Viral metagenomic analysis to complement the viral risk assessment and adventitious agent testing of live virus vaccines

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Analytical Research and Development

MSD

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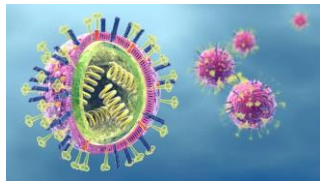
Outline

1. Viral Metagenomic Analysis (VMA) to de-risk adventitious agents in live virus vaccines
2. HIVE has publicly-available tools for metagenomic data analysis
3. ViruScreen is a streamlined internal application for metagenomic data analysis
4. Benchmarking ViruScreen with HIVE
5. Comparison of different alignment workflows

Viral metagenomic analysis (VMA) for adventitious virus detection in products

- **Viral metagenomics** uses high throughput sequencing (HTS) or next generation sequencing (NGS) to identify contaminating viruses in biological products.
- NGS is unbiased and has shown increased sensitivity and detection capabilities
- The ICH Q5A Revision 2 includes recognition and support of NGS virus testing for inclusion in regulatory submissions for biotechnology products in scope.
- Several guidelines encourage NGS testing to replace *in vivo* testing.
- NGS testing can supplement gaps in traditional adventitious agent testing of live virus vaccines or as part of an early de-risking strategy for clinical studies.



Basic steps of viral vaccine metagenomic analysis study workflow

Sample processing → Nucleic acid library preparation → Nucleic acid sequencing → Data analysis using bioinformatics

- Study samples: Harvested virus fluids and control cell fluids as well as cells and media as controls.
- Viromic and/or transcriptomic analysis is performed based on risk assessment.
- Specificity and sensitivity are determined by spiking with a virus panel to determine recovery.
- High throughput sequencing: Illumina short reads, paired end, 2x150 bases.
- Bioinformatic application results are followed-up with manual analyses.

Spike viruses used to determine recovery of representative virus species

- Well-characterized virus stocks prepared by FDA/CBER available from ATCC for spiking into VMA samples
- Demonstrate broad virus detection in different matrices.
- Represent virus families of potential safety concern and with various physical and chemical properties.
- Have also used human rhinovirus 14 (+ssRNA, 7.2 kb, unenveloped) and porcine circovirus 2 *in silico* for analysis.

Description	Abbreviation	Genome type	Genome length	Virus particle size	Virus envelope	Chemical resistance
Porcine circovirus 1 (ATCC- FDA)	PCV1	DNA, ss, circular	1.8 kb	16-18 nm	No	High
Mammalian orthoreovirus type 1, strain Lang (ATCC- FDA)	Reo1 ("REO")	RNA, ds, linear (segmented)	23.6 kb (1196-3915 nt)	80 nm	No	Medium-high
Feline leukemia virus, strain Thielen (ATCC- FDA)	FeLV	RNA, ss, linear (dimeric)	8.5 kb	80-100 nm	Yes	Low
Human respiratory syncytial virus, strain A2 (ATCC- FDA)	RSVA2 ("HRSV")	RNA, ss, linear	15 kb	150-200 nm	Yes	Low-medium
Epstein-Barr virus (HHV-4), strain B95-8 (ATCC- FDA)	EBV ("HHV4")	DNA, ds, linear	172 kb	122-180 nm	Yes	Low-medium

Proposed 1st International Virus Reference Standards for Adventitious Virus Detection in Biological Products by Next-Generation Sequencing (NGS) Technologies (CBER-5). Arifa S. Khan and Study Group Participants. WHO Expert Committee on Biological Standardization. Geneva, 19-23 October 2020.

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Overview of the short read alignment (SRA) workflow for Illumina sequences

Sample preparation and extraction.

Illumina high-throughput sequencing.

Trim adapter and low-quality sequences.

Virus harvests only: Stringent alignment to vaccine virus

Align to curated viral reference database and vaccine virus

Best hits within taxa reviewed across samples

Counterscreen 'Putative' positives

Identify bioinformatically-confirmed hits of interest

Launch investigation to confirm virus presence

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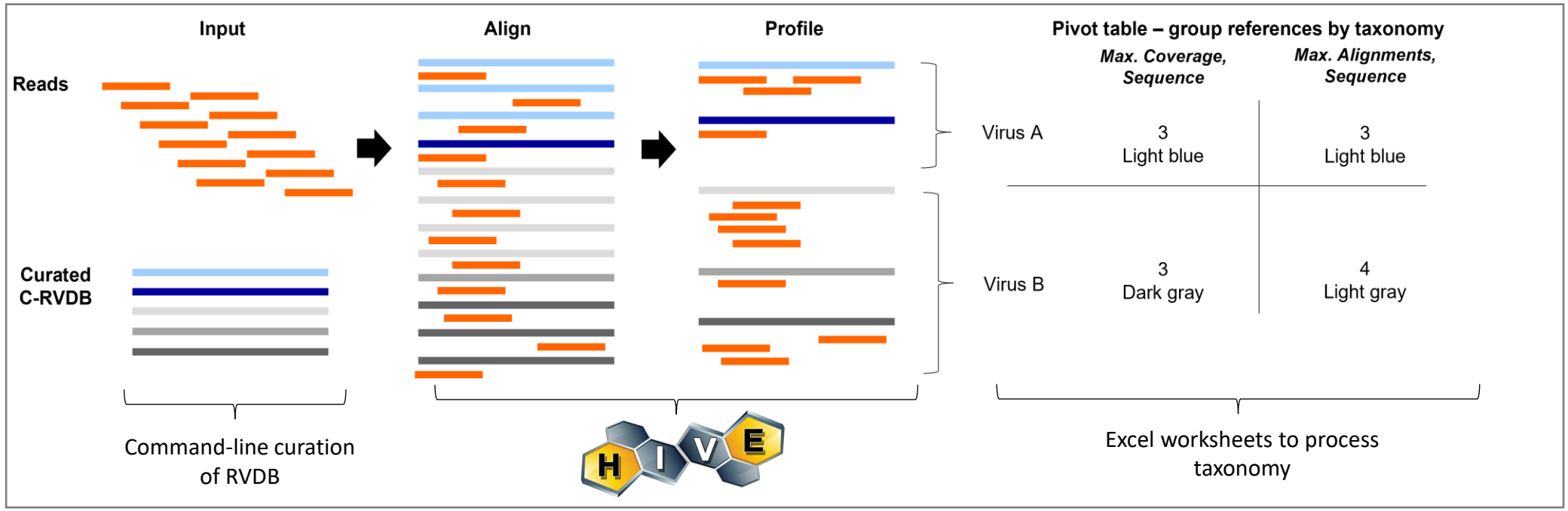
Bioinformatic analysis environment used for characterization tests in regulatory filings

- HIVE:
  - High-performance Integrated Virtual Environment
  - HIVE Hexagon: HTS read aligner
  - HIVE Heptagon: Alignment profiler
- Highlights of HIVE:
  - Instances: US FDA, public domain
  - GWU maintenance, expert development, secure access
  - Well-known and published tools available in public instance
  - Generates BioCompute objects for pipeline communication

Analysis workflow using HIVE and manual processing: short read alignment to reference database and best hits filtering



- Several publications on HIVE: hive.biochemistry.gwu.edu/home
- FDA reference viral database: https://rvdb.dbi.udel.edu/

