Our Top 5 Quality Control (QC) Metrics Every NGS User Should Know

https://blog.horizondiscovery.com/diagnostics/the-5-ngs-qc-metrics-you-should-know

We all know how vital quality control is for our samples. A lot of research has gone in to developing useful QC metrics for genomics experiments – primarily due to their high cost. Skipping this step will waste both time and money.

There are 3 main areas where QC can be applied to NGS:

- On the starting nucleic acids
- After Library preparation
- Post-Sequencing

Ultimately the best QC of your NGS experiments is likely to come from the sequence data and, because of this, many labs will run a QC lane first.

NGS QC metrics:

1. Sample Quality Control:

Usually you would start an NGS experiment with high-quality DNA and/or RNA samples. However, many experiments are performed with degraded nucleic acids. Often you rely on spectrophotometric (Nanodrop), fluorimetric (Pico- and Ribo-Green) and gel electrophoretic methods (Bioanalyser) to QC starting material. Agilent's RNA Integrity Number (RIN) provides a robust and non-subjective method for RNA QC, with most experiments using samples with RIN>7.

2. Library Quality Control:

Before sequencing most NGS libraries are checked on the Bioanalyser. This verifies the insert size is as expected and that there are no contaminating adapter-dimers. These can be a major issue on the new exclusion-amplification clustering chemistry for the HiSeq 400 and X Ten.

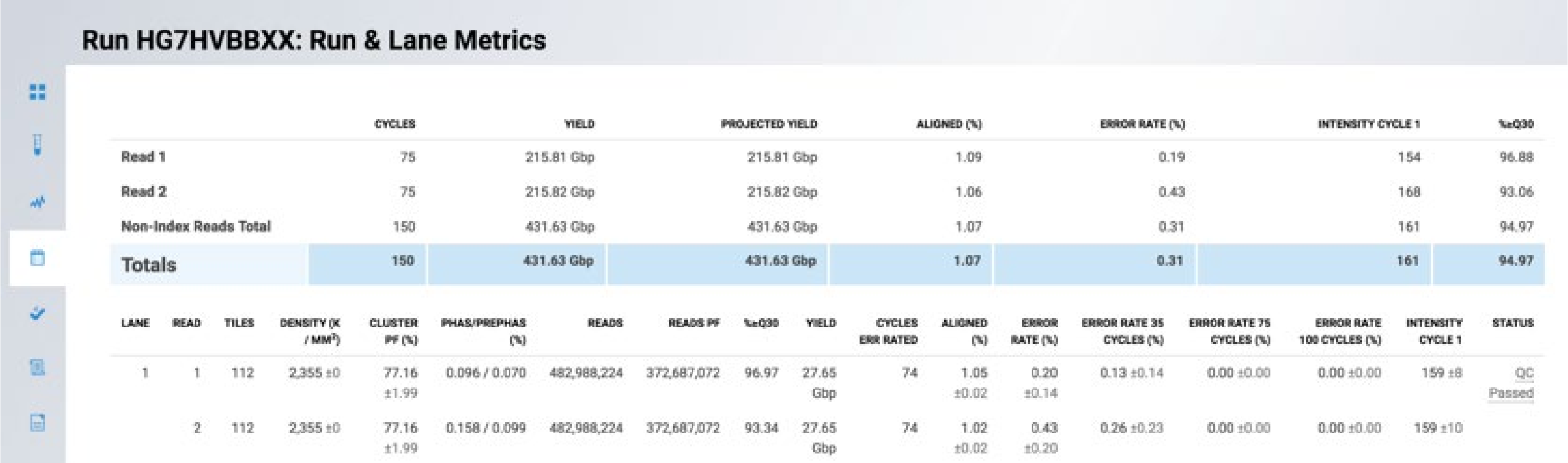
3. Sequencing Quality Control:

There are several commonly used sequencing QC metrics. **Sequence Analysis Viewer** (**SAV**) and **FastQC** analysis reports for each sequencing lane.

