

Variant calling from RNA-seq data using the GATK joint genotyping workflow

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Abstract

Put the abstract here.

1 Introduction

Since the introduction of RNAseq, many researchers have seen the opportunity to use this data not only for differential expression analysis, but also for calling variants Piskol et al. [2013]. Examples of such researches include a, b, c and d. Whereas trusted bioinformatic protocols exist for detecting sequence variants on a variety of DNAseq samples (germline DNA, whole-exome sequencing etc.) that come from distinct contexts Koboldt [2020], environmental samples), protocols designed to handle RNAseq data are scarce Piskol et al. [2013]. At present the gold-standard for variant calling on RNAseq data is the GATK Per-sample workflow although an updated detailed workflow documentation for calling variants in RNAseq data is in the roadmap of the GATK experts GATK [2021a].

Currently, researchers interested in performing GATK variant calling on RNAseq data have the option of using the fully validated Per-sample workflow GATK [2021b] or using an in-progress advanced workflow designed for cloud computing GATK [2021c]. As mentioned, the Per-sample approach has several drawbacks (cite myself)

An appealing alternative would be to follow most of the GATK Best practices relative to RNAseq data and to take advantage of joint genotyping approach which is available in version 3 and 4 of GATK for germline short variants and indels (ref). The joint-genotyping method has proven to be more sensitive, more flexible and to reduce computational challenges relative to the traditional calling approach GATK [2021d]. In addition, the latter approach has the advantage to facilitate the incremental discovery of variants that origin from distinct cohorts of samples. Technically, this can be achieved by combining parts of the GATK RNAseq workflow and parts of the GATK joint genotyping workflow.

In spite that the protocol described here largely use workflows and concepts developed by the GATK team, the reader should be aware that it has not been

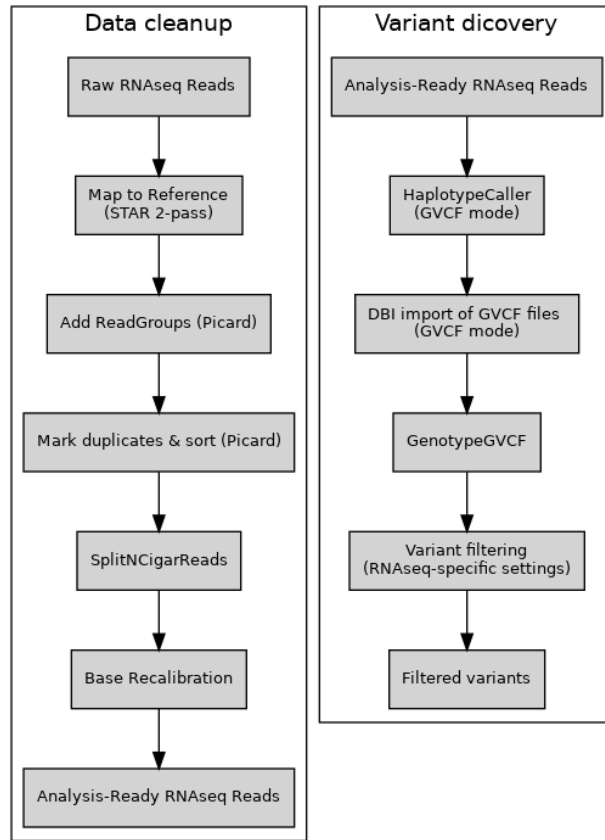


Figure 1: long desc.

validated by GATK experts. We have shown previously that a similar workflow has and we did not have experienced any problem (Brouard)

Here we present GATK4 and fully updated version of this approach.

an update end-to-end analysis of...

Figure 1 present the workflow proposed here.

In the next section, we describe how the diverse programs required to perform the whole analysis can be installed.

2 Materials

2.1 Environment

The vast majority of commands in this tutorial have been carefully tested and fully executed on a remote linux server working with the Sun Grid Engine (SGE) workload manager. Obviously, batch scripts will need to be slightly adapted if another workload manager is in place on your computer cluster or if you intend to perform the analysis locally on a linux machine.

2.2 Installing bioinformatic programs

2.2.1 The GATK suite

It is suggested to install the gatk4 suite in a separate conda environment. Assuming that you are familiar with the conda package management system, you could install all GATK programs in a environment called 'gatk4' with the following command:

```
conda create -n gatk4 -c bioconda gatk4
```

2.2.2 The SRA toolkit programs

You can easily download public sequences from the NCBI Sequence Read Archive (SRA) using the NCBI SRA toolkit. Detailed instructions about this tool can be found at https://trace.ncbi.nlm.nih.gov/Traces/sra/sra.cgi?view=toolkit_doc.. However, before using it, do not forget to configure the SRA toolkit program (<https://github.com/ncbi/sra-tools/wiki/03.-Quick-Toolkit-Configuration>). Once installed, export the SRA toolkit programs in you PATH:

```
export PATH=$PATH:$PWD/sratoolkit.2.10.9-ubuntu64/bin
```

You can make this change persistent, by adding the previous line to your .bashrc file.

2.2.3 The STAR aligner

The best practices from GATK recommend to align RNA-seq reads with STAR (ref).