Automation and streamlining of data analysis for GMP or release testing

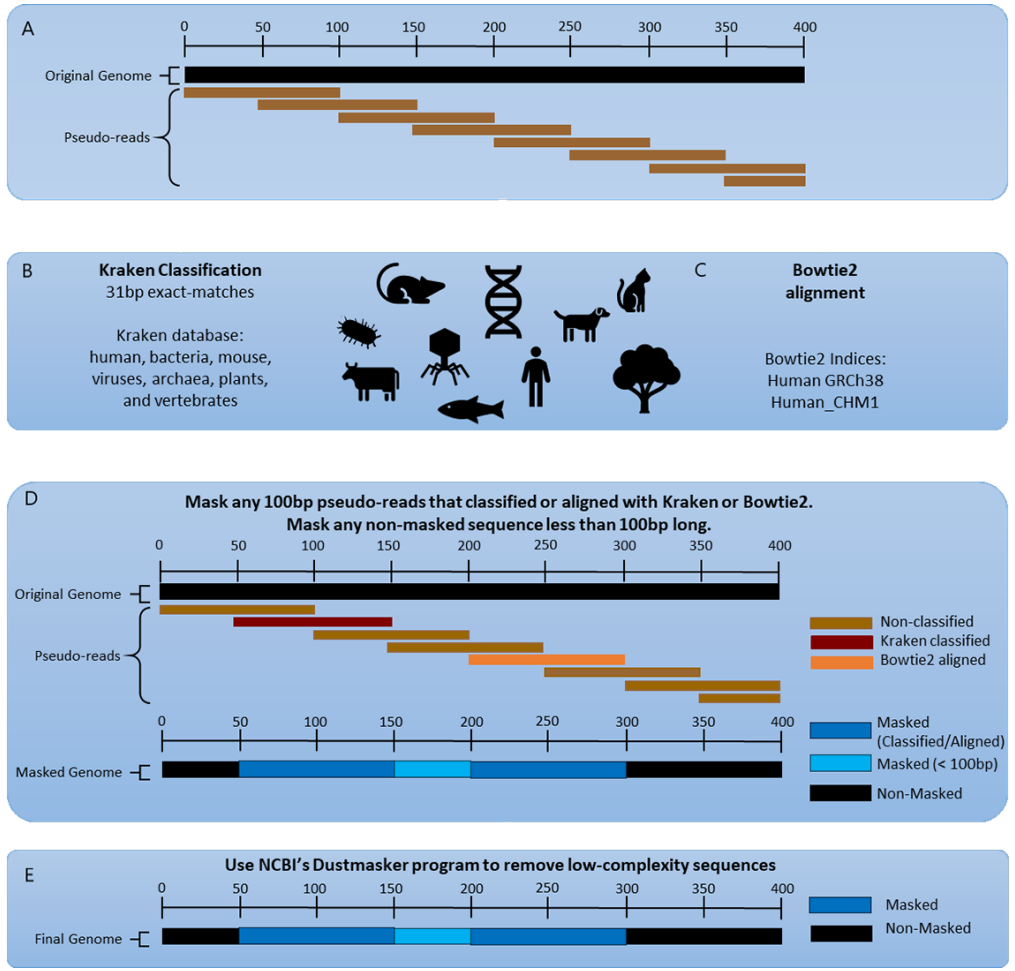
ViruScreen* is an internally-developed bioinformatic analysis application for VMA

- ✓ No computer programming skills required – user friendly
- ✓ Within company firewall – fewer copies of data
- ✓ Dedicated IT and Bioinformatics - customizable workflows
- ✓ Non-GMP and GMP environment for HTS data set analysis
- ✓ Downloadable report is generated

- Multiple workflows are available in ViruScreen for Illumina paired-end reads
  - FastQC
  - **Database curation** – Kraken, Dustmasker, custom
  - **Short read alignment (SRA)** – Bowtie 2, Magic-BLAST
  - ***De novo* assembly alignment –** Megahit, Magic-BLAST
  - Protein alignment – DIAMOND
  - Single nucleotide variant analysis – Lofreq2

*Patent application submitted 2023.

Curation of FDA clustered RVDB in UI to reduce false positive hits



- Clustered Reference Viral Database (cRVDB) generated by FDA
  - Diverse virus sequences; also includes retro-elements, etc
- Bulk vaccine virus harvest
  - Contains sequences from host cells
- Database curation is based on masking undesired regions
  - Described in Kenney, *et al*., adapted from Lu and Salzberg.
  - Split sequences into pseudoreads,
  - Taxonomically classify and align to custom database of human, bacteria, vectors, etc
  - Dustmasker used for low complexity regions
  - Undesired nucleotides converted to "N"
  - Retains same number of records

Lu J, Salzberg SL (2018) Removing contaminants from databases of draft genomes. *PLoS Comput Biol* 14(6): e1006277.

Kenney JG, et al. (2024) Communicating computational workflows in a regulatory environment. *Drug Discov Today*. 2024 Mar;29(3):103884

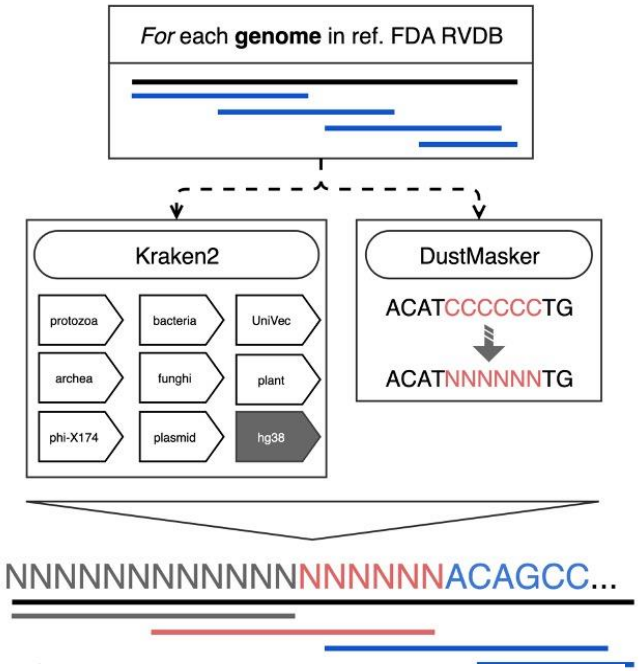
Curation statistics depicted in the user interface: cRVDB example

Communicating computational workflows in a regulatory environment

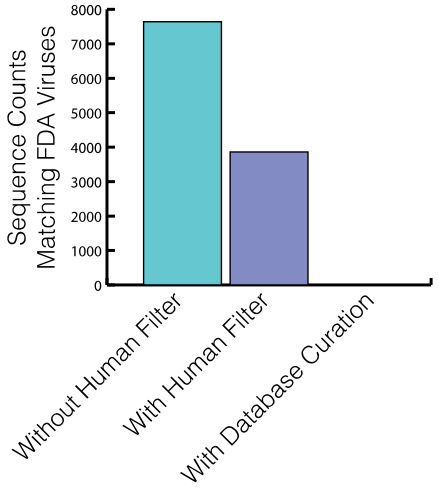
Jonathon G. Keeney [1,5,*], Naila Gulzar [1,5], Jack B. Baker [2], Ondrej Klempir [3], Geoffrey D. Hannigan [4], Danny A. Bitton [3], Julia M. Maritz [4], Charles H.S. King IV [1], Janisha A. Patel [1], Paul Duncan [2], Raja Mazumder [1]

[1] Department of Biochemistry and Molecular Medicine, School of Medicine and Health Sciences, The George Washington University, Washington, DC, USA
[2] Merck & Co., Kenilworth, NJ, USA
[3] R&D Informatics Solutions, MSD Czech Republic, Prague, Czech Republic
[4] Exploratory Science Center, Merck & Co., Cambridge, MA, USA

