# Project:

# (From Raw Reads to Gene Insights)

# RNA-Seq Analysis of Human LCLs (Lymphoblastoid cell lines)

## Subtitle :

An exploration of gene expression in Yoruba individuals using public RNA-Seq data

## Course :

Bioinformatics with Advance AI (Pulse, AIIMS Delhi)

## Date :

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## Presented by :

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# Project Overview :

**Organism:** ***Homo sapiens*** **(Human)**

**Sample: YRI Lymphoblastoid Cell Line**

**SRA Project ID: PRJNA207555**

**SRA Run ID: SRR19762473**

# Why Chose This Dataset:

✅ **Population-Specific Insight**
The dataset represents Yoruba individuals in Ibadan, Nigeria (YRI), which is one of the most studied African populations in genomics — valuable for population genetics and expression variability.

✅ **Well-Characterized Samples**
Lymphoblastoid cell lines (LCLs) are widely used in functional genomics because they're standardized, renewable, and consistent across studies.

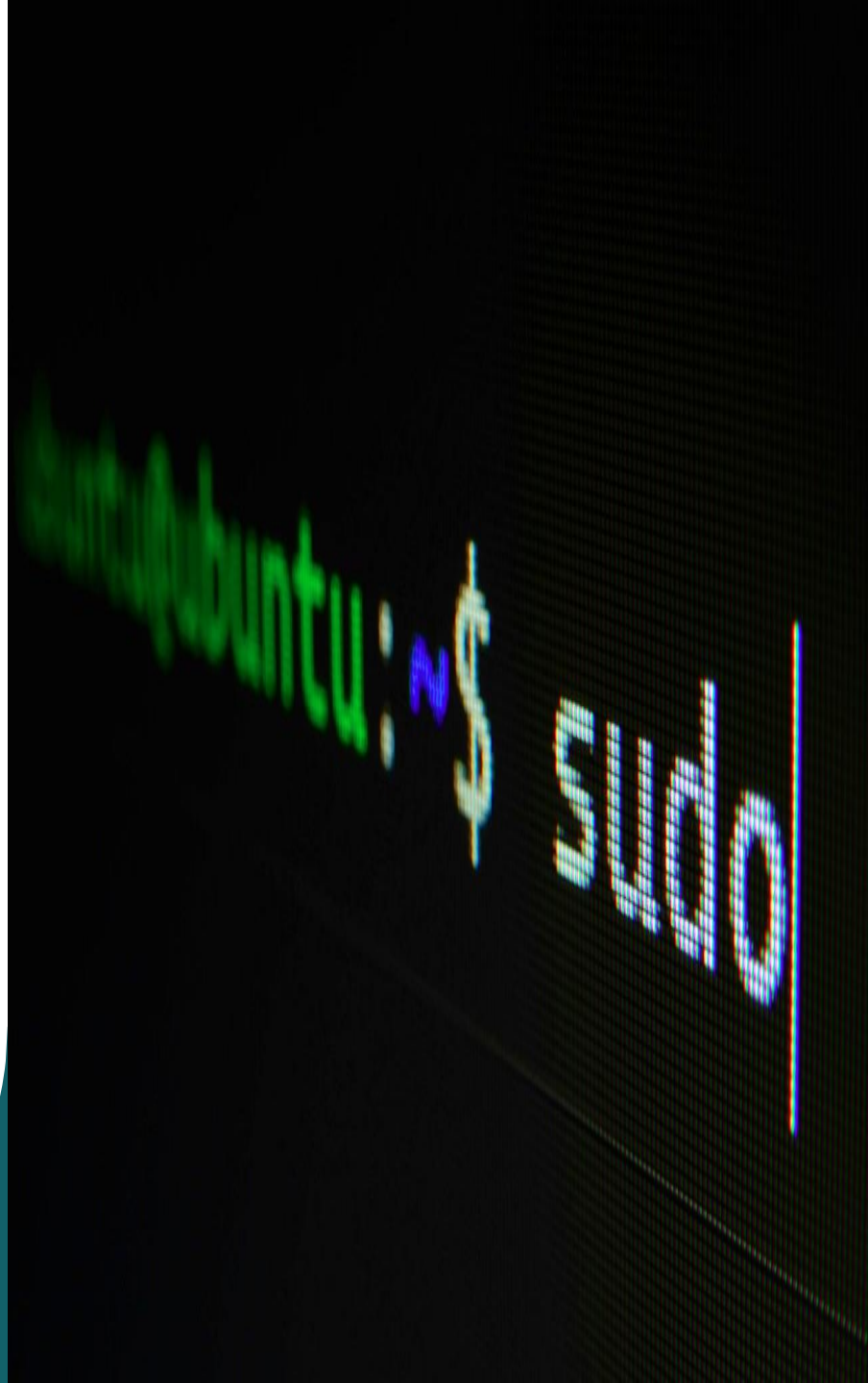
✅ **Part of the 1000 Genomes Project**
This makes the data reliable and extensively annotated, allowing for comparison with genomic variation data.

