Introduction to DNA-Seq processing

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In this workshop, we will present the main steps that are commonly used to process and to analyze sequencing data. We will focus only on whole genome data and provide command lines that allow detecting Single Nucleotide Variants (SNV), for a question of time we will only present the rational for the detection of Structural Variant (SV including CNV). This workshop will show you how to launch individual steps of a complete DNA-Seq pipeline

We will be working on a 1000 genome sample, NA12878. You can find the whole raw data on the 1000 genome website: http://www.1000genomes.org/data

For practical reasons we subsampled the reads from the sample because running the whole dataset would take way too much time and resources.

Original Setup

The initial structure of your folders should look like this:

```
<ROOT>
|-- raw_reads/               # fastqs from the center (down sampled)
    `-- NA12878              # One sample directory
        |-- runERR_1         # Lane directory by run number. Contains the fastqs
        `-- runSRR_1         # Lane directory by run number. Contains the fastqs
```

Cheat file

- You can find all the unix command lines of this practical in the file: commands.sh

Environment setup

```
export PATH=$PATH:/usr/local/scr/tabix-0.2.6/:u/igvtools_2.3.31/
export PICARD_HOME=/usr/local/bin/
export SNPEFF_HOME=/usr/local/bin/
export GATK_JAR=/usr/local/bin/GenomeAnalysisTK.jar
export BVATOOLS_JAR=/usr/local/bin/bvatools-1.4-full.jar
export TRIMMOMATIC_JAR=/usr/local/bin/trimmomatic-0.32.jar
export REF=/home/mBourgey/kyoto_workshop_WGS_2015/references/

cd $HOME
rsync -avP /home/mBourgey/cleanCopy $HOME/workshop
cd $HOME/workshop/
```

Software requirements

These are all already installed, but here are the original links.

- [Trimmomatic]()
- [BVATools]()
- [SAMTools]()
- [IGV]()
- [BWA]()
- [Genome Analysis Toolkit]()
- [Picard]()

- SnpEff

First data glance

So you’ve just received an email saying that your data is ready for download from the sequencing center of your choice.

What should you do ? solution

Fastq files

Let’s first explore the fastq file.

Try these commands

```
zless -S raw_reads/NA12878/runSRR_1/NA12878.SRR.33.pair1.fastq.gz
```

Why was it like that ? solution

Now try these commands:

```
zcat raw_reads/NA12878/runSRR_1/NA12878.SRR.33.pair1.fastq.gz | head -n4
zcat raw_reads/NA12878/runSRR_1/NA12878.SRR.33.pair2.fastq.gz | head -n4
```

What was special about the output ?

Why was it like that? Solution

You could also just count the reads

```
zgrep -c "^@SRR" raw_reads/NA12878/runSRR_1/NA12878.SRR.33.pair1.fastq.gz
```

We should obtain 15546 reads

Why shouldn’t you just do ?

```
zgrep -c "^@" raw_reads/NA12878/runSRR_1/NA12878.SRR.33.pair1.fastq.gz
```

Solution

Quality

We can’t look at all the reads. Especially when working with whole genome 30x data. You could easily have Billions of reads.

Tools like FastQC and BVATools readsqc can be used to plot many metrics from these data sets.

Let’s look at the data:

```
mkdir originalQC/
java -Xmx1G -jar ${BVATOOLS_JAR} readsqc \
  --read1 raw_reads/NA12878/runSRR_1/NA12878.SRR.33.pair1.fastq.gz \
  --read2 raw_reads/NA12878/runSRR_1/NA12878.SRR.33.pair2.fastq.gz \
  --threads 2 --regionName SRR --output originalQC/

java -Xmx1G -jar ${BVATOOLS_JAR} readsqc \
  --read1 raw_reads/NA12878/runERR_1/NA12878.ERR.33.pair1.fastq.gz \
  --read2 raw_reads/NA12878/runERR_1/NA12878.ERR.33.pair2.fastq.gz \
  --threads 2 --regionName ERR --output originalQC/
```

Copy the images from the originalQC folder to your desktop and open the images.

```
scp -r <USER>@www.genome.med.kyoto-u.ac.jp:~/workshop/originalQC/ ./
```

Open the images

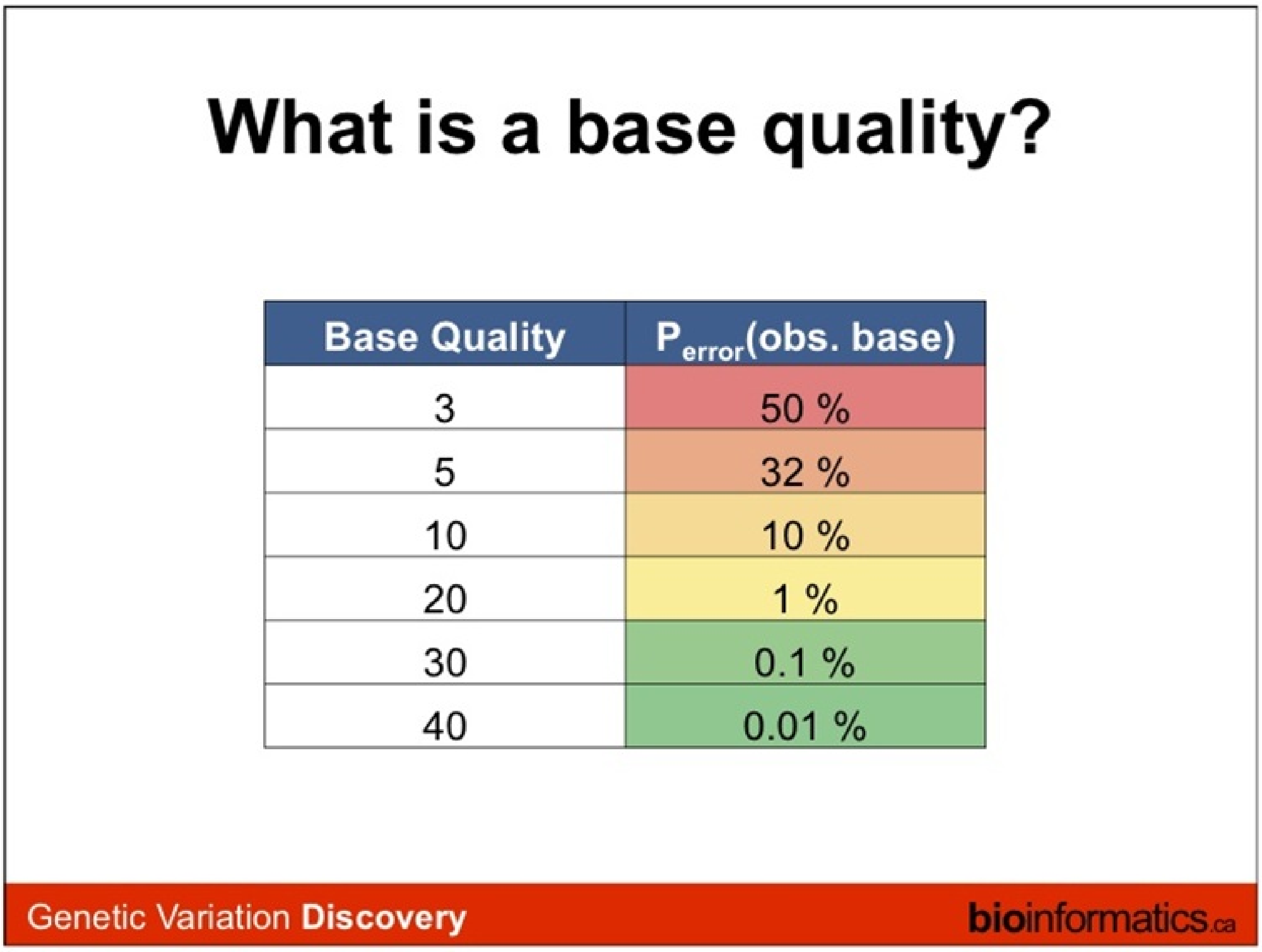
What stands out in the graphs ? Solution