Database curation

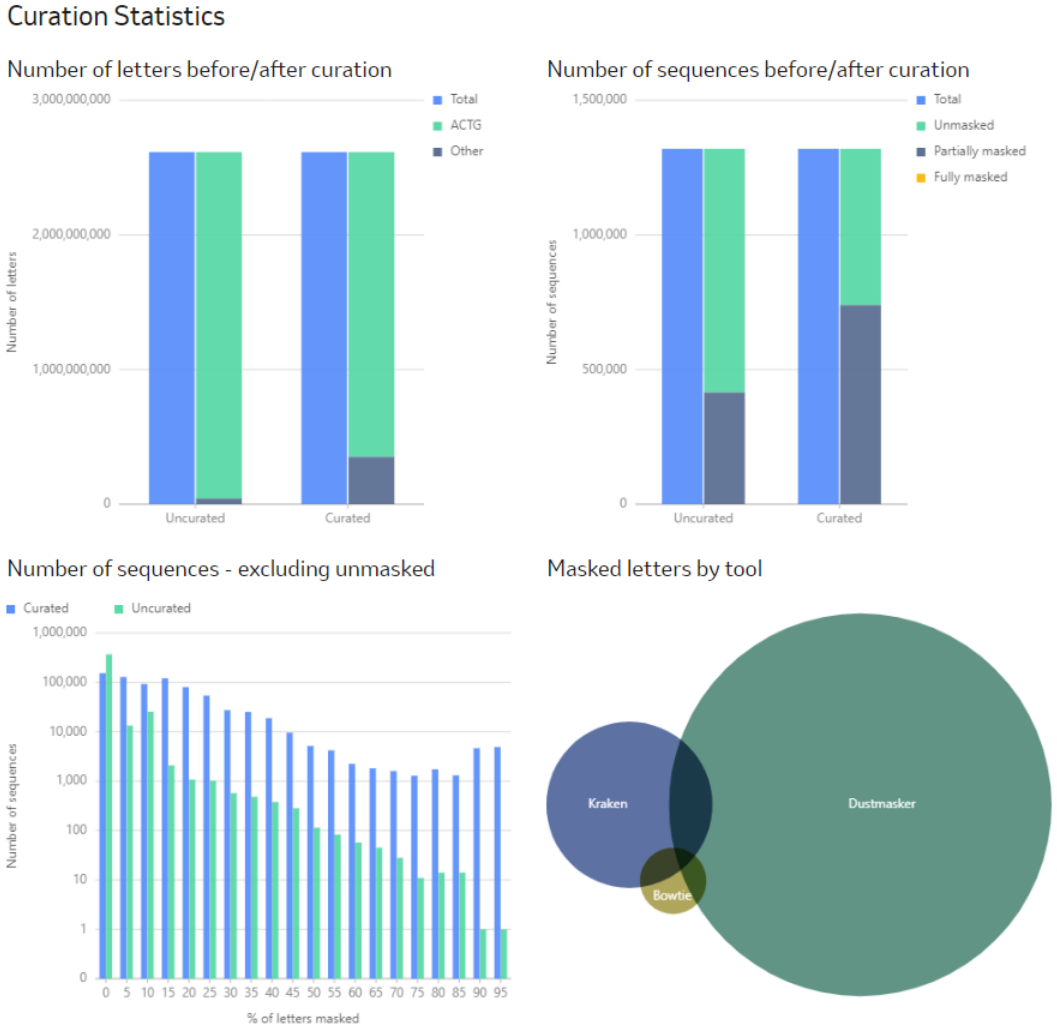


Simulated dataset results



Drug Discovery Today • Volume 29, Number 3 • March 2024

Output example from recent cRVDB version: ViruScreen UI and report



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Align to curated viral reference database and vaccine virus

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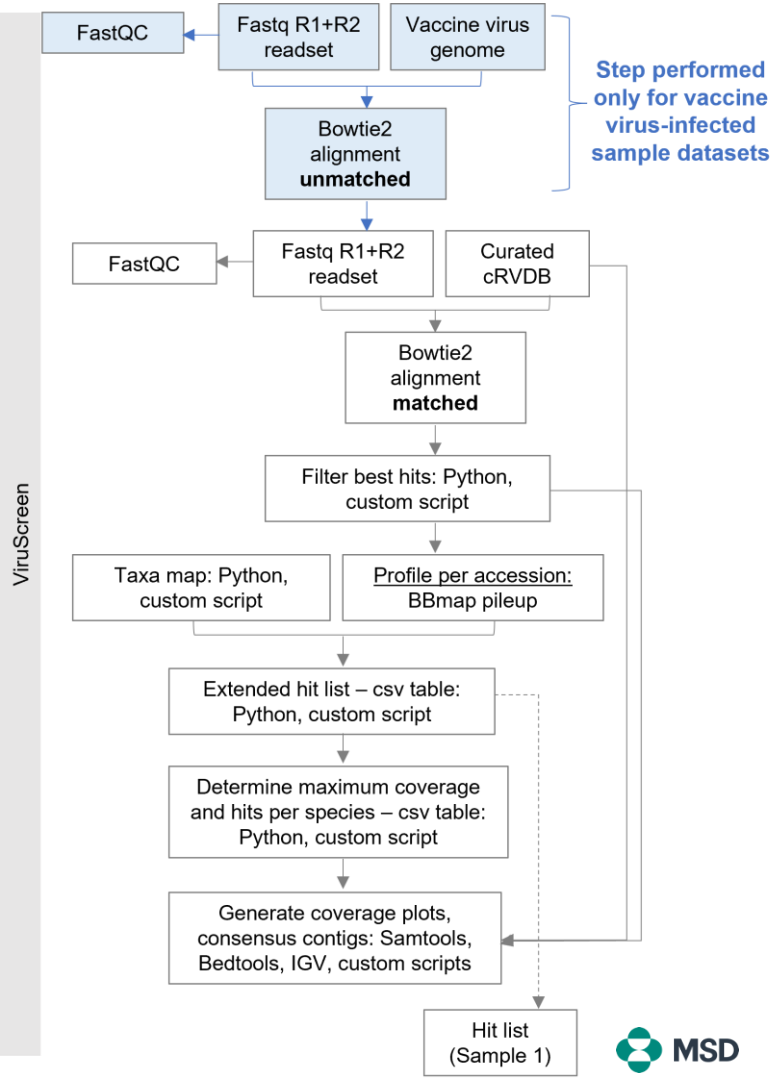
Automated SRA workflow in ViruScreen



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SRA case-study: Benchmarking ViruScreen GMP against HIVE

- **Purpose**: Analyze data sets representing different types of data and sample preparations to inform on the sensitivity and specificity across HIVE and ViruScreen workflows.
- **Data**: Research datasets spiked with known virus amounts or virus sequences for comparison.
- **Analysis**: Perform short read alignments (SRA) of different spiked datasets to the cRVDBv20 with added curation.
  - HIVE alignments performed with Hexagon
  - ViruScreen alignments performed with Bowtie2
- **Results**: Determine the recovery of the spiked viruses / virus sequences by maximum total hits and coverage length

Datasets used in the HIVE–ViruScreen comparison study

In silico-spiked data

- Concentrated uninfected media
- Background sequence reads
- Spiked *in silico* with 20 virus sequences from five virus genomes.
- ~22 M reads dataset

Internal data

- Research samples corresponding to harvest control fluids **(HCF)** and harvest virus fluids **(HVF)** from an early-phase SARS-CoV2 live viral vaccine generated in **Vero cells**
- Spiked with ~1e5 genome copies / mL of 5 virus panel.
- ~13 M HCF and ~50 M HVF reads

Published data

- **HeLa cells**
- Spiked with four mixed viruses at three levels of viral particles per cell.
- AVDTIG collaborative study. (Lab-C, RNA datasets).
- ~300 M reads each dataset

Khan AS and Study Group Participants. Proposed 1st International Virus Reference Standards for Adventitious Virus Detection in Biological Products by Next-Generation Sequencing (NGS) Technologies (CBER-5). 7 Sep 2020. WHO/BS/2020.2394.

Khan AS, Ng SHS, Vandeputte O, Aljanahi A, Deyati A, Cassart JP, Charlebois RL, Taliaferro LP. A Multicenter Study To Evaluate the Performance of High-Throughput Sequencing for Virus Detection. mSphere. 2017 Sep 13;2(5):e00307-17. PMID: 28932815.

