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A Collaborative Study to Evaluate the Proposed First WHO International Reference Panel for Adventitious Virus Detection in Biological Products by High-throughput Sequencing (HTS) Technologies

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NOTE:
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Summary

Ten laboratories from seven countries have participated in a collaborative study for evaluating the suitability of 7 reference virus stocks proposed as the 1st WHO international reference panel for adventitious virus detection in biological products by high-throughput sequencing (HTS). Five participating laboratories have provided short-read HTS data for timely submission of this report, which supports the establishment of the 7 virus stocks as a reference panel for platform evaluation and validation of HTS for adventitious virus detection. The viruses were selected to represent diverse virus families with distinct physicochemical and genome properties for evaluating the capabilities of HTS for broad adventitious virus detection in biologics. The virus panel includes Epstein-Barr virus (EBV; also known as human herpesvirus 4), mammalian orthoreovirus type 1 (REO), human respiratory syncytial virus (RSV), feline leukemia virus (FeLV), porcine circovirus type 1 (PCV1), human betacoronavirus OC43 (hCoV), and minute virus of mice (MVM). The addition of the hCoV and MVM in the virus panel extends the previous 5 WHO reference reagents for HTS adventitious virus detection (established in October, 2020) and the greater number of vials in the virus panel provides long-term availability of HTS reference standards. Similar to the previous study for establishment of the 5 WHO reference reagents, the 7 viruses were spiked together at 10^4 genome copies per mL of each virus into 10^9 genome copies per mL of adenovirus 5 (Ad5) to evaluate the breadth of virus detection by HTS (using targeted bioinformatics analysis) in a high-titer virus back ground mimicking a low-complexity biological sample (with reduced cellular material), for example a viral vaccine seed or a virus vector preparation.

Each laboratory used a common protocol for preparing the spiked samples and then followed their own protocols for the HTS workflow (sample processing, cDNA synthesis, library preparation, sequencing, and bioinformatics analysis). The results presented in this report, indicated that all 7 viruses were detected at the 10^4- 10^5 spiked level by the 5 laboratories. The difference in virus detection between the labs was a reflection of the different protocols used in the HTS workflow. No significant differences were seen in the results from two labs which performed three independent HTS experiments indicating reproducibility of the technology. The study demonstrates the suitability of the 7-virus panel for platform evaluation and validation studies using HTS for broad viral adventitious agents detection of known and unknown viruses. Furthermore, with the increased interest in using HTS since the publication of the revised ICH

Q5A(R2), the use of international virus reference standards is critical to facilitate development of optimized HTS protocols and to aid global harmonization of regulatory review of submissions with HTS data.

Introduction

High-throughput sequencing (HTS; commonly referred to as next-generation sequencing) has demonstrated capabilities of this advanced technology for broad virus detection including known and novel viruses [1, 2]. The discovery of porcine circovirus type 1 (PCV1) in a licensed rotavirus vaccine [3] and finding of a novel rhabdovirus in the Sf9 insect cell line used for baculovirus-expressed products [4] brought HTS technologies to the attention of regulators and industry as a potential new method for detection of known and novel adventitious viruses for the viral safety evaluation of biologics. The use of HTS as an alternative method for the conventional adventitious virus detection assays is encouraged in guidelines from the WHO [5] and European Pharmacopeia [6, 7] and there is also flexibility provided in the FDA guidance [8]. Additionally, the recently published ICH Q5A(R2) includes the use of a validated HTS method for replacing the *in vivo* animal assays and PCR assays, and for replacing or supplementing the *in vitro* cell culture assays [9]. The use of HTS to replace the *in vivo* assays also accomplishes the goal of the 3Rs regarding reducing animal use [10].

The use HTS as an alternative method for assessing the viral safety of biologics has been discussed in the International Alliance for Biological Standardization (IABS) meetings on HTS technologies that have covered human biologics and veterinary products and were attended by regulatory agencies, industry (biologics and biotherapeutics), contract research organizations (CROs), and academia [11]. In fact, the 3rd IABS meeting discussed HTS for replacing the *in vivo* animal assays and the *in vitro* assays particularly to address some of the limitations, such as when vaccine virus cannot be neutralized. Furthermore, replacement of the *in vivo* and *in vitro* assays by HTS can reduce the testing period, which can take 1 month or longer [12]. The critical use of HTS standards for qualification and validation was recognized and this was also discussed in detail at the NIST/FDA workshop in which different types of viral standards were discussed including live viruses which can test the entire HTS workflow as well as other types of standards (non-infectious) that could be used to test various steps in the HTS workflow [13].