Although you can retrieve and install the STAR aligner with conda, it can be installed easily by just downloading the latest STAR source from releases:

```
wget https://github.com/alexdobin/STAR/archive/2.7.7a.zip
unzip 2.7.7a.zip
cd STAR-2.7.7a/
```

You can then safely use the pre-compiled STAR executables located in the bin/ subdirectory. It is convenient to add the executables to your PATH:

```
export PATH=$PATH:$PWD/bin/Linux_x86_64
```

2.2.4 The Picard tools

We will use the Picard tools to mark duplicated reads. One could find more information about the picard tools at: <https://broadinstitute.github.io/picard/>. One can download the pre-build java program with:

```
wget https://github.com/broadinstitute/picard/releases/download/2.25.0/picard.jar
```

It is recommend to set up an environment variable to act as a shortcut. To make it persistent, simply, add a line to your .bashrc file:

```
export PICARD=/home/AAFC-AAC/brouardjs/bioinfo_programs/picard.jar
```

Then, you would be able to call Picard tools with:

```
java -jar $PICARD
```

2.2.5 Samtools, BCFtools and HTSlib

The Samtools web site (<http://www.htslib.org/>) contains a plenty of informations about the samtools suite of gold-standard programs bioinformatic programs designed to read, write, edit, index and view alignments files in the SAM, BAM and CRAM format. Less known, but just as powerful, the BCFtools are the best option to manipulate sequence variants stored in the BCF2, VCF and gVCF format. Finally, The HTSlib are a C library designed to read and write high-throughput sequencing data that is used by both the Samtools and the BCFtools. The HTSlib also contains the tabix and bgzip utilities that are mandatory to work with VCF files.

Mention the 2 2021 references that can be found on the [htslib/documentation](https://github.com/htslib/htslib). Since the Samtools, the BCFtools and the HTSlib projects are now divided in three separated repositories, the most straightforward way to make use of these three distinct packages is to build them independently.

Use the commands below to install the Samtools (and similarly for the BCFtools and HTSlib):

```
wget https://github.com/samtools/samtools/releases/download/ \
1.11/samtools-1.11.tar.bz2
bzip2 -d samtools-1.11.tar.bz2
tar -xvf samtools-1.11.tar
cd samtools-1.11
./configure --prefix=$HOME/bioinfo_programs/bcftools-1.12
```

And you may wish to add the bin directory to your \$PATH:

```
export PATH=$PATH=/home/AAFC-AAC/brouardjs/bioinfo_programs/bcftools-1.12/bin
```

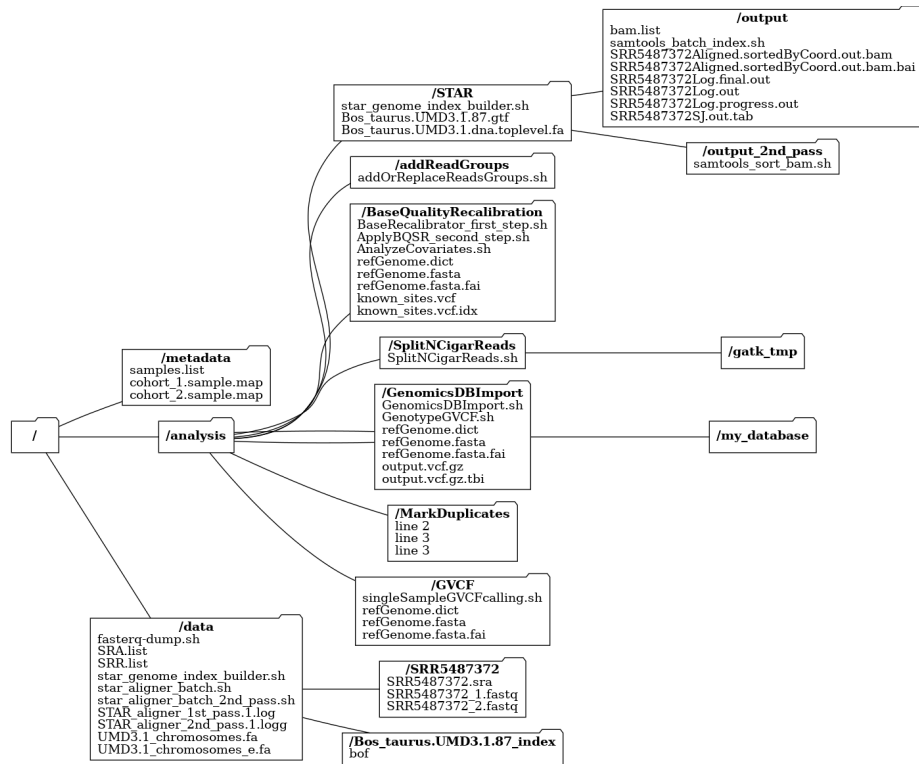


Figure 2: long desc.

3 Methods

3.1 Downloading scripts used in this tutorial

If one is interested to reproduce the workflow presented here, a good starting point would be to download the following GitHub repository:

```
git clone https://github.com/soda460/RNAseq_GATK_JGW.git
```

It contains all scripts described in this section as well as several text files that allow to easily reproduce the analysis presented here. Note that some scripts use relative paths. Therefore using the files/folder organization presentend in figure 2 will help to reproduce the results presented here with minimal changes. contained will also make the whole process easier.

