LazypipeX: Customizable Virome Analysis Pipeline Enabling Fast and Sensitive Virus Discovery from NGS data

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ABSTRACT

Metagenomic next-generation sequencing (mNGS) is pivotal for detecting known and novel viruses in diverse samples; however, its efficacy relies on robust bioinformatics pipelines. We present LazypipeX with multiple advanced features, including flexible annotation strategies that can be adjusted to match different datasets and applications. Using a synthetic benchmark, we show that a simple two-round annotation strategy designed for speed and low false positive rate required in virus diagnostics can reduce execution time to a fraction of the BLASTN search without any loss in accuracy. Additionally, using real data, we show that annotation strategies based on combinations of fast-to-accurate homology searches reduce execution time from 5- to 20-fold compared to the BLASTN/BLASTP baseline. Using one of these "annotation chains", we characterized multiple novel complete viral genomes that were missed by the Lazypipe v1/v2 and CZ ID analyses. LazypipeX is a highly efficient and versatile tool for virome analysis, offering customizable and transparent workflows that can facilitate virus discovery and identification in diverse mNGS applications.

Key words: bioinformatics pipeline; metagenomics; mNGS; virus discovery; virome

1 INTRODUCTION

Rapid developments in mNGS have enabled virologists to assess the true diversity of viruses in various samples (1). This is particularly true for viruses that do not propagate in cell culture and for novel or emerging viruses that fail to be detected using conventional PCR- or antigen-based methods. mNGS is a high-throughput, unbiased technology that can identify both known and novel pathogens and has great potential for viral discovery, surveillance, and broad-spectrum clinical diagnostics (1–3).

Virome identification with mNGS is heavily dependent on bioinformatic analysis, and it is increasingly recognized that applied bioinformatic methods will significantly influence the results (3, 4).

Bioinformatics analysis of mNGS data is an actively evolving field, with a wide range of tools available for individual steps and pipelines that support end-to-end workflows (1, 5). These pipelines can be categorized into three primary functions: characterizing viral diversity and abundance, virus identification or diagnostics, and facilitating novel virus discovery. In this study, we focused on pipelines for virus diagnostics and discovery. Open source pipelines that have been applied in virus diagnostics include metaMix (6), RIEMS (7), Kaiju (8), VIP (9), DAMIAN (2), Jovian (10), and CZ ID (3), with an excellent comparative evaluation presented by De Vries et al. (4). Pipelines for virus discovery focus on sensitivity and, as suggested by several authors, benefit from integrating protein alignments and/or protein domain annotation to identify highly divergent emerging and novel viruses, although at the cost of heavier computation (2, 3, 11, 12). Based on this notion, most pipelines that include protein-level alignments or protein domain annotations are applicable to virus discovery. There are also a number of open-source pipelines that were specifically designed to support the discovery of novel pathogens from mNGS data, such as VirusSeeker (11), DAMIAN (2), CZ ID (3) and Microseek (12). We have previously published two mNGS pipelines, Lazypipe1 and Lazypipe2, primarily designed for virus discovery (13, 14), which have been successfully applied to characterize viromes and identify novel viruses in clinical samples (15), ticks (16–18), mosquitoes (19, 20), snakes (18), farm animals (21, 22), and wildlife (23–27).

Here, we describe LazypipeX with multiple new features, including the novel concept of annotation strategies that can incorporate multiple nucleotide-, protein- and hidden Markov model (HMM)-based searches into contig annotations. Using a synthetic benchmark, we showed that a conceptually simple two-round strategy, where the first round uses a fast Minimap2 search (28) against a virus-only database, followed by a second round using BLASTN (29) against larger databases to filter false positives, can achieve a 4- to 8-fold speedup compared to a one-round BLASTN search, without any loss in accuracy. When combined with background filtering, the speedup achieved was 30-fold, with minimal loss in precision. Using real data, we showed that combining varying fast-to-accurate homology searches reduced the execution time by 5- to 20-times compared to the BLASTN/P baseline. Usability for virus discovery was illustrated by using LazypipeX to characterize multiple divergent novel viral genomes from arthropod data.

2 MATERIAL AND METHODS

2.1 LazypipeX outline

LazypipeX is a highly customizable UNIX-based pipeline for automated assembly and taxonomic profiling of metagenomic libraries, building upon the original Perl, C++, and R architectures to enable high-throughput virus identification and discovery. The workflow begins with quality preprocessing using fastp (30), followed by nontarget background read filtering using BWA-MEM (31), Sambamba (32), and SeqKit (33). Post-assembly via MEGAHIT (34) or SPADES (35), the pipeline introduces a modular "annotation strategy" concept, allowing users to arrange in chains (and rounds) multiple fast-to-accurate homology searches — such as Minimap2, BLASTN/P/X, DIAMOND, and HMMscan — against

precompiled, regularly updated NCBI NT, UniRef100, and viral HMM databases (e.g., Pfam and NeoRdRp). Taxonomic binning is performed using a bitscore-weighted model to generate detailed abundance tables and interactive Krona graphs (36).

2.2 Improved background filtering

LazypipeX supports multiple background filters to remove host or contaminant eukaryotic reads and contigs. This pre-assembly and post-assembly filtering accelerates annotation and allows for a smaller reference database. Multiple filters can be applied in tandem for pooled samples (e.g. arthropods) or samples contaminated by multiple sources. Because human nucleic acids often contaminate libraries, filtering *Homo sapiens* along with other backgrounds is recommended. The pipeline includes precompiled background filters for ~20 species, including human, mosquitoes, ticks, bats, and various mammals. Users can also add custom filters by providing a FASTA file path in the configuration file.

2.3 Flexible and transparent annotation strategies

LazypipeX introduces the concept of an "annotation strategy", which can be tailored for different use cases. Users can select strategies that chain homology searches to improve sensitivity or strategies that arrange them in rounds to boost precision. We benchmarked several predefined one-round, two-round, and chained strategies for virus identification and discovery, all accessible via the pipeline command line.

