HTSeqQC: **A Flexible and One-Step Quality Control Software for High-throughput Sequence Data Analysis**

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ABSTRACT

Use of high-throughput sequencing (HTS) has become indispensable in life science research. Raw HTS data contains several sequencing artifacts, and as a first step it is imperative to remove the artifacts for reliable downstream bioinformatics analysis. Although there are multiple stand-alone tools available that can perform the various quality control steps separately, availability of an integrated tool that can allow one-step, automated quality control analysis of HTS datasets will significantly enhance handling large number of samples parallelly. Here, we developed HTSeqQC, a stand-alone, flexible, and easy-to-use software for one-step quality control analysis of raw HTS data. HTSeqQC can evaluate HTS data quality and perform filtering and trimming analysis in a single run. We evaluated the performance of HTSeqQC for conducting batch analysis of HTS datasets with 322 sample datasets with an average ~1M (paired end) sequence reads per sample. HTSeqQC accomplished the QC analysis in ~3 hours in distributed mode and ~31 hours in shared mode, thus underscoring its utility and robust performance. In addition to command-line execution, we integrated HTSeqQC into the free, open-source, CyVerse cyberinfrastructure resource as a GUI interface, for wider access to experimental biologists who have limited computational resources and/or programming abilities.

INTRODUCTION

Advancements in high throughput sequencing (HTS) technologies transformed biological research. HTS largely replaced conventional low-throughput Sanger-based sequencing technologies for genome-scale studies. Multiple genome sequencing approaches (DNA-seq, RAD-seq, GBS, AgSeq) are being used to study genetic variations, discovery of novel genes, high-throughput genotyping, biomarker discovery, and precision medicine [1-5]. Similarly, transcriptome-level sequencing approaches (RNA-seq) allows determining the steady-state expression of genes, identification of novel transcripts and isoforms, alternatively splicing patterns, polymorphisms, gene co-expression networks, allele-specific expressions, and long non-coding RNAs (lincRNA) [6-9]. Illumina's benchtop and production-scale sequencers, which are by far the most widely used HTS platforms, can generate up to 1 and 20 billion sequence reads per run, respectively. This sequencing output is expected to rapidly increase due to further advancements in the Illumina technologies. As such advanced bioinformatics tools are necessary to accelerate the analysis and efficiently manage the large volumes of data generated by the Illumina sequencing platforms.

In all HTS experiments, a critical first step is raw data quality assessment, filtering and trimming. HTS raw data often contains sequences of poor quality along with adapter or primer contaminations, and uncalled bases (N), which if not removed can significantly hamper the downstream bioinformatics analysis leading erroneous conclusions [10-12]. Several standalone software tools are available for quality control analysis, trimming, and filtering of HTS data such as FastQC (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/), NGS QC [13], FASTX-Toolkit (http://hannonlab.cshl.edu/fastx_toolkit/), NGS QCbox [14], Trimmomatic [15], fastp [16], and QC-Chain [11]. However, most of them have limitations. For instance, FastQC performs only quality check of the data, and does not filter or trim of raw sequences (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Conversely, FASTX-Toolkit although is equipped to perform quality filtering does not support parallel computing to handle large-scale batch analysis. Other software tools such as NGS QC, QC-Chain, Trimmomatic, and NGS QCbox have limited features for quality filtering, handles few samples at a time, dependent on other open-source software tools, and have a need to be run separately for different quality control features. Even though fastp has advantages over other tools, it does not offer support handling reads with excessive uncalled bases and does not support multiplexing or batch analysis

[16]. With HTS becoming more accessible and affordable, it is crucial to develop a flexible and integrative tool that can not only perform thorough quality control analysis, but can handle several hundred samples parallelly, with the least number of data handling steps.

Here, we present HTSeqQC, which is an open-source and easy-to-use quality control analysis software tool for cleaning raw HTS datasets generated from Illumina sequencing platforms. HTSeqQC is a flexible, one-step quality control software tool and can handle large number of samples. HTSeqQC integrates filtering and trimming modules for single and paired end HTS data and supports parallel computing for batch quality control analysis. In addition to the quality filtering and trimming analysis, HTSeqQC generates statistical summaries and visualization to assess the quality of the HTS datasets. HTSeqQC can be used as command-line interface (CLI) as well as GUI. The GUI is available through CyVerse [17,18] Discovery Environment (https://cyverse.org/).

RESULTS AND DISCUSSION

Case studies

To evaluate the performance of HTSeqQC for quality control analysis, we analyzed several datasets using a 64 GB RAM and 20 CPUs computing node. First, we analyzed a single-end raw RNA-seq dataset generated on Illumina HiSeq 2000 platform corresponding to cotton, a dicot plant (BioProject accession PRJNA275482 and SRA ID: SRR1805340, Table 1) [19,20]. HTSeqQC analysis was performed with the default parameters. HTSeqQC automatically detected the Illumina sequence quality variant and filtered out the reads with Phred quality score ≤ 20. In total, HTSeqQC filtered out ~5 M reads (Fig.3 and Supplementary File 1A).