All the generated graphics have their uses. But 2 of them are particularly useful to get an overal picture of how good or bad a run went. - The Quality box plots - The nucleotide content graphs. - The Box plot shows the quality distribution of your data.

The quality of a base is computated using the Phread quality score. notes

The quality of a base is computated using the Phread quality score. $Q_{\text{sanger}} = -10 \log_{10} p$

In the case of base quality the probability use represents the probability of base to have been wrongly called



What is a base quality?

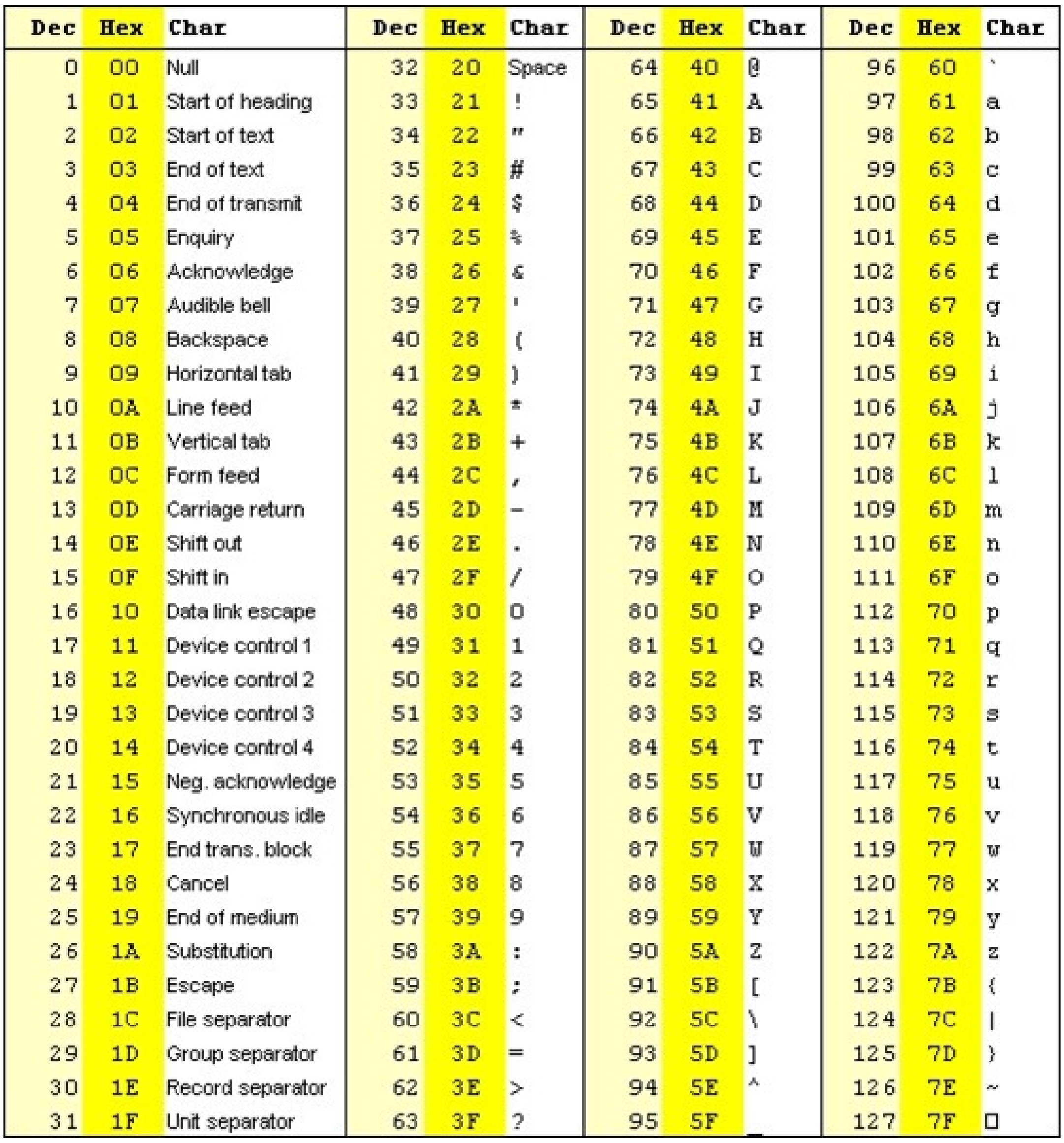
Base Quality	P_{error}(obs. base)
3	50 %
5	32 %
10	10 %
20	1 %
30	0.1 %
40	0.01 %

Genetic Variation **Discovery** **bio**informatics.ca

The formula outputs an integer that is encoded using an ASCII table.

The way the lookup is done is by taking the the phred score adding 33 and using this number as a lookup in the table.

Older illumina runs were using phred+64 instead of phred+33 to encode their fastq files.