2.3.1 Fast one-round strategies

Minimap.NT$_{Vi}$ and Minimap.RefSeq$_{Vi}$: Minimap2 all contigs against NCBI NT or RefSeq viruses. These provide efficient preliminary annotations at the cost of higher false-positive rates.

2.3.2 Two-round strategies for virus identification

Two-round strategies combine a fast initial search against a virus-only database (e.g., Minimap2) with a sensitive secondary search (BLASTN) against a broader database to prune false positives. This reduces the search space for the slower algorithm. For example, if the execution time scales linearly with the size of the query and reference data, the execution time for the annotation of all contigs with a slower search against the complete database can be expressed as:

1) $T_1 = S(Q_{all})\, S(R_{all}) / V_{slow}$

And the execution time for annotating only virus contigs in two-rounds can be expressed as:

2) $T_2 = S(Q_{all})\, S(R_{vi}) \,/\, V_{fast} + S(Q_{vi})\, S(R_{all}) \,/\, V_{slow}$

where $S(Q_{all})$ is the size of the assembly, $S(Q_{vi})$ is the size of the virus part of the assembly, $S(R_{all})$ is the size of the reference database, $S(R_{vi})$ is the size of the virus part of the reference, V_{slow} and V_{fast} are the speeds of the slower and faster search, respectively.

The ratio of execution times can be expressed as:

3) $$\frac{T_2}{T_1} \approx \frac{S(Q_{all})\, S(R_{vi})\, / \, V_{fast} + S(Q_{vi})\, S(R_{all})\, / \, V_{slow}}{S(Q_{all})\, S(R_{all})\, / \, V_{slow}} = \frac{S(R_{vi})\, V_{slow}}{S(R_{all})\, V_{fast}} + \frac{S(Q_{vi})}{S(Q_{all})}$$

For example, if $V_{slow}/V_{fast} \approx 10\%$, and if viruses make up ~10% of the query data and 15% of the reference data, then T2/T1 ≈ 0.115, leading to a ~9-fold speedup. We argue that these assumptions are realistic. For example, in the NT release from 2024/01/01, virus sequences constituted 15.3% of the database, and the proportion of assembled viral data in most metagenomic datasets is below 10% (e.g. in (37) library from rRNA depleted tissue RNA this was 1.68%). Furthermore, Minimap2, although not specifically evaluated against BLASTN, has been reported to be tens of times faster than long-read genomic mappers.

We tested three strategies using this logic:

- *Minimap.NT$_{Vi}$ -Blastn.NT$_{ABV}$:* In the first round, all contigs are mapped with Minimap2 against NT viruses (NT$_{Vi}$; database descriptions are provided in Table 1). In the second round, pre-labelled viral contigs are annotated with BLASTN against NT archaea, bacteria, and viruses (NT$_{ABV}$). This strategy includes background filtering and assumes that most eukaryotic sequences are removed before the annotation step. This allows to reduce the database for the second round to NT$_{ABV}$.
- *Minimap.RefSeq$_{Vi}$-Blastn.RefSeq$_{ABV}$:* In the first round, all contigs are mapped with Minimap2 against RefSeq virus (RefSeq$_{Vi}$). In the second round, pre-labelled viral contigs are annotated using BLASTN against RefSeq archaea, bacteria, and viruses (RefSeq$_{ABV}$). This strategy also included background filtering.
- *Minimap.NT$_{Vi}$-Blastn.NT:* In the first round, all contigs are mapped using Minimap2 against *NT$_{Vi}$*. In the second round, the pre-labelled viral contigs are annotated using BLAST against the complete NT database.

The first two require background filtering and utilize archaea-bacteria-virus (ABV) segments of NT and RefSeq. The third strategy omits filtering and uses complete NT, making it suitable for samples with complex backgrounds (e.g., fecal or environmental samples).

129 *Table 1 List of reference databases supported by LazypipeX.*

Database	Description
Minimap.NT_{ABV}, Minimap.NT_{Vi}	Fasta and accession-to-taxid files for annotations with Minimap2. NCBI NT archaea-bacteria-viruses and NT viruses.
Blastn.NT_{ABV} and Blastn.NT_{Vi}	BLASTN index for NT archaea-bacteria-viruses and NT viruses.
Minimap.$RefSeq_{ABV}$ and Minimap.$RefSeq_{Vi}$	Fasta and accession-to-taxid files for annotations with Minimap2. RefSeq archaea-bacteria-viruses and RefSeq viruses.
Blastn.$RefSeq_{ABV}$ and Blastn.$RefSeq_{Vi}$	BLASTN index for RefSeq archaea-bacteria-viruses and RefSeq viruses
Blastp.$UniRef100_{ABV}$ and Blastp.$UniRef100_{Vi}$	BLASTP index for UniRef100 archaea-bacteria-viruses and viruses
Diamond.$UniRef100_{ABV}$ and Diamond.$UniRef100_{Vi}$	Diamond index for UniRef100 archaea-bacteria-viruses and viruses
HMMscan.$PFAM_{Vi}$	Viral HMM profiles from PFAM database (Mistry et al., 2021)
HMMscan.RdRp	NeoRdRp2 database (Sakaguchi et al., 2024)

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131 ### *2.3.3 Chained strategies for virus discovery*

132 Chained annotation strategies maximize sensitivity by passing contigs with no hits through
133 progressively more sensitive (and often slower) search engines. Strategies *Vi.chain1–4* require
134 eukaryotic background filtering and utilize archaea-bacteria-virus (ABV) segments of NT and
135 UniRef100. Vi.chain3.env omits filtering and uses complete databases, making it suitable for samples
136 with complex backgrounds (e.g., fecal or environmental samples).