3.2 Downloading the MAP dataset from Sequence Read Archive

We will use the complete RNA-seq raw sequences from [Ariel et al., 2021]. However, rather than using the 72 samples presented in the article, we will focus on 24 representative samples of this study:

The **SRA.list** file contains the SRA identifiers of these 24 samples:

```
SRS2153774
SRS2153779
SRS2153781
SRS2153786
SRS2153787
SRS2153791
SRS2153793
SRS2153797
SRS2153799
SRS2153803
SRS2153805
SRS2153809
SRS2153811
SRS2153815
SRS2153817
SRS2153821
SRS2153823
SRS2153827
SRS2153829
SRS2153833
SRS2153835
SRS2153839
SRS2153841
SRS2153845
```

Navigate to the /data directory and download the 24 samples with the the following command from the SRAToolkit:

```
prefetch --option-file SRA.list
```

To extract fastq files from .sra files, the fasterq-dump command is required. For example, the following command will produce SRR5487396_R1.fastq.gz and SRR5487396_R2.fastq.gz files from the SRR5487396.sra archive.

```
fasterq-dump --split-files SRR5487396/SRR5487396.sra
```

In practice, you will want to extract all downloaded sequence read archives, which are nested in distinct folders. Navigate to the /data folder and use the following qsub command to launch the faster-qdump commands sequentially:

```
qsub faster-qdump.sh
```

where **faster-qdump.sh** is a bash script containing instructions for the SGE workload manager and the code to iterate on all downloaded sequence read archives and produce the forward and reverse fastq files:

```
#!/bin/bash
#$ -S /bin/bash
#$ -cwd
#$ -N 'fasterq-dump'
#$ -pe smp 12
#$ -o ./qsub_log.txt
#$ -e ./qsub_err.txt

for i in `ls -d SRR*`; do
cd $i
fasterq-dump --split-files $i.sra
cd ..
done
```

However, execute the latter script would take a lot of times. To take advantage of the capacity of the cluster, one should may consider launching array tasks (task in parallel). To learn more about this, one can visit this page (SGE). Before using the faster-qdump_parallel.sh, we need to create a simple list file of the 24 SRR* folders present in the /data folder:

```
ls -d1 SRR5487???>SRR.list
```

Be sure that the SRR.list file contains the 24 SRR identifiers (without the .sra extension) on separates lines before launching the parallel version of fasterq.dump.sh which look like:

```
#!/bin/bash
#$ -V
#$ -N fasterq-dump
#$ -S /bin/bash
#$ -cwd
#$ -j y
#$ -b n
#$ -e ./qsub_err.txt
#$ -o ./qsub_log.txt
#$ -q all.q
#$ -t 1-24
#$ -pe smp 4

input=$(head -n $SGE_TASK_ID SRR.list | tail -n 1)

fasterq-dump --split-files $input/$input.sra
```

Figure x show how a computationnaly intense task, namely the decompressing of an .sra archive, can be run at the same time with different input files. This can be done by using the SGE task array capabilities. Technically, the same script is run multiple times, with different values taken by the single environment variable \$SGE_TASK_ID. Since many bioinformatic tasks used in this tutorial are computationally intensive, most of the scripts presented thereafter will be base on this model.

3.3 Performing STAR alignment

3.3.1 Generating genome indexes files

STAR genomes are available for a limited number of genomes on the gingeras lab. We will use the for *Bos taurus* UMD3.1.87 annotations file since the BosTau7 version of the genome was used in Ariel et al. [2021]. The GTF file describe all exons whereas the .dna.toplevel.fa fasta file contains the corresponding sequences.

```
wget http://labshare.cshl.edu/shares/gingeraslab/www-data/dobin/\
STAR/STARgenomes/Old/ENSEMBL/bos_taurus/Bos_taurus.UMD3.1.87.gtf;
```

```
wget http://labshare.cshl.edu/shares/gingeraslab/www-data/dobin/\
STAR/STARgenomes/Old/ENSEMBL/bos_taurus/Bos_taurus.UMD3.1.dna.toplevel.fa;
```

To prepare genome index files for STAR, use the genomeGenerate built-in STAR command. Note that you have to create a directory where STAR could build the index.

```
mkdir Bos_taurus.UMD3.1.87_index
```

as in the following script:

```
cat Bos_taurus.UMD3.1.87_index/chrName.txt | head -n 34
```

```
star_genome_index_builder.sh
```

```
#!/bin/bash
#$ -S /bin/bash
#$ -cwd
#$ -N 'STAR_genome_builder'
#$ -pe smp 6
#$ -o ./qsub_log.txt
#$ -e ./qsub_err.txt
STAR --runThreadN 6 \
--runMode genomeGenerate \
--genomeDir Bos_taurus.UMD3.1.87_index \
--genomeFastaFiles ./Bos_taurus.UMD3.1.dna.toplevel.fa \
--sjdbGTFfile ./Bos_taurus.UMD3.1.87.gtf \
--sjdbOverhang 99
```


