Whole Exome Sequencing Data analysis tutorial

Step 1 : Data retrieval

Database - Introduction

The Sequence Read Archive (SRA) stores raw sequence data from "next-generation" sequencing technologies including Illumina, 454, IonTorrent, Complete Genomics, PacBio and OxfordNanopores. In addition to raw sequence data, SRA now stores alignment information in the form of read placements on a reference sequence. The SRA is NIH's primary archive of high-throughput sequencing data and is part of the International Nucleotide Sequence Database Collaboration (INSDC) that includes at the NCBI Sequence Read Archive (SRA), the European Bioinformatics Institute (EBI), and the DNA Database of Japan (DDBJ). Data submitted to any of the three organizations are shared among them.

Resources

SRA

https://www.ncbi.nlm.nih.gov/sra

SRA_Toolkit

https://www.ncbi.nlm.nih.gov/sra/docs/toolkitsoft/

open terminal

1. create and change working directory

```
$mkdir WES (create a directory named “WES”)
$cd WES (change working directory to “WES”)
$mkdir fastq (create a directory named “fastq”)
```

2. To download fastq files from SRA

```
$fastq-dump SRR5858211 –split-files -o fastq (this command will download fastq files with the id
SRR5858211 from SRA, the argument –split-files will separate the reads into SRR5858211_1 and
SRR5858211_2)
```

Step 2: Quality check and Read Preprocessing

Resources

FastQC

http://www.bioinformatics.babraham.ac.uk/projects/fastqc/

Fastp

https://github.com/OpenGene/fastp

Introduction

All the methods in this tutorial are better applicable to Illumina data sets only. Although Illumina high throughput sequencing provides highly accurate sequence data, several sequence artifacts, including base-calling errors and small insertions/deletions, poor quality reads and a primer/adapter contamination, are quite common in the high throughput sequencing data, including substitution errors. The error rates can vary from 0.5-2.0% with errors mainly rising in frequency at the 3’ ends of reads.

One way to investigate sequence data quality is to visualize the quality scores and other metrics in a compact manner to get an idea about the quality of a read data set. Read data sets can be improved by post processing in different ways like trimming off low-quality bases, cleaning up any sequencing adapters, and removing PCR duplicates. We can also look at other statistics such as, sequence length distribution, base composition, sequence complexity, presence of ambiguous bases etc. to assess the overall quality of the data set.

Highly redundant coverage (>15X) of the genome can be used to correct sequencing errors in the reads before assembly and errors. Various k-mer based error correction methods exist but are beyond the scope of this tutorial.

1. create directories for fastqc output
```
$mkdir fastqc_output
```

2. to check the quality of the reads
```
$fastqc -t 30 reads/SRR5858211_1.fastq -o fastqc_output
$fastqc -t 30 reads/SRR5858211_2.fastq -o fastqc_output
```

(after fastqc the 1st argument is -t 30 which specifies the number of threads to use for preocessing. 2nd argument is the fastq file to be checked for quality. 3rd argument is -o the location for fastqc reports)