Second, we evaluated multiple paired-end raw RNA-seq datasets generated on Illumina HiScanSQ platform corresponding to sugarcane, a monocot plant (BioProject accession PRJNA291816 and SRA IDs: SRR2165176, SRR2165177, SRR2165178) [6,21]. Initially, we ran HTSeqQC on one paired-end dataset (SRR2165176) using default parameters (Table 1). HTSeqQC was able to analyze the Illumina sequence quality variants and filtered out the reads with Phred quality score ≤ 20 (Supplementary File 1B). For instance, the HTSeqQC filtered out the ~250 K sequence reads which were below the quality threshold (Supplementary File 1B). Next, we ran the HTSeqQC with customized parameters for filtering adapter sequences, quality thresholds, and uncalled bases. In total, HTSeqQC filtered out ~451 K reads and trimmed ~20 K

reads (Supplementary File 1C). All the HTSeqQC commands used for performing the above analyses are provided in the README file.

In addition to quality control analysis, we also evaluated the processing time and batch handling of HTSeqQC using default parameters. HTSeqQC took ~9 min to perform the quality filtering analysis of ~9 M paired-end sequence reads, and ~40 min for ~82 M single-end sequence reads with 18 CPUs (Table 1).

Lastly, we analyzed 322 paired-end genotyping-by-sequencing (GBS) datasets corresponding to tomato [5]. These datasets had ~1M (×2) sequence reads per sample. For this experiment, we also compared parallel computing in shared vs. distributed mode with 18 CPUs per computing node using Nextflow. We were able to analyze the 322 datasets in ~1855 min (~31 hours) and ~157 min (~3 hours) in the shared and distributed computing modes, respectively (Table 1).

Advantages of HTSeqQC over existing quality control analysis software

We compared the advantages of HTSeqQC with prevailing quality control analysis tools such as the FastQC (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/), NGS QC [13], QC Chain [11], FASTX-Toolkit (http://hannonlab.cshl.edu/fastx_toolkit/), fastp [16] and NGS QCbox [14] on various important quality control features (Table 2). Many of the current tools are not as integrated as HTSeqQC (Table 2).

For instance, programs such as FastQC only provides the quality control statistics of the raw HTS data and cannot be used for quality filtering. The FASTX-Toolkit does quality filtering, but the various trimming, filtering options are not integrated, and each analysis must be performed separately. The NGS QCbox comes close, however only allows sequence read trimming based on a quality threshold for specific window size and it is highly dependent on other open-source software tools for quality control analysis [14]. NGS QC allows filtering and trimming of low-quality reads similar to FASTX-Toolkit, however these software tools are independent and must be run separately. The fastp provides single run quality control analysis of FASTQ files similar to HTSeqQC but does not offer key features such as multiplex/batch quality control analysis and handling of sequence reads containing uncalled bases (N). fastp does offer other features which are not included in HTSeqQC such as poly tail trimming, UMI

preprocessing, and basic support for the long-read sequence data. Hence, in this context, the two tools can be complementary to each other for a range of applications.

The removal of sequence reads containing uncalled bases (N) is critical to improve the accuracy of the sequence reads [22]. HTSeqQC has this feature of filtering the sequence reads based on the content of the uncalled bases (N). Although NGS QC allows this analysis, it is not integrated, and must be run separately [13]. In addition to overcoming several of these limitations, HTSeqQC supports common features with NGS QC and NGS QCbox such as allowing inputs in the compressed GZIP file format for seamless input of large datasets (Table 2). Furthermore, similar to NGS QC, HTSeqQC has an integrated function to automatically detect the FASTQ quality variants (Table 2). Only HTSeqQC provides a parameter to output the cleaned FASTQ data in FASTA format, avoiding using additional file format conversion tools. Lastly, in addition to the command line execution, we integrated HTSeqQC as a graphic user interface that is accessible freely for everyone to access through CyVerse.

CONCLUSION

HTSeqQC is an open-source, integrated, and easy-to-use software designed for one-step quality control visualization and quality filtering of raw HTS data generated using the Illumina sequencing platforms. The flexiblility to detect and remove the low-quality sequences, adapter or primer contamination, uncalled bases, in a single run, greatly enhances the automation of HTS data analysis projects. Because HTSeqQC can be implemented by parallel computing, it enables batch handling of large number (>300) of datasets. In addition to the command line interface, HTSeqQC is available as graphic user interface that is accessible freely through CyVerse. The later should significantly facilitate its use among biologists without much prior bioinformatics or command line computing experience.