The need for viral standards for the implementation of HTS as an alternative assay for adventitious virus detection in biologics was recognized early on by the FDA/Industry-led Advanced Virus Detection Interest Group (coordinated by the Parenteral Drug Association, PDA) [14]. An initial collaborative study involving three laboratories used commercially-available, high-titer, virus stocks to test feasibility of the selected viruses as reference materials for demonstrating broad virus detection by different HTS platforms in different matrices [15]. The viruses were selected to represent virus families with diverse physical and chemical

properties including: enveloped and non-enveloped viruses with low, moderate, and high chemical resistance to test the efficiency of nucleic acid extraction methods; different genome types (double and single-stranded DNA and RNA, linear and circular) to test the cDNA synthesis and library preparation steps; and different genome sizes (large and small) to test capabilities of the sequencing platforms and bioinformatics tools and pipelines [16, 17]. The results from this study, which indicated that similar virus detection was obtained by the three laboratories regardless of their independent protocols and pipelines, provided a scientific basis for the preparation of well-characterized five virus reference virus stocks for HTS qualification and validation to facilitate its implementation for adventitious virus detection in biologics. The establishment of these viruses as WHO reference reagents for HTS adventitious virus detection by the WHO/ECBS in October, 2020, was based on the report from an international collaborative study involving eight laboratories that used independent protocols for the entire HTS workflow to evaluate the maximum differences that could be expected for virus detection, without having established shared protocols for the technical and bioinformatics steps in HTS. The results indicated all five viruses were detected using HTS targeted bioinformatics analysis at the test spiked level of 10^4 genome copy per milliliter (GC/mL) in a high-titer virus background of 10^9 GC/mL adenovirus 5 (Ad5), mimicking a vaccine or viral vector sample and supports the use of the five reference virus stocks to demonstrate the capabilities of HTS for broad virus detection for HTS standardization and validation studies.

There has been a growing interest from manufacturers to use HTS for adventitious virus detection in biologics. The availability of the current WHO reference reagents has enabled qualification and validation studies of HTS for implementation of HTS as an alternative assay to replace the *in vivo* assays and PCR assays and to replace or supplement the *in vitro* cell culture assays. An increase in demand for the virus standards is expected due to the publication of the ICH Q5A(R2), which encourages the use of HTS for adventitious virus detection, particularly for replacement of the animal assays. A larger stock of HTS virus standards will provide long-term availability and meet the global demands for HTS implementation.

Aims of the study

This study was designed to evaluate the suitability of the proposed 1st WHO international reference panel comprising of 7 virus stocks to demonstrate the capability of HTS for broad adventitious virus detection in biological samples. The panel represents diverse virus families with distinct physical, chemical and genome properties for validating HTS and expands the virus families of the currently available 5 WHO reference reagent viruses. The study evaluated different HTS platforms for broad virus detection in a sample mimicking a vaccine viral seed or viral vector preparation. This was accomplished by spiking the 7 viruses in the panel as a mix at 10^4 GC/mL into a high titer background (10^9 GC/mL) of Ad5. The study design was the same as

that used for the establishment of the WHO reference reagent viruses. To test the real-world situation for manufacturers applying HTS for viral safety in biologics, the study participants prepared their spiked samples based on a shared SOP and then followed their own independent in-house SOPs for testing the entire HTS workflow.

Study Group

The collaborators for the spiking study group were invited from the Advanced Virus Detection Technologies Interest Group (AVDTIG) and represented different countries/continents, organizations and use of different HTS technologies and platforms to ensure diversity. They included: 1 large manufacturer, 3 contract research organizations (CROs), 2 academic labs, and 4 regulatory/control lab agencies. The individual participants, laboratories, countries and role in the current study are listed in Appendix A.

