NanoR: An user-friendly R package to analyze and compare nanopore sequencing data

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Abstract

MinION and GridION X5 from Oxford Nanopore Technologies are devices for real-time DNA and RNA sequencing. While MinION is the only real-time, low cost and portable sequencing device and, thanks to its unique properties, is becoming more and more popular among biologists, GridION X5, mainly for its costs, is less widespread but highly suitable for researchers with large sequencing projects. Despite the fact that Oxford Nanopore Technologies' devices have been increasingly used in the last few years, there is a lack of high-performing and user-friendly tools to handle the data outputted by both MinION and GridION X5 platforms. Here we present NanoR, a cross-platform R package designed with the purpose to simplify and improve nanopore data visualization. Indeed, NanoR is built on few functions but overcomes the capabilities of existing tools to extract meaningful informations from MinION sequencing data; in addition, as exclusive features, NanoR can deal with GridION X5 sequencing outputs and allows comparison of both MinION and GridION X5 sequencing data in one command. NanoR is released as free package for R at https://github.com/davidebolo1993/NanoR.

Introduction

During the last decade, second-generation sequencing technologies have definitely improved our ability to detect genetic variants in any organism. However, the short reads (100-500 base pairs) generated by these platforms do not allow to detect complex structural variants such as copy number variations [1]. In the last few years, third-generation sequencing (TGS) technologies have risen to prominence; Oxford Nanopore Technologies (ONT) and Pacific Biosciences [2] allow single-molecule and long-read sequencing with different strategies: while Pacific Biosciences uses a single-molecule real-time approach, ONT's devices (MinION, GridION X5 and PromethION) analyze the transit of each DNA molecule through a nanoscopic pore and measure its effect on an electric current.

Among ONT's devices, MinION and GridION X5 are noteworthies.

MinION is the only low-cost (commercially available by paying a starter-pack fee of $1,000), portable ($\sim$ 10 cm in length and weighs under 100 g) and real-time DNA and RNA sequencer: each MinION Flow Cell, which contains the proprietary sensor array Application-Specific Integrated Circuit (ASIC) and 2048 R9.4 (for sequencing kits 1D)

or R9.5 (for sequencing kits $1D^2$) nanopores arranged in 512 channels, is capable to generate 10–20 Gb of DNA sequence data over a 48 hours sequencing run [3]. For its unique properties, from the launch of MinION Access Programme (April 2014), MinION has become increasingly popular among biologists.

Although it is definitely more expensive (∼$50,000.00), GridION X5 fills the gap between MinION and ONT's top-of-the-range PromethION. Indeed, GridION X5 is a small benchtop sequencer that allows to use up to five MinION Flow Cells at the same time; as result, GridION X5 drastically increases the ouput of a single MinION experiment to more than 100 Gb of DNA sequence data over a 48 hours sequencing run, making this instrument suitable for researchers with broader scope projects.

While library preparation is fast and easy when working with ONT's devices, the biggest problem researchers have to face when dealing with these TGS technologies is certainly the analysis of their outputs.

ONT's MinION outputs binary *hdf5* files [4] organized in a hierarchical structure with groups, datasets and attributes; these raw data are processed in real time by the local base caller MinKNOW and, finally, stored again in *hdf5* format with the *.fast5* extension, splitted into *Pass* (*i.e.* basecalled *.fast5* files with mean quality score ≥ 7), *Fail* (*i.e.* not successfully basecalled *.fast5* files with mean quality score ¡ 7) and *Skip* (*i.e.* reads for which insufficient resources were available for basecalling) subclasses.

On the other hand, in ONT's GridION X5 MinKNOW performs device control and data acquisition while the software dogfish is used for passing reads to the basecalling resource Guppy in real-time. By default, GridION X5 outputs *.fast5* files with no basecalled information added, *.fastq* files and sequencing summary *.txt* files that describe each sequenced read.

In order to allow user-friendly analysis and comparison of both MinION and GridION X5 1D sequencing data within acceptable time frames, we have developed NanoR, a package for the cross-platform statistical language and environment R [5], which is growing more and more popular with researchers, tested on all the three main computer operating systems: Linux, MacOSX and Windows.

Results

NanoR is a cross-platform package for the statistical language and environment R, requiring the following additional packages:

- ggplot2 [6]
- seqinr [7]
- reshape2 [8]
- scales [9]
- RColorBrewer [10]
- gridExtra [11]
- rhdf5 [12]
- ShortRead [13]

Further informations on the versions of the aforementioned packages needed to run NanoR are available in the *Availability and requirements* section. NanoR is built on 4 main functions:

- *NanoPrepare()*: prepares your MinION/GridION X5 data for the following analyses
- *NanoTable()*: generates a table that contains metadata for every sequenced read
- *NanoStats()*: plots statistics for nanopore data
- *NanoFastq()*: extract *.fastq* sequences from basecalled *.fast5* reads

Each of these functions exists both in a version for MinION (M) and in a version for GridION X5 (G) data analysis (*e.g.* for *NanoPrepare()* exists both the *NanoPrepareM()* and the *NanoPrepareG()* version). In addition, we provided *NanoCompare()* to allow comparison of both MinION and GridION X5 data in one command and *FastqFilterG()* to allow GridION X5 *.fastq* files filtering when working with *.fastq* files and sequencing summary files returned by this platform. Below are described in more detail two possible workflows for users wishing to analyze their MinION or GridION X5 data.