✅ **Publicly Available & High Quality**
The data is open-access, peer-reviewed, and associated with detailed metadata, which supports reproducibility.

✅ **Suitable for RNA-Seq Analysis**
The data includes paired-end RNA-seq reads — ideal for studying gene expression, alternative splicing, and transcript assembly.

# Workflow Steps :

1. **Download SRR files** from NCBI SRA
2. **Convert** .sra → .fastq using fasterq-dump
3. **Quality check** with **FASTQC** (before trimming)
4. **Trim** low-quality reads and adapters with **Trimmomatic**
5. **Re-check quality** with FASTQC (after trimming)
6. **Align reads** to human genome (**hg38**) using **BWA**
7. **Convert & sort** alignment files using **SAMtools**
8. **Visualize** alignments in **IGV**
9. Perform **variant calling**
10. **Reflect** and report biological insights



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# FASTQC Analysis

**Quality Assessment of Raw Sequencing Reads**

# Step 1: Download the data using prefetch

➢ *prefetch -O ./test_fqc SRR19762473*

- **Tool**: *prefetch* (from NCBI SRA Toolkit)
- **Purpose**: Downloads SRA data from NCBI to your local computer.
- *-O ./test_fqc*: Specifies the output directory (*test_fqc*) where the data should be saved.
- *SRR19762473*: The accession number of the sample to be downloaded.

**Output**:

- It confirmed a successful download using HTTPS.
- The data is saved in *./test_fqc/SRR19762473* in SRA's proprietary format (*.sra*)

# Step 2: Convert SRA to FASTQ using fasterq-dump

➢ *fasterq-dump ./test_fqc/SRR19762473*

- **Tool**: *fasterq-dump* (also from SRA Toolkit)
- **Purpose**: Converts the *.sra* file to standard **FASTQ** format used in downstream tools.
- Since this is **paired-end data**, it generated two files:

*i.* *SRR19762473_1.fastq* (forward reads)

*ii.* *SRR19762473_2.fastq* (reverse reads)

**Output**:

*spots read : 23,278,323*

*reads read : 46,556,646*

- **"Spots"**: Each spot usually represents one fragment (i.e. a DNA insert).
- Since this is paired-end, each spot has 2 reads, hence 46 million reads from 23 million spots.

# Step 3: Moving the FASTQ files into the correct folder

➢ *cd ..*

*mv SRR19762473_*.fastq ~/test_fqc/*

- Moved the two FASTQ files into *test_fqc* folder.
- Now *test_fqc* folder has:

*i.* *SRR19762473* (original downloaded .sra)

*ii.* *SRR19762473_1.fastq*

*iii.* *SRR19762473_2.fastq*

# Step 4: Quality Check using FastQC

➢ *fastqc SRR19762473_1.fastq SRR19762473_2.fastq*

- **Tool**: FastQC
- **Purpose**: Performs a **quality check** on the raw FASTQ files. This includes –

i. Per base sequence quality

ii. GC content

iii. Adapter content

iv. Overrepresented sequences

v. Sequence duplication levels

## **Output**:

- The progress lines like:

*Approx 25% complete for SRR19762473_1.fastq*

indicate the analysis is running.