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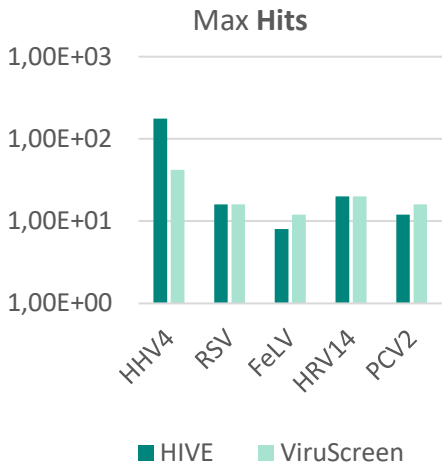
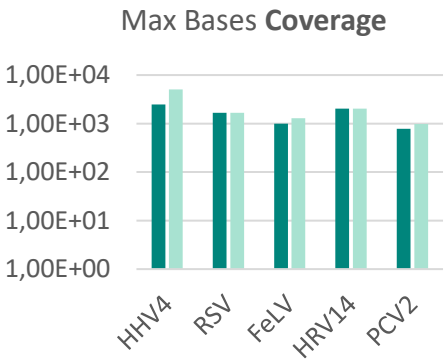
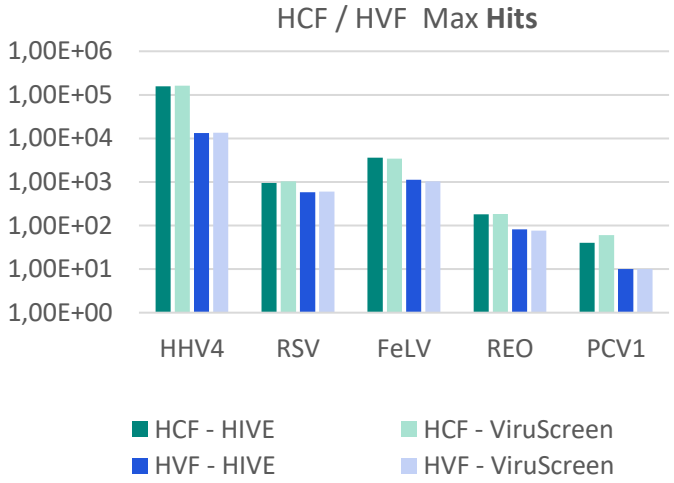
ViruScreen and HIVE workflows result in similar spike virus recovery

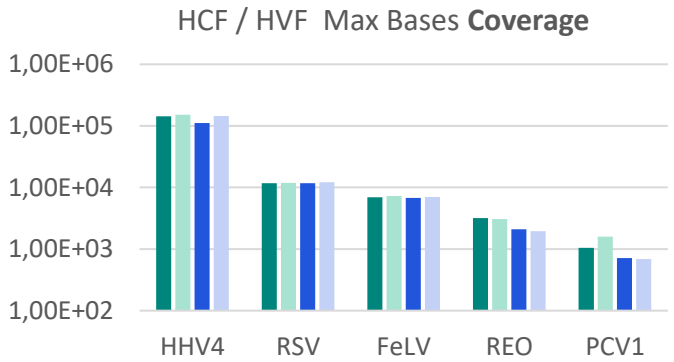
Results of *in silico* virus sequences



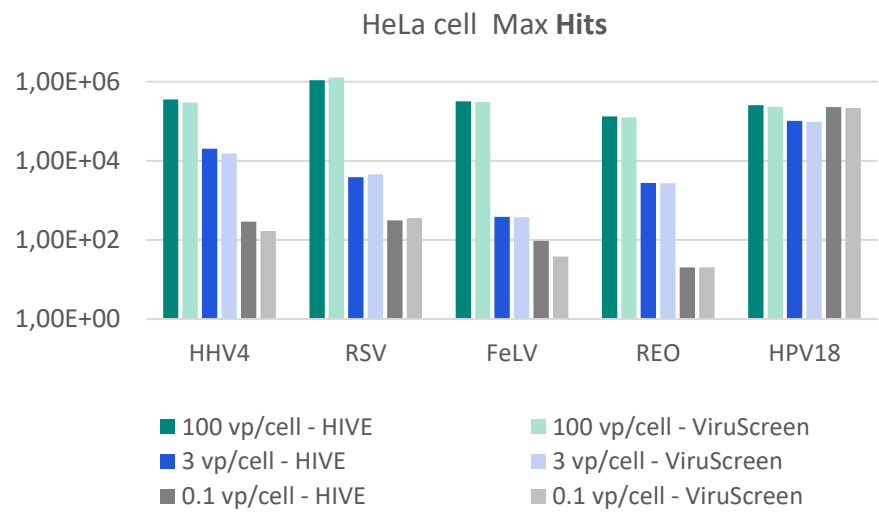


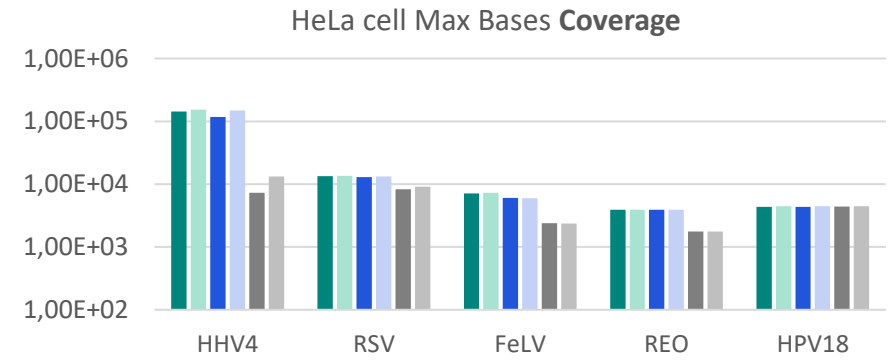
Virus panel spiked into CoV2 live virus vaccine samples





AVDTIG Lab-C datasets (virus spiked into HeLa cells)

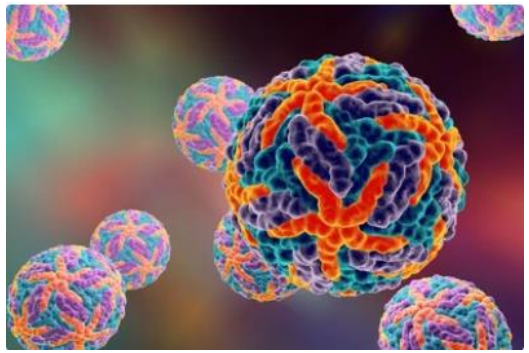




- **Both workflows led to similar recovery (~10^3 bases) from *in silico* reads corresponding to different viruses.**
- **Viruses spiked into samples and processed internally or those from a published study also exhibited similar recovery.**

HIVE-ViruScreen research study using dengue live virus vaccine samples

- V181 is an investigational live attenuated quadrivalent dengue vaccine produced in Vero cells
- Non-GMP samples of V181 dengue 3 vaccine and controls used for
  - A spike recovery study using the WHO/FDA/CBER virus panel
  - Unspiked samples used to generate research results to understand the workflows
- Short read alignments (SRA) of readsets to a curated, clustered RVDBv25 using HIVE and ViruScreen
- *De novo* assembly alignments performed in ViruScreen to compare with SRA
  - Assemble short reads into contigs with Megahit
  - Align contigs to the reference using Magic-BLAST