Dec	Hex	Char	Dec	Hex	Char	Dec	Hex	Char	Dec	Hex	Char
0	00	Null	32	20	Space	64	40	@	96	60	`
1	01	Start of heading	33	21	!	65	41	A	97	61	a
2	02	Start of text	34	22	"	66	42	B	98	62	b
3	03	End of text	35	23	#	67	43	C	99	63	c
4	04	End of transmit	36	24	$	68	44	D	100	64	d
5	05	Enquiry	37	25	%	69	45	E	101	65	e
6	06	Acknowledge	38	26	&	70	46	F	102	66	f
7	07	Audible bell	39	27	'	71	47	G	103	67	g
8	08	Backspace	40	28	(	72	48	H	104	68	h
9	09	Horizontal tab	41	29	)	73	49	I	105	69	i
10	0A	Line feed	42	2A	*	74	4A	J	106	6A	j
11	0B	Vertical tab	43	2B	+	75	4B	K	107	6B	k
12	0C	Form feed	44	2C	,	76	4C	L	108	6C	l
13	0D	Carriage return	45	2D	-	77	4D	M	109	6D	m
14	0E	Shift out	46	2E	.	78	4E	N	110	6E	n
15	0F	Shift in	47	2F	/	79	4F	O	111	6F	o
16	10	Data link escape	48	30	0	80	50	P	112	70	p
17	11	Device control 1	49	31	1	81	51	Q	113	71	q
18	12	Device control 2	50	32	2	82	52	R	114	72	r
19	13	Device control 3	51	33	3	83	53	S	115	73	s
20	14	Device control 4	52	34	4	84	54	T	116	74	t
21	15	Neg. acknowledge	53	35	5	85	55	U	117	75	u
22	16	Synchronous idle	54	36	6	86	56	V	118	76	v
23	17	End trans. block	55	37	7	87	57	W	119	77	w
24	18	Cancel	56	38	8	88	58	X	120	78	x
25	19	End of medium	57	39	9	89	59	Y	121	79	y
26	1A	Substitution	58	3A	:	90	5A	Z	122	7A	z
27	1B	Escape	59	3B	;	91	5B	[	123	7B	{
28	1C	File separator	60	3C	<	92	5C	\	124	7C	|
29	1D	Group separator	61	3D	=	93	5D	]	125	7D	}
30	1E	Record separator	62	3E	>	94	5E	^	126	7E	~
31	1F	Unit separator	63	3F	?	95	5F	_	127	7F	□

ACII table

Of the raw data we see that:

- Some reads have bad 3' ends.
- Some reads have adapter sequences in them.

Why do we see adapters in SRR ? solution

Although nowadays this doesn't happen often, it does still happen. In some cases, miRNA, it is expected to have adapters.

Trimming

Since adapter are not part of the genome they should be removed

To do that we will use Trimmomatic.

The adapter file is in your work folder.

```
cat adapters.fa
```

Why are there 2 different ones ? Solution

trimming with trimmomatic:

```
mkdir -p reads/NA12878/runSRR_1/
mkdir -p reads/NA12878/runERR_1/
```

```
java -Xmx2G -cp $TRIMMOMATIC_JAR org.usadellab.trimmomatic.TrimmomaticPE -threads 2 -
phred33 \
  raw_reads/NA12878/runERR_1/NA12878.ERR.33.pair1.fastq.gz \
  raw_reads/NA12878/runERR_1/NA12878.ERR.33.pair2.fastq.gz \
  reads/NA12878/runERR_1/NA12878.ERR.t20l32.pair1.fastq.gz \
  reads/NA12878/runERR_1/NA12878.ERR.t20l32.single1.fastq.gz \
  reads/NA12878/runERR_1/NA12878.ERR.t20l32.pair2.fastq.gz \
  reads/NA12878/runERR_1/NA12878.ERR.t20l32.single2.fastq.gz \
  ILLUMINACLIP:adapters.fa:2:30:15 TRAILING:20 MINLEN:32 \
  2> reads/NA12878/runERR_1/NA12878.ERR.trim.out

java -Xmx2G -cp $TRIMMOMATIC_JAR org.usadellab.trimmomatic.TrimmomaticPE -threads 2 -
phred33 \
  raw_reads/NA12878/runSRR_1/NA12878.SRR.33.pair1.fastq.gz \
  raw_reads/NA12878/runSRR_1/NA12878.SRR.33.pair2.fastq.gz \
  reads/NA12878/runSRR_1/NA12878.SRR.t20l32.pair1.fastq.gz \
  reads/NA12878/runSRR_1/NA12878.SRR.t20l32.single1.fastq.gz \
  reads/NA12878/runSRR_1/NA12878.SRR.t20l32.pair2.fastq.gz \
  reads/NA12878/runSRR_1/NA12878.SRR.t20l32.single2.fastq.gz \
  ILLUMINACLIP:adapters.fa:2:30:15 TRAILING:20 MINLEN:32 \
  2> reads/NA12878/runSRR_1/NA12878.SRR.trim.out

cat reads/NA12878/runERR_1/NA12878.ERR.trim.out reads/NA12878/runSRR_1/NA12878.SRR.trim.out
```

note on trimmomatic command

What does Trimmomatic says it did ? Solution

Let's look at the graphs now

```
mkdir postTrimQC/
java -Xmx1G -jar ${BVATOOLS_JAR} readsqc \
  --read1 reads/NA12878/runERR_1/NA12878.ERR.t20l32.pair1.fastq.gz \
  --read2 reads/NA12878/runERR_1/NA12878.ERR.t20l32.pair2.fastq.gz \
  --threads 2 --regionName ERR --output postTrimQC/
java -Xmx1G -jar ${BVATOOLS_JAR} readsqc \
  --read1 reads/NA12878/runSRR_1/NA12878.SRR.t20l32.pair1.fastq.gz \
  --read2 reads/NA12878/runSRR_1/NA12878.SRR.t20l32.pair2.fastq.gz \
  --threads 2 --regionName SRR --output postTrimQC/
```

How does it look now ? Solution

Alignment

The raw reads are now cleaned up of artefacts we can align each lane separatly.