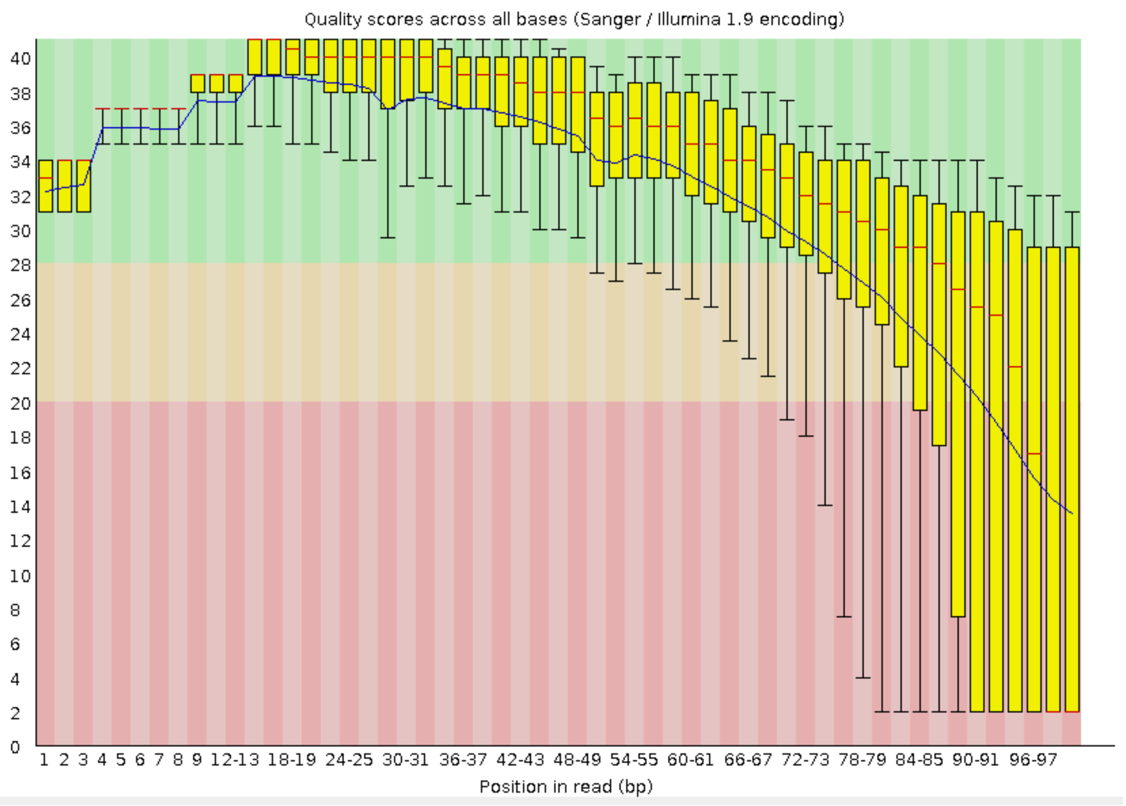
3. To view the fastqc reports use any browser
```
$firefox fastqc_output/SRR5858211_1_fastq.html
```

The report file will have a Basic Statistics table and various graphs and tables for different quality statistics. E.g.:

Table 1: FastQC Basic Statistics table

File name	**input.fastq**
File type	Conventional base calls
Encoding	Sanger / Illumina 1.9
Total Sequence	40000
Filtered Sequence	0
Sequence length	100
%GC	48

Figure 1: Per base sequence quality plot for example .fastq



A Phred quality score (or Q-score) expresses an error probability. In particular, it serves as a convenient and compact way to communicate very small error probabilities. The probability that base A is wrong (P (∼ A)) is expressed by a quality score, Q(A), according to the relationship:

$Q(A) = -10\log10(P(\sim A))$

fastp is a powerful and efficient FASTQ preprocessor designed for high-throughput sequencing data. It combines quality control, adapter trimming, and filtering into a single, fast, and user-friendly tool.

1. create directory for fastp output

```
$mkdir trimmed_reads
```

2. use fastp

```
$fastp -i reads/SRR5858212_1.fastq -I reads/SRR5858212_2.fastq -o
trimmed_reads_fastq/SRR5858212_1_trimmed.fastq.gz -O
trimmed_reads_fastq/SRR5858212_2_trimmed.fastq.gz
```

(the arguments -i and -I are for read1 and read2 respectively
the arguments -o and -O are for outputs of read1 and read2 respectively)

3. quality check using fastqc on trimmed reads

```
$mkdir fastp_fastqc_output ( make directory for fastqc output)
```

```
$fastqc -t 30 trimmed_reads_fastq/SRR5858212_1_trimmed.fastq.gz -o fastp_fastqc_output
```

```
$fastqc -t 30 trimmed_reads_fastq/SRR5858212_2_trimmed.fastq.gz -o fastp_fastqc_output
```