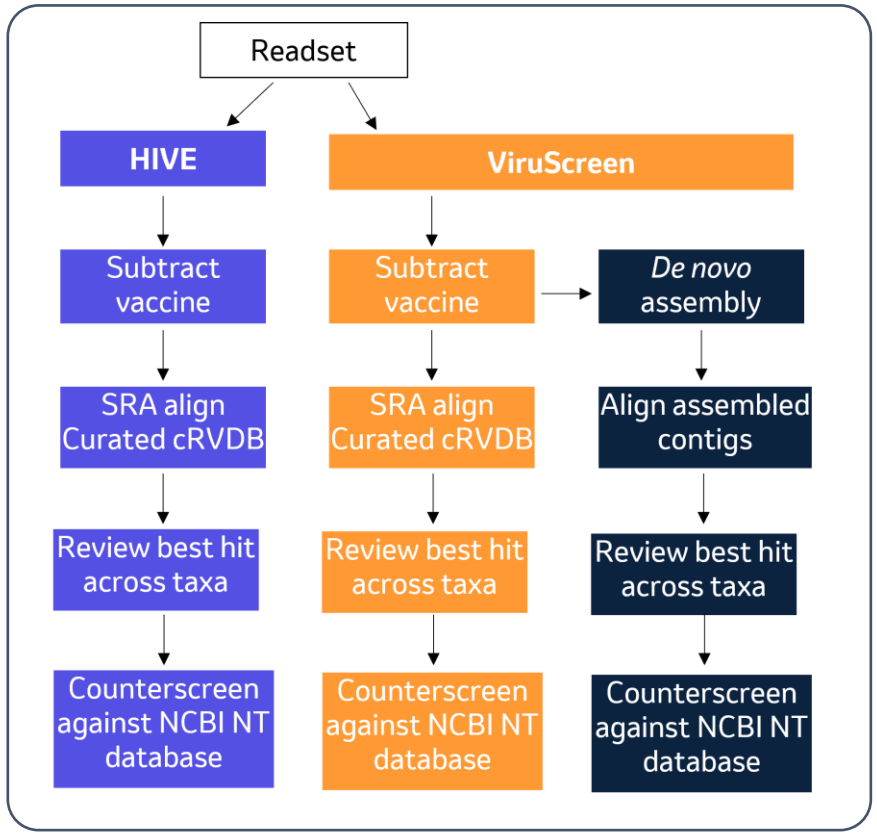

Dengue virus vaccine VMA research study design

Samples and controls to be analyzed in research study

Sample name	Condition*	Purpose	Sample treatment	Extraction method	Library
Harvest virus fluids (HVF)	Unspiked	Secreted or released viruses	+/- Virus spikes Benzonase digestion	Phenol:Chloroform: Isoamyl alcohol (total nucleic acids)	**NexteraXT**
	Spiked ~10^3 gc/mL				
	Spiked ~10^4 gc/mL				
	Spiked ~10^5 gc/mL				
Harvest control fluids (HCF)	Unspiked				
	Spiked ~10^3 gc/mL				
	Spiked ~10^4 gc/mL				
	Spiked ~10^5 gc/mL				
Control cell pellet	Unspiked	Transcriptome analysis	Trypsinization Wash 2x	Qiagen RNeasy mini kit (RNA) + Ribosomal depletion	**TruSeq mRNA Stranded**
	Spiked (pre-extraction HRSV RNA ~1 gc/cell)				
Medium control	Unspiked	Reagent control	+/- Virus spikes Benzonase digestion	Phenol:Chloroform: Isoamyl alcohol (total nucleic acids)	**NexteraXT**
Buffer control	Unspiked				

*gc/mL: genome copies per mL of fluid sample

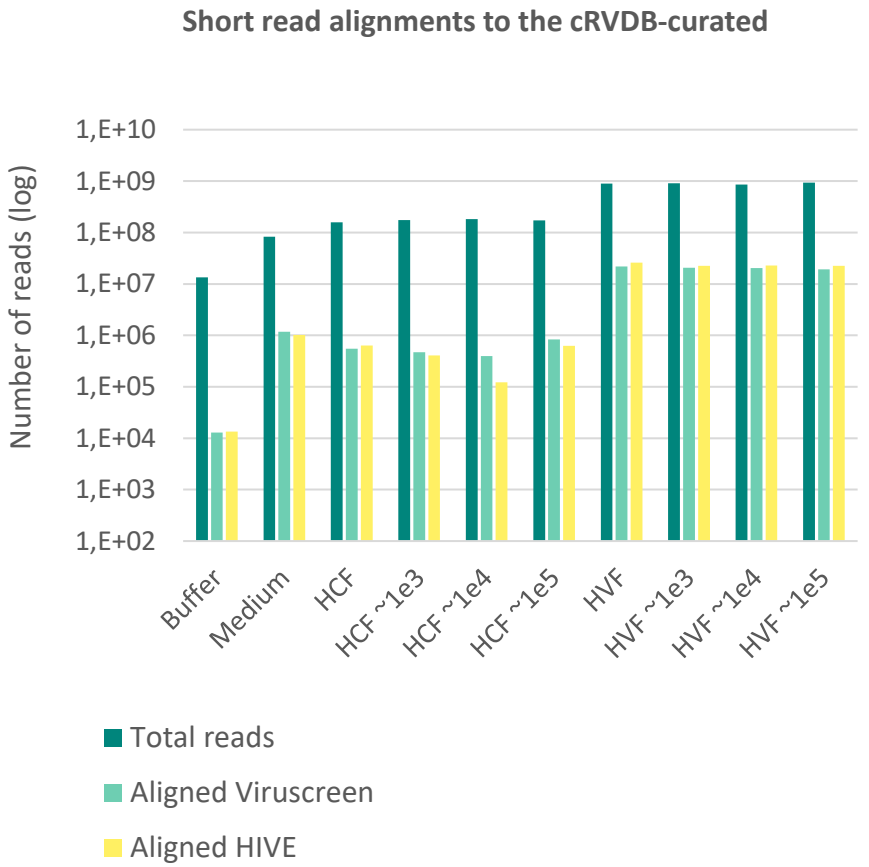
Study workflows



- **Evaluate readsets in SRA: run in parallel in both HIVE and ViruScreen.**
- **Assess *de novo* assembled contig analysis in ViruScreen.**

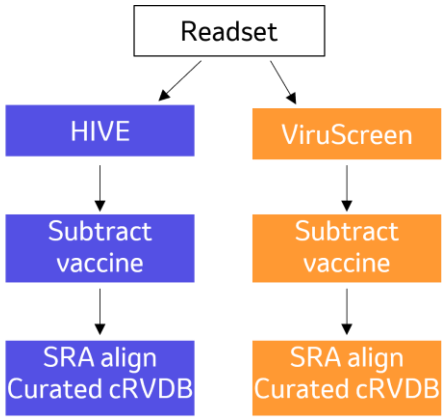
Sequencing yield and short read alignments

Sample name	Condition	Total Reads	ViruScreen vaccine-subtracted unaligned	ViruScreen aligned to cRVDB	HIVE vaccine-subtracted unaligned	HIVE aligned to cRVDB
Harvest virus fluids (HVF)	Unspiked	897,683,136	350,189,438	21,816,410	315,625,265	26,075,925
	Spiked ~10^3 gc/mL	902,878,748	400,753,954	20,561,490	365,580,953	22,570,006
	Spiked ~10^4 gc/mL	862,157,904	320,467,052	20,276,366	286,466,278	22,799,702
	Spiked ~10^5 gc/mL	931,119,204	396,219,060	19,321,400	366,443,715	22,406,014
Harvest control fluids (HCF)	Unspiked	157,024,866	n/a	554,054	n/a	638,168
	Spiked ~10^3 gc/mL	174,846,764	n/a	469,026	n/a	406,406
	Spiked ~10^4 gc/mL	182,711,880	n/a	394,130	n/a	122,296
	Spiked ~10^5 gc/mL	173,280,070	n/a	831,432	n/a	625,580
Control cell pellet	Unspiked	835,769,224	n/a	18,514	n/a	N.P.
	Spiked (pre-extraction HRSV RNA ~1 gc/cell)	843,169,168	n/a	21,004	n/a	N.P.
Medium control	Unspiked	82,745,728	n/a	1,182,448	n/a	1,013,066
Buffer control	Unspiked	13,405,896	n/a	12,966	n/a	13,480