Why should this be done separatly? Solution

Why is it important to set Read Group information ? Solution

Alignment with bwa-mem

```
mkdir -p alignment/NA12878/runERR_1
mkdir -p alignment/NA12878/runSRR_1

bwa mem -M -t 2 \
  -R '@RG\tID:ERR_ERR_1\tSM:NA12878\tLB:ERR\tPU:runERR_1\tCN:Broad Institute\tPL:ILLUMINA'
\
  ${REF}/b37.fasta \
  reads/NA12878/runERR_1/NA12878.ERR.t20l32.pair1.fastq.gz \
  reads/NA12878/runERR_1/NA12878.ERR.t20l32.pair2.fastq.gz \
  | java -Xmx2G -jar ${PICARD_HOME}/SortSam.jar \
  INPUT=/dev/stdin \
  OUTPUT=alignment/NA12878/runERR_1/NA12878.ERR.sorted.bam \
  CREATE_INDEX=true VALIDATION_STRINGENCY=SILENT SORT_ORDER=coordinate
MAX_RECORDS_IN_RAM=500000

bwa mem -M -t 2 \
  -R '@RG\tID:SRR_SRR_1\tSM:NA12878\tLB:SRR\tPU:runSRR_1\tCN:Broad Institute\tPL:ILLUMINA'
\
  ${REF}/b37.fasta \
  reads/NA12878/runSRR_1/NA12878.SRR.t20l32.pair1.fastq.gz \
  reads/NA12878/runSRR_1/NA12878.SRR.t20132.pair2.fastq.gz \
  | java -Xmx2G -jar ${PICARD_HOME}/SortSam.jar \
  INPUT=/dev/stdin \
  OUTPUT=alignment/NA12878/runSRR_1/NA12878.SRR.sorted.bam \
  CREATE_INDEX=true VALIDATION_STRINGENCY=SILENT SORT_ORDER=coordinate
MAX_RECORDS_IN_RAM=500000
```

Why did we pipe the output of one to the other ? Solution

Could we have done it differently ? Solution

We will explore the generated BAM latter if we get enough time.

Lane merging

We now have alignments for each of the sequences lanes:

- This is not practical in it's current form.
- What we wan't to do now is merge the results into one BAM.

Since we identified the reads in the BAM with read groups, even after the merging, we can still identify the origin of each read.

```
java -Xmx2G -jar ${PICARD_HOME}/MergeSamFiles.jar \
  INPUT=alignment/NA12878/runERR_1/NA12878.ERR.sorted.bam \
  INPUT=alignment/NA12878/runSRR_1/NA12878.SRR.sorted.bam \
  OUTPUT=alignment/NA12878/NA12878.sorted.bam \
  VALIDATION_STRINGENCY=SILENT CREATE_INDEX=true
```

You should now have one BAM containing all your data.

Let's double check

```
ls -l alignment/NA12878/
samtools view -H alignment/NA12878/NA12878.sorted.bam | grep "^@RG"
```

You should have your 2 read group entries.

**Why did we use the -H switch? ** Solution

Try without. What happens? Solution

lane merging note

Cleaning up alignments

We started by cleaning up the raw reads. Now we need to fix some alignments.

The first step for this is to realign around indels and snp dense regions.

The Genome Analysis toolkit has a tool for this called IndelRealigner.

It basically runs in 2 steps:

1. Find the targets
2. Realign them

GATK IndelRealigner

```
java -Xmx2G  -jar ${GATK_JAR} \
  -T RealignerTargetCreator \
  -R ${REF}/b37.fasta \
  -o alignment/NA12878/realign.intervals \
  -I alignment/NA12878/NA12878.sorted.bam \
  -L 1

java -Xmx2G -jar ${GATK_JAR} \
  -T IndelRealigner \
  -R ${REF}/b37.fasta \
  -targetIntervals alignment/NA12878/realign.intervals \
  -o alignment/NA12878/NA12878.realigned.sorted.bam \
  -I alignment/NA12878/NA12878.sorted.bam
```

How could we make this go faster ? Solution

How many regions did it think needed cleaning ? Solution

FixMates

Why ?

- Some read entries don't have their mate information written properly.

We use Picard to do this:

```
java -Xmx2G -jar ${PICARD_HOME}/FixMateInformation.jar \
  VALIDATION_STRINGENCY=SILENT CREATE_INDEX=true SORT_ORDER=coordinate
MAX_RECORDS_IN_RAM=500000 \
  INPUT=alignment/NA12878/NA12878.realigned.sorted.bam \
  OUTPUT=alignment/NA12878/NA12878.matefixed.sorted.bam
```

Mark duplicates

What are duplicate reads ? Solution

What are they caused by ? Solution

What are the ways to detect them ? Solution

Here we will use picards approach:

```
java -Xmx2G -jar ${PICARD_HOME}/MarkDuplicates.jar \
  REMOVE_DUPLICATES=false CREATE_MD5_FILE=true VALIDATION_STRINGENCY=SILENT
CREATE_INDEX=true \
  INPUT=alignment/NA12878/NA12878.matefixed.sorted.bam \
  OUTPUT=alignment/NA12878/NA12878.sorted.dup.bam \
  METRICS_FILE=alignment/NA12878/NA12878.sorted.dup.metrics
```

We can look in the metrics output to see what happened.

```
less alignment/NA12878/NA12878.sorted.dup.metrics
```

How many duplicates were there ? Solution

We can see that it computed separate measures for each library.

Why is this important to do not combine everything ? Solution

Note on Duplicate rate

Base Quality recalibration

Why do we need to recalibrate base quality scores ? Solution

GATK BaseRecalibrator:

```
java -Xmx2G -jar ${GATK_JAR} \
  -T BaseRecalibrator \
  -nct 2 \
  -R ${REF}/b37.fasta \
  -knownSites ${REF}/dbSnp-137.vcf.gz \
  -L 1:47000000-47171000 \
  -o alignment/NA12878/NA12878.sorted.dup.recalibration_report.grp \
  -I alignment/NA12878/NA12878.sorted.dup.bam

java -Xmx2G -jar ${GATK_JAR} \
  -T PrintReads \
  -nct 2 \
  -R ${REF}/b37.fasta \
  -BQSR alignment/NA12878/NA12878.sorted.dup.recalibration_report.grp \
  -o alignment/NA12878/NA12878.sorted.dup.recal.bam \
  -I alignment/NA12878/NA12878.sorted.dup.bam
```

Extract BAM metrics

Once your whole bam is generated, it’s always a good thing to check the data again to see if everything makes sens.

Compute coverage If you have data from a capture kit, you should see how well your targets worked

Insert Size It tells you if your library worked

Alignment metrics It tells you if your sample and you reference fit together

Compute coverage

Both GATK and BVATools have depth of coverage tools.

Here we’ll use the GATK one

```
java  -Xmx2G -jar ${GATK_JAR} \
  -T DepthOfCoverage \
  --omitDepthOutputAtEachBase \
  --summaryCoverageThreshold 10 \
  --summaryCoverageThreshold 25 \
  --summaryCoverageThreshold 50 \
  --summaryCoverageThreshold 100 \
  --start 1 --stop 500 --nBins 499 -dt NONE \
  -R ${REF}/b37.fasta \
  -o alignment/NA12878/NA12878.sorted.dup.recal.coverage \
  -I alignment/NA12878/NA12878.sorted.dup.recal.bam \
  -L 1:47000000-47171000
```

note on DepthOfCoverage command

Coverage is the expected ~30x

Look at the coverage:

```
less -S alignment/NA12878/NA12878.sorted.dup.recal.coverage.sample_interval_summary
```

Is the coverage fit with the expectation ? solution

Insert Size

It corresponds to the size of DNA fragments sequenced.