137 The chains are structured as follows (for database abbreviations see Table 1):

138 - *Vi.chain0* (Baseline): BLASTN (NT_{ABV}) → BLASTP ($UniRef100_{ABV}$)
139 - *Vi.chain1*: Minimap2 (NT_{ABV}) → BLASTN (NT_{ABV}) → BLASTP ($UniRef100_{ABV}$)
140 - Vi.chain2: Minimap2 (NT_{ABV}) → BLASTP ($UniRef100_{ABV}$)
141 - Vi.chain3: Minimap2 (NT_{ABV}) → DIAMOND blastp ($UniRef100_{ABV}$)
142 - Vi.chain4: Minimap2 (NT_{ABV}) → SANSparallel (UniProtKB)
143 - Vi.chain3.env: Minimap2 (NT) → DIAMOND blastp (UniRef100)

144 ### *2.3.4 Supported searches engines*

145 These include Minimap2, BLASTN/P/X, DIAMOND blastp/x, SANSparallel, and HMMscan (28, 38–40).
146 All searches utilized bitscore or e-value cut-offs defined in the main configuration file (default values
147 given in Table 2). For protein-level searches (BLASTP, DIAMOND blastp, SANSparallel, HMMscan),
148 ORFs ≥72 nt were extracted using MGA (default) or orfipy (41).

149 *Table 2 LazypipeX default search engine parameters.*

Tool	Key Options	Filter/Selection
Minimap2	-x asm20 --secondary yes –cs -s 200	peak_DP_alignment_score>=200, top hits, no ties
BLASTN/P/X	-evalue 10 -max_target_seqs 5	bitscore >= 200, top hits, no ties
DIAMOND blastp/x	--evalue 10 --max_target_seqs 5 --min-orf 72 --very-sensitive	bitscore>= 200, top hits, no ties
HMMscan	-E 0.01	Top hits with ties
SANSparallel	-m SANStopHtaxid --SANS_H 5 -R	bitscore >= 200, top hits, no ties

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151 *2.3.5 Supported reference databases*

152 These are listed in Table 1 and can be installed to local volumes using *install_db.pl* utility. The default
153 nucleotide database is NCBI NT, which was also the most common reference database employed by
154 eight out of 13 pipelines examined by de Vries et al. (4) and by CZ ID (3). For the default protein
155 database, we chose UniRef100, similar to GenomeDetective, which uses UniRef90 (42). Other potential
156 options include NCBI NR (e.g., DAMIAN (2) and CZ ID (3)) and RefSeq proteins (e.g., metaMix ((6)).
157 However, we selected UniRef100 because it is highly representative, while remaining an order of
158 magnitude smaller than the NR.

159 Supported databases were built from RefSeq (release 228), NT (version 2024/01/01), and UniRef100
160 (version 2024/11/27) by filtering archaeal, bacterial, and viral sequences using get_species_taxids.sh
161 (43) and SeqKit (33), followed by default indexing calls. The Minimap2 database comprised FASTA
162 files combined with accession-to-taxid TSV files. *HMMscan.PFAM$_{Vi}$* was compiled by downloading all
163 viral HMM profiles from Pfam (44); we also used the original NeoRdRp 2.1 database (45).

164 **2.4 Additional features**

165 LazypipeX now integrates the ICTV VMR metadata (46), including *host.source* and
166 *genome.*composition, into annotation tables. It also adds support for DIAMOND blastp/blastx (40) and
167 HMMscan (47), with new ABV and Vi databases derived from UniRef100, Pfam (44) and NeoRdRp 2.1
168 (45).

169 LazypipeX can output IGV reports (48) for the identified viral contigs. The pipeline bins contigs by
170 species, aligns them to the top reference hits via Minimap2, and produces interactive IGV visualizations
171 of genome coverage and variants. These reports facilitate the identification of contig overlaps, sequence
172 similarities, and gene coverage (e.g., polymerases) in fragmented assemblies. Example IGV reports for
173 ten mosquito pools are available at https://doi.org/10.5281/zenodo.18348371.

174 **2.5 Benchmarking**

175 *2.5.1 Benchmarking background filtering*

176 We evaluated LazypipeX background filtering on two datasets: a simulated human metagenome from
177 MetaShot (49) and two *Rattus norvegicus* liver libraries (37). For the MetaShot benchmark, reads were
178 filtered using a human reference genome (acc. GCF_000001405.40) and validated against the
179 sequence labels included in the benchmark. For the rat samples, reads were filtered against the *R.*

norvegicus reference (GCF_036323735.1) and validated by comparing results to LazypipeX BLASTN-based binning. Performance was assessed by iterating over the BWA MEM (31) alignment score (AS) and mapping quality (mapq) thresholds.

2.5.2 Benchmarking strategies for virus identification on simulated data

We evaluated LazypipeX with one- and two-round strategies using a MetaShot simulated human metagenome (20.5M PE 150bp reads, 80 viral and 70 bacterial pathogens) (49).

To assess time savings versus accuracy, we compared two-round strategies (*Minimap.NT*$_{Vi}$-*Blastn.NT*$_{ABV}$ and *Minimap.NT*$_{Vi}$-*Blastn.NT*) against their one-round equivalents (*Blastn.NT*$_{ABV}$ and *Blastn.NT*). We also tested *Minimap.RefSeq*$_{Vi}$-*Blastn.RefSeq*$_{ABV}$, standalone Minimap2 rounds (*Minimap.NT*$_{Vi}$*, Minimap.RefSeq*$_{Vi}$), and a common metagenomic approach, DIAMOND blastp against complete NR (*Diamondp.NR)*.

For performance baselines, we included Lazypipe v2.1 (Minimap against NT) and the CZ ID pipeline (Illumina v8.3, NCBI index dated 2024/02/06 with human host subtraction) (3). Most local runs utilized human background filtering (GCF_000001405.40). The databases used included RefSeq (rel. 228), NT (2024/01/01), UniRef100 (2024/11/27), and NR (2025/08/07). For all compared pipelines, reported taxids were linked to the NCBI taxonomy dated 2025/09/10 using a taxonkit (50). For CZ, ID read counts were selected from the NT count field when this was non-zero, and from the NR count field when the NT count was zero. For all pipelines, taxa with fewer than 10 reads and taxa labelled as bacteriophages were ignored. All local runs were performed on a Linux/Unix CPU supercluster with 30 cores, with each running at 2.1 GHz.

