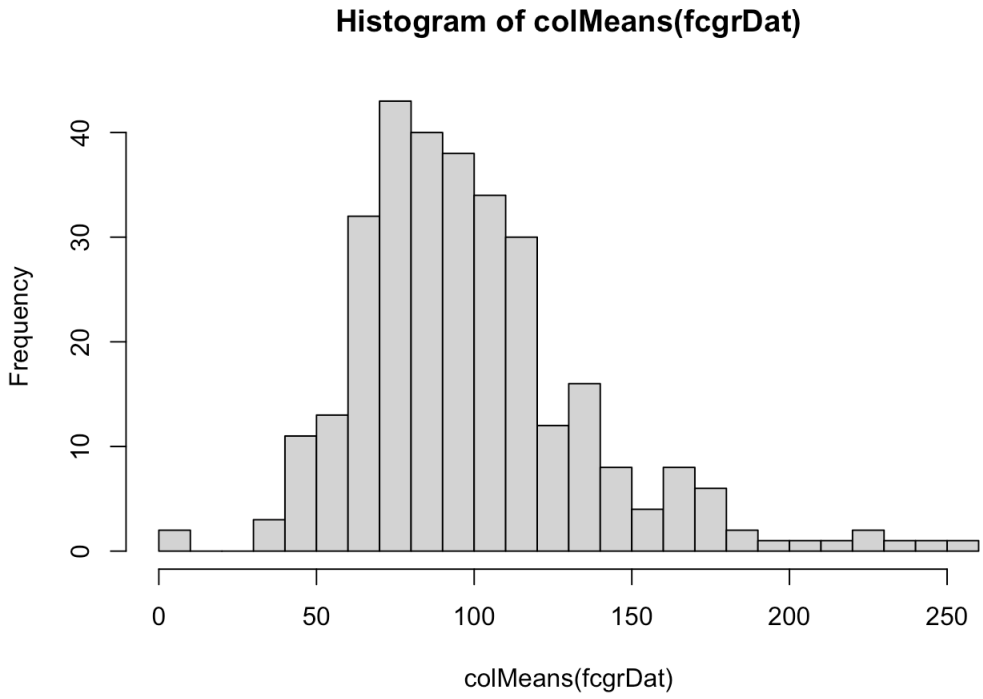
Similary, the `colSums()` function will calculate the sum for every column

```
## Calculate column sums for first 10 samples:
colSums( fcgrDat[,1:10] )
```

```
## NA06984 NA06985 NA06986 NA06989 NA06994 NA07000 NA07037 NA07048 NA07051 NA07056
##   62151  111187  101223   45200   33785   66438   50123   38034   32550   38135
```

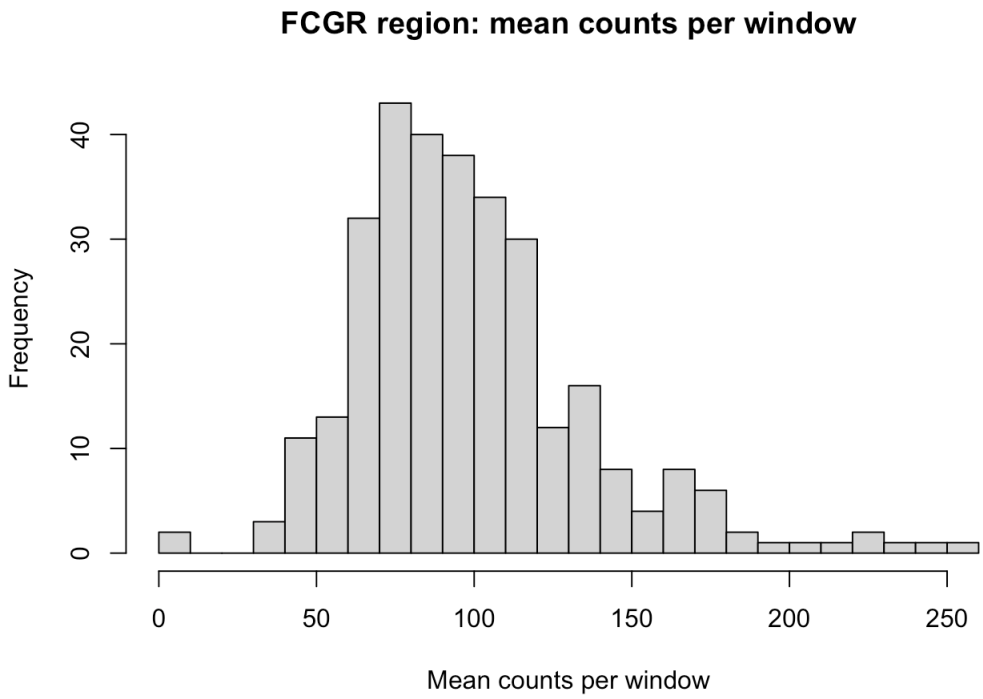
We can examine the distribution of the mean per-window counts for each sample by plotting a histogram:

```
## Create histogram of mean per-window counts per sample
## The "20" parameter indicates the approximate number of bins to use in the plot
hist( colMeans(fcgrDat), 20)
```



