A Comparison of Performance for Different SARS-Cov-2 Sequencing Protocols

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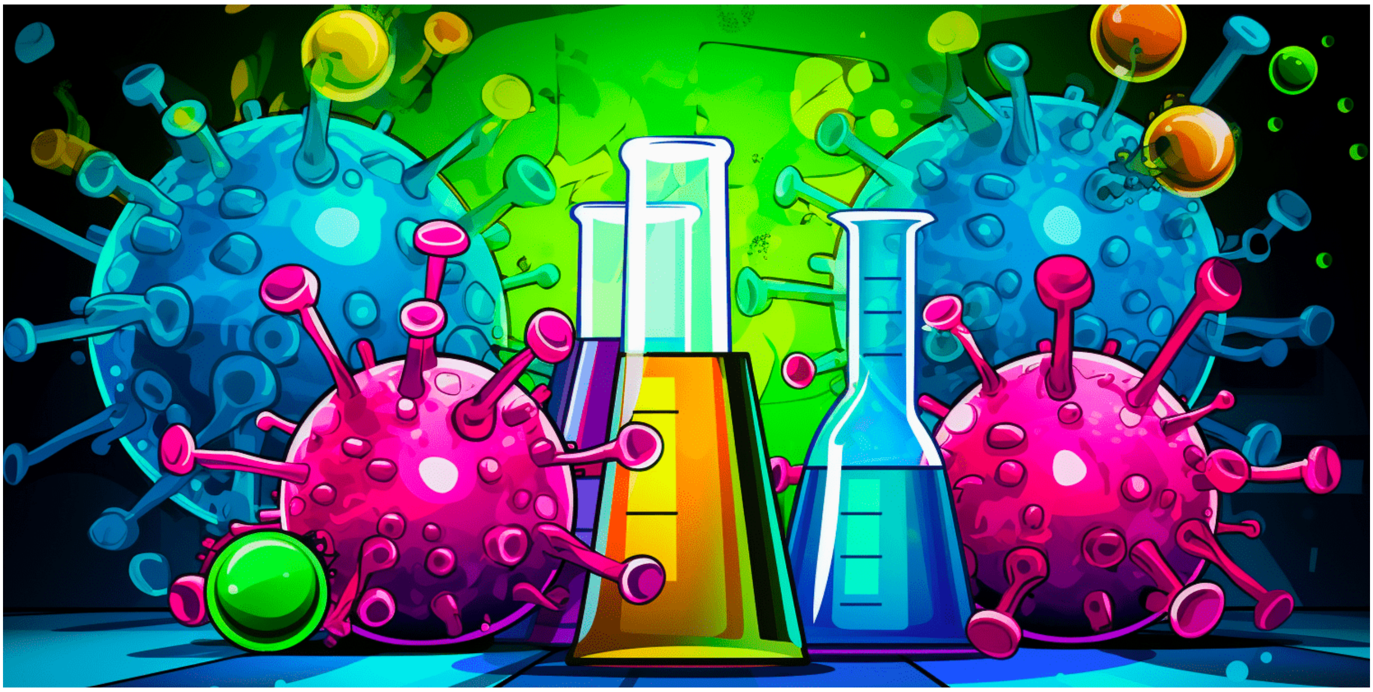

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A Comparison of Performance for Different SARS-Cov-2 Sequencing Protocols

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SARS-Cov-2 genome sequencing has been identified as a fundamental tool for fighting the COVID-19 pandemic. It is used, for example, for identifying new variants of the virus and for elaborating phylogenetic trees that help to trace the spread of the virus. In the present study, we provide a comprehensive comparison between the quality of the assemblies obtained from different sequencing protocols. We demonstrate how some protocols actively promoted by different high-level administrations are inefficient and how less-used alternative protocols show a significantly increased performance. This increase in performance could lead to cheaper sequencing protocols and therefore to a more convenient escalation of the sequencing efforts around the world.

COVID-19, SARS-Cov-2, genome assembly, virus genome, genome sequencing, de novo genome assembly, sequencing protocols, ARTIC protocol

Introduction

There are two basic strategies to recreate a genome departing from the data obtained by the actually available sequencing machines:

1. Recreate the genome with no prior knowledge using de novo sequence assembly

2. Recreate the genome using prior knowledge with reference-based alignment/mapping

It is generally accepted that each strategy has its own advantages and drawbacks. The quality of reference-based assembly is heavily dependent upon the choice of a close-enough reference: identification of some variants can be missed if the sample is not close enough to the reference. On the other hand, de novo genome assembly is more computationally exigent and not always possible from the available data.