MinION data analysis

The MinION local basecaller MinKNOW stores passed, failed and skipped *.fast5* reads in different folders. As passed *.fast5* reads can be considered the high-quality reads from the experiment, NanoR is designed to take into account failed and skipped *.fast5* reads only for calculating their number and percentage with respect to the total number of reads produced during the experiment while the other statistics are computed on passed *.fast5* reads.

Data preparation

In order to prepare MinION sequencing data for further analyses, we provide *NanoPrepareM()*,which reads all the *.fast5* files in the *Pass*, *Fail* and *Skip* directories and organizes data so that they can be used by the other functions from NanoR. The user must provide to *NanoPrepareM()* the path to basecalled *.fast5* reads stored in the *Pass* directory and a label that will be used to name the experiment, while the paths to failed and skipped *.fast5* reads are optional parameters (if paths to failed and skipped *.fast5* files are not provided, the number of failed and/or skipped *.fast5* reads produced by the MinION experiment is considered to be 0 at this level of the analysis). *NanoPrepareM()* returns an object of class list required by other functions from this package.

Extraction of *.fast5* files metadata

Each basecalled *.fast5* file contains a number of key statistics (*i.e.* metadata) such as the read identifier, the number of both channel and mux that generate a certain read, its generation time as well as its length and quality score; *NanoTableM()* extracts all these informations from each passed *.fast5* read, in addition to its identifier and GC content (GC content calculation is optional). As metadata extraction from *.fast5* sequences can be slow for large datasets, *NanoTableM()* can be accelerated using multiple cores. The user must provide to *NanoTableM()* the list returned by *NanoPrepareM()*, the path to a folder where the results of the analysis will be saved and can provide the number of cores to be used for *.fast5* metadata extraction (1, by default) and a logical expression indicating whether *NanoTableM()* has to compute GC content or not (by default, GC content calculation is set to "TRUE"); *NanoTableM()* returns a table with 7 columns that describes each passed *.fast5* read.

Extraction of *.fastq* and *.fasta* files

Each passed *.fast5* file contains sequence information in *.fastq* format that the user may wish to extract for further analyses by using *NanoFastqM()*. *NanoFastqM()* takes, as inputs, the path to the *Pass .fast5* files folder, the path to a folder where the results of the *.fastq* extraction will be saved, the number of cores to be used for *.fastq* extraction (1, by default) and a logical expression indicating whether *NanoFastqM()* has to write *.fasta* file in addition to *.fastq* file in the user-defined folder (by default, *.fasta* extraction is set to "FALSE").

Plot statistics

NanoStatsM() is the main function of the NanoR package for MinION data analysis; the user must provide to *NanoStatsM()* the object of class list returned by *NanoPrepareM()*, the table returned by *NanoTableM()* and the path to the same folder specified for *NanoTableM()*. As results, *NanoStatsM()* returns 4 tables (that contains informations about reads and base pairs number, reads length and reads quality and are required by the function *NanoCompare()* from this package) and 5 *.pdf* files contaning:

- plots of cumulative reads and base pairs produced during the experiment
- plots of reads number, base pairs number, reads length (min, average and max) and reads quality (min, average and max) calculated every half an hour of experiment. (Fig 1)
- plot of read length and read quality compared jointly (Fig 2)
- plots of channels and muxes activity with respect to their real disposition on the MinION flowcell (these plots are useful to diagnose problems in particular areas of the flowcell) (Fig 3)
- plots that summarize number and percentage of passed and failed/skipped *.fast5* reads as well as GC content count (if computed by *NanoTableM()*) calculated for passed ones

Fig 1. Reads number, base pairs number, reads length and reads quality per-time bin.The plots show the number of reads (**Panel A**) and basepairs (**Panel B**), the maximum, average and minimum length of reads in $\log_{10}$ scale (**Panel C**) and the maximum, average and minimum quality of reads (**Panel D**), all calculated every 30 minutes of an experimental MinION run

Fig 2. Reads length and reads quality compared jointly.The plot shows the correlation between length (x axis) and quality (y axis) for $\sim$ 100000 MinION reads. The regression line highlights that longer the reads are, higher their quality score is.

Fig 3. Heatmap of channels and muxes activity. The plots show the base pairs productivity of channels (**Panel A**) and muxes (**Panel B**) with respect to their real disposition on the Flow Cell; inactive channels and muxes are grey-colored.

NanoR with MinION non-classified data

Although NanoR is designed to work with passed MinION *.fast5* files, sometimes users may have to deal with a mix of passed and failed/skipped *.fast5* reads stored in the same folder. In this case, the path to the folder contaning the mixed *.fast5* files can be given to *NanoPrepareM()* instead of the path to the passed *.fast5* files and, subsequently, the list returned by *NanoPrepareM()* can be given to *NanoTableM()*. Indeed, *NanoTableM()* looks for the *.fastq* sequence inside each *.fast5* file: if the *.fastq* sequence is found, the *.fast5* file is classified as "passed" and metadata are extracted from this sequence, otherwise the *.fast5* file is classified as "failed" and no informations are extracted. After that, the object of class list returned by *NanoPrepareM()* and the table returned by *NanoTableM()* can be given, as inputs, to *NanoStatsM()*: this function automatically filters out sequences for which no informations are avaialable and compute statistics for the others.

NanoFastqM() is designed to deal with mixed *.fast5* files likewise *NanoTableM()* is: indeed, if the *.fastq* sequence is found inside the *.fast5* file, it is extracted and saved, otherwise the *.fast5* file is classified as "failed" and filtered out.