Different from the gap size (= distance between reads) !

These metrics are computed using Picard:

```
java -Xmx2G -jar ${PICARD_HOME}/CollectInsertSizeMetrics.jar \
  VALIDATION_STRINGENCY=SILENT \
  REFERENCE_SEQUENCE=${REF}/b37.fasta \
  INPUT=alignment/NA12878/NA12878.sorted.dup.recal.bam \
  OUTPUT=alignment/NA12878/NA12878.sorted.dup.recal.metric.insertSize.tsv \
  HISTOGRAM_FILE=alignment/NA12878/NA12878.sorted.dup.recal.metric.insertSize.histo.pdf \
  METRIC_ACCUMULATION_LEVEL=LIBRARY
```

look at the output

```
less -S alignment/NA12878/NA12878.sorted.dup.recal.metric.insertSize.tsv
```

There is something interesting going on with our library ERR.

Can you tell what it is? Solution

Alignment metrics

For the alignment metrics, samtools flagstat is very fast but with bwa-mem since some reads get broken into pieces, the numbers are a bit confusing.

We prefer the Picard way of computing metrics:

```
java -Xmx2G -jar ${PICARD_HOME}/CollectAlignmentSummaryMetrics.jar \
  VALIDATION_STRINGENCY=SILENT \
  REFERENCE_SEQUENCE=${REF}/b37.fasta \
  INPUT=alignment/NA12878/NA12878.sorted.dup.recal.bam \
  OUTPUT=alignment/NA12878/NA12878.sorted.dup.recal.metric.alignment.tsv \
  METRIC_ACCUMULATION_LEVEL=LIBRARY
```

explore the results

```
less -S alignment/NA12878/NA12878.sorted.dup.recal.metric.alignment.tsv
```

Do you think the sample and the reference genome fit together ? Solution

Variant calling

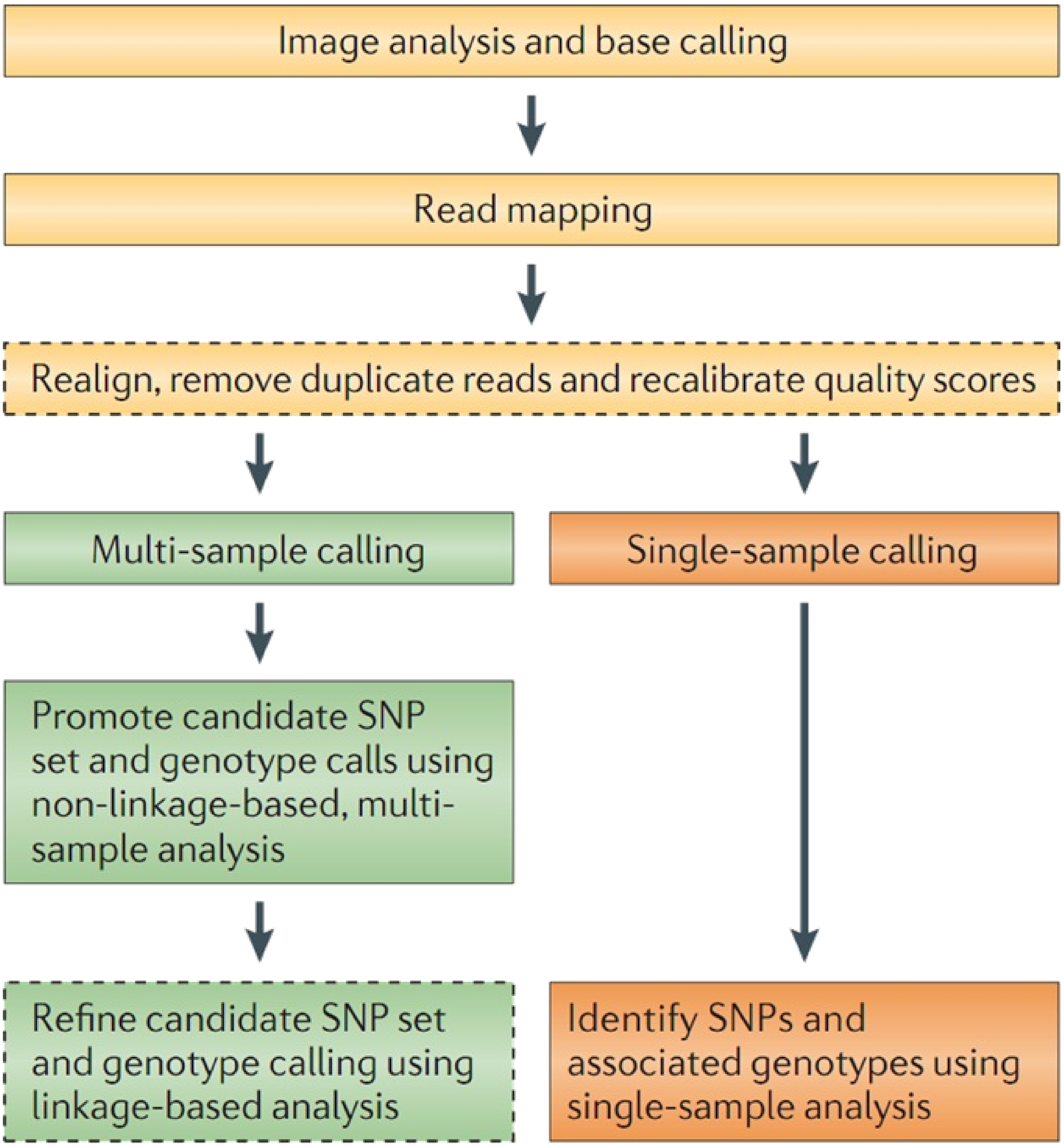


Image analysis and base calling
Read mapping
Realign, remove duplicate reads and recalibrate quality scores
Multi-sample calling
Single-sample calling
Promote candidate SNP set and genotype calls using non-linkage-based, multi-sample analysis
Refine candidate SNP set and genotype calling using linkage-based analysis
Identify SNPs and associated genotypes using single-sample analysis
SNV call summary workflow

Most of SNV caller use either a Baysian, a threshold or a t-test approach to do the calling

I won’t go into the details of finding which variant is good or bad since this will be your next workshop.