We can make the plot more informative by improving the labels. The `main` parameter specifies the plot title, while `xlab` and `ylab` specify the x and y axis labels.

```
hist( colMeans(fcgrDat), 20, main="FCGR region: mean counts per window",
      xlab="Mean counts per window")
```



Use the commands you've learned so far to generate a histogram of average read depth across the FCGR region for all samples.

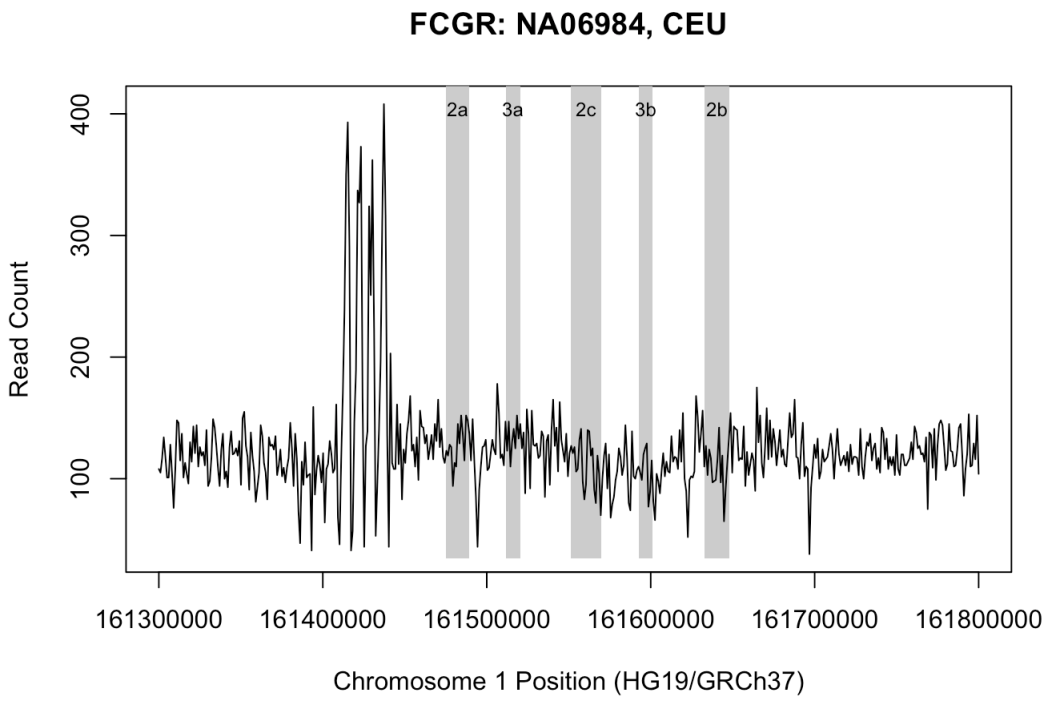
Exploring copy number variation

The file `plotCNV.R` contains a function for plotting the count data for a sample across the region of interest. The file can be read into R via

```
source('plotCNV.R')
```

The following command plots the data for sample NA06984 across the FCGR region:

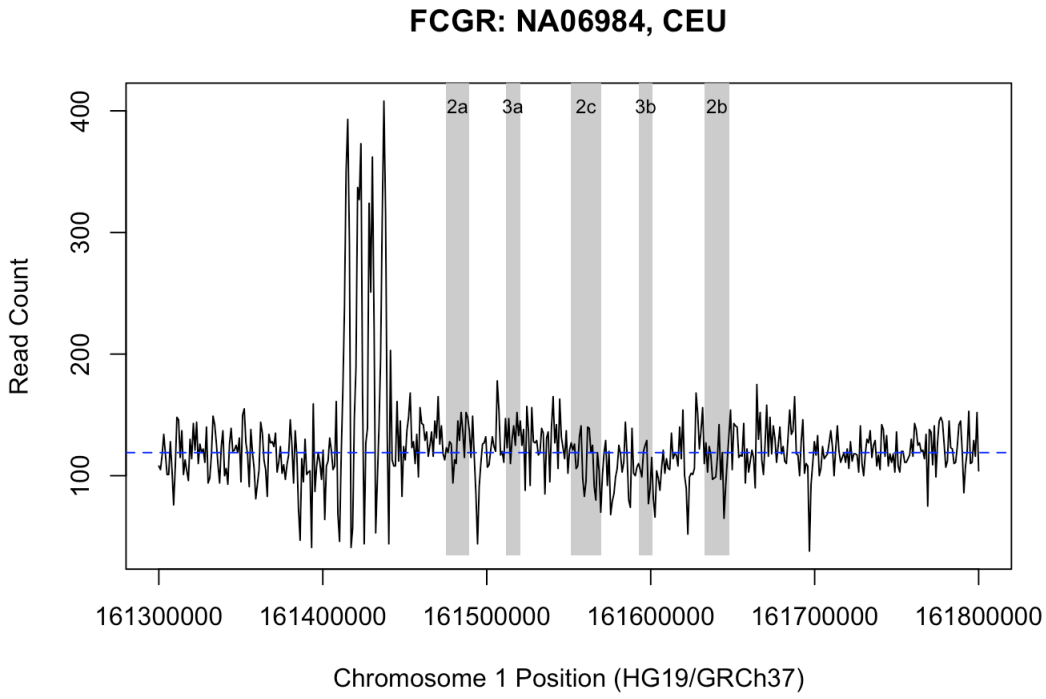
```
plotCNV( fcgrDat, "NA06984", "FCGR" )
```



The gray vertical bars mark the FCGR genes, and the black trace depicts the per-window counts across the region. The large “spiky” area to the left of the FCGR genes relates to a region of repetitive DNA where sequence alignment is difficult.

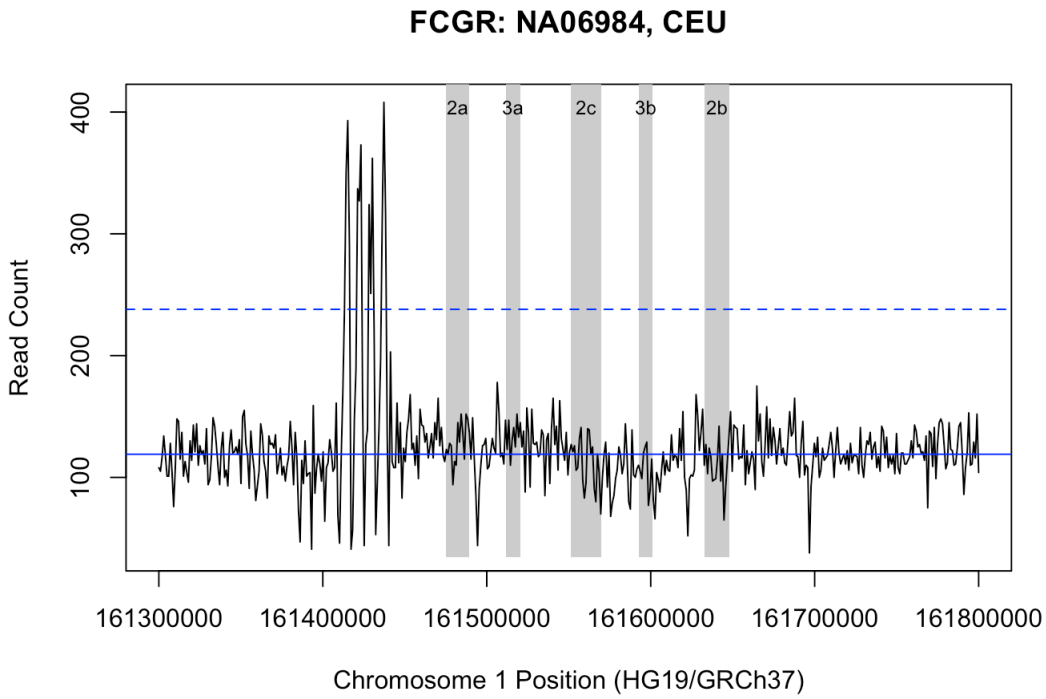
We can add information about the median to the plot using the `abline()` function (the “h” parameter denotes a horizontal line, “col” idicates colour, and “lty” selects the line type):

```
plotCNV( fcgrDat, "NA06984", "FCGR" )
abline( h = median(fcgrDat[, "NA06984"]), col='blue', lty=2 )
```