GridION X5 data analysis

While MinION is the most popular among ONT devices and the format of the outputted data has become highly standardized from the launch of MinION Access Programme, GridION X5 is definitely less widespread and some issues with the outputted data have yet to be fixed. By default, GridION X5 outputs *.fast5* reads without basecalled informations inside, *.fastq* files and sequencing summary *.txt* files that describes each read[1]. Due to the fact that *.fastq* sequences and sequencing summary files are generated for both failed and passed reads, NanoR, during the analysis, will filter out failed reads (only their number and percentage will be taken into account) and compute statistics only on the passed ones.

Data preparation

In order to prepare GridION X5 sequencing data, we provide *NanoPrepareG()*. The user has to give to *NanoPrepareG()*, as inputs, the path to the folder that contains *.fastq* files and sequencing summary files and a label that will be used, together with the Flow Cell identifier, to name the experiment; *NanoPrepareG()* takes, as optional inputs, the path to the skipped *.fast5* files and the number of cores (1, by default) to be used to accelerate sequencing summary files reading. This function returns an object of class list required by other functions from this package.

Metadata selection

The sequencing summary files returned by GridION X5 contain the metadata required to compute significant statistics on the sequenced reads. *NanoTableG()* takes, as inputs, the list returned by *NanoPrepareG()* and the path to a user-defined folder in which the result of this function will be saved. As result, *NanoTableG()* simply selects the most meaningful metadata from the sequencing summary files previously arranged by *NanoPrepareM()* and extracts GC content from *.fastq* files[2]: the resulting table contains 7 columns that describes each GridION X5 outputted sequence.

[1] these data are outputted inside the */data/basecalled/[ExperimentName]/[FlowCellId]* folder, while the skipped reads (*i.e.* reads for which *.fastq* files extraction and sequencing summary files generation was not successful) are stored in the */data/reads/[FlowCellId]/[ExperimentId]/fast5/[FolderNumber]* folder

[2] Sometimes, *.fastq* files returned by GridION X5 are ill-formatted: this problem was reported by some members of the Oxford Nanopore community and was theoretically solved with the release of

Filter *.fastq* files

As previously said, the *.fastq* files returned by GridION X5 contains informations for both passed and failed sequences. We provide *FastqFilterG()* to filter out failed *.fastq* files; the user must provide to *FastqFilterG()* the path to the folder that contains *.fastq* files and sequencing summary files, the path to a folder in which pass *.fastq* files will be stored[1] and a label that will be used, together with the Flow Cell identifier, to name the experiment. By default, *FastqFilterG()* returns only a *.fastq* file for passed sequences but, if the user wish, it can also return the *.fastq* file for both passed and failed sequences and tanslate into *.fasta* files the previously generated *.fastq* files.

Plot statistics

NanoStatsG(), as it is the counterpart of *NanoStatsM()* for the GridION X5 data analysis, is the main function of NanoR package for GrdiION X5 data analysis; it takes exactly the same inputs required by *NanoStatsM()* and returns exactly the same outputs with the only exception that, as there are no informations about the active muxes in the sequencing summary files, only the channels activity heatmap will be plotted.

NanoR with GridION X5 basecalled *.fast5* files

NanoR functions for GridION X5 data analysis can also deal with basecalled *.fast5* files. Indeed, when analyzing basecalled GridION X5 *.fast5* files, the user has simply to set to "TRUE" the *BasecalledFast5* parameter of *NanoPrepareG()* and give to the *Data* parameter of this function the path to the folder that contains the basecalled *.fast5* files (*e.g* the path to the *Pass .fast5* files folder returned by ONT's official command-line basecaller Albacore);the user can also give to *NanoPrepareG()* the path to the *Fail .fast5* files, that will be considered only to calculate their number and percentage, while the downstream analyses will take into account only passed *.fast5* reads. The object of class list returned by *NanoPrepareG()* can be given to *NanoTableG()* to automatically extract metadata while giving to *NanoFastqG()* the same parameters accepted by *NanoFastqM()* results in the extraction of *.fastq*/*.fasta* sequences from the inputted *.fast5* files (extraction of both metadata and *.fastq*/*.fasta* sequences can be accelerated using multiple cores). Statistics for the inputted*.fast5* files can be computed using *NanoStatsG()*, giving to this function the same parameters described before for *NanoStatsM()*: when dealing with *b*asecalled *.fast5* sequences, *NanoStatsG()* plots both channels and muxes activity heatmaps.

Compare MinION/GridION X5 data

When dealing with 2 or more MinION/GridION X5 experiments, users may wish to compare their sequencing runs: to this puprose we provide *NanoCompare()*. *NanoCompare()* takes, as inputs, a vector containing the paths to the folders where results from *NanoStatsM()*/*NanoStatsG()* are stored, an ordered vector contaning the identifier used to name each experiment (for MinION data, the labels chosen when running *NanoStatsM()*; for GridION X5 data, the labels used when running *NanoStatsG()* together with the Flow Cell identifier, separated with an underscore character), the path to the folder in which *NanoCompare()* plots will be saved and a logical expression indicating whether GC content count have been computed for your data (if GC content have not been computed for all the experiments the user wish to compare, the *GCC* parameter of this function has to be set to "FALSE"). *NanoCompare()* returns 2 *.pdf* files contaning:

Guppy 1.4.0. If NanoR encounter one or more corrupted *.fastq* files, GC content calculation and *.fastq* files filtering will stop and NanoR will print the number of the ill-formatted *.fastq* file

- violin plots that compare reads number, base pairs number, reads length and reads quality of the given experiments every 10 hours of sequencing run-time (the default sequencing run-time is 48 hours) (Fig 4)
- histogram plots that compare reads length, reads quality and, optionally, reads GC content count

Fig 4. Violin plots for comparison between experiments. From top to bottom, the plots show the comparison between 4 GridION X5 experiments in term of reads number, base pairs number, reads mean length and reads mean quality. Comparison is done every 10 hours of experimental run, as the standard duration of a MinION/GridION X5 run is 48 hours.