MATERIALS AND METHODS

Implementation

HTSeqQC is a standalone open-source command-line software tool developed using Python 3 for quality control analysis of HTS data generated from Illumina sequencing platforms. The current HTSeqQC version (v1.0) was developed specifically for Illumina generated FASTQ datasets, however, it could be utilized with other sequencing platforms given the input is FASTQ

and supports same quality formats as Illumina. We focused on Illumina mainly because it is the most-widely utilized platform. We will continue development and subsequent versions of HTSeqQC will be released to expand its use to other platforms such as PacBio and/or Nanopore Sequencing. At its core, HTSeqQC consists of two main modules that are intended to filter and trim single and paired-end sequence datasets. Both modules were implemented using parallel computation to increase the performance of quality control analysis by allocating the input workload to the multiple CPUs. By default, there are only two CPUs, however this number can be changed as per user preferences. To some extent, HTSeqQC works similar to MapReduce where it splits the large sequence file into smaller chunks, distributes the input data to multiple CPUs, and combines the input from each process to produce a final output.

HTSeqQC checks quality issues in the raw HTS datasets and performs the quality filtering and/or trimming in a single run for removing low-quality bases, adapter contamination, and uncalled bases (N) as per the settings of the user. The entire quality control can be completed in a single input command. By default, HTSeqQC filters out the sequence reads with Phred quality score ≤ 20. We did not add the feature of duplicate reads removal in HTSeqQC as this feature is directly related to the gene quantification and estimation of expression [7]. A flowchart HTSeqQC analysis is shown in Fig. 1.

HTSeqQC can handle batch analysis of multiple sequencing datasets at a time. It accepts FASTQ file format as input and produces FASTQ or FASTA file format as output. HTSeqQC also generates summary statistics and visualization outputs for the filtered cleaned HTS datasets. All the outputs by default are saved in the same directory containing the raw RNA-seq input datasets. HTSeqQC was primarily designed to run on the Linux and Mac operating systems as command-line interface CLI (Fig. 2), however, it can also run on the Windows operating systems using virtual machine. In addition, we made HTSeqQC publicly available as GUI (Fig. 2) on CyVerse [17,18], which would be useful for experimental biologists with minimal bioinformatics training. CyVerse is an open-access, scalable, comprehensive, data analysis and management infrastructure for large-scale data analysis developed for life science research. CyVerse can be accessed through world-wide-web, and users can register for free at https://user.cyverse.org/register. To use the HTSeqQC as GUI on CyVerse, it is necessary to have CyVerse account. Additionally, we have also provided the docker image and Nextflow template for running the HTSeqQC.

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Author contributions

RB developed the software, platform and conducted the analysis. CA, and KM supervised the study, data analysis and interpretation. All authors have read, reviewed, and approved the manuscript.

Competing interests

All authors declare no competing interests.

Availability

HTSeqQC software, Docker image and Nextflow template are available for download at https://github.com/reneshbedre/HTSeqQC and graphical user interface (GUI) is available at CyVerse Discovery Environment (DE) (https://cyverse.org/). Documentation available at https://reneshbedre.github.io/blog/htseqqc.html and https://cyverse-htseqqc-cyverse-tutorial.readthedocs-hosted.com/en/latest/. The HTSeqQC is also available on Anaconda cloud (https://anaconda.org/bioconda/htseqqc) and can be installed using biconda channel.

Supplementary data

Supplementary information provided in Supplementary File 1.

Figure legends

Fig. 1: **Flowchart of HTSeqQC analysis.** HTSeqQC includes two main modules for quality control analysis of single and paired-end HTS datasets generated from Illumina sequencing platforms. HTSeqQC filters and trims the raw HTS datasets to remove low-quality bases, adapter or primer contamination, and uncalled bases to generate high-quality datasets for downstream bioinformatics analysis.

Fig. 2: Command-line interface (CLI) and Graphic-user interface (GUI) of HTSeqQC. HTSeqQC can be launched as CLI or GUI modules. For CLI, the HTSeqQC need to installed on a local system, whereas GUI is preinstalled and ready to use online at CyVerse (https://cyverse.org/).

Fig. 3: The sequence quality evalaution of HTS datatsets performed by HTSeqQC. A) The sequence quality distribution among the raw (unfiltered) and cleaned (filtered) sequence data, B) The distribution of percenatges of sequence reads with quality score, C) The percentage GC content distribution among the raw and cleaned sequence data, D) The content of nucleotide bases in raw and cleaned sequence data.

Table 1: Summary of the quality control analysis of single and paired-end datasets with single and multiple samples performed using HTSeqQC CLI.