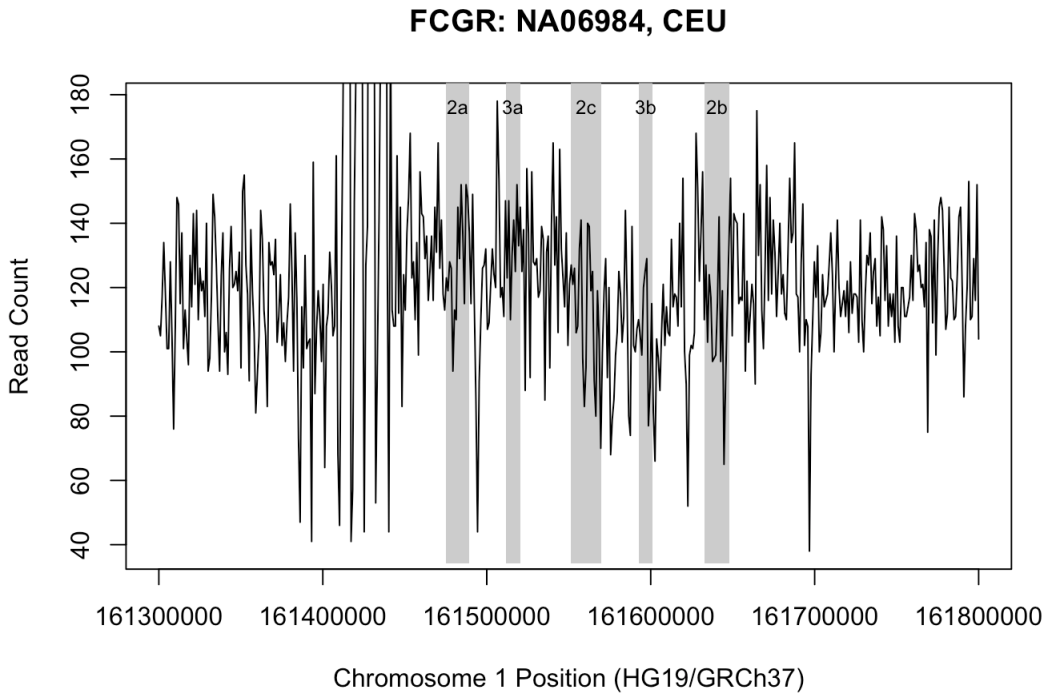
"Normal" copy number for the FCGR region is 2, so for an individual with 2 copies of this region we would expect the per-window read count to fluctuate around the median value. An individual with a duplication would have higher values, while an indiviudal with a deletion would have lower values. The following plot adds a line at twice the median - the level you would expect to see the per-window counts rise to in an area where four copies existed in a sub-region of the FCGR locus.

```
plotCNV( fcgrDat, "NA06984", "FCGR" )
abline( h = median(fcgrDat[, "NA06984"]), col='blue' )
abline( h = 2*median(fcgrDat[, "NA06984"]), col='blue', lty=2 )
```