3.3.2 Alignment with the STAR aligner

At this step, we align our raw .fastq files with the STAR aligner.

```
#!/bin/bash
#$ -V
#$ -N 'STAR_aligner'
#$ -S /bin/bash
#$ -cwd
#$ -j y
#$ -b n
#$ -e e
#$ -o STAR_aligner_1st_pass.$TASK_ID.log
#$ -q all.q
#$ -t 1-24
#$ -pe smp 4

input=$(head -n $SGE_TASK_ID SRR.list | tail -n 1)

mkdir -p ../analysis/STAR/output/$input

STAR --genomeDir ./Bos_taurus.UMD3.1.87_index \
--runThreadN 12 \
--readFilesIn ./input/$input"_1.fastq" ./input/$input"_2.fastq" \
--outFileNamePrefix ../analysis/STAR/output/$input \
--outSAMtype BAM SortedByCoordinate \
--outSAMunmapped Within \
--outSAMattributes Standard
```

3.3.3 Adding Splice junctions (2nd mapping pass)

GATK workflow recommend to perform 2-pass mapping with the STAR aligner. The 2nd mapping pass is identical to the first alignment except that all splice junctions discovered in the first pass are given as input to the programs, allowing to XXXX. Simply specify a list of all splice junctions files after the `-sjdbFileChrStartEnd` argument.

```
----outTmpDir ../analysis/STAR/output_2nd_pass/_STARtmp_$TASK_ID \

#!/bin/bash
#$ -V
#$ -N 'STAR_aligner_2nd_pass'
#$ -S /bin/bash
#$ -cwd
#$ -j y
```

```

## -b n
## -e e
## -o STAR_aligner_2nd_pass.$TASK_ID.log
## -q all.q
## -t 1-24
## -pe smp 4

input=$(head -n $SGE_TASK_ID SRR.list | tail -n 1)

mkdir -p ../analysis/STAR/output_2nd_pass/$input

STAR --genomeDir ./Bos_taurus.UMD3.1.87_index \
--runThreadN 4 \
--readFilesIn ../$input/$input"_1.fastq" ../$input/$input"_2.fastq" \
--outFileNamePrefix ../analysis/STAR/output_2nd_pass/$input \
--outSAMtype BAM SortedByCoordinate \
--outSAMunmapped Within \
--outSAMattributes Standard \
--outTmpDir ../analysis/STAR/output_2nd_pass/_STARtmp_$SGE_TASK_ID \
--sjdbFileChrStartEnd \
../analysis/STAR/output/SRR5487372SJ.out.tab \
../analysis/STAR/output/SRR5487384SJ.out.tab \
../analysis/STAR/output/SRR5487396SJ.out.tab \
../analysis/STAR/output/SRR5487408SJ.out.tab \
../analysis/STAR/output/SRR5487420SJ.out.tab \
../analysis/STAR/output/SRR5487432SJ.out.tab \
../analysis/STAR/output/SRR5487376SJ.out.tab \
../analysis/STAR/output/SRR5487388SJ.out.tab \
../analysis/STAR/output/SRR5487400SJ.out.tab \
../analysis/STAR/output/SRR5487412SJ.out.tab \
../analysis/STAR/output/SRR5487424SJ.out.tab \
../analysis/STAR/output/SRR5487436SJ.out.tab \
../analysis/STAR/output/SRR5487378SJ.out.tab \
../analysis/STAR/output/SRR5487390SJ.out.tab \
../analysis/STAR/output/SRR5487402SJ.out.tab \
../analysis/STAR/output/SRR5487414SJ.out.tab \
../analysis/STAR/output/SRR5487426SJ.out.tab \
../analysis/STAR/output/SRR5487438SJ.out.tab \
../analysis/STAR/output/SRR5487382SJ.out.tab \
../analysis/STAR/output/SRR5487394SJ.out.tab \
../analysis/STAR/output/SRR5487406SJ.out.tab \
../analysis/STAR/output/SRR5487418SJ.out.tab \
../analysis/STAR/output/SRR5487430SJ.out.tab \
../analysis/STAR/output/SRR5487442SJ.out.tab

```

3.3.4 Sorting alignment files

After you align your sequences. You will want that your .bam files to be indexed and sorted by coordinates. As mentioned earlier, STAR allows the output to be sorted by coordinates. If you have carefully followed this tutorial, there is no need to sort again your bam files and you can safely ignore the next step and jump to the next subheading. However, if for any reasons, your alignments files are not sorted adequately, the sort option of samtools is as easy as :

```
samtools sort file.bam -o file_sorted.bam
```

In a folder containing unsorted alignment, first prepare a list of bam files to be sorted with:

```
ls -1 *.bam > bam.list
```

For our data, a qsub script would allow to re-sort our alignments files:

```
#!/bin/bash
#$ -V
#$ -N samtools_sort
#$ -S /bin/bash
#$ -cwd
#$ -j y
#$ -b n
#$ -e e
#$ -o o
#$ -q all.q
#$ -t 1-24
#$ -pe smp 2

input=$(head -n $SGE_TASK_ID bam.list | tail -n 1 | xargs basename -s '.bam')

samtools sort $input.bam -o $input"_sorted.bam"
```

Note that this listing could be used as a template to perform a variety of samtools subcommands that involve renaming the output files.

