Next-Generation Sequencing: Quality Control

BaRC Hot Topics – January 2017
Bioinformatics and Research Computing
Whitehead Institute

http://barc.wi.mit.edu/hot_topics/

Why QC?

- Do you want to include the reads with low quality base calls?
- Why are so little reads mapped? Could it be because of adaptor, other species/vectors contamination?
- How is your library preparation? How to identify samples with low library complexity? For RNA-seq, are the high gene expression levels due to real biological signal or to PCR artefacts ?

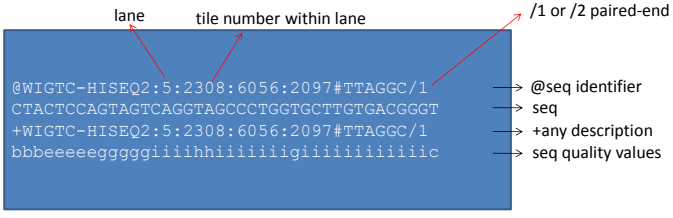
QC

- Before mapping:
  - How to identify and remove reads with low base calls?
  - How to identify and remove reads with linkers/adaptors ?
  - How to screen for potential species/vector/ribosomal contamination?
  - How is your library complexity?
- After Mapping:
  - What is percentage of reads aligned?
  - Is your sequencing library stranded or unstranded?
  - How could I know if the high expression levels are due to real biological signal or to PCR artefacts?

Illumina data format

- Fastq format:

http://jura.wi.mit.edu/genomecorewiki/index.php/SequencingFormats



Input qualities	Illumina versions
--solexa-quals	<= 1.2
--phred64	1.3-1.7
--phred33	>= 1.8

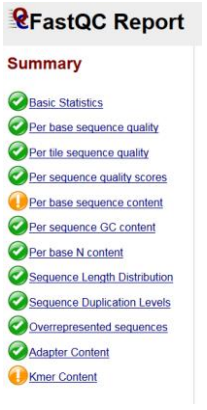
Check read quality with fastqc

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

1. Run fastqc to check read quality

   $ bsub –q 14 fastqc sample.fastq

2. Open output file: "fastqc_report.html"



Output from fastqc

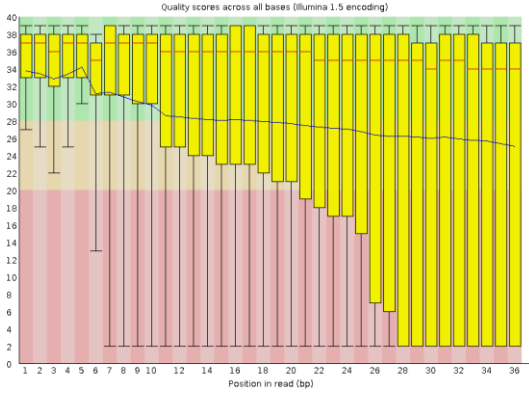
Basic Statistics

Measure	Value
Filename	sample.fastq
File type	Conventional base calls
Encoding	Illumina 1.5
Total Sequences	9053
Filtered Sequences	0
Sequence length	36
%GC	50

We have to know the quality encoding to use the appropriate parameter in the mapping step.

Note: sample.fastq is 0.05% of original fastq

FastQC: per base sequence quality



Red: median blue: mean yellow: 25%, 75% whiskers: 10%, 90%

remove reads with lower quality

-i: input file
-o: output file
-v: report number of sequences

$ fastq_quality_filter –h # usage information

$ bsub –q 14 fastq_quality_filter -v -q 20 -p 75 -i sample.fastq -o sample_good.fastq

-q: Minimum quality score
-p: Minimum percent of bases that must have [-q] quality

Check job status:

$ bjobs

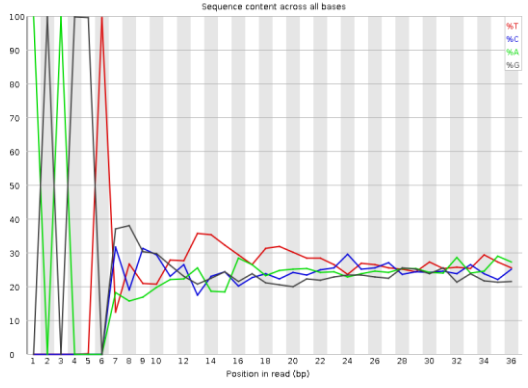
Look at your email to see the number of discarded reads