Discussion

NanoR is designed to be intuitive, even for biologists with little knowledge of R language, and produces meaningful data within acceptable time frames; in order to prove that, we compared the user waiting-time when extracting metadata from MinION passed *.fast5* files with 3 different packages that runs in the R environment: the NanoR package (using *NanoTableM()*), with and without GC content calculation), the poRe [14] package (using *extract.fast5()*, taken from the poRe parallel GUI and used to extract only metadata[3]) and the IONiseR [15] package (using *readFast5Summary()* and *readInfo()*[4]); in particular, we calculated the user waiting-time when analyzing increasing number of reads (25000,50000,100000,500000,1000000). The aforementioned functions were parallelized with the R "parallel" package using 10 Intel®Xeon®CPU E5-46100 @ 2.40GHz cores on a 48 cores SUSE Linux Enterprise Server 11.

As expected, the user waiting-time increases, for all the three packages, when increases the number of the reads analyzed, with IONiseR that turned out to be the slowest and NanoR the fastest, if it doesn't have to read the *.fastq* information to compute GC content.(Fig 5) We have also assessed the user waiting-time when retrieving metadata from reads of different size, as the length-independent speed of analysis can be an useful feature when dealing with ultra-long reads [16]; in this case, all the three tools perform equally, as there isn't, as expected, an increase of the user-waiting time when the size of the reads increases (except for NanoR if it had to read the *.fastq* information to compute GC content: in that case, the user-waiting time increases in a length-dependent manner). NanoR has a number of features in common with other tools built to deal with MinION sequencing data such as the already-mentioned poRe and IONiseR, poretools [17] and HPG pore [18]. Table 1 summarizes NanoR features and compares them to those implemented in poRe, IONiseR, poretools and HPG pore.

Conclusion

MinION and GridION X5 from ONT are real-time sequencing devices capable to generate very long reads required for precise characterisation of complex structural

[3]The modified version of the poRe function we used extracts the .fast5 filename, the channel number, the read number, the read start time, the length of the sequence, the run identifier, the read identifier and the experiment start time and assesses the read quality returning a logical expression indicating whether the read is passed (T) or not (F).

[4]The *readInfo()* function from IONiseR returns the filename, the number of both channel and mux that generate a certain sequence and its pass/fail status.

Fig 5. Time comparison between NanoR (blue), poRe (red) and IONiseR (green) when extracting metadata informations from increasing number of *.fast5* reads (25000,50000,100000,500000,1000000) coming from our laboratory, using 10 Intel®Xeon®CPU E5-46100 @ 2.40GHz cores. Under the same conditions (*i.e.* without GC content calculation, solid lines), NanoR is the fastest in extracting metadata informations for all the groups of *.fast5* file considered (*e.g.* NanoR takes 59 minutes to extract metadata from 1000000 *.fast5* files, poRe takes 80 minutes and IONiseR takes over 10 hours) while the extraction of metadata together with GC content computation (dashed line) makes NanoR working slightly slower (it takes approximatley 100 minutes to both extract metadata informations from 1000000 *.fast5* files and calculate their GC content).

Table 1. Comparison between NanoR, poRe, IONiseR, poretools and HPG Pore

Feature	NanoR	poRe	IONiseR	poretools	HPG Pore
Extract *.fastq*[*]	✓	✓	✓	✓	✓
Extract *.fasta*[*]	✓	✓		✓	✓
Extract metadata[*]	✓	✓	✓	✓	✓
Calculate/plot GC content[*]	✓				✓
Plot yield[*]	✓	✓	✓	✓	✓
Plot reads and base pairs number per time bin[*]	✓				
Plot min/mean/max reads quality and length per time bin[*]	✓				
Plot reads length versus reads quality[*]	✓				
Plot reads length histograms[*]	✓[5]	✓		✓	✓
Plot reads quality histograms[*]	✓[6]				
Plot channels and muxes occupancy[*]	✓		✓	✓[7]	
Plot reads and base pairs number per channel histograms[*]		✓			✓
Plot pass/fail/skip *.fast5* files number and percentage[*]	✓				
Extract informations and plots statistics from GridION X5 outputs[**]	✓				
Compare MinION/GridION X5 data	✓				

[5] NanoR plots reads length histograms for each experiment when comparing experiments with *NanoCompare()*
[6] NanoR plots reads quality histograms for each experiment when comparing experiments with *NanoCompare()*
[7] Poretools does not plot muxes occupancy
[*] These features refers to MinION data analysis
[**] NanoR computes statistics described for MinION data also for GridION X5 data

variants such as copy number alterations. While library preparation is not the "hard part" when working with these devices, researchers may have some difficulties in analyzing their outputted data. Here we presented NanoR, a cross-platform package for the widespread statistical language R, that allows easy and fast analysis of both MinION and GridION X5 outputs. Currently, NanoR has been extensively tested on (and can deal with) the following 1D sequencing data:

- MinION basecalled *.fast5* files, returned by both MinION local basecaller MinKNOW and ONT's official command-line basecaller Albacore
- GridION X5 default output files (*.fastq* files and sequencing summary *.txt* files)
- GridION X5 basecalled *.fast5* files returned by Albacore

NanoR can be easily improved in the future in order to be up-to-date with the expected

releases of MinION and GridION X5 softwares and to extend its data analysis capability (*e.g.* we have already plan to make NanoR capable to deal with $1D^2$ sequencing data as soon as possible) and, even though there are other programs that can be used to analyze MinION data such as poRe, IONiseR, poretools and HPG pore, we believe that the exclusive features of NanoR described in this article make this tool extremely useful for researchers that work with ONT's sequencing data.