Here we will just call and view the variants using the samtools mpileup function:

```
mkdir variants
samtools mpileup -L 1000 -B -q 1 -g \
    -f ${REF}/b37.fasta \
    -r 1:47000000-47171000 \
    alignment/NA12878/NA12878.sorted.dup.recal.bam | bcftools call -c -  \
    > variants/mpileup.vcf
```

note on samtools mpileup and bcftools command

Now we have variants from all three methods. Let’s compress and index the vcfs for futur visualisation.

```
tabix -p vcf variants/mpileup.vcf.gz
```

Let’s look at the compressed vcf.

```
zless -S variants/mpileup.vcf.gz
```

Details on the spec can be found here: http://vcftools.sourceforge.net/specs.html

Fields vary from caller to caller.

Some values are are almost always there:

- The ref vs alt alleles,
- variant quality (QUAL column)
- The per-sample genotype (GT) values.

note on the vcf format fields

Annotations

We typically use snpEff but many use annovar and VEP as well.

Let’s run snpEff:

```
java  -Xmx6G -jar ${SNPEFF_HOME}/snpEff.jar \
  eff -v -c ${SNPEFF_HOME}/snpEff.config \
  -o vcf \
  -i vcf \
  -stats variants/mpileup.snpeff.vcf.stats.html \
  GRCh37.74 \
  variants/mpileup.vcf \
  > variants/mpileup.snpeff.vcf
```

Look at the new vcf file:

```
less -S variants/mpileup.snpeff.vcf
```

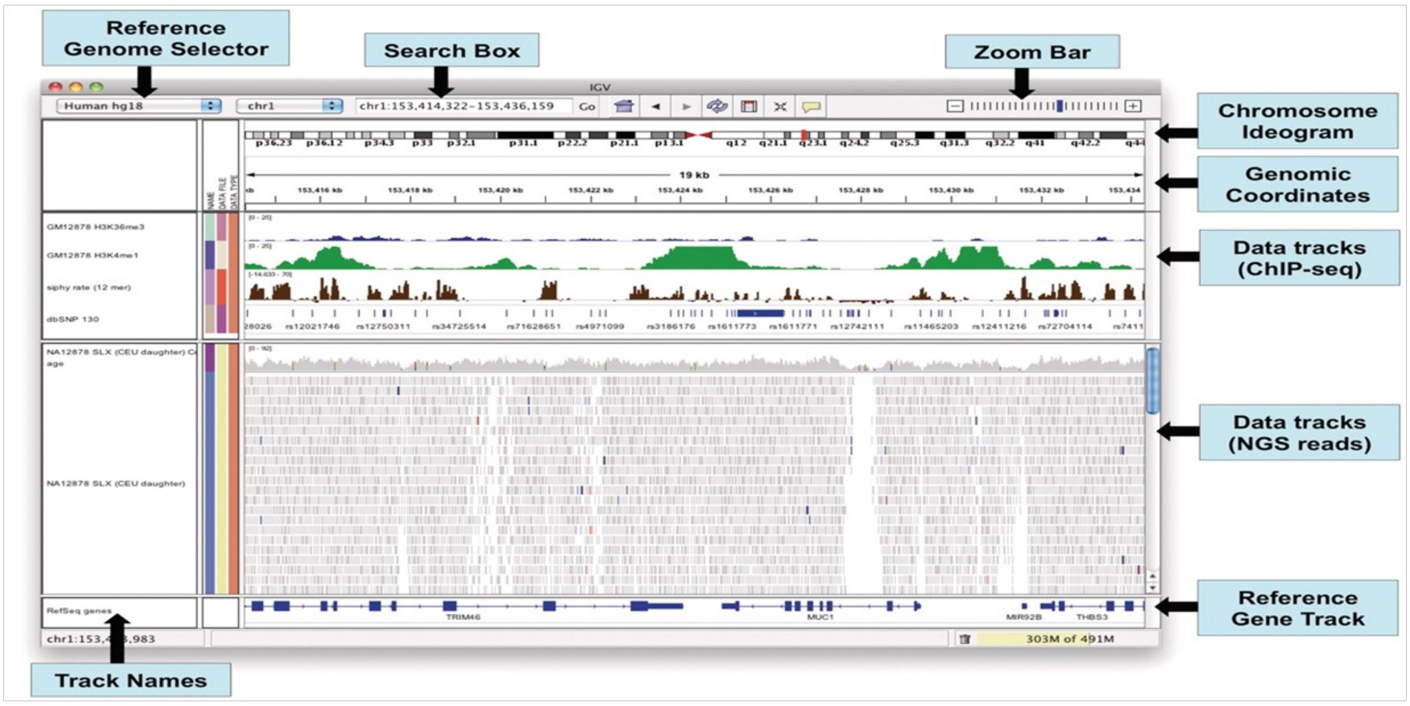
Can you see the difference with the previous vcf ?solution

For now we will not explore this step since you will be working with gene annotations in your next workshop.

You could also take a look at the HTML stats file snpEff created: it contains some metrics on the variants it analyzed.

Data visualisation

The Integrative Genomics Viewer (IGV) is an efficient visualization tool for interactive exploration of large genome datasets.



IGV browser presentation

Before jumping into IGV, we'll generate a track IGV can use to plot coverage:

```
igvtools count \
  -f min,max,mean \
  alignment/NA12878/NA12878.sorted.dup.recal.bam \
  alignment/NA12878/NA12878.sorted.dup.recal.bam.tdf \
  b37
```

Then:

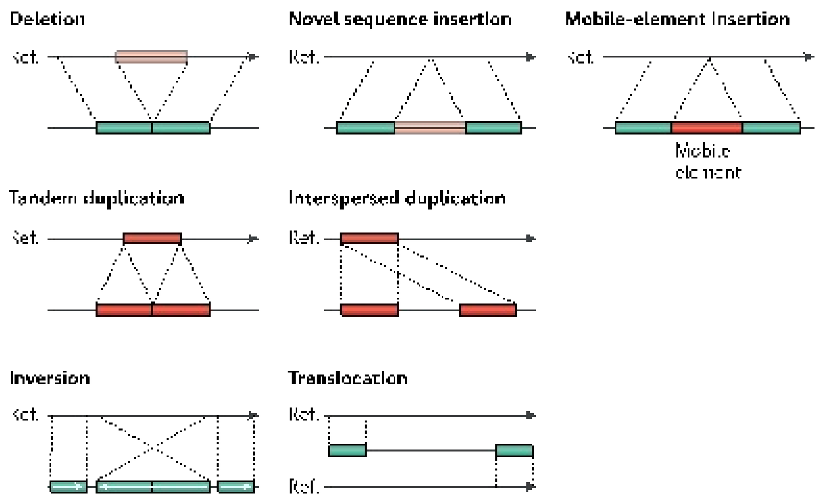
1. Open IGV
2. Chose the reference genome corresponding to those use for alignment (b37)
3. Load bam file
4. Load vcf file