Because the repetitive sequence before the FCGR region can affect the range of the plot, the `plotCNV()` function includes the option to truncate the height of the y axis (this only works for the FCGR region):

```
plotCNV( fcgrDat, "NA06984", "FCGR", truncate=TRUE)
```



In order to create plots for multiple samples, we can use a “for loop”. In the example below the variable `i` takes the values 1,2,…,6 through each iteration through the loop, and prints the value of `i` each time:

```
for(i in 1:6){
  print(i)
}
```

```
## [1] 1
## [1] 2
## [1] 3
## [1] 4
## [1] 5
## [1] 6
```

We can use this to automate data processing - the following example calculates the mean for each of the first 6 samples:

```
for(i in 1:6){
  print( mean(fcgrDat[,i]) )
}
```

```
## [1] 124.302
## [1] 222.374
## [1] 202.446
## [1] 90.4
## [1] 67.57
## [1] 132.876
```

This is the same as the result obtained from applying the `colMeans()` function to the first six columns of the `fcgrDat` data object:

```
colMeans( fcgrDat[,1:6] )
```

```
## NA06984 NA06985 NA06986 NA06989 NA06994 NA07000
## 124.302 222.374 202.446  90.400  67.570 132.876
```

In order to use this functionality to automate plot generate, it is useful to turn on a setting that asks us to hit “enter” before each plot is displayed (`par()` is a function to set graphics parameters):

```
par(ask=TRUE)
```

Use the commands you have learned so far to generate plots of the FCGR region for the first 20 samples (or all of them if you feel like hitting "enter" a lot!).

HINT: the following code might help you:

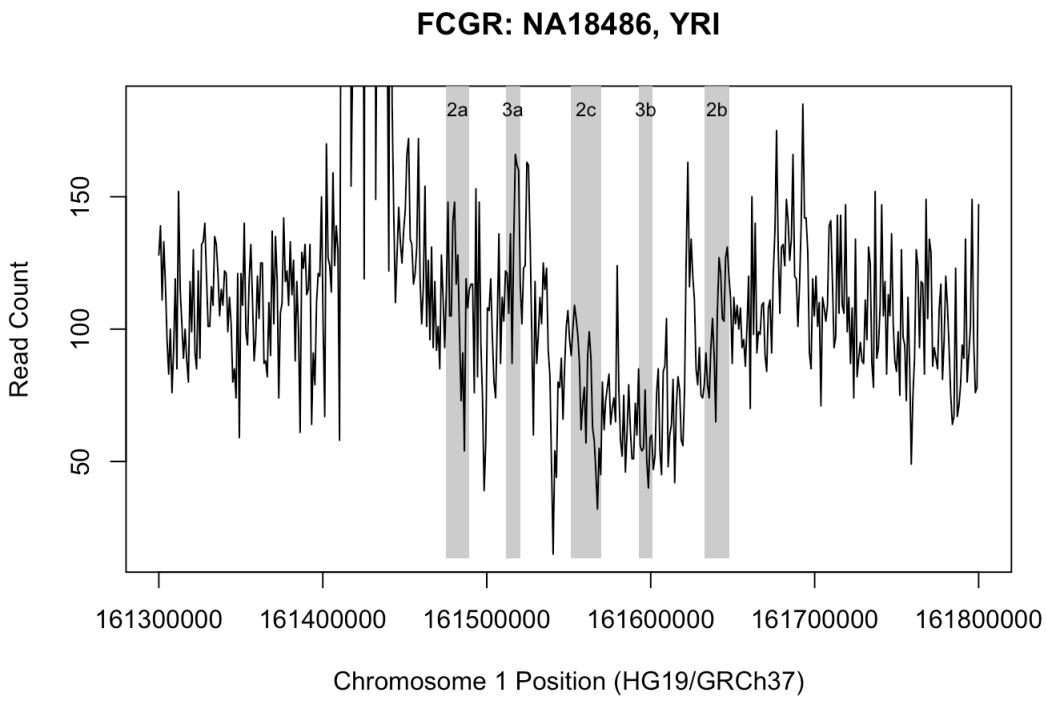
```
i = 1
plotCNV( fcgrDat, colnames(fcgrDat)[i], "FCGR", truncate=TRUE)
```

Other populations

The first 99 samples are all from the CEU population. To examine the data from other populations, you need to select samples relating to that population. The relevant column ranges are listed in the table in the "Data generation parameters and samples" section of this document.