Problem solved? Re-run quality control on filtered reads:

$ bsub fastqc sample_good.fastq

Use your browser to look at the sample_good_fastqc.html

Output from fastqc



About 100% of the first six bases are AGAGGT

Trim the read sequence

delete the first 6nt from 5'

$ fastx_trimmer –h # usage information

-f: First base to keep
-l: Last base to keep
-i: input file
-o: output file
-v: report number of sequences

$ bsub fastx_trimmer -v -f 7 -l 36 -i sample_good.fastq -o sample_good_trimmed.fastq

# Problem solved? Check trimmed reads
$ bsub fastqc sample_good_trimmed.fastq

Use your browser on your laptop to look at the sample_good_trimmed_fastqc.html

Output from fastqc can show you the adaptor contamination

Overrepresented sequences

Sequence	Count	Percentage	Possible Source
TGGAATTCTCGGGTGCCAAGGAACTCCAGTCACTTAGGCA	7360116	82.88507591015895	RNA PCR Primer, Index 3 (100% over 40bp)
GCGAGTGCGGTAGAGGGTAGTGGAATTCTCGGGTGCCAAG	541189	6.094535921273932	No Hit
TCGAATTGCCTTTGGGACTGCGAGGCTTTGAGGACGGAAG	291330	3.2807783416601866	No Hit
CCTGGAATTCTCGGGTGCCAAGGAACTCCAGTCACTTAGG	210051	2.365464495397192	RNA PCR Primer, Index 3 (100% over 38bp)

Remove adapter/Linker

$ cutadapt # usage
$ bsub " cutadapt -a TGGAATTCTCGGGTGCCAAGGAACTCCAGTCACTTAGGCA foo.fastq | fastx_artifacts_filter > no_adapter.fastq"

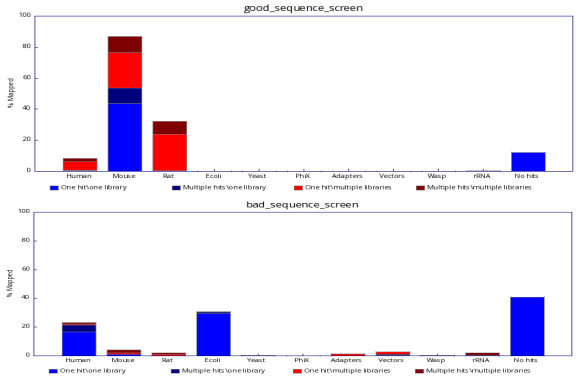
-a: Sequence of an adapter that was ligated to the 3' end.
-e : max. error rate (default =0.1)
fastx_artifacts_filter: filter reads with all but 3 identical bases

cutadapt: http://code.google.com/p/cutadapt/
fastx artifacts filter: http://hannonlab.cshl.edu/fastx_toolkit/galaxy.html#fastx_artifacts

Recommendation for preprocessing

- Treat all the samples the same way.
- Watch out for preprocessing that may result in very different read length in the different samples as that can affect mapping.
- If you have paired-end reads, make sure you still have both reads of the pair after the processing is done.
- Run fastqc on the processed samples to see if the problem has been removed.

Identify species/vectors contamination fastq_screen:



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

fastq_screen

- $ bsub fastq_screen --illumina1_3 --aligner bowtie2 sample_good_trimmed.fastq

  --aligner *Specify 'bowtie' or bowtie2' to use for the mapping*

  --illumina1_3 *Assume that the quality values are in encoded in Illumina v1.3 format. Defaults to Sanger.*

  --conf *Manually specify a location for the configuration file to be used for this run.*

  On local tak server: /usr/local/bin/fastq_screen.conf contains the source index files. You can create your own configuration file to include other potential contamination.

```
DATABASE    Human   /nfs/genomes/human_hg38_dec13_no_random/bowtie/hg38
DATABASE    rRNAs_human     /nfs/genomes/human_rRNAs/rRNAs
DATABASE    Mouse   /nfs/genomes/mouse_mm10_dec_11_no_random/bowtie/mm10
DATABASE    Arabidopsis     /nfs/genomes/a.thaliana_TAIR_10/bowtie/tair10
DATABASE    Fly     /nfs/genomes/d.melanogaster_apr_06/bowtie/dm3
DATABASE    Fish    /nfs/genomes/zfish_danRer7/bowtie/danRer7
DATABASE    Yeast   /nfs/genomes/sgd_2010/bowtie/sacCer3
DATABASE    PhiX174 /nfs/genomes/phiX174/phiX174
DATABASE    Ecoli   /nfs/genomes/e_coli/e_coli
DATABASE    adapters_FastQC /nfs/genomes/NGS_adapters_primers/adapters_primers
```