Explore/play with the data:

- find an indel
- Look around…

Rational on Structural Variant calling methods

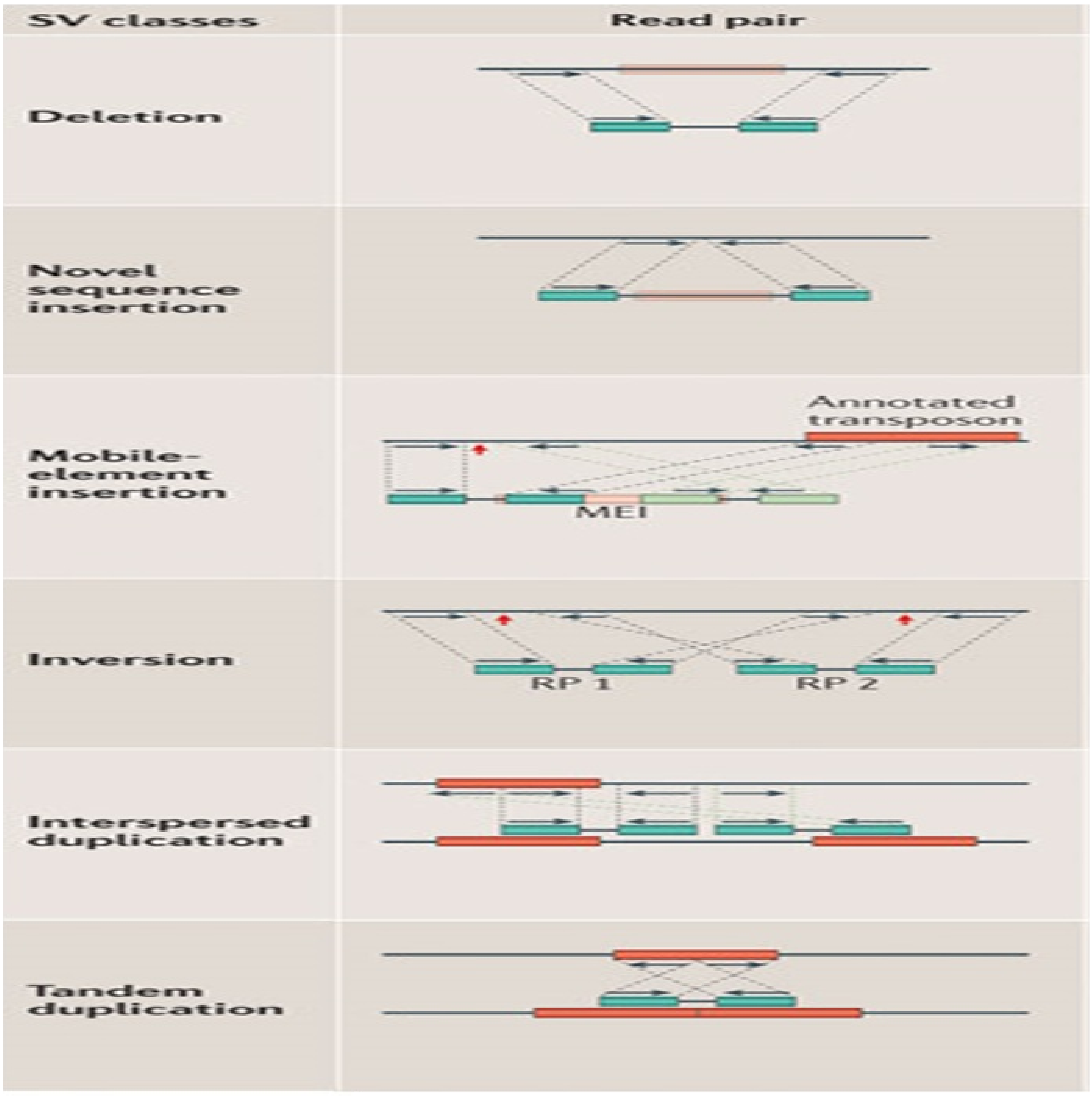
What are structural variants ?



SV examples

Read pair methods

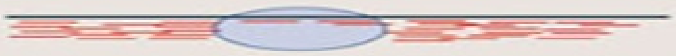
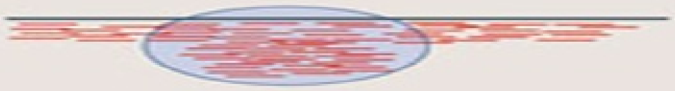
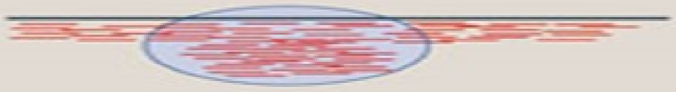
Identification of read pairs clusters with abnormal inserts size or orientation