SRA Accession or Samples	Read Type (# Samples)	# Sequence reads (File size in GB[a])	Read length (bp)	# CPUs (Parallel computing)	Run time (Min)
SRR2165176	Paired (1)	8,583,424×2 (5.6)	100	18 (Shared)	9
SRR2165176 SRR2165177 SRR2165178	Paired (3)	8,583,424×2 (5.6) 9,282,222×2 (6) 9,918,081×2 (6.4)	100 100 100	18 (Shared)	28
SRR1805340	Single (1)	82,059,811 (27)	100	18 (Shared)	40
Tomato GBS[b]	Paired (322)	~ 1,000,000×2 (206)	150	18 (Shared) 18 (Distributed)	1855 157

[a]Combined file size of two files for paired-end sequence reads

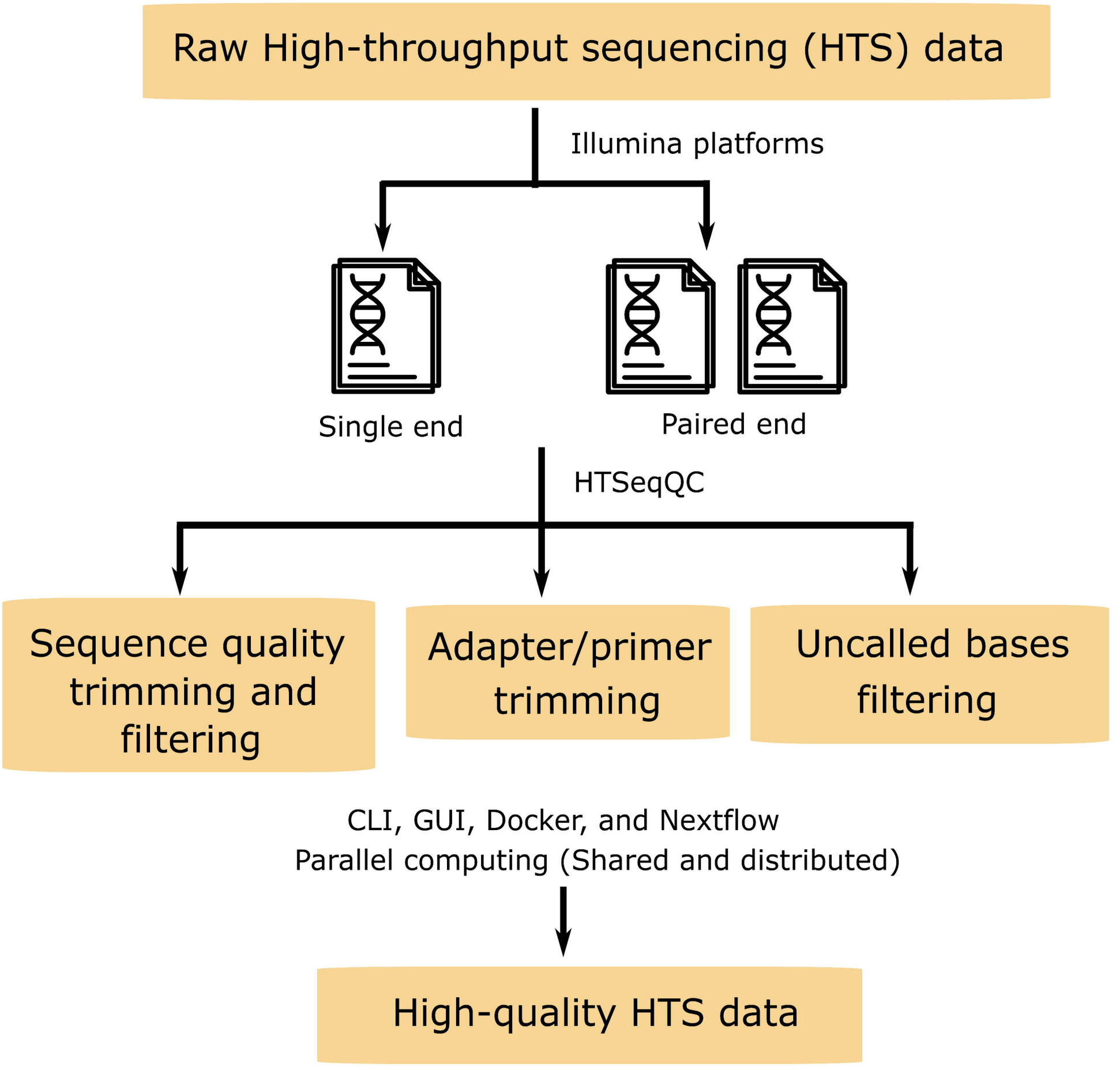
[b]322 tomato genotypes were sequenced using low-coverage whole genome sequencing by Illumina HiSeq 4000 [5]. The number of sequences reads are average of all the datasets.

Table 2: The key sequence quality features comparison of HTSeqQC with other leading and equivalent quality control software tools.