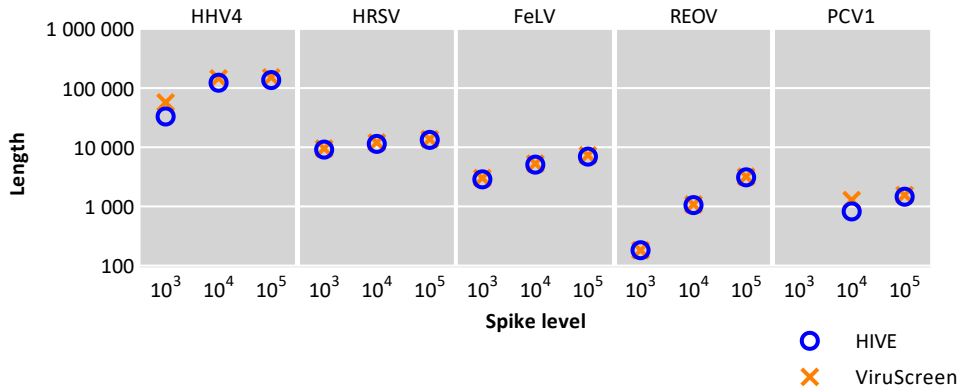


- **The readsets generated for the benchmarking study exhibited similar alignment to both the vaccine virus, where applicable, and to the curated cRVDB**

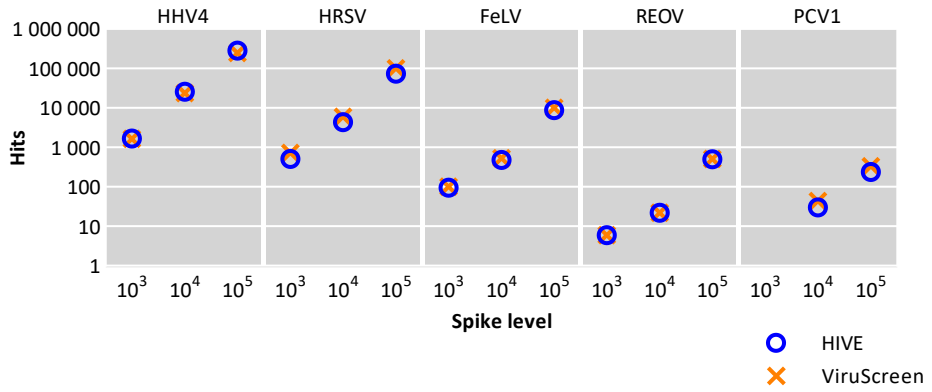
Spike virus analysis of dengue HVF and HCF in HIVE and ViruScreen



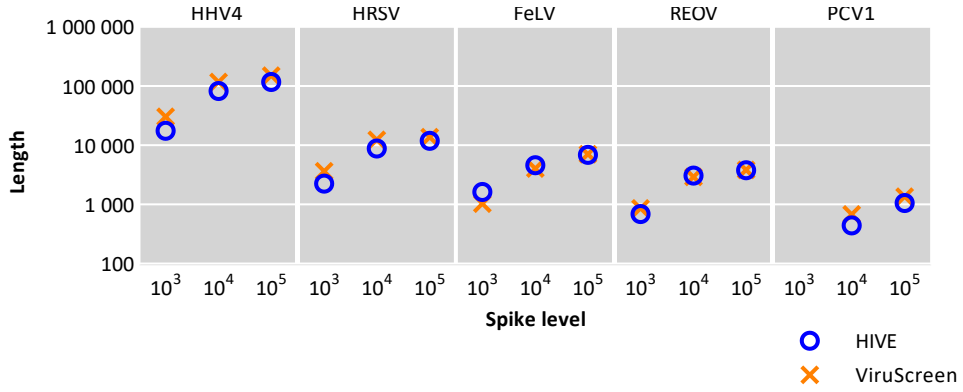
Harvested control fluids **max bases covered**



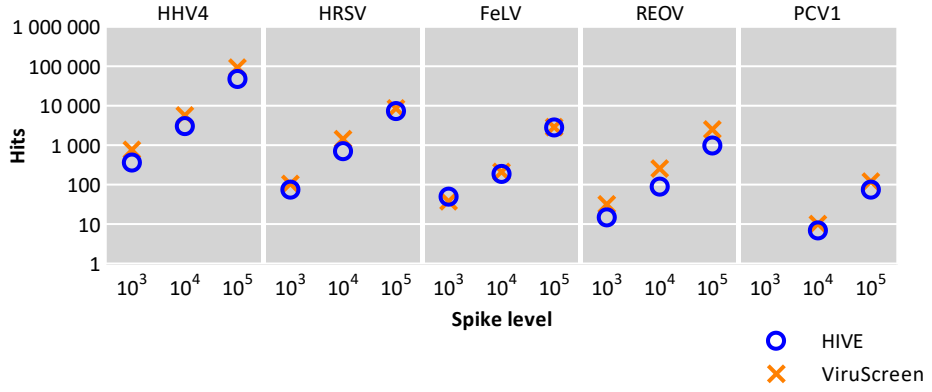
Harvested control fluids **max hits**



Harvested Virus fluids **max bases covered**

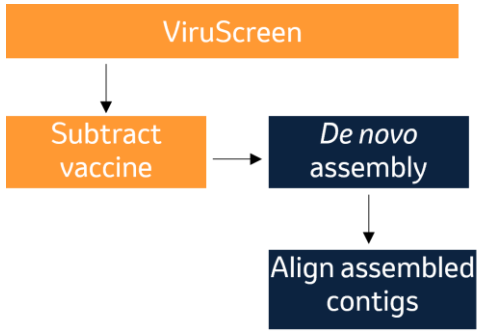


Harvested virus fluids **max hits**



- **Limit of detection is ~10^3 spike level for all viruses except PCV1 (~10^4 genome copies / ml) across both HIVE and ViruScreen for both uninfected and infected cell fluids.**