4. Sequence Analysis Viewer (SAV):

SAV is an Illumina tool used to monitor sequencing during, or check QC after a run (Illumina's SAV user guide).

BaseSpace contains useful data presented in the “Run and Lane Metrics” table. These include yield, error rate, %Q30, Density (K/mm^2), Cluster PF(%) and Phas/Prephas (%) (Fig1).



Run HG7HVBBXX: Run & Lane Metrics

	CYCLES	YIELD	PROJECTED YIELD	ALIGNED (%)	ERROR RATE (%)	INTENSITY CYCLE 1	%≥Q30
Read 1	75	215.81 Gbp	215.81 Gbp	1.09	0.19	154	96.88
Read 2	75	215.82 Gbp	215.82 Gbp	1.06	0.43	168	93.06
Non-Index Reads Total	150	431.63 Gbp	431.63 Gbp	1.07	0.31	161	94.97
Totals	150	431.63 Gbp	431.63 Gbp	1.07	0.31	161	94.97

LANE	READ	TILES	DENSITY (K / MM^2)	CLUSTER PF (%)	PHAS/PREPHAS (%)	READS	READS PF	%≥Q30	YIELD	CYCLES ERR RATED	ALIGNED (%)	ERROR RATE (%)	ERROR RATE 35 CYCLES (%)	ERROR RATE 75 CYCLES (%)	ERROR RATE 100 CYCLES (%)	INTENSITY CYCLE 1	STATUS
1	1	112	2,355 ±0	77.16 ±1.99	0.096 / 0.070	482,988,224	372,687,072	96.97	27.65 Gbp	74	1.05 ±0.02	0.20 ±0.14	0.13 ±0.14	0.00 ±0.00	0.00 ±0.00	159 ±8	QC Passed
	2	112	2,355 ±0	77.16 ±1.99	0.158 / 0.099	482,988,224	372,687,072	93.34	27.65 Gbp	74	1.02 ±0.02	0.43 ±0.20	0.26 ±0.23	0.00 ±0.00	0.00 ±0.00	159 ±10	

4.1. Yield:

Yield is the number of bases generated in the run. Yield is important to all users, but is usually something your service provider will guarantee so you don’t need to worry about it.

4.2. Error Rate:

Refers to the percentage of bases called incorrectly at any one cycle. It is calculated from the reads that are aligned to Illumina’s PhiX control. If this was not used then %Q30 is your best tool to check base quality. Error rate increases along the length of the read.

4.3. %Q30:

The percentage of bases with a quality score of 30 or higher, respectively (see “Quality Scores Explained” below). Most Illumina runs will generate >70-80% Q30 data. This value is an average across the whole read length, and error rate increases towards the end of the reads. Because of this a run can “fail” at the end of a long-read, but pass Illumina’s specs for the run with respect to Q30 – if a read is Q40 for bases 1-100, and Q10 for bases 101-150 it will pass the Q30 spec, but if you need the ends of the reads to be high quality, you may be disappointed.

4.4. Density (K/mm^2):

The density of clusters on the flowcell (in thousands per mm^2). On MiniSeq, MiSeq, NextSeq and HiSeq 2500 this is an important metric to evaluate if the data are low-quality. It should be assessed in tandem with %PF as the two together can diagnose problems with over- or under-loading your library. On HiSeq 4000 and X density is a fixed value and diagnosing issues with library loading are much more difficult to fix.

4.5. Cluster PF (%):

In Illumina clustering a single molecule should generate a single cluster with a clear signal in the base being sequenced. The %PF is the number of clusters that passed Illumina's "Chastity filter". The clusters that do not pass this filter are generally removed from downstream analysis.

The Chastity filter works by calculating the ratio of the highest base intensity to the sum of the 1st and 2nd highest, anything less than 0.6 is filtered out. If a cluster was formed from a single-molecule then the chastity score will be 1; if it were formed from two molecules the signal would be equal and the chastity score will be 0.5.