to view the report

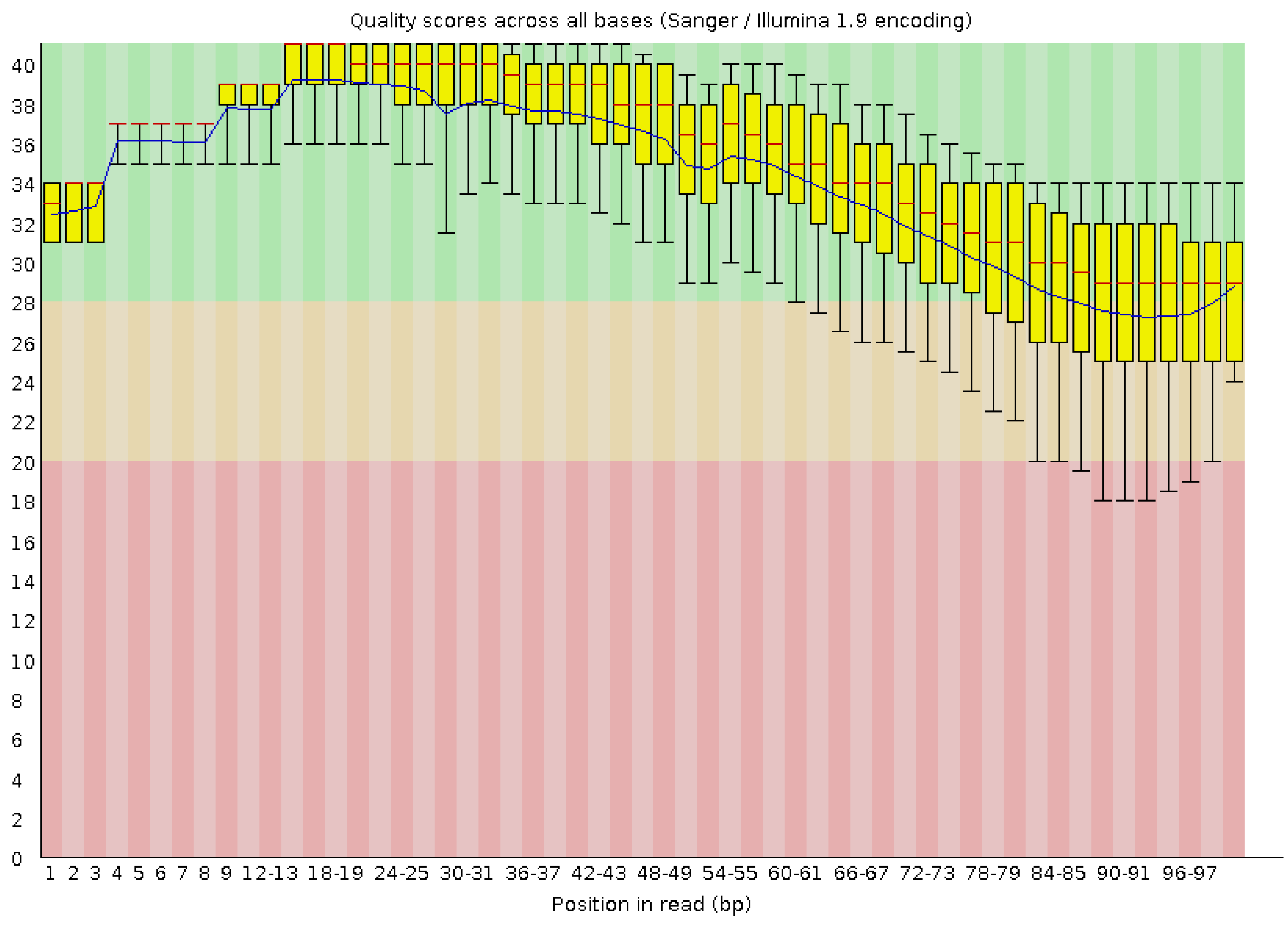
```
$firefox fastp_fastqc_output/SRR5858211_1_trimmed_fastqc.html
```

The output should be like:

Table 2: FastQC Basic Statistics table

File name	**output_trimmed_fastqc**
File type	Conventional base calls
Encoding	Sanger / Illumina 1.9
Total Sequence	38976
Filtered Sequence	0
Sequence length	50-100
%GC	48

Figure 2: Per base sequence quality plot for the quality-trimmed reads



Step 3 : READ Alignment and preprocessing

Resources

BWA

http://bio-bwa.sourceforge.net/

Samtools

http://samtools.sourceforge.net/

Introduction

The goal of this hands-on session is to perform an unspliced alignment for a small subset of raw reads. We will align raw sequencing data (after preprocessing) to the human genome using BWA and then we will manipulate the SAM output in order to visualize the alignment on the IGV browser.