2.5.3 Benchmarking virus discovery on real data

We benchmarked chained strategies (*Vi.chain0-4* and *Vi.chain3.env*) on Illumina libraries from 90 *Ochlerotatus* mosquito pools (acc. PRJNA852425) previously analyzed using Lazypipe1 and downstream BLASTX (19). Background was filtered using the human (GCF_000001405.40) and four mosquito reference genomes (GCF_002204515.2, GCF_035046485.1, GCF_016801865.2, and CA_964187845.1). The results were compared against *Vi.chain0* baseline and Lazypipe2.

A novel horsefly (*Tabanidae sp.*) library was used to test HMMscan integration. Filtering utilized a related *Chrysops caecutiens* reference (acc. GCA_963971475.1). The annotation chain followed *Vi.chain1* (Minimap2 → BLASTN → BLASTP), supplemented with HMMscan (Pfam → NeoRdRp v2.1).

Finally, LazypipeX was compared against CZ ID (v8.3) using the horsefly and two mosquito samples (SRR19859382–3). All local runs used default settings on a 30-core (2.1 GHz) Linux cluster.

3 RESULTS

3.1 Background filtering

The results of background filtering on the simulated data are presented in Table 3. Filtering by mapping quality (mapq) was less effective than filtering by alignment score (AS). At the default threshold (AS ≥ 100), 99.998% of human reads were removed, while all non-retroviral reads were retained. Notably, 96.375% of human endogenous retroviral (HERVs) reads were removed, likely due to the presence of these sequences in the reference genome. For accurate ERV binning, we recommend strategies that bypass background filtering (see Discussion). The results for the rat samples (Supp. Table 1) similarly favored AS filtering; the default threshold (AS ≥ 100) removed 63.1-70.2% of rodent reads while retaining 99.95-100.00% viral and ~100.00% bacterial reads.

Table 3 Benchmarking background filtering on simulated data from (49). Selected filter highlighted. Filter, applied filter with AS representing alignment score and mapq mapping quality threshold, Total, total fraction of reads filtered, Homo sapiens, human reads filtered, Bacteria, bacterial reads filtered, Viruses, all viral reads filtered, HERV, reads from Human Endogeneous Retroviruses filtered, Viruses excl. Retroviridae, filtered viral reads excluding Retroviridae reads.

Filter	Total	Homo sapiens	Bacteria	Viruses	HERV	Viruses excl. Retroviridae
AS0	99.587 %	100.000 %	91.696 %	97.529 %	99.544 %	96.804 %
AS30	94.687 %	100.000 %	0.021 %	21.982 %	96.453 %	0.151 %
AS50	94.686 %	99.999 %	0.002 %	21.913 %	96.450 %	0.046 %
AS75	94.685 %	99.999 %	0.001 %	21.887 %	96.444 %	0.008 %
AS100	94.684 %	99.998 %	0.000 %	21.861 %	96.354 %	0.000 %
AS125	94.681 %	99.997 %	0.000 %	21.701 %	95.649 %	0.000 %
AS150	76.305 %	80.631 %	0.000 %	11.793 %	51.980 %	0.000 %
mapq0	99.587 %	100.000 %	91.696 %	97.529 %	99.544 %	96.804 %
mapq10	90.708 %	94.938 %	16.278 %	26.474 %	52.932 %	20.282 %
mapq20	89.851 %	94.855 %	1.954 %	12.879 %	50.600 %	2.122 %
mapq30	89.159 %	94.219 %	0.382 %	10.595 %	45.364 %	0.459 %
mapq40	89.043 %	94.117 %	0.038 %	10.147 %	44.656 %	0.022 %
mapq50	88.564 %	93.623 %	0.005 %	8.680 %	38.240 %	0.005 %
mapq60	88.285 %	93.331 %	0.001 %	8.266 %	36.431 %	0.000 %

3.2 Virus identification from simulated data

We evaluated LazypipeX (several strategies), Lazypipe2, and CZ ID using a human-simulated metagenome (49). The species-level precision, recall, F1-score (harmonic mean of precision and recall), execution time, memory usage, and erroneous predictions are detailed in Table 4.

The highest accuracy was achieved by LazypipeX annotations against the complete NT database, without host filtering. Both one-round (*Blastn.NT*) and two-round (*Minimap.NT*$_{Vi}$*-Blastn.NT*) strategies yielded identical errors: two false negatives (FN) – *ML29* (misclassified as *Mammarenavirus lassaense*) and *Orthobornavirus bornaense* (contig unannotated), and two false positives (FP) – *Orthopoxvirus cowpox* (misclassified *Vaccinia virus*) and *ASM Simian foamy virus* (misclassified *Human spumaretrovirus*). The two-round version achieved a 4.3X speedup (~27h vs. ~117h).

LazypipeX annotations against NT_{Vi}/NT_{ABV} (with background filtering) reported two FPs and three FNs – two filtered endogenous retroviruses and *ML29*. The execution time dropped from ~32h (one-round) to ~4h (two-round), an 8.4X speedup. LazypipeX annotations against the smaller RefSeq database (*Minimap.RefSeq*$_{Vi}$*-Blastn.RefSeq*$_{ABV}$ and *Minima.RefSeq*$_{Vi}$) had higher error rates, resulting in three FPs and six FNs. Overall, transitioning from one-round *BLASTN.NT* to two-round NT_{ABV} annotations (*Minimap.*NT_{Vi}*-Blastn.*NT_{ABV}) provided ~30X speedup with identical recall and minimal precision loss.

Annotating with DIAMOND Blastp against NR (*Diamondp.NR*) showed low accuracy reporting five FPs and failed to detect 15 viral pathogens (Table 4). Lazypipe2 was hindered by low precision, primarily due to a high number of unlabeled bacteriophages. The CZ ID also reported multiple FPs, including four FP eukaryotic viruses and 14 unlabeled bacteriophages. The FNs for CZ ID included two retroviruses, likely filtered with the host genome, as well as *Betapapillomavirus 2* and *Bocaparvovirus primate1* (Table 4). The latter two were likely misclassified as *Human papillomavirus* and *Human bocavirus*, which may be attributed to discrepancies between the applied taxonomies.