3.3.5 Indexing alignment files

BAM files (the binary analog of Sequence Alignment Mapping (SAM) files) are very efficient bioinformatic files designed to store high-throughput alignment data. Typically, they contain the result of the alignment of millions of sequencing reads against a reference genome and greatly benefit from being accompanied with index files for faster random access to the aligned reads. In practice most programs will complain if in the absence of index files. Note that BAM files need to be sorted before being indexed. To index all bam files

present in the STAR output _2nd_pass folder, first prepare a list of bam files to be indexed with:

```
ls -1 *.bam > bam.list
```

then, you can index all alignements in parallel with:

```
qsub samtools_batch_index.sh
```

where the latter script looks like:

```
#!/bin/bash
#$ -V
#$ -N samtools_index
#$ -S /bin/bash
#$ -cwd
#$ -j y
#$ -b n
#$ -e e
#$ -o o
#$ -q all.q
#$ -t 1-24
#$ -pe smp 2

input=$(head -n $SGE_TASK_ID bam.list | tail -n 1)

samtools index $input
```

3.4 Adding Read groups

Contrarily to fastq, SAM files have the capacity to handle large amount of metadata. A good practice would be to include informations about the reference genome and the samples in alignment files. Informations about the processing steps could also be stored in alignment files. Many programs, such as GATK, will add this metadata information when manipulating SAM/BAM files.

Before running any GATK workflow, one will need to add Read groups. This step ensure that relevant informations about the sequencing process are assigned to each read in an alignment file. When this step is done carefully, it allow to mitigate the consequences of the sequencing biais that might arise due to how the sequencing process was performed GATK [2021e]. For example, when samples are multiplexed, it is important to known which reads origin from the same library, which were sequenced on the same flowcell, etc. The GATK engine requires the presence of several read group fields to run without errors. To learn more about the Read groups as understant by the GATK team and to learn how to derive this information from read names, one should consult this page: <https://gatk.broadinstitute.org/hc/en-us/articles/360035890671-Read-groups>.

Here, we will set the flowcells, sequencing lanes and sample barcode in the PU (Platform Unit) tag. We will also set the PL (Platform/technology used to produce the read) and LB (DNA preparation library identifier) tags. Note that we do not need to set any ID (Read group identifier) is overridden by the PU tag for base recalibration when it is defined.

Before running the main script, we will extract the first and fifth columns from our metadata file and place them in separate files, namely RGLB.txt and RGPU.txt, that will be further used to populate the LB and PU read groups fields:

```
# Corresponding to the sample name
cut -f 1 -d ',' ../../metadata/metadata.txt | tail -n +2 > RGLB.txt
```

```
# Corresponding to the PU tag FLOW CELL
cut -f 5 -d ',' ../../metadata/metadata.txt | tail -n +2 > RGPU.txt
```

where /metadata/metadata.txt looks like:

```
sample,cowID,SRAID,Run,RGPU
A_CTL24,cowA,SRS2153774,SRR5487372,C5NL3ACXX.1.CAGATC
A_MAP24,cowA,SRS2153779,SRR5487376,C5NL3ACXX.1.TGACCA
B_CTL24,cowB,SRS2153781,SRR5487378,C5NL3ACXX.3.TGACCA
B_MAP24,cowB,SRS2153786,SRR5487382,C5NL3ACXX.3.GTGAAA
C_CTL24,cowC,SRS2153787,SRR5487384,C5K8FACXX.3.AGTCAA
C_MAP24,cowC,SRS2153791,SRR5487388,C5K8FACXX.3.GTCCGC
D_CTL24,cowD,SRS2153793,SRR5487390,C5K8FACXX.1.TGACCA
D_MAP24,cowD,SRS2153797,SRR5487394,C5K8FACXX.1.GTGAAA
E_CTL24,cowE,SRS2153799,SRR5487396,C547FACXX.1.AGTCAA
E_MAP24,cowE,SRS2153803,SRR5487400,C547FACXX.1.GTCCGC
F_CTL24,cowF,SRS2153805,SRR5487402,C547FACXX.3.TGACCA
F_MAP24,cowF,SRS2153809,SRR5487406,C547FACXX.3.GTGAAA
G_CTL24,cowG,SRS2153811,SRR5487408,C5NL3ACXX.5.AGTCAA
G_MAP24,cowG,SRS2153815,SRR5487412,C5NL3ACXX.5.GTCCGC
H_CTL24,cowH,SRS2153817,SRR5487414,C5NL3ACXX.7.TGACCA
H_MAP24,cowH,SRS2153821,SRR5487418,C5NL3ACXX.7.CTTGTA
I_CTL24,cowI,SRS2153823,SRR5487420,C5K8FACXX.5.AGTTCC
I_MAP24,cowI,SRS2153827,SRR5487424,C5K8FACXX.5.GTGAAA
J_CTL24,cowJ,SRS2153829,SRR5487426,C5K8FACXX.7.TGACCA
J_MAP24,cowJ,SRS2153833,SRR5487430,C5K8FACXX.7.GTGAAA
K_CTL24,cowK,SRS2153835,SRR5487432,C547FACXX.5.AGTCAA
K_MAP24,cowK,SRS2153839,SRR5487436,C547FACXX.5.GTCCGC
L_CTL24,cowL,SRS2153841,SRR5487438,C547FACXX.7.AGTCAA
L_MAP24,cowL,SRS2153845,SRR5487442,C547FACXX.7.GTCCGC
```