1. Make directory for reference genome

```
$mkdir reference
```

Download the reference genome, here we have used GRCh38.

https://ftp.ncbi.nlm.nih.gov/genomes/all/GCF/000/001/405/GCF_000001405.40_GRCh38.p14/GCF_000001405.40_GRCh38.p14_genomic.fna.gz

2. Index the reference genome

Before starting mapping, first we first need to index the reference genome. Indexing means arranging the genome into easily searchable chunks.

```
$bwa index reference/hg38.fa
```

(bwa index will output some files with a set of extensions (.amb, .ann, .bwt, .pac, .sa)

3. Alignment using BWA

make directory for alignment files

```
$mkdir BWA_alignment
```

```
$bwa mem -t 30 reference/hg38.fa trimmed_reads/SRR5858212_1_trimmed.fastq.gz
trimmed_reads/SRR5858212_2_trimmed.fastq.gz > BWA_alignment/SRR5858212_alignment.sam
```

(we are using the bwa mem algorithm to align the reads. -t 30 specifies number of threads to be used. Next argument is fastq file of read1 then read2 and final argument is for output file in sam format)

4. Convert SAM to BAM

SAM stands for Sequence alignment mapping and BAM is its binary equivalent. This reduces storage requirements in downstream analysis.

Make directory for output

```
$mkdir BWA_alignment_conversion
```

SAM to BAM

```
$samtools view -@ 30 -bS BWA_alignment/SRR5858212_alignment.sam -o BWA_alignment_conversion/SRR5858212_alignment.bam
```

(-@ 30 for number of threads used, -bS specifies input file is in SAM format and output file should be in BAM format. -o specifies the output BAM file)

5. Sort BAM files

When you map and align the reads to the reference, the resulting read alignments are in random order with respect to their position in the reference genome. In other words, the .bam file is in the order that the sequences occurred in the input .fastq file. So the BAM files must be sorted.

6. BWA_alignment_sort

Make directory for output

```
$mkdir BWA_alignment_sort
```

```
$samtools sort -@ 30 BWA_alignment_conversion/SRR5858212_alignment.bam -o BWA_alignment_sort/SRR5858212_alignment_sort.bam
```

(sort function, 1st argument is the bam file then -o specifies the output file)

Step 4: Remove PCR duplicates and add read group

Resources

https://broadinstitute.github.io/picard/

Accurate variant calling requires the removal of PCR duplicates to avoid false positives and ensure reliable identification of true genetic variations. Removing PCR duplicates improves the overall quality of the sequencing data by reducing noise and increasing the accuracy of downstream analyses.

Make directory for output

```
$mkdir PCR_duplicates_removed
```

```
$java -Xmx100g -jar /home/bioinformatics/Desktop/Workshop/Tools/picard-tools-1.141/picard.jar
MarkDuplicates INPUT=BWA_alignment_sort/SRR5858212_alignment_sort.bam
OUTPUT=PCR_duplicates_removed/SRR5858212_alignment_PCR.bam
REMOVE_DUPLICATES=true METRICS_FILE=SRR5858212_alignment_PR.Metrics
VALIDATION_STRINGENCY=SILENT
```

(-Xmx100g allocates 100GB of memory so that there are enough memory for processing large BAM files. -jar ../picard.jar path to the picard jar file. MarkDuplicates is the tool name, INPUT is the sorted bam file. Output is user defined, also a bam file. REMOVE_DUPLICATESis set to true, which will remove the duplicates from output file. METRIC_FILE will have information on duplicates identified and removed. VALDIATION_STRINGENCY is set to silent to suppress warnings and errors. Useful for large datasets)