Table 4 Accessing LazypipeX accuracy of virus taxon retrieval, execution time and memory consumption on simulated data from (Fosso et al., 2017). Compared pipelines are ordered by the descending F1-score. Column headers: TP, number of true positive species, FP, number of false positive species, FN, number of false negative species, F1, F1-score, Maxrss, maximum memory consumption.

[1]Other false negatives for Diamond.NR: Alphainfluenzavirus influenzae, Cytomegalovirus humanbeta5, Morbillivirus hominis, Orthoebolavirus zairense, Orthohepadnavirus hominoidei, Orthomarburgvirus marburgense, Phlebovirus napoliense, TTV-like mini virus and Uukuvirus uukuniemiense.
[2]Other false positives for Lazypipe2: Bacteriophage sp., Bifidobacterium phage Bbif-1, Caudoviricetes sp., Escherichia phage Lambda_ev058 and phi458, Herelleviridae sp., Inoviridae sp., Maltschvirus maltsch, Myoviridae sp. ctk251 and ctZhz2, RockefellervirusIPLA5, Siphoviridae sp. ctX581, Streptococcus phage IPP16, Streptococcus satellite phage Javan375 and uncultured human fecal virus.
[3]Other false positives for CZID: Biseptimavirus BU01, Caudoviricetes sp., Crassvirales sp., Myoviridae sp. ctk251/ctuJM17, Phietavirus B236, Podoviridae sp. ct7Ex2, Siphoviridae sp. ctCCX1/ctGz830/ctSqC25/ctX581/ctxvK3, uncultured human fecal virus and virus sp. ctJrn16.

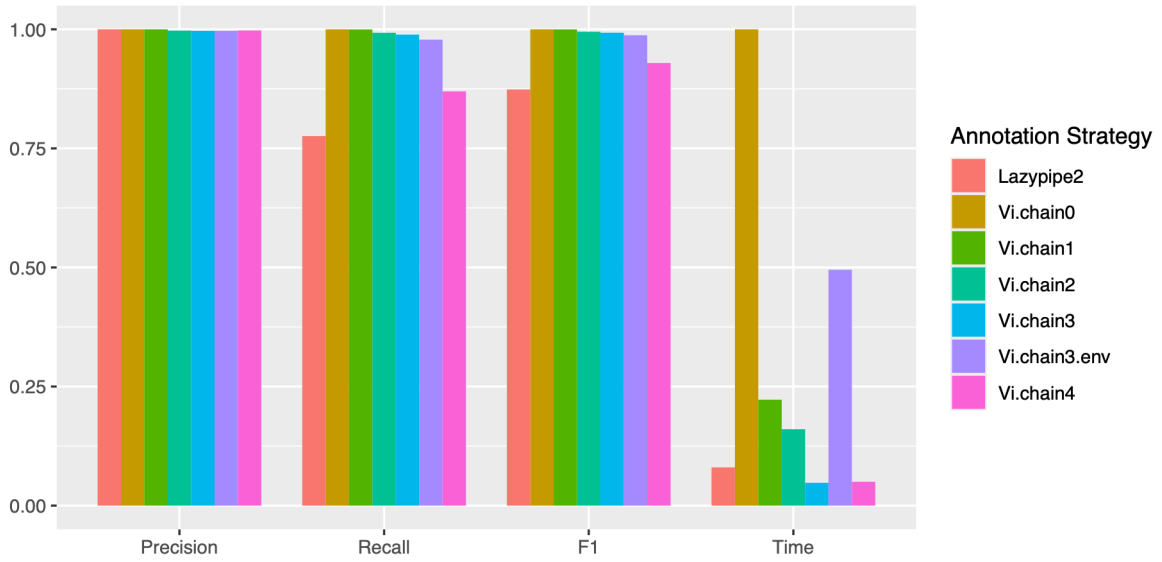
Strategy	TP	FP	FN	Precision	Recall	F1	Execution Time	Maxrss (MB)	Betapapillomavirus 2	Betaretrovirus mas pfimon	Bocaparvovirus primate1	Human endogenous retrovirus K	Human endogenous retrovirus	Human endogenous retrovirus W	Mopeia Lassa virus reassortant 29	Orthobornavirus bornaense	Other False Negatives	AGM simian foamy virus	Betaretrovirus squmon	Borna disease virus	Human bocavirus	Human papillomavirus	Orthobunyavirus khatangaense	Orthopneumovirus bovis	Orthopoxvirus cowpox	Phlebovirus toscanaense	Similspumavirus chlaet	Similspumavirus pantrotro	Simplexvirus humanalpha1	Spleen focus-forming virus	unknown Hepeviridae	Other False Positives
Minimap.NT_{Vi} - Blastn.NT	80	2	2	97.6 %	97.6 %	97.6 %	1:03:09:17	110,985							FN	FN		FP								FP						
Blastn.NT	80	2	2	97.6 %	97.6 %	97.6 %	4-21:17:37	25,857							FN	FN		FP								FP						
Minimap.NT_{Vi} - Blastn.NT_{ABV}	79	2	3	97.5 %	96.3 %	96.9 %	3:53:04	20,827					FN	FN	FN										FP	FP						
Blastn.NT_{ABV}	79	2	3	97.5 %	96.3 %	96.9 %	1-08:28:43	25,713					FN	FN	FN										FP	FP						
Minimap.NT_{Vi}	79	2	3	97.5 %	96.3 %	96.9 %	1:08:28	12,166					FN	FN	FN										FP					FP		
Minimap.$RefSeq_{Vi}$ - Blastn.$RefSeq_{ABV}$	77	3	5	96.3 %	93.9 %	95.1 %	0:50:09	10,374		FN		FN	FN	FN	FN				FP								FP	FP				
Minimap.$RefSeq_{Vi}$	77	3	5	96.3 %	93.9 %	95.1 %	0:45:51	10,066		FN		FN	FN	FN	FN				FP								FP	FP				
Lazypipe2 --ann minimap	79	16	3	83.2 %	96.3 %	89.3 %	3:43:05	70,548					FN	FN	FN										FP							FP[2]
Diamondp.NR	66	5	15	93.0 %	81.5 %	86.8 %	1-07:50:35	33,550		FN	FN		FN	FN	FN	FN	FN[1]	FP	FP	FP								FP			FP	
CZID NT/NR	78	20	4	79.6 %	95.1 %	86.7 %	NA	NA	FN		FN		FN	FN								FP	FP	FP			FP		FP		FP	FP[3]