- *null* lines at the top can be ignored — possibly just standard error output.

After analysis, **FastQC** generates:

➢ An *.html* report for each input file (*SRR19762473_1_fastqc.html*)

i. https://muskan-fastqc-report-1.netlify.app/

ii. https://muskan-fastqc-report-2.netlify.app/

➢ A *.zip* file with raw data.

# Summary of Tools Used:

| Step | Tool | Purpose |
|---|---|---|
| **1** | *prefetch* | Download *.sra* file from SRA database |
| **2** | *fasterq-dump* | Convert *.sra* to *.fastq* |
| **3** | *mv* | Move files to organize them |
| **4** | *FastQC* | Assess quality of raw reads |

# Trimmomatic Analysis

**Adapter Removal and Quality Trimming of Paired-End Reads**

# Step 1: Update & Install Trimmomatic

- *sudo apt update*
- *sudo apt install trimmomatic*

- *sudo apt update*: Updates the list of available packages.
- *sudo apt install trimmomatic*: Installs Trimmomatic, a tool for trimming sequencing reads.
- By default, Trimmomatic was installed in a *.jar* file: */usr/share/java/trimmomatic.jar.*

- *java -jar /usr/share/java/trimmomatic.jar –version*

- Use *java –jar* to run the *.jar* file because *trimmomatic* isn't a shell command by itself.
- Output *0.39* confirms it's installed correctly.

# Step 2: Downloaded the Adapter File

- *wget https://raw.githubusercontent.com/timflutre/trimmomatic/master/adapters/TruSeq3-PE.fa -O TruSeq3-PE.fa*
- Trimmomatic needs the adapter file *TruSeq3-PE.fa*
- This downloaded the adapter file to home directory.

- *realpath ~/TruSeq3-PE.fa*
- This returned */home/piggyubuntu/TruSeq3-PE.fa*

# Step 3: Run Trimmomatic

- *java -jar /usr/share/java/trimmomatic.jar PE -threads 4 *

  *SRR19762473_1.fastq SRR19762473_2.fastq *

  *SRR19762473_1_paired.fastq SRR19762473_1_unpaired.fastq *

  *SRR19762473_2_paired.fastq SRR19762473_2_unpaired.fastq *

  *ILLUMINACLIP:/home/piggyubuntu/TruSeq3-PE.fa:2:30:10 *

  *LEADING:3 TRAILING:3 SLIDINGWINDOW:4:20 MINLEN:36*

# What Did This Trimmomatic Command Do?

| Parameter | Description |
|---|---|
| *PE* | Paired-end mode |
| *-threads 4* | Use 4 CPU threads |
| Input files | *SRR19762473_1.fastq and SRR19762473_2.fastq* |
| Output (paired reads) | **_1_paired.fastq, *_2_paired.fastq* |
| Output (unpaired reads) | **_1_unpaired.fastq, *_2_unpaired.fastq* |
| *ILLUMINACLIP:* | Remove adapter contamination using adapter file |
| *LEADING:3* | Trim low-quality bases (below 3) from the start |
| *TRAILING:3* | Trim low-quality bases (below 3) from the end |
| *SLIDINGWINDOW:4:20* | Trim if average quality over 4 bases drops below 20 |
| *MINLEN:36* | Drop reads shorter than 36 bases |

# 📊 Results of Trimmomatic

**From the output:**

*Input Read Pairs: 23278323*

*Both Surviving: 19338632 (83.08%)*

*Forward Only Surviving: 3514321 (15.10%)*

*Reverse Only Surviving: 237256 (1.02%)*

*Dropped: 188114 (0.81%)*

- ✅ **Paired** reads retained: ~83%
- 📦 **Unpaired** reads (only one mate survived): ~16%
- 🗑️ **Dropped** reads: less than 1%