ADD read group

Read groups provide metadata that helps organize and track large datasets, especially when dealing with multiple samples, libraries, and sequencing runs.

```
java -Xmx100g -jar /home/bioinformatics/Desktop/Workshop/Tools/picard-tools-1.141/picard.jar
AddOrReplaceReadGroups INPUT=PCR_duplicates_removed/SRR5858212_alignment_PCR.bam
OUTPUT=Add_read_group/SRR5858212_alignment_RG.bam SORT_ORDER=coordinate
RGID=SRR5858212 RGLB=SRR585812 RGPL=illumina RGPU=SRR5858212
RGSM=SRR5858212 CREATE_INDEX=true VALIDATION_STRINGENCY=SILENT
```

(AddOrReplaceReadGroups is the tool name, INPUT is the PCR duplicates removed bam file. Output is also a bam file. SORT_ORDER set to coordinate. RGID, RGLB, RGPL, RGPU and RGSM are the readgroup ID, Library, Platform and sample respectively. CREATE_INDEX is set to true, which will create index file for the output bam file. VALIDATION_STRINGENCY is set to SILENT to suppress warnings.)

Step 5: Variant calling and Annotation

Resource

https://github.com/broadinstitute/gatk/releases

Once you have aligned file against the human reference genome, you detected nucleotidie level changes in the raw reads by comparing the reference genome using variant caller tools. There are several best performing tools exist, such as DeepVariant, GATK, samtools and the Strelka etc.

GATK is a fast and accurate variant caller optimized for germline and somatic variants detection. In this tutorial, we used a germline method to detect all variants from the retinoblastoma samples.

1. Make directory for output

```
$mkdir variant_calling
```

2. GATK HaplotypeCaller

```
$/home/bioinformatics/Downloads/gatk-4.2.0.0/gatk HaplotypeCaller --java-options "-Xmx100g"
-R ../../reference/hg38.fa -I Add_read_group/SRR5858212_alignment_RG.bam -O
variant_calling/SRR5858212_GATK.vcf.gz
```

(HaplotypeCaller is the tool used to call variants, -Xmx100g specifies 100GB of memory allocation, -R for the reference genome file in fasta format. -I input BAM file. -O output VCF file.

Variant filtering based on desired genomic regions

By filtering the VCF file based on a BED file, you can focus on specific regions of the genome, such as coding regions, exons, or regions of interest for a particular study. Here we use the tool tabix

```
$tabix -h -R ../../Covered_region.bed variant_calling/SRR5858212_GATK.vcf.gz >
variant_calling/SRR5858212_GATK_Covered.vcf
```

(tabix is the tool, -h specifies to use the index to query the VCF file, -R specifies the BED file containing the genomic region of interest. Next argument is the input VCF file containing variant calls then ">" followed by the output vcf file.

Variant annotation

ANNOVAR is a rapid, efficient tool to annotate the functional consequences of genetic variation from high-throughput sequencing data.

Make directory for output files

```
$mkdir variant_annotation
```

variant annotation

```
$perl /home/bioinformatics/Documents/Tools/Annovar_37/table_annovar.pl
variant_calling/SRR5858212_GATK_Covered.vcf
/home/bioinformatics/Music/eyeVarP/Refence/humandb/ -buildver hg38 -out
variant_annotation/SRR5858212_annovar -otherinfo -remove -protocol
refGene,esp6500siv2_all,1000g2015aug_all,cosmic70,esp6500siv2_all,exac03,gnomad30_genom
e,nci60,clinvar_20210123,avsnp150,ljb26_all,dbnsfp41a,dbscsnv11,intervar_20180118,mcap,revel
,regsnpintron -operation g,f,f,f,f,f,f,f,f,f,f,f,f,f,f,f,f -nastring . -vcfinput
```

(table_annovar.pl is a perl script and should be executed with a perl interpreter. Next argument is the input vcf file. Then location to humandb directory which contains all the preprocessed databases. Then the reference genome build version, -out for the output prefix for annotated vcf file. -otherinfo for additional information. -remove to remove temporary file. -protocol defines the set of databases and annotations to use. -operation which operation to use for each protocol. G means gene based. G means filter based. -nastring . Will fill fields with no annotation with a "." -vcfinput specifies that the input is in vcf file format.