The RGLB.txt file will therefore hold the library name for each 24 samples (e.g. C_MAP24, which design a RNAseq library done on the cow C 24h post-infection with the MAP pathogen). The RGPU.txt hold the Platform Unit tag, which

is made of three types of informations: the flowcell, sequencing lane and barcode identifiers, with the following format: FLOWCELL_BARCODE.LANE.SAMPLE_BARCODE. We use the following script to add read groups in our alignments files. Note that bash arrays are zero-indexed. It is why, we use the value given by \$SGE_TASK_ID -1 (0-23) to fetch the correct values in RGLB and RGPU arrays.

```
#!/bin/bash

#$ -V
#$ -N AddOrReplaceReadGroups
#$ -S /bin/bash
#$ -cwd
#$ -j y
#$ -b n
#$ -e e
#$ -o logfile.txt
#$ -q all.q
#$ -t 1-24
#$ -pe smp 1

eval "$(conda shell.bash hook)"
conda activate gatk4

SAMPLES="$HOME/jsb/springer2/RNAseq_GATK_JGW/metadata/samples.list"
BAMPATH="$HOME/jsb/springer2/RNAseq_GATK_JGW/analysis/STAR/output_2nd_pass"
OUTPUT="$HOME/jsb/springer2/RNAseq_GATK_JGW/analysis/addReadGroups"

readarray -t RGLB < ./RGLB.txt
readarray -t RGPU < ./RGPU.txt

input=$(head -n $SGE_TASK_ID $SAMPLES | tail -n 1)

java -jar $PICARD AddOrReplaceReadGroups \
I=$BAMPATH/$input"Aligned.sortedByCoord.out.bam" \
O=$OUTPUT/$input".bam" \
RGLB=${RGLB[$SGE_TASK_ID -1]} \
RGPL=ILLUMINA \
RGPU=${RGPU[$SGE_TASK_ID -1]} \
RGID=${RGPU[$SGE_TASK_ID -1]} \
RGSM=$input

conda deactivate
```

It is highly recommended to validate such editing tasks. Using a simple UNIX trick allows one to see the read groups of a .bam files:

```
samtools view -H $i.bam | grep '@RG'
```

With our data, we can iterate on all .bam files and ensure that the reads groups have been correctly set:

```
for i in `tail -n +2 ../../metadata/metadata.txt | cut -f 4 -d ','`; do echo $i; samtools v
```

3.5 MarkDuplicates

Duplicate reads can arise from PCR duplication artifacts that take place during the library construction or from reading errors that occur during the sequencing process (optical duplicates). Regardless of their origin, these reads need to be identified in alignment files. The MarkDuplicate program from the Picard tools have many options to deal with these issue and output some metrics. For example, the program offer the possibility to completely remove the duplicate reads and to make the distinction between optical and PCR duplicates. Here we will simply identified the duplicate reads in our .bam files before proceeding to the next step:

```
#!/bin/bash
#$ -V
#$ -N samtools_index
#$ -S /bin/bash
#$ -cwd
#$ -j y
#$ -b n
#$ -o logfile.$TASK_ID.log
#$ -q all.q
#$ -t 1-24
#$ -pe smp 2

SAMPLES="$HOME/jsb/springer/metadata/samples.list"
BAMPATH="$HOME/jsb/springer/analysis/addReadGroups"
OUTPUT="$HOME/jsb/springer/analysis/MarkDuplicates"

input=$(head -n $SGE_TASK_ID $SAMPLES | tail -n 1)

java -jar $PICARD MarkDuplicates \
I=$BAMPATH/$input".bam" \
O=$OUTPUT/$input"_marked_duplicates.bam" \
M=$OUTPUT/$input"_marked_dup_metrics.txt"
```

3.5.1 The gff3 file

With the command below, one can see the number of features annotated for each ID in the gff3 file:

```
{bash}
cat Ensembl179_UMD3.1_genes.gff3 | cut -f 1 | uniq -cd
```

The result of this command is:

```
21287 GK000001.2
18819 GK000030.2
26235 GK000002.2
29793 GK000003.2
29598 GK000005.2
18201 GK000004.2
15375 GK000006.2
16534 GK000008.2
28038 GK000007.2
24860 GK000011.2
12861 GK000009.2
23581 GK000010.2
9839 GK000012.2
17254 GK000015.2
11207 GK000014.2
18771 GK000013.2
17134 GK000016.2
14916 GK000017.2
8554 GK000020.2
13262 GK000021.2
26279 GK000018.2
30597 GK000019.2
7463 GK000024.2
15631 GK000022.2
14696 GK000023.2
10159 GK000026.2
14729 GK000029.2
8317 GK000028.2
5227 GK000027.2
17365 GK000025.2
14 GJ058430.1
31 GJ058425.1
3 GJ059486.1
4 GJ058433.1
16 GJ058437.1
15 GJ058424.1
12 GJ060129.1
12 GJ059670.1
124 AY526085.1
24 GJ059556.1
9 GJ059463.1
13 GJ060118.1
19 GJ060120.1
16 GJ060032.1
```



```
23 GJ059509.1
3 GJ058729.1
3 GJ060027.1
3 GJ058256.1
```

Therefore, to ensure that the gff3 chromosome IDs match those present in our alignment files, we need to slightly edit this file to replace the pattern used for the chromosome IDs (GK + 0000 + chromosome number) by solely the chromosome number. This could be done easily with sed:

```
sed -r s'/^GK[0]+([0-9]+).2/\1/'g Ensembl79_UMD3.1_genes.gff3 > Ensembl79_UMD3.1_genes_e.gff3
```

Obviously it is good idea to inspect the edited file to ensure that the desired changes have been done properly.