Spike virus analysis of *de novo* assembled contig alignments in ViruScreen



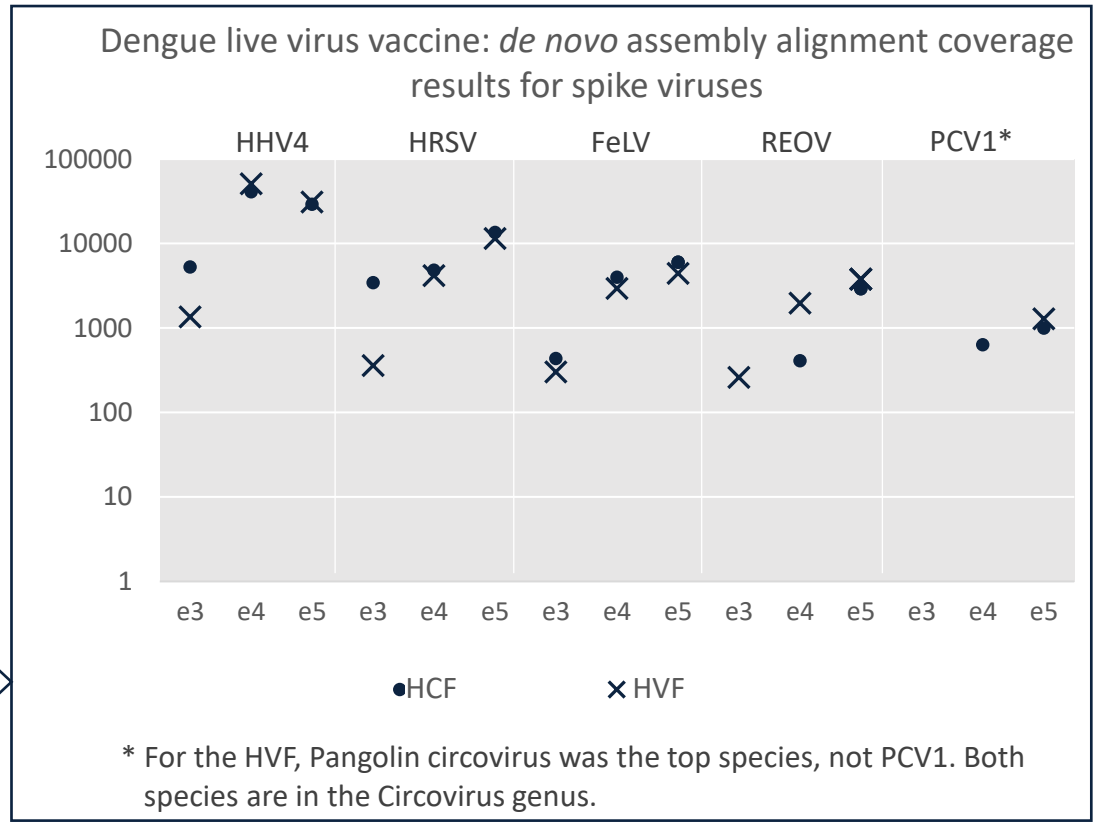
Assembled alignments

Limit of detection:

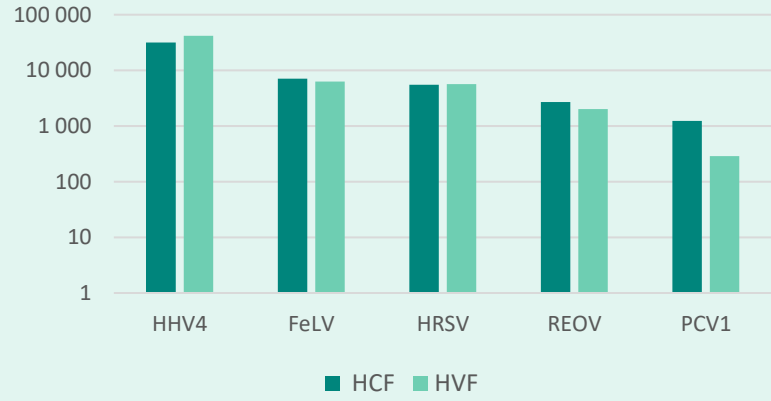
~10^3 gc/mL: HHV4, HRSV, FeLV

~ 10^4 gc/mL: REOV

~ 10^5 gc/mL: Circovirus



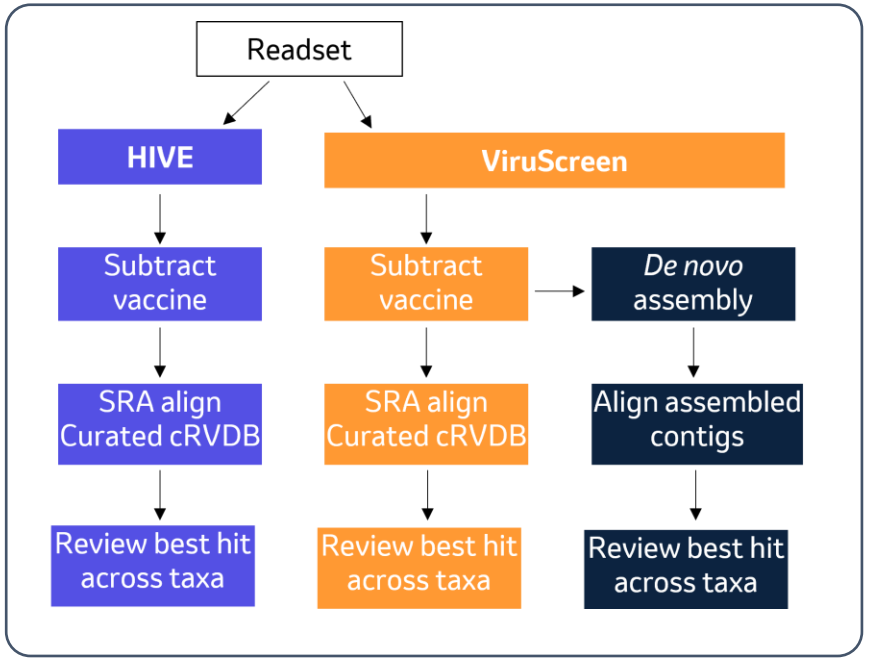
* For the HVF, Pangolin circovirus was the top species, not PCV1. Both species are in the Circovirus genus.



Analysis of the CoV2 live vaccine readsets (~10^5) used in the SRA benchmarking study indicates that PCV1 species is recovered following *de novo* contig alignment to the curated cRVDB, in addition to other Circovirus spp.

- **Across sample readsets, spike recovery following *de novo* assembly is not as sensitive as short read alignments.**
- **For *de novo*-assembled CoV2 live vaccine sample readsets, PCV1 was detected at ~ 10^5 gc/mL**

Analysis across unspiked samples for different applications and workflows

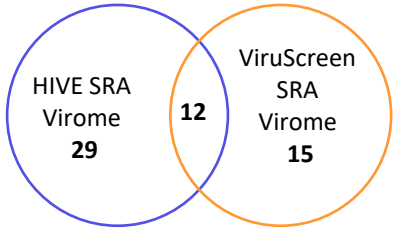


Number of best hits across taxa: species and genus

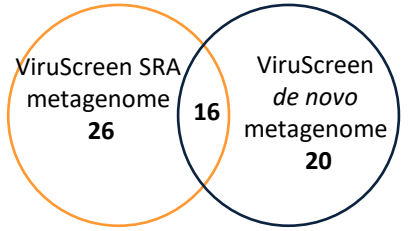
	HIVE SRA	ViruScreen SRA	ViruScreen *de novo* alignments
Number of *species* – virome	29	15	6
Number of genera – virome	11	8	6
Number of *species* –metagenome	n.d.	26	20
Number of genera – metagenome	n.d.	11	10

n.d. not determined

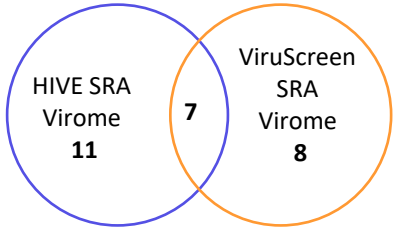
Number of species



Number of species



Number of genera



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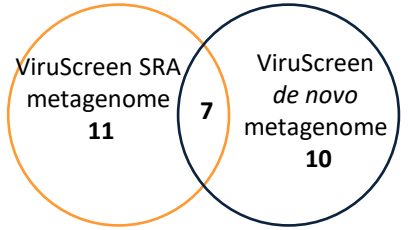
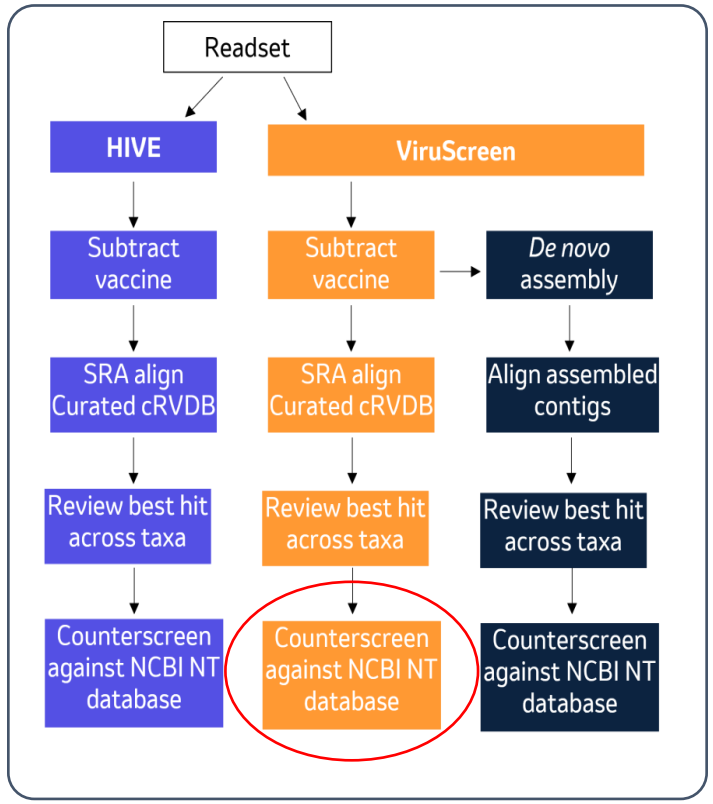