Software availability and requirements

- Project name: NanoR
- Project home page:https://github.com/davidebolo1993/NanoR.
- Operating systems: Linux, Windows, MacOSX
- Programming language: R
- Other requirements: $R \geq 3.1.3$, $ggplot2 \geq 2.2.1$, $reshape2 \geq 1.4.3$, $RColorBrewer \geq 1.1.2$, $scales \geq 0.5.0$, $seqinr \geq 3.4.5$, $gridExtra \geq 2.3$, $ShortRead \geq 1.24.0$, $rhdf5 \geq 2.14$
- License: GPLv3

A small dataset of MinION passed *.fast5* files supporting the results of this article, the complete set of plots returned by NanoR as well as exhaustive installation istructions and examples of how to run NanoR functions and a folder containing all the functions on which NanoR is built can be found at https://github.com/davidebolo1993/NanoR.

Declarations

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Competing interests

The authors declare that they have no competing interests.

Author's contributions

Davide Bolognini wrote and tested the R code. Niccolò Bartalucci and Alessandra Mingrino prepared MinION and GridION X5 libraries. Alberto Magi and Alessandro Maria Vannucchi supervised the project. Davide Bolognini and Alberto Magi wrote the manuscript.

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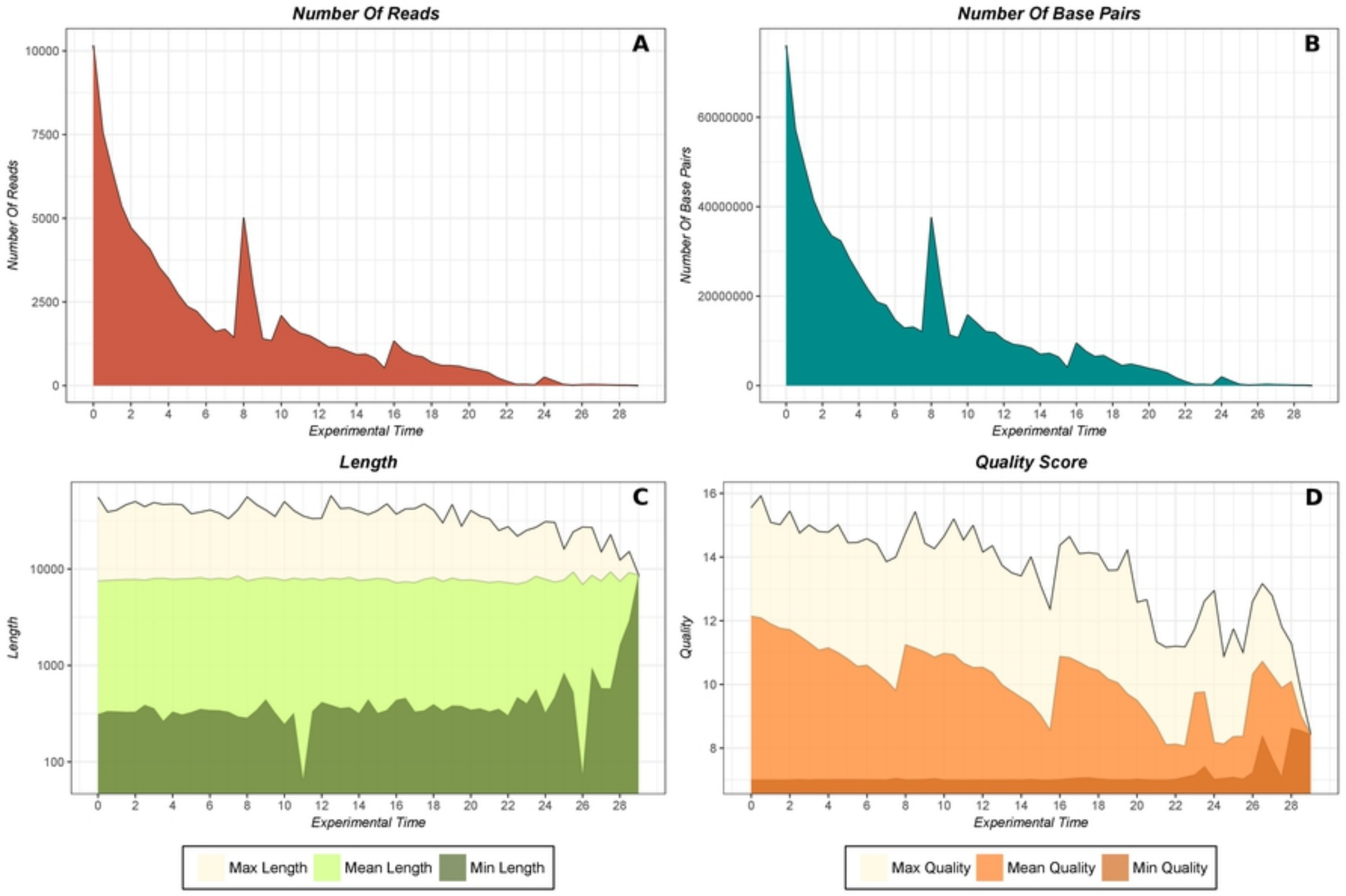
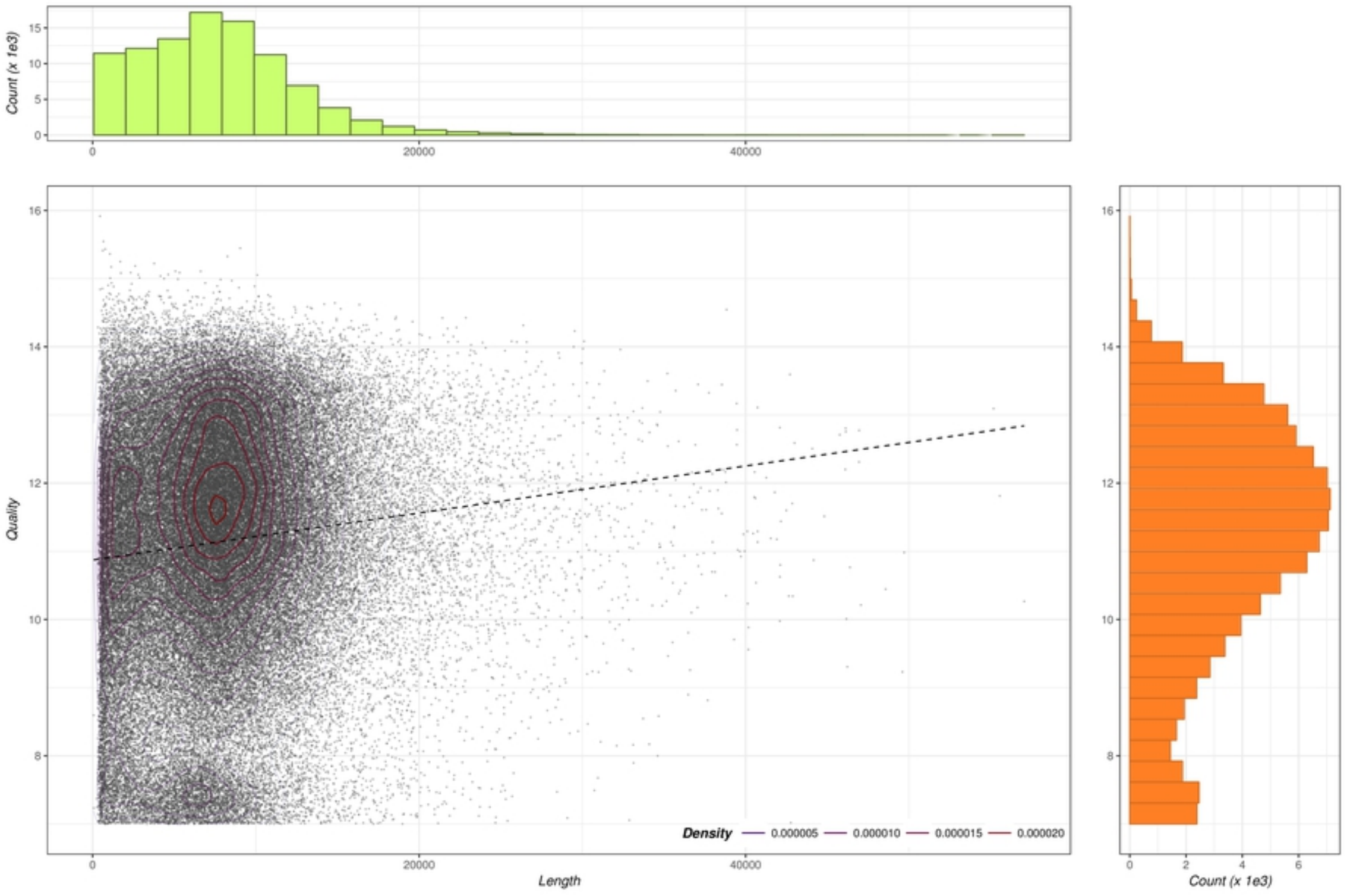

Number Of Reads
A
Number Of Reads
Experimental Time
Number Of Base Pairs
B
Number Of Base Pairs
Experimental Time
Length
C
Length
Experimental Time
Max Length
Mean Length
Min Length
Quality Score
D
Quality
Experimental Time
Max Quality
Mean Quality
Min Quality

Figure1


Count (x 1e3)
15
10
5
0
0
20000
40000
Quality
16
14
12
10
8
Density
0.000005
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0.000020
Length
Count (x 1e3)
0
2
4
6