Step 6: VarP

VarP is a variant prioritization model that gives a score to each variant based on their pathogenicity, Allele frequency and other user defined parameters.

Make output directory

```
$mkdir VarP
```

replace all the “=.” to “-999”.

```
$sed 's/=.;/=-999;/g' variant_annotation/SRR5858212_annovar.hg38_multianno.vcf > variant_annotation/SRR5858212_VarP.vcf
```

(sed executes the stream editor, s for substitution, / is the delimiter. In this command it searches for the patter “=.;” and changes them all to “-999;” . g is for global replacement. Then input and output file names)

VarP input

```
$printf 'variant_annotation/SRR5858212_VarP.vcf\tSRR5858212' > variant_annotation/SRR5858212.txt
```

this text file will be the input file for VarP it contains the path to the vcf file after the substitution mentioned in the previous step separated by a tab and the name of the sample.

VarP

```
$python3 /home/bioinformatics/Music/eyeVarP/Docker/VarP/VarP.py priority varp/SRR5858212_varp variant_annotation/SRR5858212.txt /home/bioinformatics/Music/eyeVarP/Docker/VarP default_0.001_variants_parameters_PPF.txt
```

(VarP is a python executed file. priority is the name of the function. Next argument is the prefix of the output file. then the input txt file. then the parameters file.)

Combine VarP with heuristic method

```
$Rscript /home/bioinformatics/Music/eyeVarP/Docker/Rscript/code/Filtering.r variant_annotation/SRR5858212_annovar.hg38_multianno.txt varp/SRR5858212_varp.txt varp/SRR5858212
```

The filtering program is an Rscript. It takes two inputs. one is the variant annotation file from annovar another one is the prioritized file from VarP. It merges information from both the files and produces a new dataset with combined annotations and scores.

```
$grep -Ew 'Gene.refGene|splicing|stopgain|nonsynonymous SNV|frameshift deletion|frameshift insertion|stoploss|startloss' varp/SRR5858212.csv > varp/SRR5858212_VarP_extracted.csv
```

this command will extract lines with the mentioned keywords after the -Ew flag from the input csv file and writes into the output csv file.

Step 7: Exomiser and eyeVarP

Resources

https://exomiser.readthedocs.io/en/latest/

eyeVarP https://doi.org/10.1016/j.gim.2023.100862

Exomiser is a powerful bioinformatics tool designed to prioritize disease-causing genetic variants identified through whole-exome or whole-genome sequencing. Exomiser incorporates patient phenotypes (described using HPO terms) into the variant prioritization process.

Exomiser needs a input yml file with the path to the input vcf file and HPO ID of the phenotypes we want to prioritize the variants based on.

```
$java -jar /home/bioinformatics/Downloads/exomiser/Exomiser/exomiser/exomiser-cli-
12.1.0/exomiser-cli-12.1.0.jar --analysis eyeVarP/test-analysis-exome.yml –
spring.config.location=/home/bioinformatics/Downloads/exomiser/Exomiser/exomiser/exomiser-cli-
12.1.0/application.properties
```

(Exomiser is a java executable jar file. --analysis specifies the analysis configuration yml file. --spring.config.location specifies the application.properties file.

eyeVarP model

eyeVarP is a A computational framework for the identification of pathogenic variants specific to eye disease.

```
$Rscript /home/bioinformatics/Music/eyeVarP/Docker/Rscript/code/eyeVarP.r
eyeVarP/SRR5858212_GATK_Covered.variants.tsv varp/SRR5858212_VarP_extracted.csv
/home/bioinformatics/Music/eyeVarP/Refence/Gene_ranking.csv
eyeVarP/SRR5858212_eyeVarP_final
```

eyeVarP is programmed in R. It takes three input files. A tsv file generated by exomiser, a VarP output file and a gene ranking file. the last argument is the prefix of the output file.