QC

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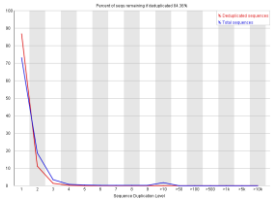
Library Complexity

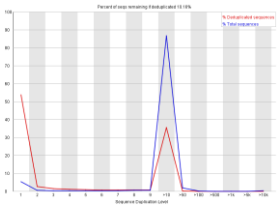
- Reasons: low-input; single cell RNA-seq
- Low library complexity may be an indicator that:
  - A new sample and a new library should be prepared.
  - We can not sequence the same sample anymore because we will not find new sequences.
- Chip-seq:
  - we have to find a better Ab to perform the IP.
  - In certain experimental settings we may expect a low library complexity. *i.e.* We are profiling a protein that binds to a small subset of the genome.

Library Complexity

- Refers to the fraction of unique fragments present in a given library.
- One proxy for library complexity is to look at the sequence duplication levels on the FastQC report:



% Complexity
85.6



% Complexity
4.95

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Mapping Statistics

- RSeQC (http://rseqc.sourceforge.net/)
  - $ bam_stat.py –i sample2.bam > sample_stat.txt

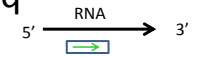
```
Total records:                 10000

QC failed:                     0
Optical/PCR duplicate:         0
Non primary hits               2121
Unmapped reads:                0
mapq < mapq_cut (non-unique):  507

mapq >= mapq_cut (unique):     7372
Read-1:                        3845
Read-2:                        3527
Reads map to '+':              5936
Reads map to '-':              1436
Non-splice reads:              7067
Splice reads:                  305
Reads mapped in proper pairs:  4566
Proper-paired reads map to different chrom:0
```

Is your library stranded or not stranded?

- Checking library preparation protocol
- Mapping your RNA-seq reads as if they were non-strand specific, "guess" how RNA-seq reads were stranded.
  - RSeQC (http://rseqc.sourceforge.net/)
  - $ infer_experiment.py -i sample.bam –r gene_model.bed
    - comparing the "strandness of reads" with the "strandness of transcripts".
    - The "strandness of reads" is determined from alignment
    - the "strandness of transcripts" is determined from annotation.

Infer_experiment.py single-end RNA-seq

Two different ways to strand reads:



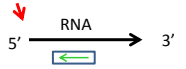
i) ++,--

read mapped to '+' strand indicates parental gene on '+' strand
read mapped to '-' strand indicates parental gene on '-' strand

ii) +-,-+

read mapped to '+' strand indicates parental gene on '-' strand
read mapped to '-' strand indicates parental gene on '+' strand

Strand-specific example:

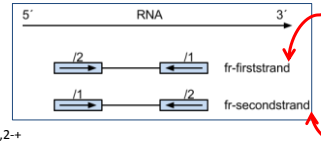


Fraction of reads failed to determine: 0.0170
Fraction of reads explained by "++,--": 0.9669
Fraction of reads explained by "+-,-+": 0.0161

Infer_experiment.py pair-end RNA-seq

Ways to strand reads:

1+-,1-+,2++,2–
read1 mapped to '+' strand indicates parental gene on '-' strand
read1 mapped to '-' strand indicates parental gene on '+' strand
read2 mapped to '+' strand indicates parental gene on '+' strand
read2 mapped to '-' strand indicates parental gene on '-' strand

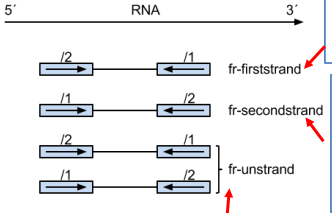


1++,1–,2+-,2-+
read1 mapped to '+' strand indicates parental gene on '+' strand
read1 mapped to '-' strand indicates parental gene on '-' strand
read2 mapped to '+' strand indicates parental gene on '-' strand
read2 mapped to '-' strand indicates parental gene on '+' strand

http://onetipperday.sterding.com/2012/07/how-to-tell-which-library-type-to-use.html