4.6. Phas/Prephas (%):

This is an important metric to pay attention to – low numbers are what you want to see e.g. 0.1/0.1. Phasing is the rate at which individual molecules in a cluster become out of sync with each other, with some falling behind (phasing), and others jumping ahead (pre-phasing). The value given is the percentage of true signal being lost in each cycle, so after 150 cycles 15% of the data is now noise. Phasing is why long-reads are difficult!

There are several possible causes for poor phasing/pre-phasing, but to estimate this correctly requires a sample with balanced base-composition (25% of each base), if you know your sample to be unbalanced then you may need to add extra PhiX control. Assuming your sample is not the problem the most likely causes are the reagents or flowcell. Check the use-by date of reagents, check that there were no problems with fluidics, and check that the temperature was not too high during the run.

5. Sequencing Reads:

If you are performing read-counting applications such as RNA-seq or ChIP-seq then this metric is likely to be more important than yield. The two can be used interchangeably by sequencing service providers, so make sure you know which is most important to you.

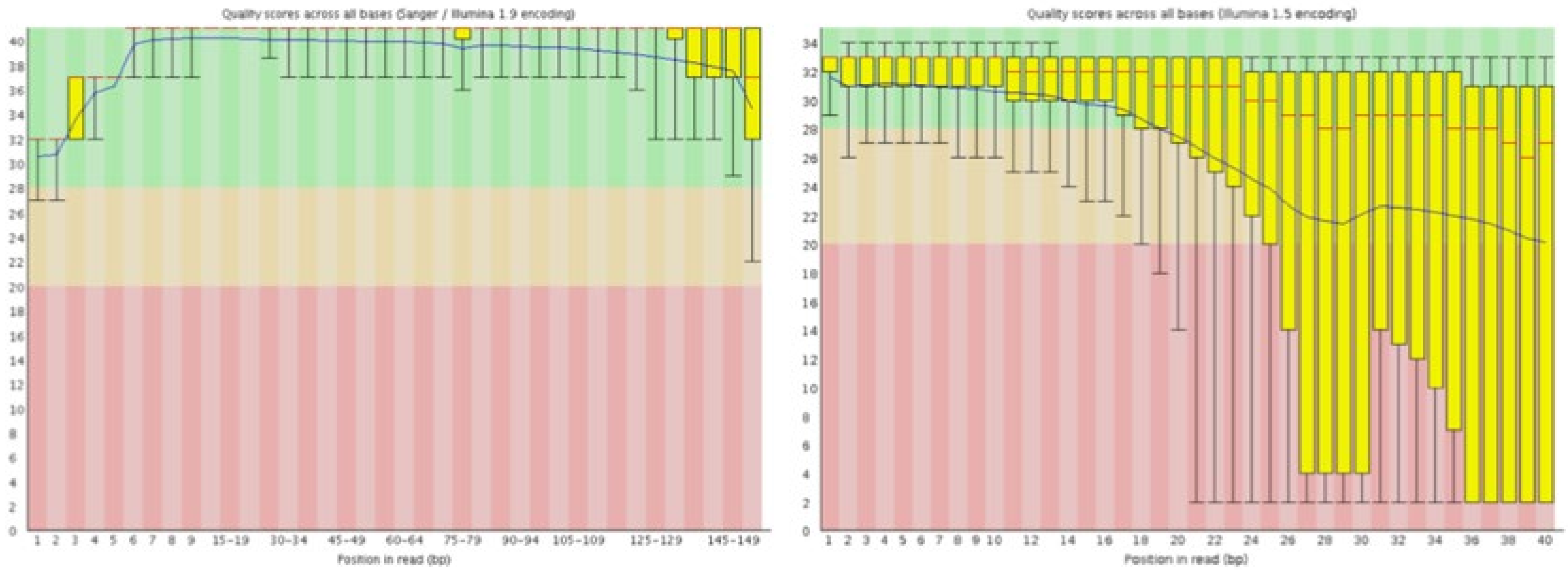
Analysis of NGS Quality Scores

"A quality score is an estimate of the probability of that base being called wrongly: $q = -10 \times \log_{10}(p)$*"*