The characterization of the individual 7 viruses in the panel was done by the CBER lab using Illumina HTS and ddPCR assays. This included generating the consensus full-length reference virus genomes, identification of virus variants and investigating adventitious viruses; quantification of viral and residual host cell DNA sequences by ddPCR assays. One-year stability study was done under an FDA/CBER contract at ATCC for infectious titer and viral GC/mL.

Materials and Methods

Candidate Virus Panel

The 7 viruses in the panel (Table 1) were selected to represent different virus families of potential safety concern in vaccines and potentially in other biologics and consisted of distinct physical, chemical and genome properties to evaluate the entire HTS workflow including virus extraction, cDNA synthesis, sequencing, and bioinformatics pipelines.

Table 1. Reference virus properties.

Virus	Genome type	Genome size	Particle size	Envelope	Chemical resistance
REO	RNA, double-strand; Linear (segmented)	23.6 kb (1,196 – 3,915 nt	80 nm	No	Medium - high

FeLV	RNA; single-strand; Linear (dimeric)	8.5 kb	80-100 nm	Yes	Low
RSV	RNA; single-strand; linear	15 kb	150-200 nm	Yes	Low - medium
PCV	DNA, single-strand; circular	1.8 kb	16-18 nm	No	High
EBV	DNA, double-strand; Linear	172 kb	122-180 nm	Yes	Low - medium
hCOV	RNA, single-strand; Linear	30.7 kb	80-120 nm	Yes	Low
MVM	DNA, single-strand; Linear	5.1 kb	26 nm	No	Medium-High

The viruses used in an initial collaborative study with 3 labs [15] were purchased from a commercial laboratory that had obtained the viruses from the American Type Culture Collection (ATCC, Herndon, VA, U.S.A.). These same five virus strains were prepared under an FDA/CBER contract with ATCC for generating the WHO international reference reagents using cell lines available at ATCC. The viruses were: Epstein-Barr virus strain B95-8 (EBV), mammalian othoreovirus type 1 strain Lang (REO), human respiratory syncytial virus strain A2 (RSV), and feline leukemia virus strain KT-FeLV-UCD-1 (FeLV)]. The same reagents (viruses and cell lines) were used for the preparation of these 5 viruses in the 7-virus panel being evaluated in this study, with additional ATCC reagents used for the betacoronavirus OC43 (hCOV) and minute virus of mice (MVM) (Table 2).

Table 2. Virus strains and cell lines for propagation.

Virus	**Strain (ATCC catalogue number)**	**Cell line used for propagation (ATCC catalogue number)**
REO	Lang (VR-230)	LLC-MK2 (CCL-7.1)
FELV	KT-FeLV-UCD-1 (persistently infected cell line-CRL-8012)	FL74-UCD-1(CRL-8012)

RSV	A2 (VR-1540)	Hep-2 (CCL-23)
PCV	Type 1 (persistently infected cell line – CCL-33)	PK-15 (CCL-33)
EBV/HHV4	B95-8 (persistently infected cell line – CRL-1612)	B95-8 (CRL-1612)
hCoV	OC43 (VR-1558)	HRT-18G (CRL-11663)
MVM	Prototype (p) (VR-1346)	A9 (CCL-1.4)

Virus preparation

The preparation and vialing of the viruses were done under an FDA/CBER contract at ATCC. Virus specifications (minimum requirements) were provided to ATCC for infectious titer and genome copy number. Each virus was grown separately under rigorously handling conditions to avoid cross-contamination. The virus strains and cells used for their propagation are indicated in Table 2. Viruses were grown in cells using culture conditions to maximize virus yield and were harvested, clarified to remove cellular debris and filtered (except RSV and PCV1 were clarified but not filtered to reduce adverse impact on the virus titer), prior to pelleting through a 20% sucrose cushion (RSV and FeLV) or without a sucrose cushion (HCOV, PCV-1, REO, and MVM); EBV was precipitated using 10% polyethylene glycol (due to the large volume of the harvest material). Viruses were further purified using discontinuous (step) sucrose density gradients (20%/60% for hCOV, REO and FELV, 30%/60% for RSV, and 20/70% for MVM, EBV, and PCV-1). All of the viruses were nuclease-treated prior to the purification step to reduce host cellular nucleic acids. Batches were pooled to obtain final specifications for infectious titer and genome copy number. Manufacturing details are provided in Appendix B-1.