“Current variant discovery approaches often rely on an initial read mapping to the reference sequence. Their effectiveness is limited by the presence of gaps, potential misassemblies, regions of duplicates with a high-sequence similarity and regions of high-sequence divergence in the reference. Also, mapping-based approaches are less sensitive to large INDELs and complex variations” (1)

“We document that 18.6% of SNP genotype calls in HLA genes are incorrect and that allele frequencies are estimated with an error greater than ±0.1 at approximately 25% of the SNPs in HLA genes. We found a bias toward overestimation of reference allele frequency for the 1000G data, indicating mapping bias is an important cause of error in frequency estimation in this dataset.” (2)

“Detecting indels is challenging for several reasons: (1) reads overlapping the indel sequence are more difficult to map and may be aligned with multiple mismatches rather than with a gap; (2) irregularity in capture efficiency and non-uniform read distribution increase the number of false positives; (3) increased error rates makes their detection very difficult within microsatellites; and (4) localization, near identical repetitive sequences can create high rates of false positives” (3)

In an ideal scenario, researchers should have both options available: reference mapping and de-novo assembly. If one of these is missed, the results do not count with the maximal possible reliability. And if there is the possibility to have both at the same cost, there is absolutely no reason for not having both.

For that reason, it is important that the libraries for sequencing SARS-Cov-2 are designed with de novo genome assembly in mind.

Some studies have already been developed to assess the performance of the most commonly used protocols (4), but these are exclusively focused on the obtained coverage of the reads and not on the quality of the de novo assemblies. This study will establish a comparison of protocols based on the quality of the de novo assembly, which is a more exigent metric to assess the performance of the protocols. The performance of mapping to a reference genome will not be analyzed as this has already been analyzed in previous studies and a superior performance in de novo assembly is already strongly correlated to a superior performance in reference-mapping.

Method

I used different search patterns at the NCBI SRA (5) website to find SARS-Cov-2 sequencing data obtained using different protocols. Despite this is not a totally reliable method (some search terms are ambiguous) I think it can help to understand the proportions.
Table 2 shows the number of matches found for every sequencing hardware technology. Despite the fact that some protocols were developed for some specific hardware, we can see how these are being used for other hardware too. For example, there are many more ARTIC (6) results for Illumina

Table 1. Queries at the NCBI portal

Protocol	Query
ARTIC	sars ARTIC
ARTIC V2	sars ARTIC V2
ARTIC V3	sars ARTIC V3
RANDOM	sars random NOT ARTICV3 NOT ARTICV2 NOT ARTIC
ALL	sars

than for Nanopore despite the protocol was initially designed for Nanopore.

See how results corresponding to the ARTIC protocol roughly correspond to 41% of all available SARS-Cov-2 runs in the SRA archive.

From the results of these queries, I randomly selected some runs and downloaded the data sets. Then I assembled the data sets using the best performing genome assembly software from SPAdes (7), rnaSPAdes (8) and metaSPAdes (I will note as xSPAdes the best result obtained from these). In case the runs contained long reads, Flye and Canu (9) were also applied. I finally assembled some of the short-read runs with Contignant s-aligner (10).

SPAdes, rnaSPAdes and metaSPAdes have been demonstrated to be the best-performing open-source software for viral genome de-novo assembly in different previous studies. Flye and canu are considered the best-performing assembly utilities for long-read data. Meanwhile, s-aligner is a new de novo genome assembler that has recently demonstrated superior performance for viral-genome assembly over the previous short-read assemblers.

Results

Table 3 shows the results obtained.
From these results, some observations can be extracted.

A. Short-read data sets outperform long-read ones.

I still have not found a long-read data set that completes a perfect assembly. Doesn't matter the library design or the technology employed (Nanopore or PacBio). The mean NG50 for long-read data sets is 7.622 while any protocol using short-reads at least doubles that.

In addition, the obtained sequences have a higher misassembly rate, which makes that data less feasible for variant detection.