3.6 SplitNCigarReads

The next step is very specific to RNAseq. Briefly, during this step reads that match align over distinct exons are split and blah.

However, before doing this step, we need to get and prepare reference genome files.

3.6.1 Prepare reference genome files

Until now, we have work with STAR reference genome which barely contains sequences that are transcribed in RNA. In order to produce alignments files that will adequately represent reads that span splice junctions, the SplitNCigarReads command require the complete reference sequence. At this point, pay attention to download the same version of the reference genome as the one that was used to prepare the STAR genome.

To get the UMD3.1 assembly, visit <https://bovinegenome.elsiklab.missouri.edu/> and download the UMD3.1_chromosomes.fa.gz and Ensembl79_UMD3.1_genes.gff3.gz files. If you inspect the headers of the UMD3.1_chromosomes.fa, you will notice that each fasta entry contains many fields (e.g. gnl , UMD3.1 Accession numbers). To inspect the fasta headers, simply use grep:

```
grep ">" UMD3.1_chromosomes.fa

>gnl|UMD3.1|GK000010.2 Chromosome 10 AC_000167.1
>gnl|UMD3.1|GK000011.2 Chromosome 11 AC_000168.1
>gnl|UMD3.1|GK000012.2 Chromosome 12 AC_000169.1
...
>gnl|UMD3.1|GJ060407.1 GPS_000344847.1 NW_003101152.1
>gnl|UMD3.1|GJ060408.1 GPS_000344848.1 NW_003101153.1
```

It is crucial that entries in the reference genome match the corresponding ones in the STAR genome. One could use the following commands to rename the complicated chromosome entries from the UMD3.1 genome to the plain chromosome numbers as what is seen in the STAR genome:

```
sed -r s'/^>.+Chromosome\s+(\S+)\s+./>\1/' UMD3.1_chromosomes.fa > temp1.fa
grep ">" temp1.fa | head -n 40
sed -r s'/^>gnl\|UMD3\.1\|(\S+)\s+./>\1/' temp1.fa > temp2.fa
grep ">" temp2.fa | tail -n +30 | head -n 10
mv temp2.fa refGenome.fasta
rm temp1.fa
```

Along with the reference genome, many GATK tools will need a dictionary file ending in .dict and an index file ending in .fai. <https://gatk.broadinstitute.org/hc/en-us/articles/360035531652-FASTA-Reference-genome-format>). These files need to share the same basename as the reference genome. You can prepare the index with:

```
samtools faidx refGenome.fasta
```

Use the GATK CreateSequenceDictionary tool to create the required .dict file:

```
gatk CreateSequenceDictionary -R refGenome.fasta
```

3.6.2 Run SplitNCigarReads

Here the listing for the SplitNCigarReads step:

```
#!/bin/bash
#$ -V
#$ -N SplitNCigarReads
#$ -S /bin/bash
#$ -cwd
#$ -j y
#$ -b n
#$ -e e
#$ -o SplitNCigarReads.$TASK_ID.log
#$ -q all.q
#$ -t 1-24
#$ -pe smp 4

eval "$(conda shell.bash hook)"
conda activate gatk4

SAMPLES="$HOME/jsb/springer/metadata/samples.list"
BAMPATH="$HOME/jsb/springer/analysis/MarkDuplicates"
OUTPUT="$HOME/jsb/springer/analysis/SplitNCigarReads"

input=$(head -n $SGE_TASK_ID $SAMPLES | tail -n 1)

gatk SplitNCigarReads \
-R refGenome.fasta \
```

```
-I $BAMPATH/$input"_marked_duplicates.bam" \
-O $OUTPUT/$input"_SplitNCigarReads.bam" \
--tmp-dir $output/gatk_tmp
```

```
conda deactivate
```

3.7 Base Quality Recalibration

This optional step is highly recommended by

3.7.1 BaseRecalibrator first step

```
#!/bin/bash
#$ -V
#$ -N BaseRecalibrator
#$ -S /bin/bash
#$ -cwd
#$ -j y
#$ -b n
#$ -e e
#$ -o BaseQualityRecalibration_${TASK_ID}.log
#$ -q all.q
#$ -t 1-24
#$ -pe smp 4

eval "$(conda shell.bash hook)"
conda activate gatk4

SAMPLES="$HOME/jsb/springer/metadata/samples.list"
BAMPATH="$HOME/jsb/springer/analysis/SplitNCigarReads"
OUTPUT="$HOME/jsb/springer/analysis/BaseQualityRecalibration"

input=$(head -n $SGE_TASK_ID $SAMPLES | tail -n 1)

gatk BaseRecalibrator \
-R ./refGenome.fasta \
-I $BAMPATH/$input"_SplitNCigarReads.bam" \
--known-sites ./jsb.vcf \
-O $OUTPUT/$input"_recal_data.table"

conda deactivate
```

In the second step of the BaseQuality recalibration, GATK use the content in the .table files to recalibrate base quality.