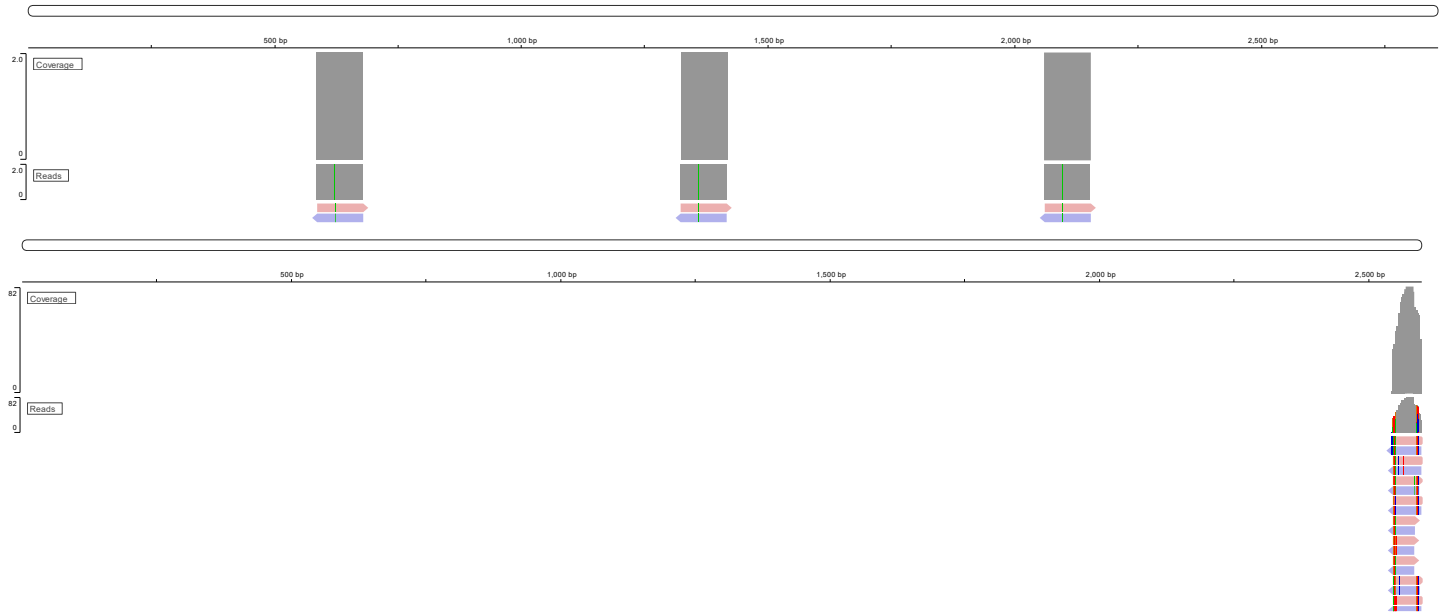
- SRA across unspiked samples HIVE returned the most hits
  - Presumably partially due to lack of paired read filtering in HIVE workflow.
- In ViruScreen, the SRA leads to higher number of putative hits to counterscreen than does the *de novo* alignment workflow.

Example I: Counterscreening of a hit that was *not* bioinformatically confirmed



Family	Genus	Best hit species within Genus	Maximum of total bases covered
			Harvest Virus Fluids
Retroviridae	Lentivirus	HIV-1	279

Example coverage plots (different accessions)



Arguments against specificity to HIV-1:

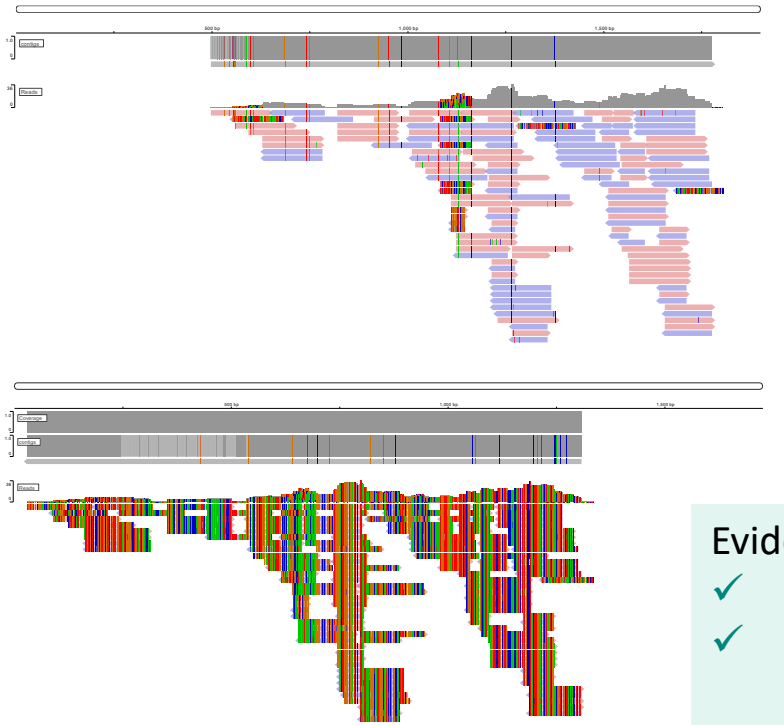
- ☒ Representative BLASTX alignments demonstrate best match to non-viral accessions.
- ☒ Representative BLASTn and BLASTx alignments to primate proteins or lentiviral cloning vector
- ☒ No coverage of uniquely-HIV-1 sequences

Example II: Counterscreening a hit of a true positive (HVF spiked sample)

Family	Genus	Best hit species within Genus	Maximum of total bases covered
			Harvest Virus Fluids spiked ~1e5 gc/mL
Circoviridae	Circovirus	Pangolin Circovirus B2	1,279
Circoviridae	Circovirus	Pangolin Circovirus T2	1,279

Spiked samples contain true positive hits – use as example to show counterscreening to confirm best match

Example coverage plots to ViruScreen results



- Perform BLASTx
- Perform BLASTn

BLASTn results taxonomy tree indicates several Circovirus species

Evidence to bioinformatically confirm the result

- ✓ Representative BLASTx alignments have best match to PCV1 replicase
- ✓ Representative BLASTn alignments demonstrate high match scores to several Circovirus species.

Conclusions

- Viral Metagenomic Analysis (VMA) studies have been undertaken to supplement the virus risk assessment for select live virus vaccines and cell banks.
- HIVE bioinformatic analyses have been successfully filed as characterization studies.
- An internal application for customized workflows was created to streamline bioinformatic analysis for potential future release of GMP materials.
- Comparison across workflows provides opportunity to benchmark bioinformatic methods.
- Large datasets from the dengue live vaccine samples were generated to complement the initial comparisons between HIVE and ViruScreen which exhibited similar performance for spiked virus recovery.
- Additionally, testing of the end-to-end workflow led to identification of successes and opportunities to leverage in studies for filing of advanced virus detection methods for biological products.
- Further automation of largely manual analysis such as counterscreening and comparative analysis in ViruScreen will facilitate future applicability for product-specific release or GMP assays.

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Thank you