For example, to plot the first sample from the YRI population, you could use the command:

```
plotCNV( fcgrDat, colnames(fcgrDat)[203], "FCGR", truncate=TRUE)
```



If you look through the first 20 CEU samples, and the first 20 YRI samples, we see about the same number of duplications at FCGR3B in each population (2 in CEU and 3 in YRI, although they are pretty hard to call). To determine whether there is a significant difference in the FCGR duplication rate between the populations, we can use the Chi-squared test. First we need to construct a simple 2x2 table representing our observtaions (2 duplications and 18 non-duplications in CEU and 3 duplications and 17 non-duplications in YRI):

```
matrix( c(2,18,3,17), 2,2)
```

```
##      [,1] [,2]
## [1,]    2    3
## [2,]   18   17
```

We can then perform the Chi-squared test on this table

```
chisq.test( matrix( c(2,18,3,17), 2,2) )
```

```
##
##  Pearson's Chi-squared test with Yates' continuity correction
##
## data:  matrix(c(2, 18, 3, 17), 2, 2)
## X-squared = 0, df = 1, p-value = 1
```

which (as you would expect from the data) shows no evidence for a difference in duplication frequency between the two populations. The same result is seen when using Fisher's Exact Test (which is probably the better test to use here, due to the small sample size, and low counts in the table):

```
fisher.test( matrix( c(2,18,3,17), 2,2) )
```

```
##
##  Fisher's Exact Test for Count Data
##
## data:  matrix(c(2, 18, 3, 17), 2, 2)
## p-value = 1
## alternative hypothesis: true odds ratio is not equal to 1
## 95 percent confidence interval:
##  0.04766622 6.29273155
## sample estimates:
## odds ratio
##  0.6369091
```

Assignment

The assignment for this module is due at 5pm on 26 April (Wednesday stream) or 27 April (Thursday stream). For your document, please provide answers to the questions below, and also the questions at the end of the week 2 and week 3 handouts. When answering each question, please provide the R code used to generate the output (if required), the output itself, and any comments/discussion needed to fully answer the question. Please keep the code, output and comments together for each question (similar to how the lab handouts are laid out).

Week one questions:

Please include the following details in your document:

1. The data being used in this lab were generated as part of the 1000 Genomes Project. Briefly describe what sort of technology has produced these data, and how we are using this information to examine gene copy number.
2. The populations used in this lab are denoted CEU, CHB and YRI. Explain what these codes mean.
3. Load the IRGM data, and display the data for sample NA18510. Explain what these numbers relate to, in terms of the original aligned read data from the 1000 Genomes Project.
4. Calculate the median of the data for sample NA18510. Assuming that two copies of the region upstream of IRGM gene are present in most individuals, what value would you expect the data to take in the region of IRGM if three copies of this region were present? What if only one copy were present?
5. Generate a plot of sample NA18510 with the median marked on the plot via the abline command. Also show the lines expected for three copies, and one copy. Based on the results of your plot, how many copies of the region upstream of IRGM do you think this individual has?
6. How many reads were aligned for sample NA18510 across this region? Assuming that the reads are each 100bp long, and the region itself spans 200,000 bases, calculate the average sequence read depth for this region.
7. Use the colSums function to calculate the total number of reads aligned for each sample. Generate a histogram showing the average read depth for each sample.
8. Examine the IRGM plots for the first 20 samples from each of the CEU, YRI and CHB populations. Which population exhibited a higher incidence of a deletion upstream of IRGM? What was the number of samples with this deletion in each of the CEU, CHB and YRI populations?
9. Perform a Fisher's Exact Test to determine whether the differences you observe in IRGM deletion frequency differ across the three populations. Based on the output of the test, what do you conclude about IRGM deletion is these populations?