Features	HTSeqQC	FastQC[a]	NGS QC	QC-Chain	FASTX-Toolkit	NGS QCbox	fastp
Low quality filtering	Yes	Yes	Yes	Yes	Yes	No	Yes
Uncalled bases (N)	Yes	No	Yes*	No	No	No	No
Adapter or primer trimming	Yes	Yes	Yes	Yes	Yes	No	Yes
Multiple adapter support	Yes	Yes	Yes	Yes	No	No	Yes
Minimum read length filtering	Yes	No	No	No	No	Yes	Yes
Low quality trimming	Yes	No	Yes*	Yes	No	Yes	Yes
Paired-end support	Yes	No	Yes	Yes	No	Yes	Yes
Paired-end sequence order	Yes	No	Yes	Yes	No	Yes	Yes
Visualization	Yes	Yes	Yes	Yes	Yes	Yes	
Parallel computing	Yes	Yes	Yes	Yes	No	Yes	Yes
Automatic quality variant detection	Yes	No	Yes	No	No	No	No
FASTA Output	Yes	No	No	No	No	No	No
Programming	Python 3	Java	PERL	C++	C	C	C/C++
GZIP FASTQ input	Yes	No	Yes	No	No	Yes	Yes
Multiple sample support (batch analysis)	Yes	Yes	Yes	No	No	Yes	No
GitHub	Yes	Yes	No	No	Yes	Yes	Yes