# Step 4: Organized Output

Moved the trimmed output files to a new folder:

- *mkdir -p ~/test_trimmomatic*
- *mv ~/test_fqc/SRR19762473_*_paired.fastq ~/test_fqc/SRR19762473_*_unpaired.fastq ~/test_trimmomatic/*

Now *~/test_trimmomatic* contains:

i. **_1_paired.fastq*

ii. **_2_paired.fastq*

iii. **_1_unpaired.fastq*

iv. **_2_unpaired.fastq*

# Step 5: Re-ran FastQC on Trimmed Data

➢ *fastqc SRR19762473_*_paired.fastq SRR19762473_*_unpaired.fastq*

This checks whether:

✓ Adapter contamination is gone

✓ Quality scores improved

✓ Low-quality reads were effectively trimmed

FastQC produces:

- .html reports for each file
- You can open them in a browser to visually inspect quality

# FastQC produces:

1. **Paired reads:**

- SRR19762473_1_paired.fastq

https://muskan-srr19762473-1-paired-fastqc.netlify.app

- SRR19762473_2_paired.fastq

https://muskan-srr19762473-2-paired-fastqc.netlify.app

**2. Unpaired reads:**

- SRR19762473_1_unpaired..fastq

https://muskan-srr19762473-1-unpaired-fastqc.netlify.app

- SRR19762473_2_unpaired.fastq

https://muskan-srr19762473-2-unpaired-fastqc.netlify.app

# Summary

| Step | Description |
|---|---|
| Installed Trimmomatic | For quality trimming and adapter removal |
| Downloaded Adapter File | Used *wget* to get *TruSeq3-PE.fa* |
| Trimmed Reads | Cleaned reads based on adapter and quality thresholds |
| Verified Output | Checked trimming results and moved files |
| Re-evaluated Quality | Ran FastQC again to confirm trimming improved quality |

# BWA Alignment and SAM/BAM Processing

**Mapping Trimmed Reads to the Human Reference Genome (hg38)**

# Step 1: Downloading the Reference Genome

➢ *wget https://hgdownload.soe.ucsc.edu/goldenPath/hg38/bigZips/hg38.fa.gz*

- Downloads the **human reference genome (hg38)** in compressed FASTA format from UCSC.
- *hg38.fa.gz* is a gzipped file containing all human chromosomes.

➢ *gunzip hg38.fa.gz*

- Uncompresses the *hg38.fa.gz* file to *hg38.fa* , which is readable by BWA.

# Step 2: Indexing the Reference Genome (Required by BWA)

➢ *bwa index hg38.fa*

- BWA builds an index from the reference genome, allowing it to **rapidly align short reads**.
- This creates several index files *(.amb, .ann, .bwt, .pac, .sa)* necessary for alignment.

# Step 3: Aligning Paired-End Reads to the Reference Genome

➢ *bwa mem hg38.fa ~/test_trimmomatic/SRR19762473_1_paired.fastq ~/test_trimmomatic/SRR19762473_2_paired.fastq > SRR19762473_aligned.sam*

- Aligns **quality-trimmed paired-end reads** to the reference genome.
- Output is in **SAM format** (Sequence Alignment/Map), a human-readable alignment file.

- *bwa mem* is preferred for reads ≥70bp, and outputs a SAM file showing where each read maps in the genome.

# Step 4: Converting SAM to BAM (Binary Format) & Sorting the BAM File

➢ *samtools view -bS SRR19762473_aligned.sam > SRR19762473_aligned.bam*

- Converts the large SAM file to a BAM file (binary, compressed version of SAM).
- BAM is faster to process and compatible with most downstream tools.