FastQC: Per base sequence quality

Good data

Bad data

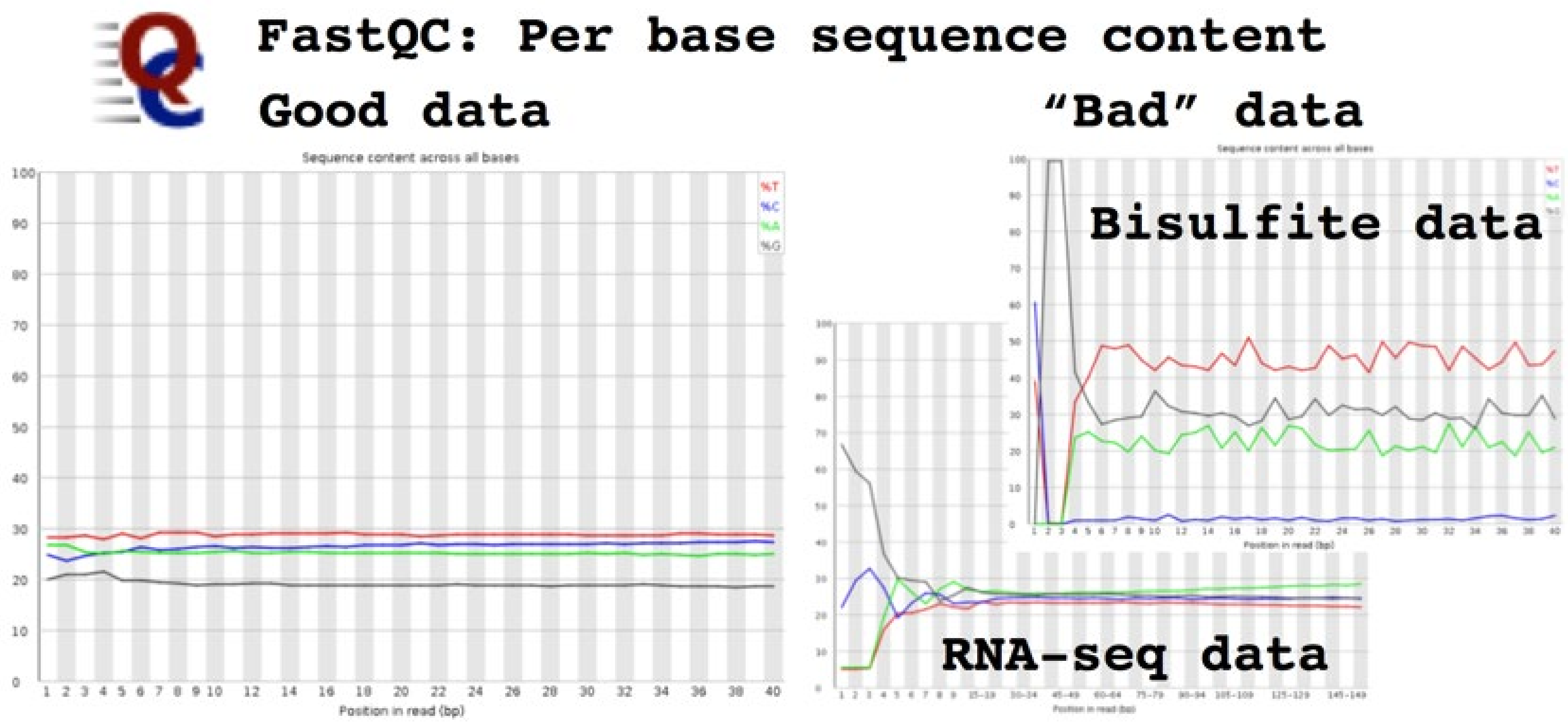


FASTQC: Is a very commonly used NGS QC package that is applied before primary biological analysis (FastQC homepage). It reports multiple QC metrics, which are reported with a traffic light warning system, normal (green), abnormal (orange) or bad (red), making it relatively easy to interpret results. These can be used to quickly identify common problems with NGS data and QC individual sequencing runs before starting biological analysis.