Figure2

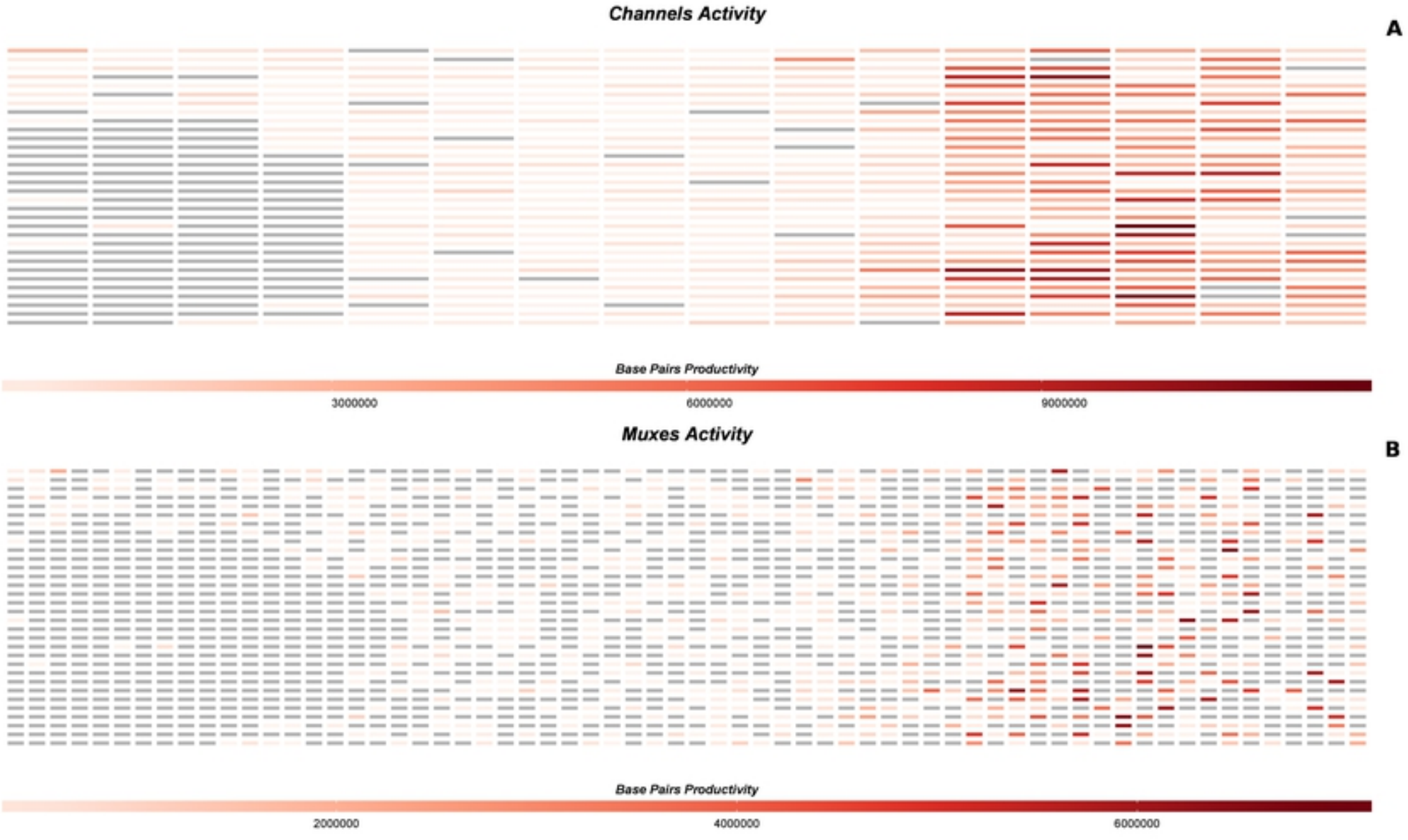
Channels Activity
A
Base Pairs Productivity
3000000
6000000
9000000
Muxes Activity
B
Base Pairs Productivity
2000000
4000000
6000000

Figure3

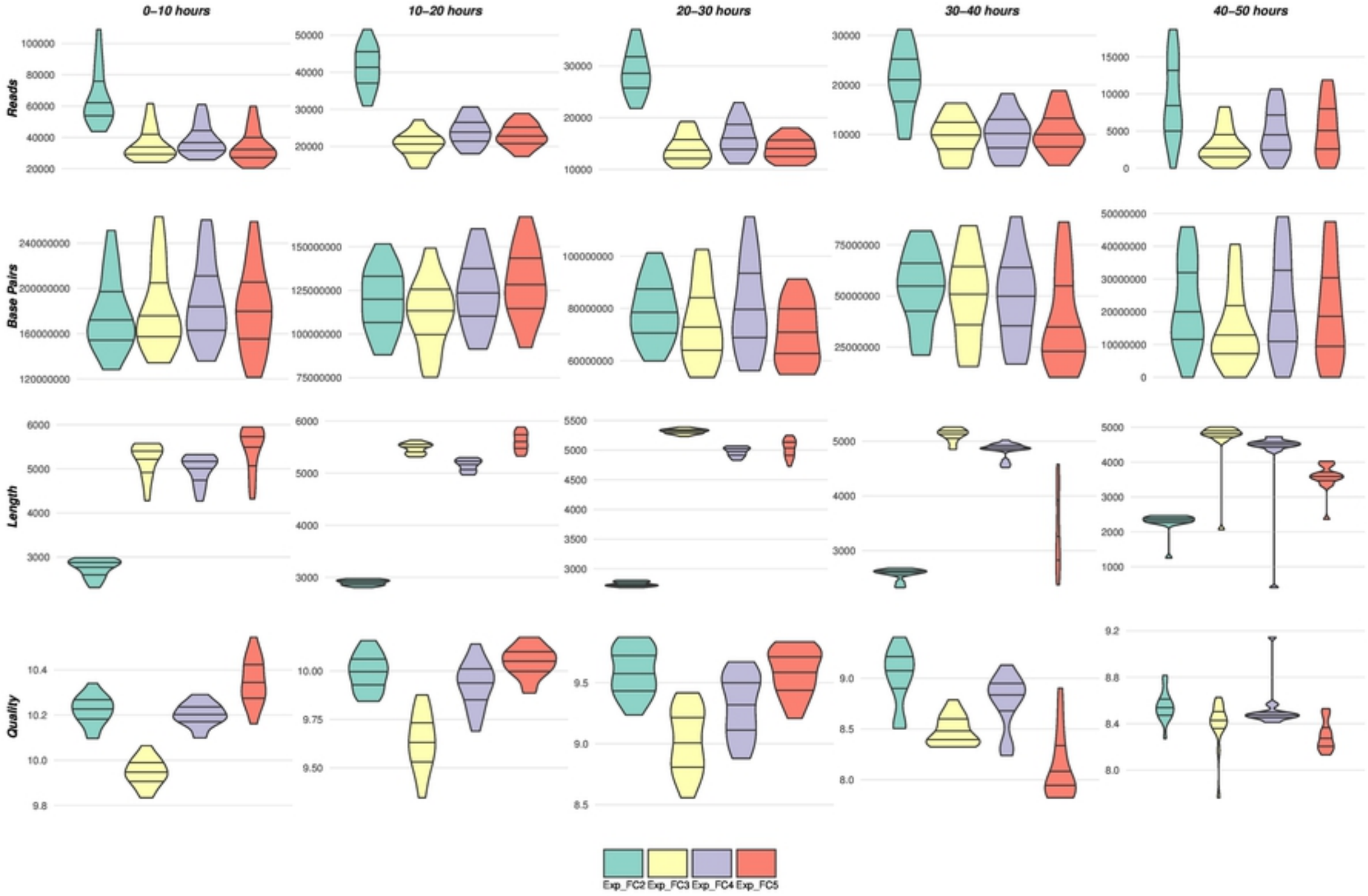
0–10 hours
10–20 hours
20–30 hours
30–40 hours
40–50 hours
Reads
Base Pairs
Length
Quality
Exp_FC2
Exp_FC3
Exp_FC4
Exp_FC5