➢ *samtools sort SRR19762473_aligned.bam -o SRR19762473_aligned_sorted.bam*

Sorts reads by their coordinates in the genome, which is essential for:

- Variant calling
- Visualization (e.g. in IGV)
- Duplicate marking

# Step 5: Indexing the Sorted BAM File

➢ *samtools index SRR19762473_aligned_sorted.bam*

- Creates an index *(.bai)* that allows rapid access to any region of the BAM file.
- Required for tools like **IGV**, **bcftools**, or **GATK** to access alignments quickly.

✅ **Result**

i. SRR19762473_aligned_sorted.bam → sorted alignment file

ii. SRR19762473_aligned_sorted.bam.bai → index for that BAM

✓ Ready for **visual inspection** (e.g., IGV) or **variant calling** (e.g., with *bcftools* or GATK)

# IGV Visualization of Aligned Reads

To visualize the aligned sequencing reads *(.bam)* on the human reference genome (**hg38**) using **Integrative Genomics Viewer (IGV)**

# Steps Performed:

1. **Launched IGV Desktop App -**

Version: *2.19.5* with built-in Java

**2. Loaded Reference Genome -**

Selected **Human hg38** from IGV genome list

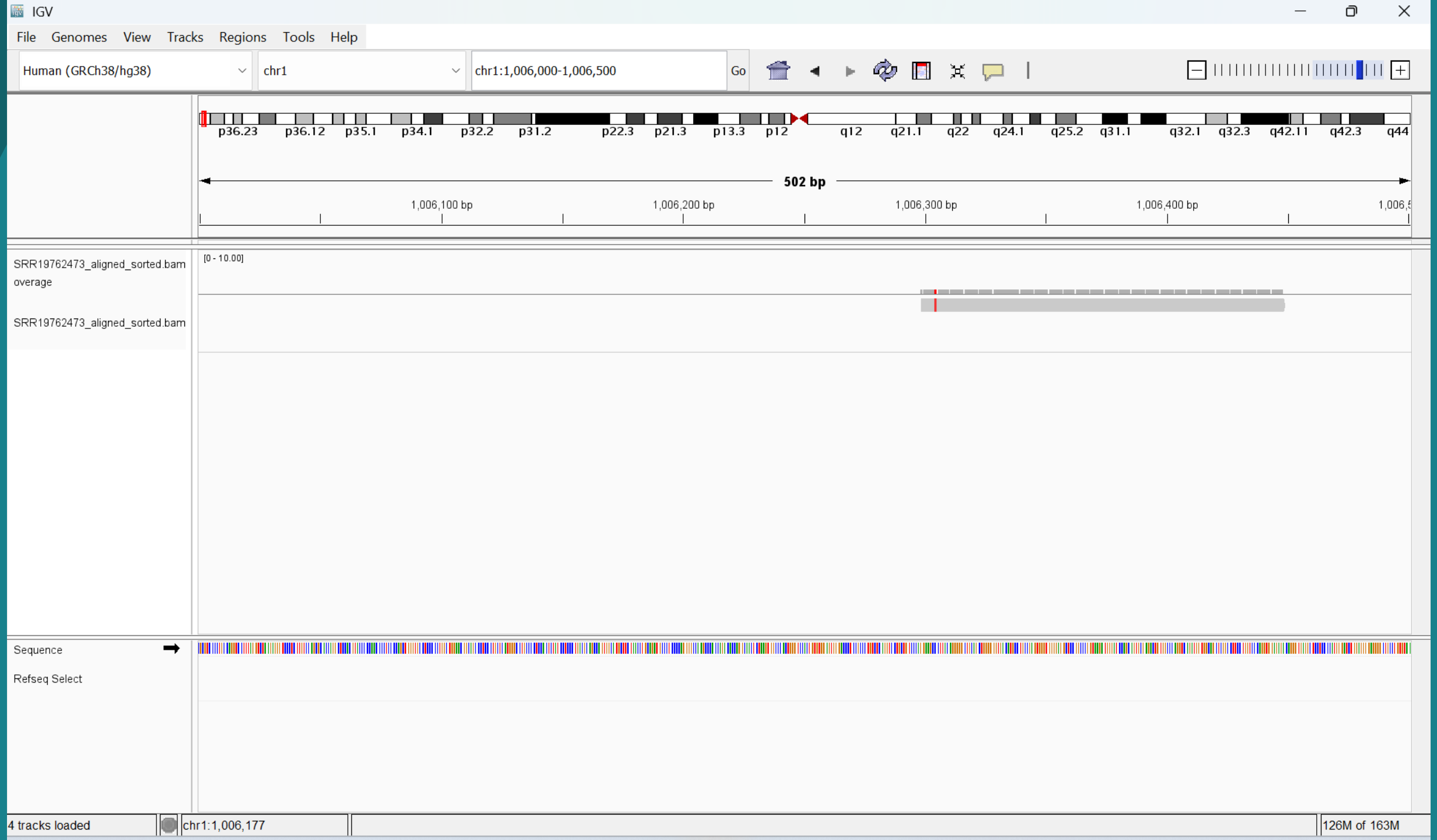
**3. Loaded Aligned Data -**

Loaded *SRR19762473_aligned_sorted.bam* and its index *.bai*

**4. Navigation –**

Jumped to region *chr1:1,006,000–1,006,500* using the search bar.

Zoomed in to observe aligned reads and mismatches.


IGV
File Genomes View Tracks Regions Tools Help
Human (GRCh38/hg38)
chr1
chr1:1,006,000-1,006,500
Go
p36.23 p36.12 p35.1 p34.1 p32.2 p31.2 p22.3 p21.3 p13.3 p12 q12 q21.1 q22 q24.1 q25.2 q31.1 q32.1 q32.3 q42.11 q42.3 q44
502 bp
1,006,100 bp
1,006,200 bp
1,006,300 bp
1,006,400 bp
SRR19762473_aligned_sorted.bam overage
[0 - 10.00]
SRR19762473_aligned_sorted.bam
Sequence
Refseq Select
4 tracks loaded
chr1:1,006,177
126M of 163M