B. The ARTIC protocol is far from delivering optimal results.

Despite being widely used (41% of runs in the SRA archive) its performance is low and far from the best-performing protocols. If we only consider results for short-read data the mean NG50 is 16.712, which is a quite bad result.

C. The ARTIC protocol doesn't outperform other protocols.

When making use exclusively of open-source assembly software, the ARTIC protocol doesn't even significantly outperform results from other protocols. Its NG50 mean is similar to the NG50 overall mean of all protocols using open-source software: 16.712 with ARTIC vs. 15.865 overall, and slightly lower than protocols using random primers (17.220).

D. Library designs with random primers largely outperform designs with fixed primers when using s-aligner.

When making use of all available software options, not only open source, designs with random primer selection largely outperform designs with fixed primer selection, like ARTIC. If we compare the NG50 mean from results for short-read data employing ARTIC and SPAdes (16.712), it is 71% lower than the NG50 mean obtained from random-primer data and s-aligner assembler (28.654). Indeed, the combination of s-aligner plus random-primer data guarantees in most cases an almost perfect assembly of the virus genome. Thirteen out of fifteen cases got as a result an almost-perfect assembly.

This observation is corroborated by the frequent presence of gaps in the reference mapping of runs obtained from fixed-primers designs. This is, indeed, something that could be expected from designs based on fixed primers. That limitation is already recognized by the WHO (11).

E. The ARTIC protocol under-performs even when using s-aligner as software for genome assembly.

S-aligner is, in general, a better tool for viral genome assembly. But even when using it, the ARTIC protocol underperforms compared to other protocols. The average NG50 using s-aligner for ARTIC data sets is 16.757, which is similar to the average NG50 with open-source software (16.712), but far from the average NG50 obtained with s-aligner for random-primer protocols (28.654).

F. No paired-read performance benefit over single-read.

When using s-aligner as assembly software with random-primer library designs, there is no significant difference between using paired-end data or single-read data: 28.394 (single) vs. 28.654 (overall).

Conclusions

There are significant differences in performance between different protocols for sequencing the SARS-Cov-2 (figure 1). The difference in performance between using the ARTIC protocol with short-read technologies and using a random-primer design with an s-aligner is statistically significant, with a p-value <0.00001. The difference in performance in the NG50 metric is on average 71.5%. In addition, when

Table 2. Sequencing runs found for every protocol and hardware

Protocol	Illumina	Nanopore	Capillary	LS454	Ion Torrent	BGISEQ	PacBio
ARTIC	78115	22909	12	4	0	0	0
ARTIC V2	2681	66	0	0	0	0	0
ARTIC V3	714	0	0	0	0	0	0
RANDOM	7525	323	0	1	26	109	0
ALL SARS	215187	34866	1762	7	536	148	25

Table 3. Sequencing results for randomly selected data-sets

Run Id	Hardware	Library design	layout	run size (MB)	Canu NG50	xSPAdes NG50	s-aligner NG50
SRR12351628	miseq	ARTIC V3	paired	84		4371	8431
SRR12819233	novaseq 6000	random	paired	382		21585	29845
SRR12445029	ion torrent	random	single	1413		4980	29299
SRR13684392	miseq	ARTIC	paired	1500		29404	
SRR11410529	miseq	ARTIC	paired	111		19294	
SRR12045777	miseq	ARTIC v3	paired	188		19338	20522
SRR13200927	nextseq 500	unspecified	single	287		9412	10610
SRR10903401	miseq	random	paired	140		4094	10610
SRR12623307	miseq	ARTIC v3	paired	11		19.283	
SRR11772204	miseq	ARTIC v2	paired	113		29.837	
SRR12045770	miseq	ARTIC v3	paired	100		1.412	19.242
SRR11410528	miseq	ARTIC	paired	76		19.291	19.294
SRR13660064	miseq	ARTIC v3	paired	10		16.463	12.708
ERR5094566	gridion	liverpool	single	37	0	4.216	
SRR13623050	miseq	ARTIC v3	paired	143		29.842	29.814
SRR13623049	miseq	ARTIC v3	paired	131		29.833	
SRR12481157	miseq	random	paired	94		23.583	29.836
ERR4182482	GridION	unknown	single	10	0	8.315	
SRR13574254	illumina	ARTIC v3	paired	435		1.000	11.459
SRR13727443	illumina	artic v3	paired	850		1.631	
SRR13731834	illumina	artic v3	paired	50		29.687	
SRR13495171	illumina	random	paired	650			29.820
SRR13380666	PacBio	hybrid	single	78	0		
ERR5094578	minion	artic v3	single	14	0	0	2.835
ERR5165938	nextseq 550	hybrid	paired	213			11.743
SRR13380665	PacBio	hybrid	single	633	15.123		
ERR5165938	nextseq 550	hybrid	paired	605		29.839	
SRR13727440	illumina	ARTIC V3	paired	1126		0	12.591
SRR12445036	ion torrent	random	single	191		5.104	29.112
ERR4971211	nextseq 500	random	paired	126			
SRR13615951	BGISEQ	random	single	48		29.858	29.846
SRR13615945	BGISEQ	random	single	8		29.852	29.797
SRR13615944	BGISEQ	random	single	3		29.852	29.829
SRR13615947	BGISEQ	random	single	5		29.856	29.837
SRR13615942	BGISEQ	random	single	40		28.307	29.754
SRR13300938	ion torrent	random	single	586			18.500
SRR12445040	ion torrent	random	single	405		5.560	29.340
SRR12445032	ion torrent	random	single	119		2.475	29.351
SRR13050769	hiseq	random	paired	3659		0	25.854
SRR13495171	illumina	random	paired	651		0	29.804