[a]FastQC only provides quality metric and does not filter or trim HTS data

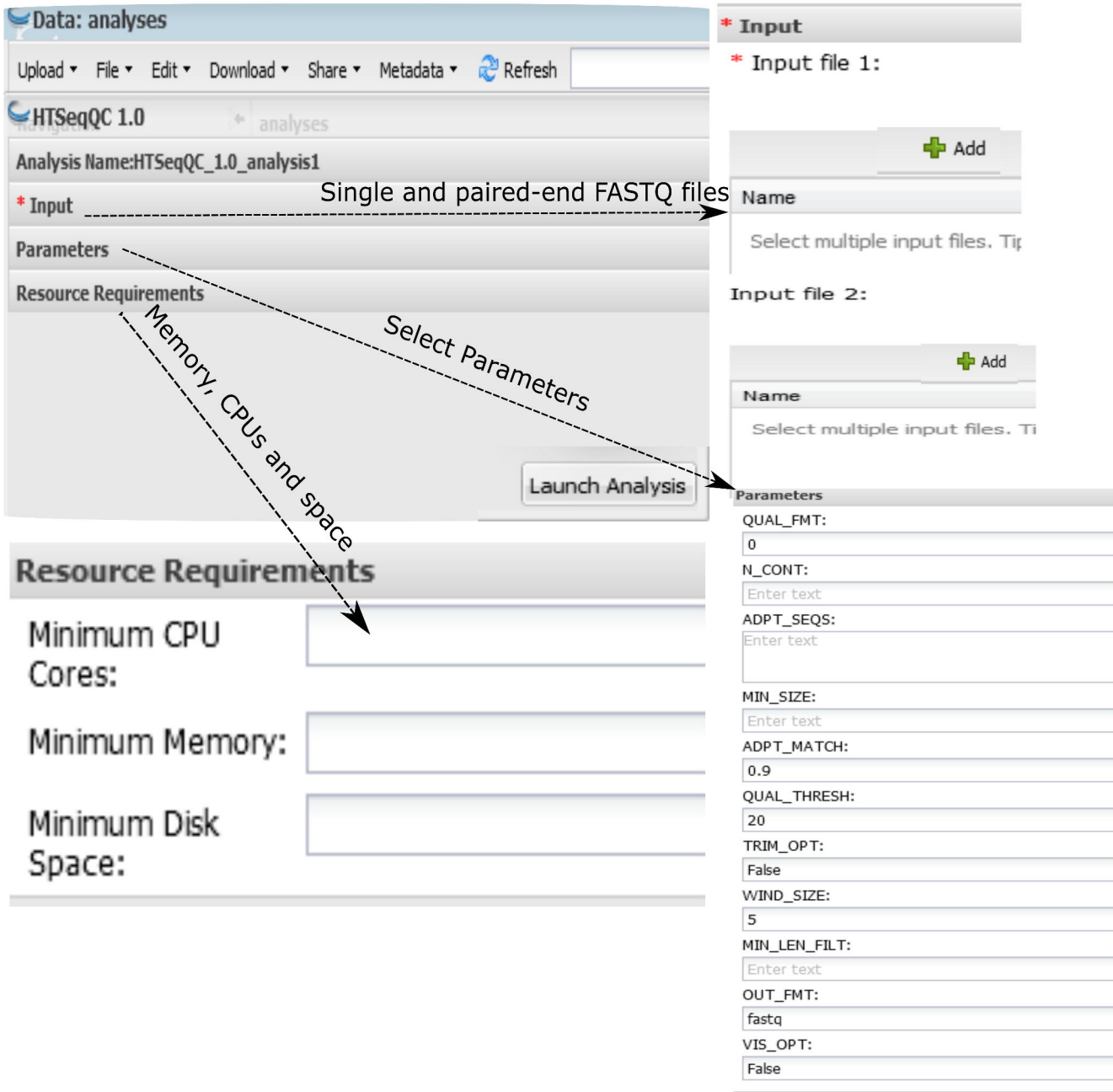
*Separate tools for given features

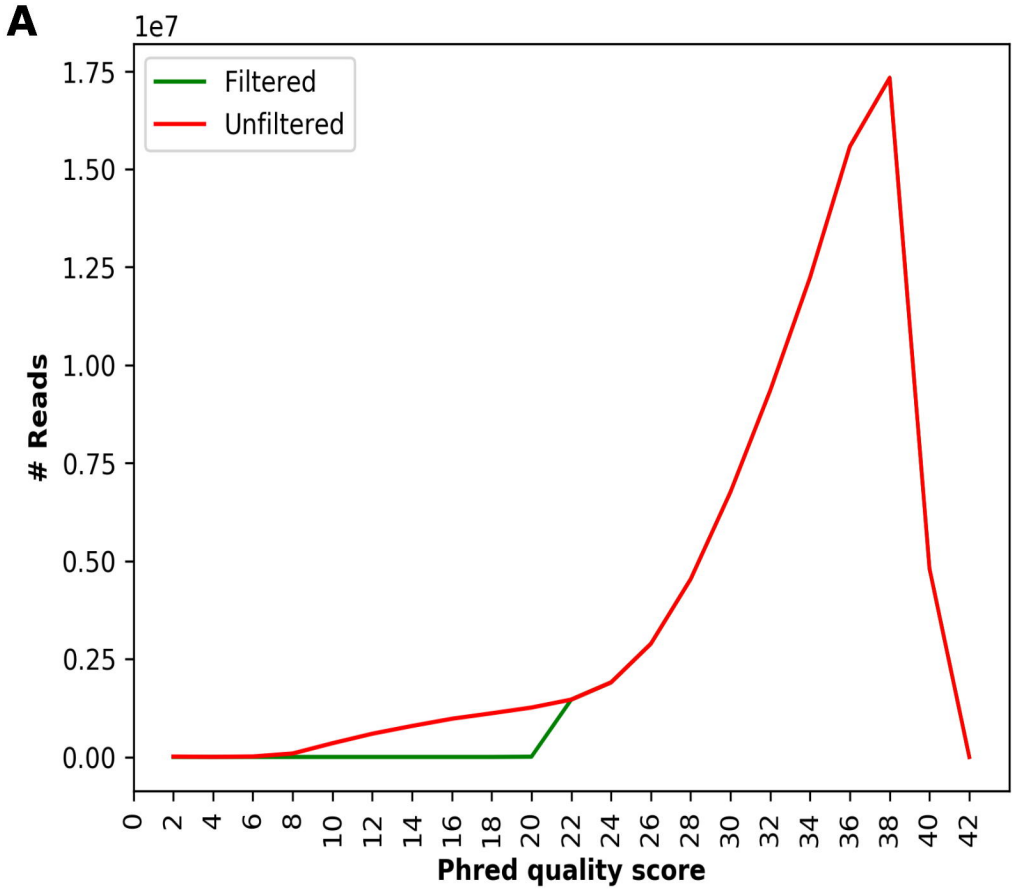

Raw High-throughput sequencing (HTS) data
Illumina platforms
Single end
Paired end
HTSeqQC
Sequence quality trimming and filtering
Adapter/primer trimming
Uncalled bases filtering
CLI, GUI, Docker, and Nextflow
Parallel computing (Shared and distributed)
High-quality HTS data

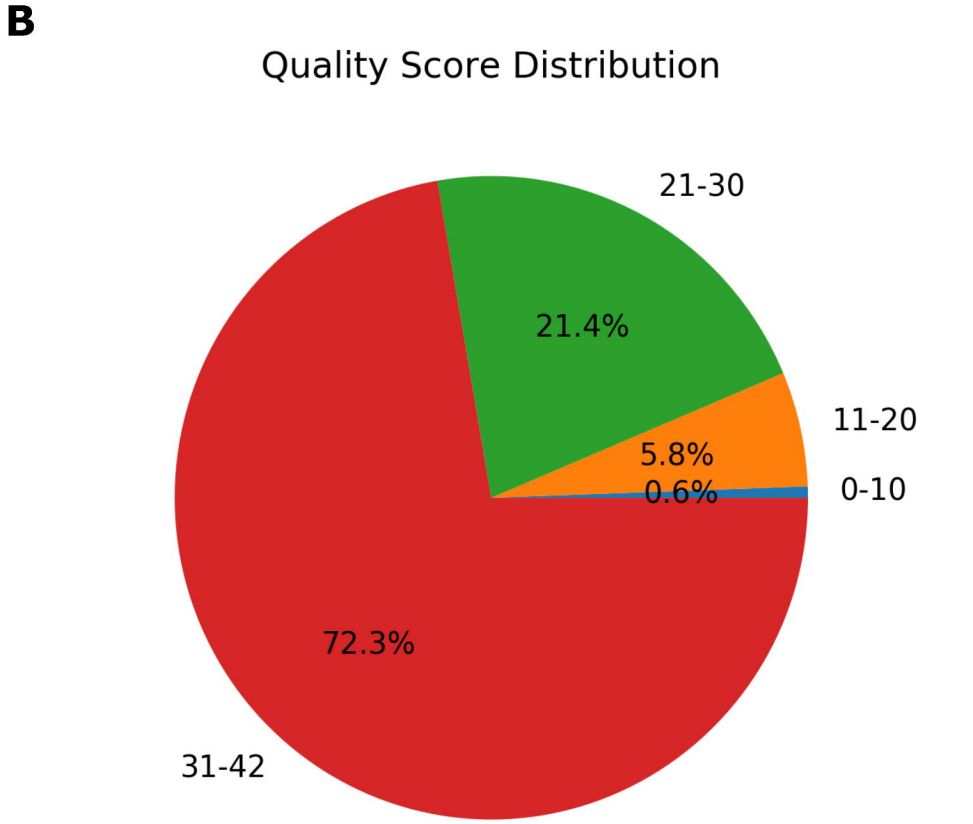
Command-line interface (CLI)