# IGV Visualization of Aligned Reads :

This image shows a zoomed-in view of **aligned sequencing reads** from the file *SRR19762473_aligned_sorted.bam* visualized using **IGV** over the **hg38 reference genome** at region *chr1:1,006,000–1,006,500.*

**Interpretation:**

- **Gray bars** represent individual reads successfully aligned to the reference genome.
- A **red bar** indicates a **mismatch** (potential SNP or sequencing error) at that base position.
- The **bottom sequence track** shows the reference genome bases.
- The **"Coverage" track** (gray histogram above the reads) is visible but shallow, indicating **low depth** (~1–10 reads) at this locus.

# Variant Calling & Filtering

Tool Used: **BCFtools**

# Step 1: Variant Calling with bcftools mpileup and call

```
bcftools mpileup -Ou -f ~/bwa_align/hg38.fa
~/bwa_align/SRR19762473_aligned_sorted.bam | \
bcftools call -mv -Ob -o ~/bwa_align/SRR19762473_variants.bcf
```

🔍 **What's happening?**

- *bcftools mpileup* examines the **aligned reads** (in your BAM file) against the **reference genome** *(hg38.fa)* and identifies **potential variant sites**.
- The *.ou* flag outputs **uncompressed BCF** data to stream directly into the next command.
- The *bcftools call* command actually **calls the variants** — identifies whether the differences are **SNPs (Single Nucleotide Polymorphisms)** or **indels (insertions/deletions)**.

📦 **Output:**

- *SRR19762473_variants.bcf* : A **compressed binary VCF** (BCF) file containing **all detected variants**, regardless of quality.

# Step 2: Converting BCF to Human-Readable VCF

```
bcftools view ~/bwa_align/SRR19762473_variants.bcf -Ov -o
~/bwa_align/SRR19762473_variants.vcf
```

🔍 **What's happening?**

- Converts the binary *.bcf* file into **standard VCF format** (.vcf), which is plain-text and easier to read or analyze further.
- *-ov* = Output VCF format, uncompressed.