3.3 Virus discovery from real data

3.3.1 Benchmarking against BLAST baseline

We compared the identification of viral sequences from 90 mosquito samples (19) using five chained strategies (*Vi.chain1-4* and *Vi.chain3.env*) and Lazypipe2. As "true" viral sequences or taxa are unknown for real data, we used *Vi.chain0* (BLASTN (NT_{ABV}) → BLASTP ($UniRef100_{ABV}$)) as the ground-truth baseline to measure the relative accuracy and time savings compared to BLAST-based workflows.

271 In this evaluation, we focused on viral sequences ≥ 500 bp, defining true positives (TP) as sequences
272 identified by both the evaluated and baseline strategies, and false positives (FP) or negatives (FN) as
273 sequences unique to the evaluated or baseline strategies, respectively. Precision, recall, and the F1-
274 metric were calculated using the total base-pair counts for these categories (Fig. 1 and Supp. Table 2).



275

276 *Figure 1 Overall performance of chained strategies (and Lazypipe2 –ann minimap) on the mosquito dataset.*
277 *Precision, recall, F1-metric and Time were calculated relative to Vi.chain0 baseline as described in the text.*

278 All LazypipeX strategies achieved ≥ 99.7% precision. *Vi.chain1* (introducing Minimap2 before BLASTN)
279 provided a 5-fold speedup with negligible loss in recall (~1 kbp of FNs). *Vi.chain2* and *Vi.chain3*
280 (substituting BLASTN with Minimap2 and BLASTP with DIAMOND) yielded 6-fold and 20-fold
281 speedups, respectively, with minimal loss in recall (~31 kbp and ~47 kbp of FNs, respectively).
282 *Vi.chain3.env* (no background filtering) doubled the FNs to ~91 kbp, likely due to contigs with close
283 eukaryotic homologs excluded from the baseline. *Vi.chain4* (substituting BLASTP with SANSparallel)
284 matched the 20-fold speedup of *Vi.chain3* but showed the lowest recall (87.0%). Lazypipe2 recall was
285 significantly lower (77.6%) because it only permitted annotation against a single database. For this
286 benchmark, the default NT database was used, which resulted in poor detection of distant homologs.

287 The average runtimes for mosquito samples (median library size 92.7 Mbp) were reduced from 20 hours
288 to as little as 1 hour (*Vi.chain3/4*), illustrating time savings of up to 95% compared to the BLAST
289 baseline.

290 *3.3.2 Identified viral sequences*

291 Here, we characterized viral sequences identified using the LazypipeX *Vi.chain1* strategy (Minimap2
292 (NT_{ABV}) → BLASTN (NT_{ABV}) → BLASTP ($UniRef100_{ABV}$)) from the mosquito dataset and compared
293 these with previously published findings (19). We also examined a novel rhabdovirus discovered in a
294 horsefly sample using *Vi.chain1* augmented with the HMMscan search. Viral discoveries were

295 compared between *Vi.chain1* and CZ ID for the horsefly and two mosquito samples (SRR19859382-
296 83). The detailed LazypipeX contig lists and CZ ID reports are provided in Supp. Table 3 and
297 https://doi.org/10.5281/zenodo.18348611, respectively.

298 For the mosquito dataset, *Vi.chain1* identified 1,242 viral contigs (≥ 1 kbp; 3,559 kbp total). Of these,
299 Minimap2 identified 78.1 %, BLASTN 1.6 % and BLASTP 20.3 %. To evaluate chained annotations for
300 virus discovery, we focused on contigs identified via Minimap2 and BLASTP.

301 *3.3.2.1 Identified rhabdoviruses*

302 Within *Vi.chain1*, Minimap2 identified 44 contigs (≥ 1 kbp; Supp. Table 3), covering all rhabdoviruses
303 previously reported in this dataset (19). BLASTP identified two additional contigs representing
304 complete rhabdovirus genomes, provisionally named *Kuusamo primrhavirus* and *Inari merhavirus*,
305 whereas HMMscan detected a 12.7 kbp contig provisionally named *Lieksa stangrhavirus* (horsefly
306 sample). Taxonomic classification and virus names were based on phylogenetic and ORF analyses
307 (Supp. Text 1), incorporating the names of the Finnish municipalities where the samples originated.

308 *3.3.2.2 Identified negeviruses*

309 Within *Vi.chain1*, Minimap2 identified 56 contigs (≥ 1 kbp; Supp. Table 3), covering all negeviruses
310 previously reported for this dataset (19). BLASTP identified four additional complete negevirus-like
311 sequences (11.1-11.4 kbp) and 21 partial sequences. Comparison of these BLASTP findings with
312 previously published negeviruses (19) confirmed a very low nucleotide (nt) level homology (qcov ≤ 3%).
313 The seven largest BLASTP findings were provisionally named as isolates of *Kuusamo* and *Hanko*
314 *negev-like viruses* based on phylogenetic and ORF analyses (Supp. Text 1), and their municipalities of
315 origin.