Infer_experiment.py pair-end RNA-seq

http://onetipperday.sterding.com/2012/07/how-to-tell-which-library-type-to-use.html



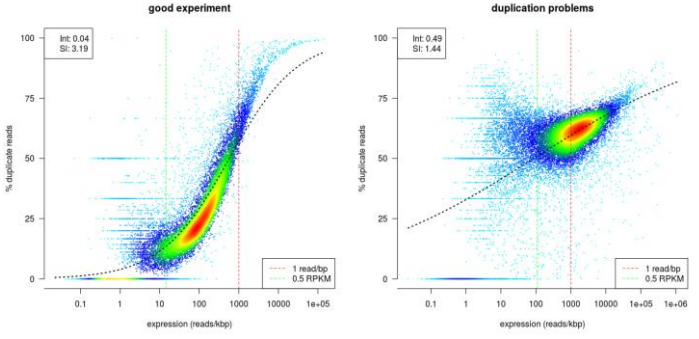
Fraction of reads explained by "1++,1--,2+-,2-+": 0.0193
Fraction of reads explained by "1+-,1-+,2++,2--": 0.9807
Fraction of reads explained by other combinations: 0.0000

Fraction of reads explained by "1++,1--,2+-,2-+": 0.9807
Fraction of reads explained by "1+-,1-+,2++,2--": 0.0193
Fraction of reads explained by other combinations: 0.000

Fraction of reads failed to determine: 0.0648
Fraction of reads explained by "1++,1--,2+-,2-+": 0.4590
Fraction of reads explained by "1+-,1-+,2++,2--": 0.4763

DupRadar

(https://www.bioconductor.org/packages/release/bioc/html/dupRadar.html)



DupRadar requirements:
alignment file: mark duplicates with bamutil or Picard MarkDuplicates
Gene model in gtf format
Strand information
Single or Paired reads

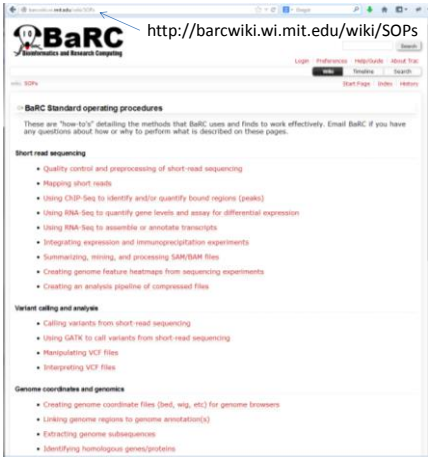
Summary

- Before mapping:
  - Quality control
    - fastqc
  - Clean up reads:
    - fastx tool kit: fastq_quality_filter, fastx_trimmer
    - Cutadapt
  - Check species/vector contamination
    - fastq_screen
- After mapping:
  - Get mapping statistics:
    - bam_stat.py
  - Check library strandness:
    - Infer_experiment.py
  - Check sequence duplication in RNA-seq samples:
    - DupRadar

References

fastqc	do quality control	http://www.bioinformatics.babraham.ac.uk/projects/fastqc
fastx Toolkit	reads editor	http://hannonlab.cshl.edu/fastx_toolkit/
	remove reads with low base call quality	fastq_quality_filter
	remove 5' or 3' reads	fastx_trimmer
	remove artifacts in reads	fastx_artifacts_filter
cutadapt	remove adaptors	https://code.google.com/p/cutadapt
fastq_screen	screen for species/vectors contamination	http://www.bioinformatics.babraham.ac.uk/projects/fastq_screen/
RSeQC	RNAseq quality control packages	http://rseqc.sourceforge.net/
	get mapping summary	bam_stat.py
	check strandness	infer_experiment.py
DupRadar	check reads duplication	http://bioconductor.org/packages/release/bioc/html/dupRadar.html
QualiMap	mapping summary, coverage distribution	http://qualimap.bioinfo.cipf.es/
Picard	package with functions on NGS data	https://broadinstitute.github.io/picard/
	Insert size of PE reads	CollectInsertSizeMetrics
	coverage across transcripts	CollectRnaSeqMetrics
multiQC	merge QC reports	http://multiqc.info/

BaRC Standard operating procedures



Coming up

- Introduction to Python:
  - By the authors of “Python For The Life Sciences” book
  - Jan. 24th and 25th @1pm
  - Registration required