📦 **Output:**

- *SRR19762473_variants.vcf* : Contains all the raw variant calls in human-readable text format.

# Step 3: Filtering High-Confidence Variants

```
bcftools filter -i 'QUAL>30 && DP>10' \
  ~/bwa_align/SRR19762473_variants.vcf \
  -Ov -o ~/bwa_align/SRR19762473_variants_filtered.vcf
```

🔍 **What's happening?**

Filters out **low-quality or low-confidence variant calls** based on:

*QUAL > 30*: Variant quality score must be >30 (Phred-scaled, meaning ~99.9% accuracy).

*DP > 10*: Read **depth** (coverage) must be greater than 10 reads at that site.

*-i* : include variants that meet this expression.

📦 **Output:**

*SRR19762473_variants_filtered.vcf*: Final set of **high-confidence variants** for downstream interpretation.

# Checking File Sizes

➢ *ls -lh ~/bwa_align/*variants**

📊 What is shown:

- *SRR19762473_variants.bcf* → **14 MB** (binary, compressed)
- *SRR19762473_variants.vcf* → **84 MB** (uncompressed, includes all calls)
- *SRR19762473_variants_filtered.vcf* → **19 MB** (filtered, high-quality calls)

## Summary

| Step | Tool | Purpose |
|---|---|---|
| 1⃣ | *bcftools mpileup + call* | Identify variants from aligned reads |
| 2⃣ | *bcftools view* | Convert binary BCF to readable VCF |
| 3⃣ | *bcftools filter* | Keep only high-quality variants |

# Visualize Variants in IGV:

1. **Open IGV on your Desktop**
2. **Set Genome to hg38 -**

Top left: Select "Genome" → "Load Genome" → "hg38“

**3. Load the BAM file -**

- Go to **File** → **Load from File...**
- Navigate to your *bwa_align* directory
- Select *SRR19762473_aligned_sorted.bam*