Results in which both assembling methods underperformed were excluded as likely due to problems in the data set.
Empty cells correspond to assemblies that were not tried because of lack of relevance for the study.

Table 4. Sequencing results for runs obtained from the ARTIC protocol

Run Id	xSPAdes NG50	s-aligner NG50
SRR12351628	4.371	8.431
SRR13684392	29.404	
SRR11410529	19.294	
SRR12045777	19.338	20.522
SRR12623307	19.283	
SRR11772204	29.837	
SRR12045770	1.412	19.242
SRR11410528	19.291	19.294
SRR13660064	16.463	12.708
SRR13623050	29.842	29.814
SRR13623049	29.833	
SRR13574254	1.000	11.459
SRR13727443	1.631	
SRR13731834	29.687	
SRR13727440	0	12.591
Mean	16.712,4	16.757,62
Variance	11.991,46	6.849,55

Table 5. Sequencing results for runs obtained with random primers amplification

Run Id	xSPAdes NG50	s-aligner NG50
SRR12819233	21.585	29.845
SRR12445029	4.980	29.299
SRR10903401	29.877	
SRR12481157	23.583	29.836
SRR12445036	5.104	29.112
SRR13615951	29.858	29.846
SRR13615945	29.852	29.797
SRR13615944	29.852	29.829
SRR13615947	29.856	29.837
SRR13615942	28.307	29.754
SRR13300938		18.500
SRR12445040	5.560	29.340
SRR12445032	2.674	29.351
SRR13050769	0	25.854
SRR13495171	0	29.804
Mean	17.220,57	28.654,93
Variance	13.063,15	2.984,97

evaluating the perfect-assembly ratio, we find that ARTIC has a 33.3 success rate, while the s-aligner-based protocol has an 86.7% success rate. With long-read data sets, the success rate of ARTIC is 0% and NG50 can't even be calculated because of lack of data.

These results suggest that the hundreds of thousands of genome sequencing being done in the world to trace the spread of the virus and detect new variants are not making use of the most reliable and efficient methods. The low NG50 and perfect-assembly ratio suggest that these methods are far from being reliable if de novo genome assembly is considered a need, as suggested by previous studies on the efficacy

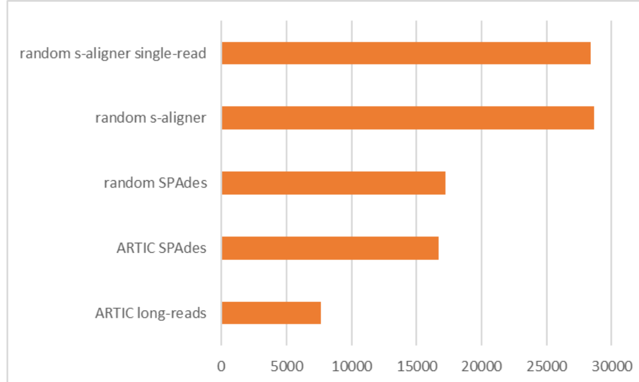

Fig. 1. NG50 for different clusters of runs.

of only-mapping assembly. Mapping the data to a reference genome is usually considered a necessary but insufficient step, and it is always preferable to have a de novo assembly, which is the only reason for not preferring the unavailability of that possibility. We demonstrate in this study that there are protocols that reliably permit us to obtain de novo genome sequencing of SARS-Cov-2: a tool that would improve the quality of the actual efforts to trace the virus worldwide.