Vialing and storage

The viruses are in a liquid final formulation of 10 mM Tris-HCl, 135 mM NaCl, 619 0.5% BSA, 5% Trehalose. Viruses were vialed individually in aliquots $\geq$ 0.5 mL and stored at -70 °C or colder at ATCC. The material was stored in 1.2 mL cryogenic screw top polypropylene tubes to withstand long-term storage at low temperature (Thermo™ Scientific Nalgene™, catalog number 5012-0012). The tubes are externally threaded (internally for the screw cap) to enhance aseptic techniques and along with the sealing ring permits secure closure. The vials are currently stored at the NIH/NIAID BEI Resources Repository, an international repository and distributor of cells and viruses, with temperature control and monitoring facilities.

At least 1000 vials of each virus were prepared and individually vialed (Table 3) to allow generation of different spiked levels by the user.

Table 3. Virus IDs.

Item no.	Product name
FSCUST-90	Purified betacoronavirus 1 (hCoV), OC43 (FDA)
FSCUST-91	Purified porcine circovirus (PCV1), type 1 (FDA)
FSCUST-92	Purified mammalian orthoreovirus (MRV), Lang (FDA)
FSCUST-93	Purified feline leukemia virus, Theilen (FDA)
FSCUST-94	Purified Epstein-Barr virus, b95-8 (FDA)
FSCUST-95	Purified respiratory syncytial virus, A2 (FDA)
FSCUST-96	Purified minute virus of mice, prototype (FDA)

Post-fill Testing and characterization

Virus testing was done on the vialed (Details are provided in Appendix B-2). This included sterility and mycoplasma testing, determination of infectious titer, and genome copy number (determined based on 9 replicates) by ATCC. Genome copy numbers were confirmed independently in the CBER lab using the same ddPCR assays. Bacterial and fungal contamination was tested using BacTALERT and mycoplasma was screened by PCR. Infectious titer was quantified in sensitive cell lines based on CPE, immunofluorescence, PCR or transformation assays based on the virus. The ATCC Certificate of Analysis (COAs) with the results for each of the viruses is attached in Appendix C. It should be noted that the cell lines used for EBV and PCV1 also produced squirrel monkey retrovirus (SMRV) [18] and porcine endogenous retrovirus (PERV) [19], respectively. The genome copy number of these "carry-over" viruses and the reference viruses were determined using RT-ddPCR assays by CBER (see footnote in Table 4).

Additionally, residual host cell DNA was quantified by ddPCR assays in the CBER lab. The consensus sequences of the virus genomes and variant analysis were done by HTS in the CBER lab as well as analysis for adventitious viruses. Total reference virus particle counts were determined using negative stain electron microscopy by SGS Vitrology (data not shown). No other viruses, virus-like particles, fungi, yeasts, mycoplasmas or bacteria were observed. The results of the characterization of the virus panel are shown in Table 4.

Table 4. Characterization of the Reference Virus Panel

Virus	Infectious titer ($TCID_{50}$/mL)	Reference Virus genome copies (GC/mL)	Host cell DNA (copies per mL)
hCOV	1.58×10^7	2.64×10^{10}	1.64×10^4
PCV1[a]	2.3×10^6	8.1×10^9	9.81×10^3
REO	8.9×10^8	1.50×10^{10}	<LOD
FELV	2.0×10^7	4.0×10^{10}	2.05×10^5

EBV[b]	1.1×10^6	2.8×10^7	9.59×10^4
RSV	5.00×10^6	5.53×10^{10}	1.54×10^6
MVM	1.58×10^7	1.18×10^{10}	3.50×10^5

[a]PERV, 1.08×10^9 GC/mL
[b]SMRV, 1.80×10^{10} GC/mL

Stability testing

Real-time stability studies are shown in Table 5. One-year stability testing of the 7 viruses in the panel was performed at ATCC for infectious titer and virus genome copies. Genome copy number was determined by ddPCR assays of 9 replicates. The results show the viruses were stable under the storage conditions (-70 °C or colder)

Table 5. Stability studies. One year stability study was done for infectious titer and genome copy number.