The most useful plots for most users are:

Per Base Sequence Quality, which plots the Q-score of the raw sequence reads as a box-plot for each cycle. Higher is always better, and a characteristic quality decay is seen in most runs.

Per Base Sequence content, which plots the proportion of each base at each cycle. In a random fragment library from a “normal” genome you would expect to see all four bases equally represented. Deviation from normal base content can indicate issues with library quality, but equally some genomes are very GC biased and some NGS applications also introduce a strong GC bias, e.g. Bis-seq.



FastQC: Per base sequence content

Good data

"Bad" data

Bisulfite data

RNA-seq data

Sequence quality should be high, generally above Q30, along the length of a good Illumina read. The profile has changed over time; there is a clear decay of read quality towards the end of the read, but read-lengths of 150bp are possible on HiSeq and up to 300bp on MiSeq. The second read in a paired-end run is always slightly lower quality than the first. Low-quality bases can be easily trimmed; lots of low quality bases may indicate a poor library, or problems with phasing. However a sudden drop in quality is likely to indicate something happened during the run, or that there are short fragments and you are reading into adapter. Tracking the quality profile can identify issues with sequencing chemistry and/or instruments, and can help.

The Illumina technology produces the best data when all four bases are equally represented e.g. whole genome sequencing. This is due to several reasons, all of which are to do with the analysis algorithms used to detect and call bases on the sequencer. As you are unlikely to be modifying these the best suggestion is to monitor base composition (FastQC plot), and to understand when it is likely vary because of the library type being sequenced e.g. RNA-seq, Nextera, or bisulfite-converted DNA.

Analysing NGS QC metrics

Many of the tools or metrics that can be used to QC NGS data are context dependent. The information above should be used as a guide for standard whole genome sequencing samples, and will be different if you are analysing RNA-seq, bisulfite, amplicon, transposase, ATAC-seq or many other data. If you are unclear about how to interpret data for your application look online at sites like SEQanswers, Compare results from published data or start to collect QC data for your project and learn to interpret it yourself.

It will also be important to consider other QC metrics after alignment and analysis. The more time and effort you spend on QC the better quality your results and conclusion will be.

Find out how one clinical laboratory used our reference material to generate and monitor the quality control metrics in the case study below.

See also:

FASTQ Processing Tools for Data Analysis

ExAmp Cluster Amplification Workflow

How Do I Achieve Consistent Quantitation? Part 1

How Do I Achieve Consistent Quantitation? Part 2

How Do I Optimize Amplicon Sequencing Data? Part 1

How Do I Optimize Amplicon Sequencing Data? Part 2

How Can I Tell If I Sequenced Through the Insert? Part 1

How Can I Tell If I Sequenced Through the Insert? Part 2

Illumina DRAGEN Bio-IT Platform: Analysis Pipeline Overview

Illumina DRAGEN Bio-IT Platform: Technology Overview

Illumina Experiment Manager

Amplicon and 16S Metagenomics Sequencing: Introduction

AmpliSeq for Illumina: Library Prep Protocol

Assessing the Success of your Amplicon Sequencing Project

Bcl2Fastq v2+: Introduction

BaseSpace Sequence Hub: Command Line Tools for Basic Analysis - Part II

BaseSpace Sequence Hub: New Features

Best Practices for Nextera Library Prep

Enrichment Data Analysis: Introduction

Enrichment Sequencing Part I: Tips for Successful Library Prep with Nextera Rapid Capture and TruSight Enrichment

Enrichment Sequencing Part II: Tips for a Successful Library Prep with TruSeq Exome and Rapid Exome

Enrichment Sequencing Part III: Sequencing and Data Analysis

DesignStudio: Introduction

DesignStudio: Illumina Custom Enrichment Panels