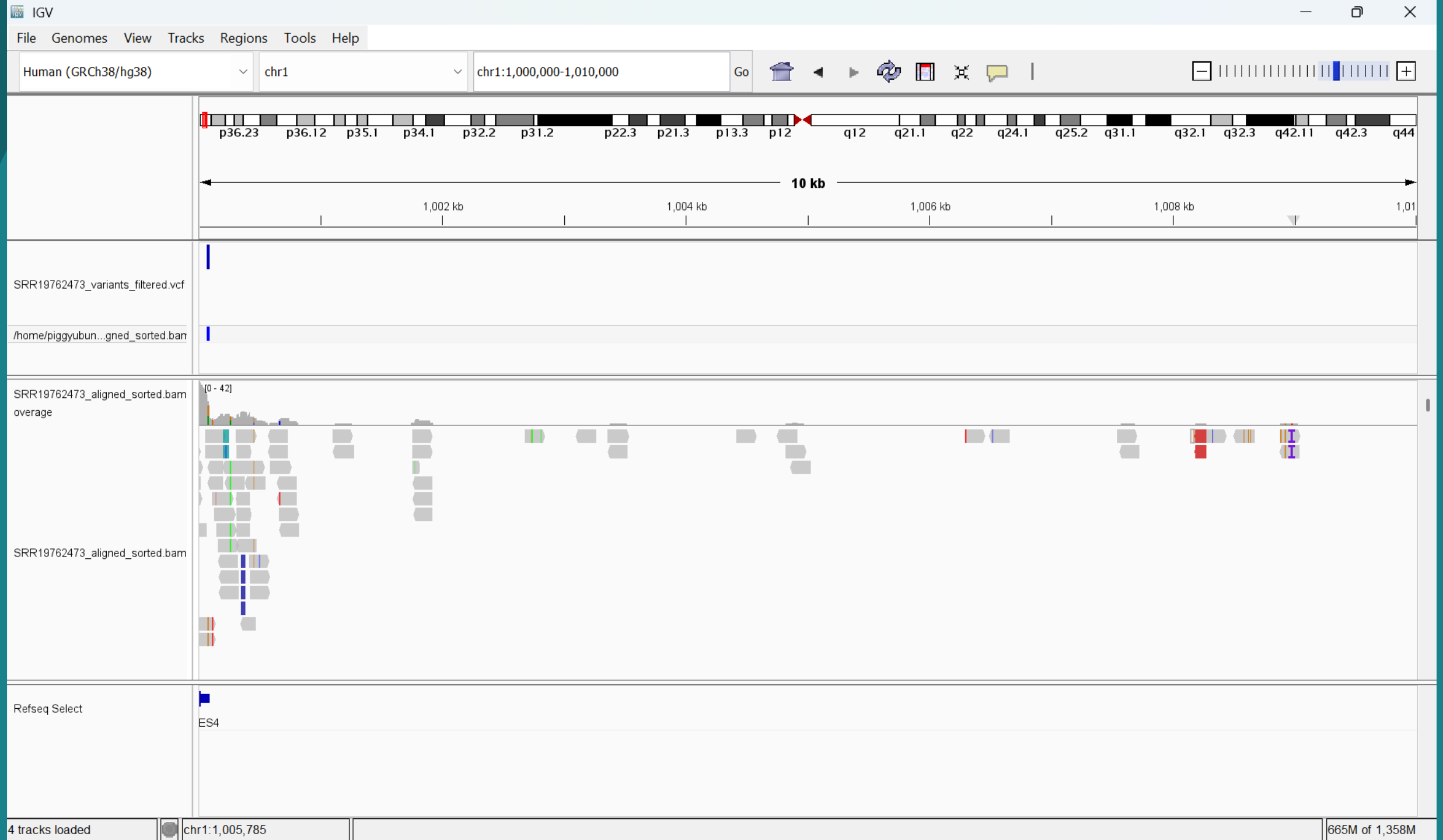
**4. Load the VCF file -**

Again go to **File** → **Load from File...**

Select *SRR19762473_variants_filtered.vcf*

**5. Navigate to a region with variants -**

- Use Ctrl+F or the **search bar** at the top (type: chr1:1000000-1010000)
- You’ll see reads in the BAM track and variant markers (colored) in the VCF track


IGV
File Genomes View Tracks Regions Tools Help
Human (GRCh38/hg38)
chr1
chr1:1,000,000-1,010,000
Go
p36.23 p36.12 p35.1 p34.1 p32.2 p31.2 p22.3 p21.3 p13.3 p12 q12 q21.1 q22 q24.1 q25.2 q31.1 q32.1 q32.3 q42.11 q42.3 q44
10 kb
1,002 kb
1,004 kb
1,006 kb
1,008 kb
SRR19762473_variants_filtered.vcf
/home/piggyubun...gned_sorted.bam
SRR19762473_aligned_sorted.bam
overage
[0 - 42]
SRR19762473_aligned_sorted.bam
Refseq Select
ES4
4 tracks loaded
chr1:1,005,785
665M of 1,358M

# 🔍 What We See in the Visualization:

- **Coverage Track** (*SRR19762473_aligned_sorted.bam*):

Displays read depth across the selected genomic region. The height of the vertical bars indicates how many sequencing reads align at each base — giving a sense of coverage.

- **Alignments Track**:

Shows individual sequencing reads as gray rectangles.

- **Colored marks** (e.g., red, blue, purple) highlight **mismatches**, **insertions**, or **deletions** compared to the reference genome.
- This helps visually confirm the presence of real variants versus sequencing errors.

- **VCF Track** (*SRR19762473_variants_filtered.vcf*):

Displays high-confidence variants called from the data.

- Only variants passing the filter *QUAL > 30 && DP > 10* are shown.
- These represent reliable single-nucleotide variants (SNVs) or indels discovered in the sample.

# Thank you

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