3.7.2 BaseRecalibrator second step

```
#!/bin/bash

#$ -V
#$ -N ApplyBQSR
#$ -S /bin/bash
#$ -cwd
#$ -j y
#$ -b n
#$ -e e
#$ -o ApplyBQSR_${TASK_ID}.log
#$ -q all.q
#$ -t 1-24
#$ -pe smp 4

eval "$(conda shell.bash hook)"
conda activate gatk4

SAMPLES="$HOME/jsb/springer/metadata/samples.list"
BAMPATH="$HOME/jsb/springer/analysis/SplitNCigarReads"
OUTPUT="$HOME/jsb/springer/analysis/BaseQualityRecalibration"

echo $SAMPLES
input=$(head -n $SGE_TASK_ID $SAMPLES | tail -n 1)

gatk ApplyBQSR \
-R ./refGenome.fasta \
-I $BAMPATH/$input"_SplitNCigarReads.bam" \
--bqsr-recal-file $OUTPUT/$input"_recal_data.table" \
-O $OUTPUT/$input"_recal.bam"

conda deactivate
```

3.8 Variant Calling

More details about the incremental discovery can be found [here](#):

3.8.1 GVCF part

```
#!/bin/bash
#$ -V
#$ -N callingGVCF
#$ -S /bin/bash
#$ -cwd
```

```

#$ -j y
#$ -b n
#$ -e e
#$ -o singleSampleCallingGVCF_${TASK_ID}.log
#$ -q all.q
#$ -t 1-24
#$ -pe smp 4

eval "$(conda shell.bash hook)"
conda activate gatk4

SAMPLES="$HOME/jsb/springer/metadata/samples.list"
BAMPATH="$HOME/jsb/springer/analysis/BaseQualityRecalibration"
OUTPUT="$HOME/jsb/springer/analysis/GVCF"

echo $SAMPLES
input=$(head -n $SGE_TASK_ID $SAMPLES | tail -n 1)

gatk --java-options "-Xmx4g" HaplotypeCaller \
-R ./refGenome.fasta \
-I $BAMPATH/$input"_recal.bam" \
-O $OUTPUT/$input".g.vcf.gz" \
-ERC GVCF

conda deactivate

```

3.8.2 Import DBI

DBI import

```

eval "$(conda shell.bash hook)"
conda activate gatk4

SAMPLES="$HOME/jsb/springer/metadata"
OUTPUT="$HOME/jsb/springer/analysis/GenomicsDBImport"

gatk --java-options "-Xmx4g -Xms4g" \
GenomicsDBImport \
--genomicsdb-workspace-path $OUTPUT/"my_database" \
-L 1 \
-L 2 \
-L 3 \
-L 4 \
-L 5 \
-L 6 \
-L 7 \

```

```

-L 8 \
-L 9 \
-L 10 \
-L 11 \
-L 12 \
-L 13 \
-L 14 \
-L 15 \
-L 16 \
-L 17 \
-L 18 \
-L 19 \
-L 20 \
-L 21 \
-L 22 \
-L 23 \
-L 24 \
-L 25 \
-L 26 \
-L 27 \
-L 28 \
-L 29 \
--sample-name-map $SAMPLES/"cohort_1.sample_map" \
--tmp-dir $HOME/jsb/tmp \
--reader-threads 5
conda deactivate

```

3.8.3 Genotype GVCF

The final step in variant calling can be done with GATK GenotypeGVCFs program.

```

#!/bin/bash
#$ -V
#$ -N GenotypeGVCF
#$ -S /bin/bash
#$ -cwd
#$ -j y
#$ -b n
#$ -e e
#$ -o GenotypeGVCF.log
#$ -q all.q
#$ -pe smp 4

eval "$(conda shell.bash hook)"

```

```
conda activate gatk4
```

```
gatk --java-options "-Xmx4g" GenotypeGVCFs \  
-R refGenome.fasta \  
-V gendb://my_database \  
-O output.vcf.gz  
conda deactivate
```

3.9 Examine and visualize alignment files

At this step, you could examine your alignments files.

Tablet (<https://ics.hutton.ac.uk/tablet/download-tablet/>) is a good program to visualize alignments files. The IVG viewer could also be used.

You should keep a copy of the vcf output file in a safe place. It is fairly common to corrupt a .gz file with compressing/decompressing operations.

```
gunzip -c output.vcf.gz > output.vcf
```

References

- Robert Piskol, Gokul Ramaswami, and Jin Billy Li. Reliable identification of genomic variants from rna-seq data. *American journal of human genetics*, 93: 641–651, October 2013. ISSN 1537-6605. doi: 10.1016/j.ajhg.2013.08.008.
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- GATK. Read groups, 2021e. URL <https://gatk.broadinstitute.org/hc/en-us/articles/360035890671-Read-groups>.