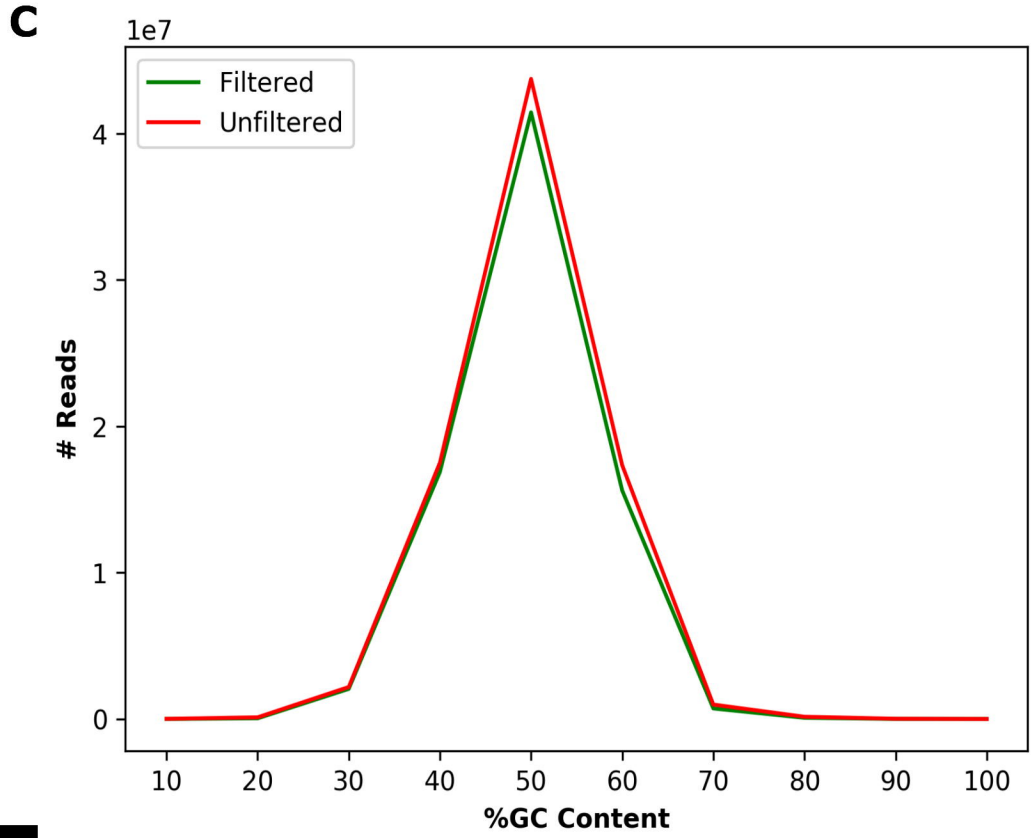
```
usage: filter.py [-h] [-a INPUT_FILES_1 [INPUT_FILES_1 ...]]
                 [-b INPUT_FILES_2 [INPUT_FILES_2 ...]] [-c QUAL_FMT]
                 [-e N_CONT] [-f ADPT_SEQS] [-d MIN_SIZE] [-g ADPT_MATCH]
                 [-i QUAL_THRESH] [-n TRIM_OPT] [-p WIND_SIZE]
                 [-r MIN_LEN_FILT] [-q CPU] [-m OUT_FMT] [-v VIS_OPT]
                 [--version]

Quality control analysis of single and paired-end sequence data

optional arguments:
  -h, --help            show this help message and exit
  -a INPUT_FILES_1 [INPUT_FILES_1 ...], --p1 INPUT_FILES_1 [INPUT_FILES_1 ...]
                        Single end input files or left files for paired-end
                        data (.fastq, .fq). Multiple sample files must be
                        separated by comma or space
  -b INPUT_FILES_2 [INPUT_FILES_2 ...], --p2 INPUT_FILES_2 [INPUT_FILES_2 ...]
                        Right files for paired-end data (.fastq, .fq).
                        Multiple files must be separated by comma or space
  -c QUAL_FMT, --qfmt QUAL_FMT
                        Quality value format [1= Illumina 1.8, 2= Illumina
                        1.3,3= Sanger]. If quality format not provided, it
                        will automatically detect based on sequence data
  -e N_CONT, --nb N_CONT
                        Filter the reads containing given % of uncalled bases
                        (N)
  -f ADPT_SEQS, --adp ADPT_SEQS
                        Trim the adapter and truncate the read sequence
                        (multiple adapter sequences must be separated by
                        comma)
  -d MIN_SIZE, --msz MIN_SIZE
                        Filter the reads which are lesser than minimum size
  -g ADPT_MATCH, --per ADPT_MATCH
                        Truncate the read sequence if it matches to adapter
                        sequence equal or more than given percent (0.0-1.0)
                        [default=0.9]
  -i QUAL_THRESH, --qthr QUAL_THRESH
                        Filter the read sequence if average quality of bases
                        in reads is lower than threshold (1-40) [default:20]
  -n TRIM_OPT, --trim TRIM_OPT
                        If trim option set to True, the reads with low quality
                        (as defined by option --qthr) will be trimmed instead
                        of discarding [True|False] [default: False]
  -p WIND_SIZE, --wsz WIND_SIZE
                        The window size for trimming (5->3) the reads. This
                        option should always set when -trim option is defined
                        [default: 5]
  -r MIN_LEN_FILT, --mlk MIN_LEN_FILT
                        Minimum length of the reads to retain after trimming
  -q CPU, --cpu CPU     Number of CPU [default:2]
  -m OUT_FMT, --ofmt OUT_FMT
                        Output file format (fastq/fasta) [default:fastq]
  -v VIS_OPT, --no-vis VIS_OPT
                        No figures will be produced [True|False]
                        [default:False]
  --version             show program's version number and exit
```