Figure4

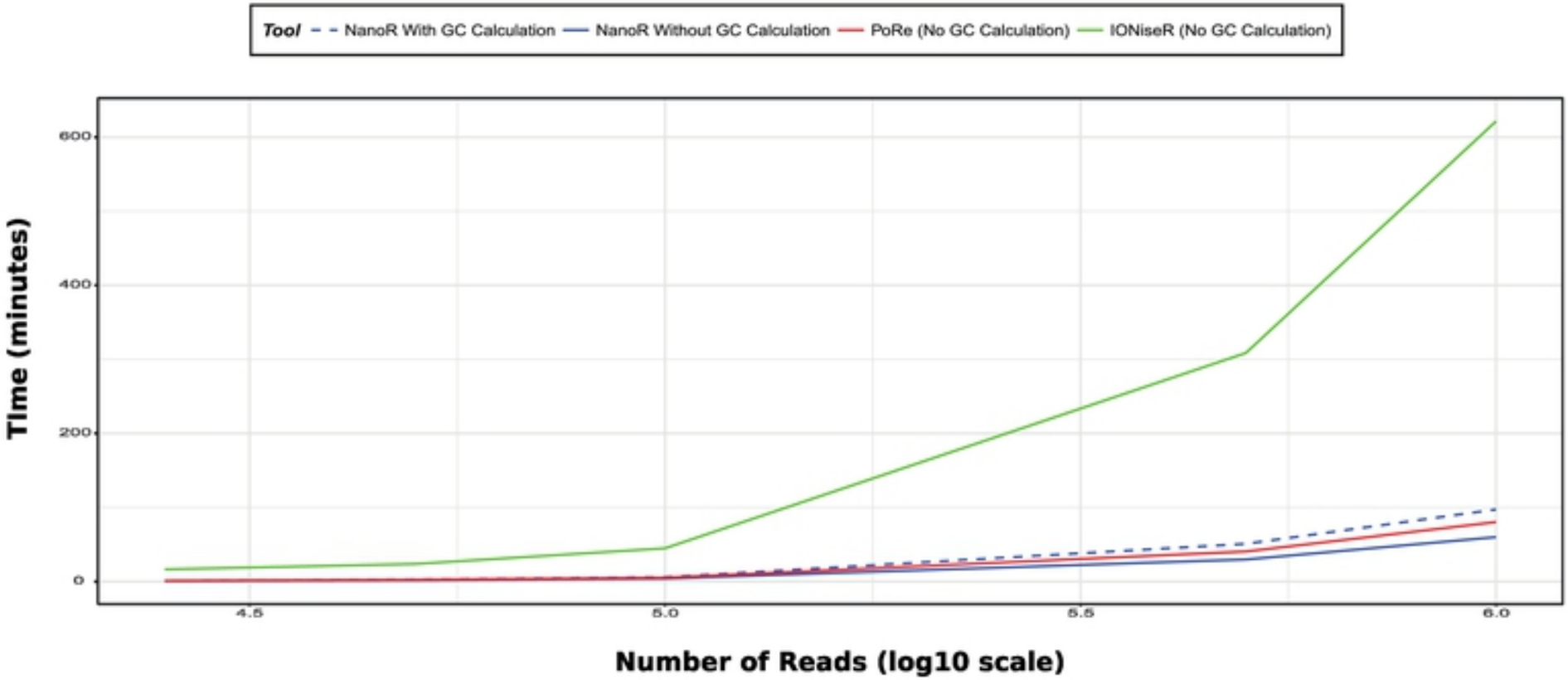
Tool
NanoR With GC Calculation
NanoR Without GC Calculation
PoRe (No GC Calculation)
IONiseR (No GC Calculation)
Time (minutes)
600
400
200
0
4.5
5.0
5.5
6.0
Number of Reads (log10 scale)

Figure5