316 *3.3.2.3 Identified totiviruses and artiviruses*

317 Within *Vi.chain1*, Minimap2 identified 315 contigs (≥ 1 kbp; Supp. Table 3), covering 49 of the 52
318 *Totiviridae* species previously reported for this dataset (19). This search failed to report *Hanko totivirus*
319 *10 (2.7 kbp), Ilomantsi totivirus 3* (1.2 kbp), and *Karstula totivirus (1.1 kbp)*, potentially due to high
320 similarity to other totivirus-like sequences. BLASTP identified five additional complete totivirus-like
321 whole genomes (7.5-7.7 kbp), which were provisionally named as isolates of *Joensuu artivirus* based
322 on phylogenetic and ORF analyses (Supp. Text 1), and their municipalities of origin. Comparison with
323 previously reported totivirus sequences (19) confirmed a very low nucleotide-level homology (qcov
324 <14%) for all five contigs.

325 *3.3.2.4 Identified flaviviruses*

326 Minimap2 identified 10 contigs (≥ 1 kbp; Supp. Table 3), covering all *Flaviviridae* species previously
327 reported for this dataset (19). BLASTP identified six additional contigs that were mapped to *Flaviviridae*.
328 These were provisionally named *Palkane flavivirus*, *Palkane-like flavivirus*, *Hameenlinna flavivirus,* and
329 *Lestijarvi flavivirus* based on phylogenetic analysis (Supp. Text 1) and their municipalities of origin.

330 Comparison with previously reported *Flaviviridae* sequences (19) revealed 72.0% nucleotide identity
331 between *Hameenlinna flavivirus* (acc. BK071037) and *Hanko virus* (acc. ON949929.1). All other
332 sequences showed no significant homology at the nucleotide level.

333 *3.3.3 Comparison of virus discovery by CZ ID and LazypipeX*

334 Comparing the CZ ID and LazypipeX findings for *Rhabdoviridae* from the horsefly and the two mosquito
335 samples (acc. SRR19859382-83) revieled that, while CZ ID identified the complete *Inari merhavirus*
336 genome, it only recovered the L-gene for *Lieksa stangrhavirus* and 138-606 bp fragments of *Kuusamo*
337 *primrhavirus* (https://doi.org/10.5281/zenodo.18348611), all of which were captured as complete
338 genomes using LazypipeX. Similarly, for negeviruses and arti- or totiviruses, CZ ID recovered
339 fragmented sequences (0.1-1.1 kbp) and failed to report the full genomes of *Kuusamo negev-like virus*
340 (isolate *FIN/PP-2018/83*) and *Joensuu artivirus* (isolate *FIN/PP-2018/83*) reported by LazypipeX. No
341 *Flaviviridae* contigs were detected in either pipeline in these samples. Notably, CZ ID did not report any
342 novel large (≥ 1 kbp) rhabdo, nege, arti, or totivirus sequences that were not captured by LazypipeX.

343 **DISCUSSION**

344 LazypipeX is a versatile and efficient pipeline for virus discovery and identification from metagenomic
345 next-generation sequencing (mNGS) data, offering significant advancements in speed, accuracy, and
346 flexibility.

347 The pipeline's background filtering enables transparent removal of host and contaminant sequences by
348 specifying database accessions and metadata in the configuration file. This approach helped achieve
349 a marked speedup for a simulated benchmark with high host-sequence backgrounds (Table 4), driven
350 by reduced data size and a smaller reference database for annotation. Benchmarking on real and
351 synthetic datasets demonstrated high filtering accuracy (Table 3 and Supp. Table 1). The pipeline
352 supports pre-built filters for common use cases, custom filter creation, and the simultaneous application
353 of multiple filters. These capabilities make LazypipeX adaptable to diverse scenarios, such as clinical
354 samples from human or animal patients, vector pools (e.g., mosquitoes and ticks), and wildlife
355 specimens lacking species-level identification.

356 Like its predecessors, LazypipeX employs *de novo* assembly to generate contigs, enhancing the
357 sensitivity and specificity of homology searches. This approach is shared by pipelines that classify
358 assemblies instead of or in addition to classifying raw reads (2, 3, 6, 7, 10). LazypipeX integrates both
359 nucleotide and protein alignments, a feature that is rare in mNGS pipelines. A recent comparison found
360 that only two of the 13 pipelines used both alignment types (Alawi et al., 2019; Vilsker et al., 2019), and
361 only DAMIAN incorporated Hidden Markov Models (de Vries et al., 2021). LazypipeX users can tailor
362 the annotation procedure by chaining homology searches (e.g., Minimap2, BLASTN/P/X, DIAMOND,
363 SANSparallel, and HMMscan) against different databases, optimizing the sensitivity or specificity
364 depending on the dataset. This flexibility supports iterative adjustment of the pipeline to match diverse
365 goals in viral metagenomics.

366 The two-round annotation strategy in LazypipeX significantly enhanced virus identification efficiency.
367 An initial fast search against a virus-only database, followed by a second round using BLASTN against
368 a larger database, achieved a four- to 8-fold speedup without compromising accuracy (Table 4). When
369 combined with eukaryotic background filtering, this approach yielded a 30-fold speedup compared to
370 single-round BLASTN against the complete NT database. Compared to Lazypipe2, DIAMOND blastp
371 on NR, and CZ ID, LazypipeX's two-round strategy (*Minimap.NT*$_{Vi}$*-Blastn.NT* and *Minimap.NT*$_{Vi}$-
372 *Blastn.NT*$_{ABV}$) demonstrated superior accuracy in identifying eukaryotic viral taxa (Table 4). For
373 example, the DIAMOND blastp missed 15 viral pathogens, likely due to high protein-level similarity
374 among related viruses. These results highlight the strength of LazypipeX's nucleotide-based searches
375 for the detection of known viruses.

376 LazypipeX two-round strategy is conceptually similar to other efforts to perform fast alignments prior to
377 more accurate but slower refinement steps. The idea was inspired by the VirusSeeker pipeline, which
378 starts with the fast identification of candidate viral sequences against virus-only nucleotide and protein
379 databases, and then removes false positives with alignments against NCBI NT and NR (11). Similarly,
380 in CZ ID, reads are first mapped with fast nucleotide and protein aligners prior to aligning contigs using
381 BLAST against targeted databases (3). In GenomeDetective, viral reads are identified using a fast
382 DIAMOND search on UniRef90 prior to assembling and running BLASTN/BLASTX on RefSeq (42).
383 Lazypipe complements these approaches by offering the flexibility to choose different search engines
384 and databases for both scanning and refinement stages.