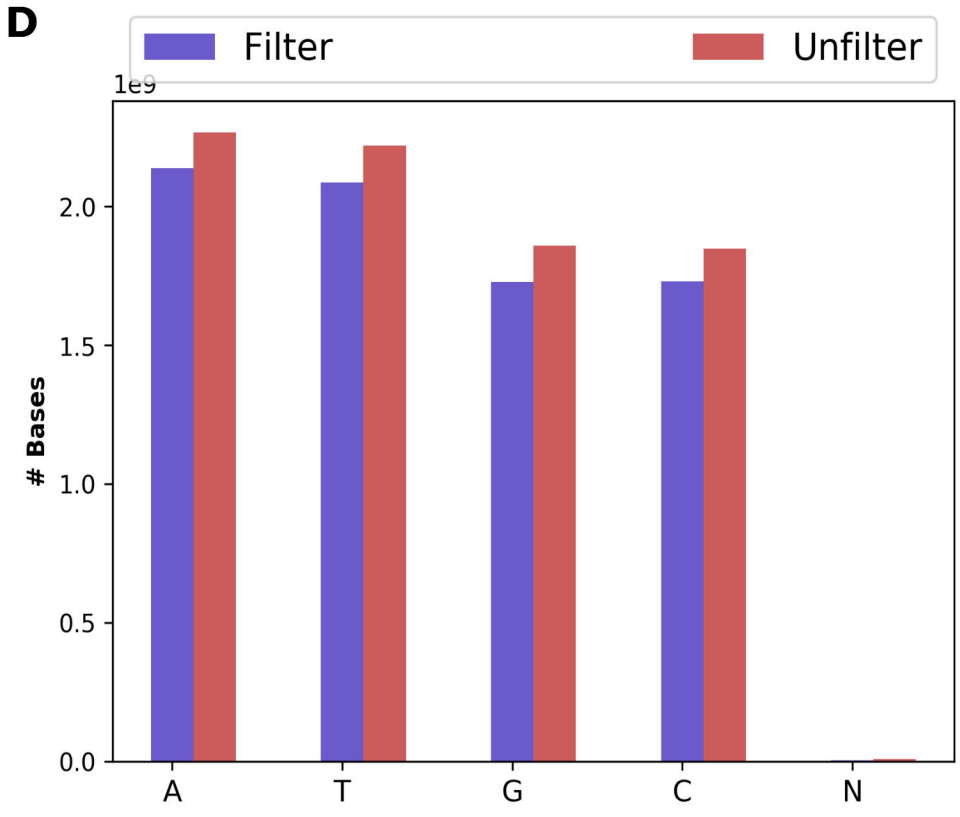
Graphical user interface (GUI) using CyVerse




A
1e7
Filtered
Unfiltered
Reads
Phred quality score


B
Quality Score Distribution
21-30
21.4%
11-20
5.8%
0.6%
0-10
72.3%
31-42


C
1e7
Filtered
Unfiltered
Reads
%GC Content


D
Filter
Unfilter
1e9
Bases
A
T
G
C
N