Discussion

Another factor for considering which protocols to use for sequencing SARS-Cov-2 is the cost. ARTIC was specifically designed to be low-cost for that reason.

When evaluating the costs of different sequencing protocols three aspects should be considered.

1. The cost of the sequencing hardware
2. The cost of the products per sample
3. The overall time expended per sample

Unfortunately, I don't have the necessary experience or access to materials to evaluate these costs. For that reason, I contacted several public health organizations, warning them of the significant lack of performance of some protocols and offering them cooperation to find better ones. You can see in Annex I a list of entities that were contacted. None of them have acceded to cooperate at the moment of the writing of this manuscript. One can guess what their motivations are, but some motivations can be firmly discarded: they are not rejecting that because they are already developing equivalent studies or because they already have the answers that such study would bring.

Even though I lack the experience to make a full analysis of the cost-effectiveness of different protocols for sequencing SARS-Cov-2, some clues can be extracted from the data in this study. We see how we can obtain reliable, almost complete, de novo genome assemblies from data sets under 10MB (therefore largely multiplexable), obtained with less-expensive hardware like Ion Torrent or BGI. Also with Illumina, we can establish cost-effective protocols making use of fewer data and single-read technology. That suggests that cost-effective protocols are possible that are also reliable under a de-novo assembly perspective and not only under a reference-mapping one. The increase in performance also suggests that a higher percentage of sequencing efforts will end up in conclusive results, therefore eliminating the cost of most inconclusive results. All that information suggests that, overall, more cost-effective protocols than ARTIC are possible and desirable.

Data availability statement

The data underlying this article are available as DOI: 10.5281/zenodo.4558343.

The s-aligner software is available for free at https://contignant.com for first-time users. It's free to use for 15 days after installation. No personal identification is required but a contact email must actually be provided for downloading it.

Competing interests

I am the developer and the owner of all the rights to the s-aligner software.

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Supplementary Note A: Institutions invited to cooperate

See in table 6 the list of public institutions that were contacted whether to warn them of a possible inefficiency in the applied protocols for sequencing SARS-Cov-2 (including an offer to cooperate) or to warn them of the existence of a new tool that could have an impact on the protocols for sequencing SARS-Cov-2 (offering them also cooperation).

Table 6. Public institutions contacted to warn them of a possible improvement in public protocols for the management of the COVID-19 crisis.

Organization	City	Contact method	Contact date	Message content	Response
Hospital Universitari de la Vall d'Hebron	Barcelona (Spain)	Cold email	Feb 15, 2021	Warning of low performance of ARTIC protocol.	They did not acknowledge reception
Hospital Clínic	Barcelona (Spain)	Cold email	Feb 15, 2021	Warning of low performance of ARTIC protocol.	They did not acknowledge reception
Hospital Sant Pau	Barcelona (Spain)	Email to a connection and cold email to leaders	Feb 15, 2021	Warning of low performance of ARTIC protocol.	Unofficially: not interested / not their scope. No official acknowledgement of reception.
Sanger Institute	Hinxton (UK)	Cold email	Feb 15, 2021	Warning of low performance of ARTIC protocol.	They acknowledged reception and opened a ticket. No further news from them.
Red Española de Investigación en Sida	Hinxton (UK)	e-mail recommended by a connection	Feb 9, 2021	Warning of low performance of SARS-Cov-2 sequencing.	Rejected: too busy.
Barcelona Supercomputer Center	Barcelona (Spain)	Cold email	Sept 2020	Warning of better performance for viruses.	They did not acknowledge reception
Elixir Spain	Barcelona (Spain)	Cold email	Sept 2020	Warning of better performance for viruses.	They did not acknowledge reception
Instituto Nacional de Biotecnología	Barcelona (Spain)	Cold email	Sept 2020	Warning of better performance for viruses.	They did not acknowledge reception

Several private institutions were also contacted. None has either responded.