385 LazypipeX's chained annotation strategies proved to be highly effective for virus discovery. In a real
386 dataset of 90 mosquito pooled samples, the *Vi.chain1* strategy (Minimap2/BLASTN/BLASTP) reduced
387 execution time 5-fold compared to the BLASTN/P baseline, with precision and recall above 99.7% and
388 98.9%, respectively. Substituting BLASTP with faster tools, like DIAMOND or SANSparallel (*Vi.chain3,*
389 *Vi.chain4*), achieved a 20-fold speedup, although *Vi.chain4* showed lower recall (87.0%). *Vi.chain1*
390 retrieved nearly all previously reported viral sequences (19) via its initial Minimap2 search, while the
391 subsequent BLASTP search identified novel divergent viruses, including multiple complete genomes.
392 The older Lazypipe (v2), which is limited to one search at a time, had a much lower recall (78.6%).
393 Incorporating HMMscan into *Vi.chain1* enabled the discovery of a novel rhabdovirus whole-genome
394 sequence in a horsefly sample, which CZ ID only partially recovered (L gene only). Comparisons with
395 CZ ID for rhabdo-, nege-, arti/toti-, and flaviviruses further demonstrated the superior recovery of novel
396 genomes by lazypipeX. Findings by BLASTP in *Vi.chain1* were characterized using ORF, homology,
397 and phylogenetic analyses, which confirmed that most of these were unrelated to viral sequences
398 previously reported for this dataset, revealing complete genomes for at least six novel viruses (Supp.
399 Text 1). These results underscore the utility of LazypipeX for detecting divergent viruses in complex
400 mNGS data.

401 Based on our benchmarking, we proposed general guidelines for selecting LazypipeX strategies across
402 various use cases (Table 5). For virus identification, we recommend two-round strategies against NT$_{ABV}$

403 for tissue samples, while the complete NT database is preferable for samples potentially containing
404 retroviral sequences or complex eukaryotic backgrounds (Table 5).

405 *Table 5 Guidelines for selecting annotation strategies.*

Sequence Data	Analysis Goals	Recommended Annotation Strategy
Illumina libraries from tissue samples and other samples with clearly defined eukaryotic background	Virus identification excluding retroviruses. BLASTN accuracy with up to 30X speedup compared to BLASTN on complete NT.	*background filter* + *Minimap.NT*$_{Vi}$ - *Blastn.NT*$_{ABV}$
	Virus identification including retroviruses. BLASTN accuracy with up to 8X speedup compared to BLASTN.	*Minimap.NT*$_{Vi}$ - *Blastn.NT*
	Virus discovery excluding retroviruses. Precision/recall at ~100%/100% of combined BLASTN/BLASTP with up to 5X speedup.	*background filter* + *Vi.chain1*
	Virus discovery excluding retroviruses. Precision/recall at ~100%/99% of combined BLASTN and BLASTP with up to 20X speedup.	*background filter* + *Vi.chain3*
Illumina libraries from environmental, fecal and other samples with complex eukaryotic backgrounds	Virus identification including retroviruses. BLASTN accuracy with up to 8X speedup compared to BLASTN.	*Minimap.NT*$_{Vi}$ - *Blastn.NT*
	Virus discovery including retroviruses. Precision/recall at ~100%/98% of combined BLASTN and BLASTP with up to 20X speedup.	*Vi.chain3.env*

406

407 For virus discovery from tissue samples, we suggest using either *Vi.chain1* or *Vi.chain3*. *Vi.chain3* is
408 particularly well suited for large datasets, as it achieved a 20-fold speedup relative to the BLASTN/P
409 baseline with only a marginal reduction in accuracy (Supp. Table 2). Conversely, *Vi.chain1* offers a 5-
410 fold speedup with virtually no loss in accuracy; this remains a viable option for analyzing smaller
411 datasets or utilizing larger high-performance computing resources. For virus discovery from
412 environmental, fecal, and other samples with complex eukaryotic backgrounds, we recommend using
413 *Vi.chain3.env*, as this will also bin eukaryotic sequences.

414 **Future perspectives**

415 We plan to extend the Lazypipe series to field-based applications, specifically targeting integration with
416 the Oxford Nanopore MinION platform. Future iterations will be optimized for both offline and online
417 processing modes, enabling rapid on-site analysis of mNGS data in remote or resource-limited settings.

418 4 Author Contributions

419 Conceptualization of the project: I.W., R.K., T.S., and O.V.; Conceptualization of the pipeline: I.W., R.K.,
420 and T.S.; Implementation, updates, and code distribution: I.W.; Reference Database Curation: I.W.;
421 Benchmarking: I.W.; Testing and Reporting: I.W., R.K., and T.S.; Writing — Original Draft Preparation:
422 I.W.; Writing — Review & Editing: O.V., R.K., and T.S.; Supervision of the study: R.K., T.S., and O.V.;
423 Funding Acquisition: R.K. and O.V.

5 Acknowledgements

The authors wish to acknowledge the CSC IT Center for Science, Finland, for providing computational resources.

6 Conflicts of Interest

The authors have no conflict of interest to declare.

7 Data Availability Statement

The LazypipeX user manual and source code are available at https://bitbucket.org/plyusnin/lazypipe/ and https://www.helsinki.fi/en/projects/lazypipe. The virus sequences produced are publicly available in the NCBI GenBank database (accession codes provided in Supp. Table 4).

8 Funding

This research was funded by the Academy of Finland (grant number 339510), PREPARE-TID (EU grant number 101137132), DURABLE (EU HERA), VEO – European Union's Horizon 2020 (grant number 874735), the Jane and Aatos Erkko Foundation, and Helsinki University Hospital Funds. The funding bodies were not directly involved in designing or implementing the research described in this manuscript.

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