PER method

Depth of coverage methods

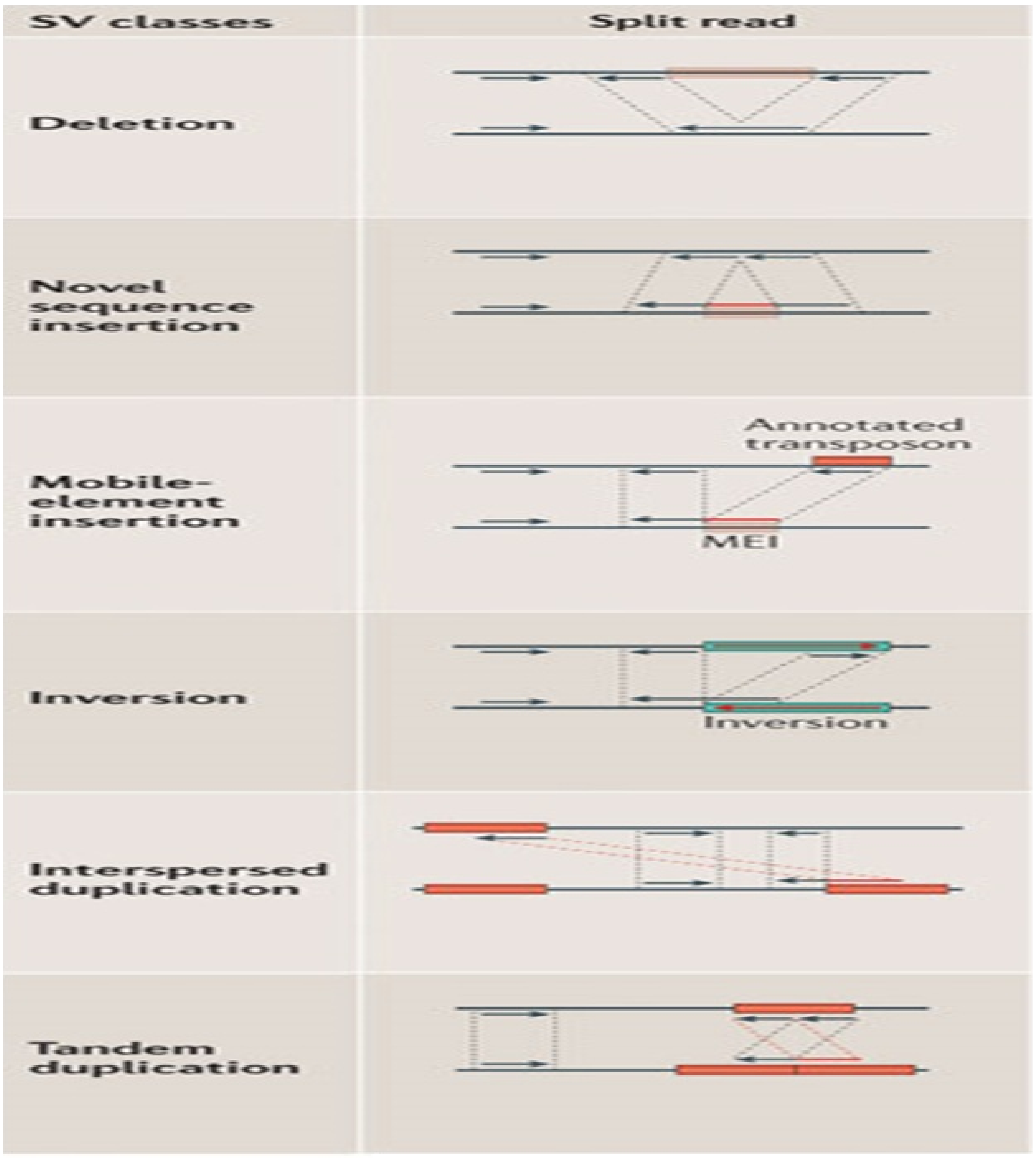
Identification of genomic regions harbouring a lack or an excess of reads

SV classes	Read depth
Deletion	
Novel sequence insertion	Not applicable
Mobile-element insertion	Not applicable
Inversion	Not applicable
Interspersed duplication	
Tandem duplication	

DOC method

Split read methods

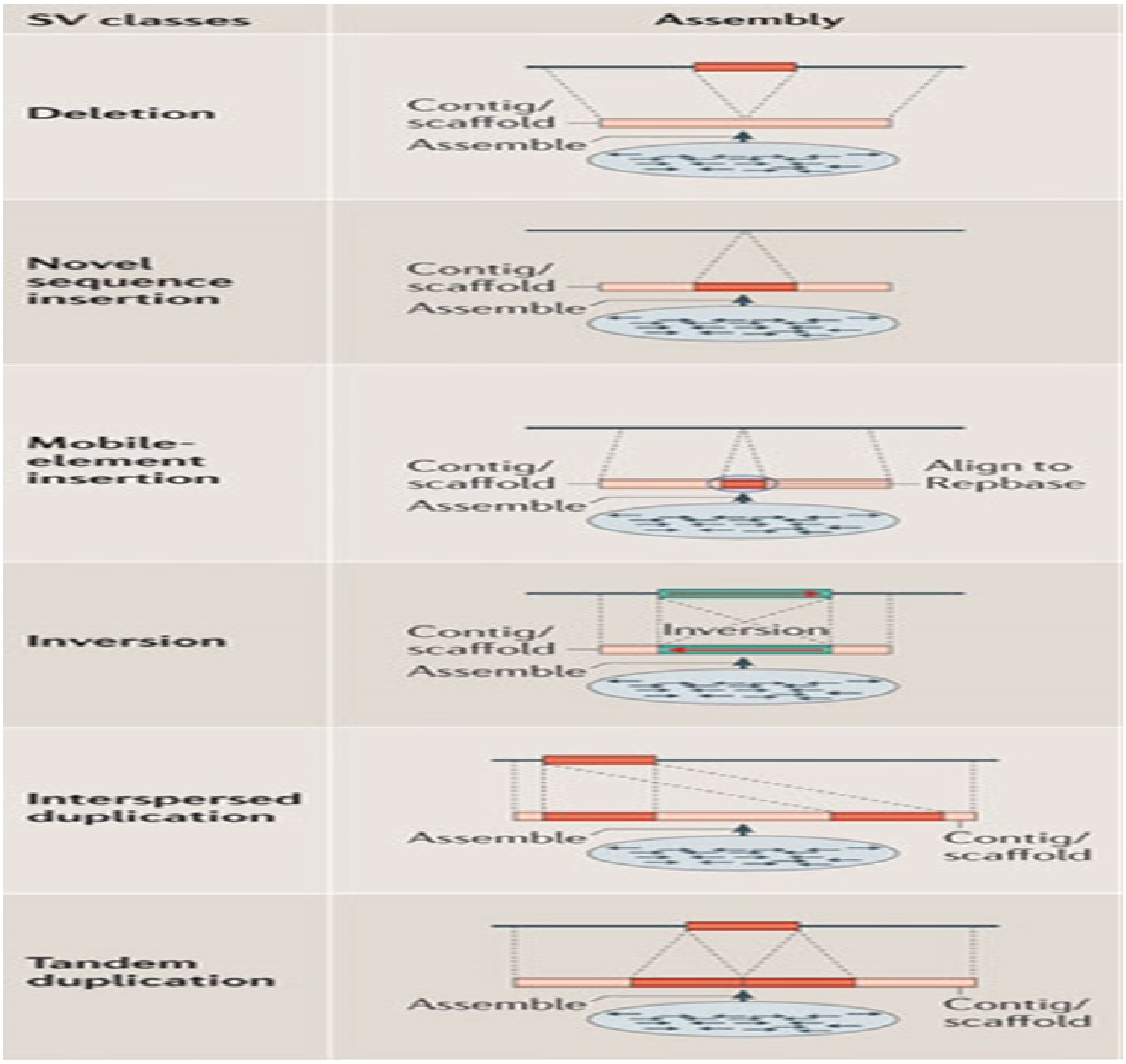
local alignment in a targeted genomic region of unmapped ends from one-end-anchored reads



SR method

Assembly methods

It performs a de novo assemblies followed by local permissive alignments



DN method

Add-on

[Additional exercice to play with sam/bam files]()

Aknowledgments

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