Virus	Infectious titer ($TCID_{50}$/mL)		Virus genome copies (GC/mL)	
	2021-2022	2022-2023	2021-2022	2022-2023
hCOV	1.58×10^7 (December 2021)	8.89×10^6 (October 2022)	2.64×10^{10} (December 2021)	2.68×10^{10} (October 2022)
PCV1	2.3×10^6 (June 2022)	1.6×10^6 (July 2023)	8.1×10^9 (June 2022)	1.5×10^{10} (June 2023)
Reo	8.9×10^8 (May 2022)	2.8×10^6 (May 2023)	1.50×10^{10} (May 2022)	1.46×10^{10} (May 2023)
FeLV	2.0×10^7 (June 2022)	1.6×10^7 (June 2023)	4.0×10^{10} (June 2022)	2.6×10^{10} (June 2023)
EBV	1.1×10^6 (April 2022)	6.32×10^5 (April 2023)	2.8×10^7 (April 2022)	3.07×10^7 (April 2023)
RSV	5.00×10^6 (December 2021)	1.58×10^6 (December 2022)	5.53×10^{10} (December 2021)	3.22×10^{10} (December 2022)
MVM	1.58×10^7 (September 2021)	1.58×10^7 (November 2022)	1.18×10^{10} (October 2021)	4.46×10^9 (November 2022)

Additionally, the 5 viruses established as WHO reference reagents have shown stability for 6 years (last time point tested) under the same storage conditions as the proposed WHO reference virus panel.

Stability at different temperatures and accelerated degradation studies are not planned for the IS since the unitage is genome copy number and nucleic acids are known to be stable at various temperatures. The viruses are provided in liquid formulation and therefore no reconstitution assessment is needed

to be performed. The storage vials are the same as those previously used for the WHO reference reagents, which have shown stability (infectious titer and GC/mL) for 6 years and therefore no vial integrity studies are planned.

Current inventory and succession plan

The primary purpose of these 7 virus stocks is to be provided for use as a reference virus panel for demonstrating specificity, sensitivity and breadth of virus detection for qualification and validation of HTS, which is needed for implementation of this technology for adventitious virus detection in biologics. So far, only 1 virus panel comprising one vial of each of the 7 viruses has been distributed to each of the study participants to conduct this collaborative study for evaluating the virus panel as the proposed 1st WHO virus reference panel for HTS adventitious virus detection. There is much interest in acquiring the virus panel due to the additional two virus families, which extend the currently used 5 WHO reference virus reagents. The number of requests are expected to increase due to the greater interest in using HTS due to its incorporation into the revised ICH Q5A(R2) guideline for replacing the *in vivo* animal assays and to supplement or replace the *in vitro* adventitious agents detection assays. Additionally, there is great interest for using HTS to shorten the adventitious virus testing time for accelerating new product development including SARS-CoV-2 and other vaccines.

The virus panel is currently in a private collection in the BEI Resources Repository (https://www.niaid.nih.gov/research/bei-resources-repository) and will be available in their public catalogue after the Expert Committee on Biological Standardization (ECBS) meeting in March 2024, where the proposed virus panel will be presented for adoption as the "1st WHO International Reference Panel for Adventitious Virus Detection by HTS". The current inventory is indicated as of Sept. 2023 (Table 6). The number of vials are less than the number prepared since some were used for QC and stability studies and characterization of the virus stocks.

Table 6. Current inventory of the proposed WHO reference virus panel.

ID	Product name	Lot no.	Physical Inventory (vials)	Notes
FSCUST-90	Purified betacoronavirus 1 (hCoV), OC43 (FDA)	70047896	953.00	>1000 vials prepared
FSCUST-91	Purified porcine circovirus (PCV1), type 1 (FDA)	70048776	953.00	>1000 vials prepared

FSCUST-92	Purified mammalian orthoreovirus (MRV), Lang (FDA)	70048775	953.00	>1000 vials prepared
FSCUST-93	Purified feline leukemia virus, Theilen (FDA)	70048774	953.00	>1000 vials prepared
FSCUST-94	Purified Epstein-Barr virus, b95-8 (FDA)	70048773	953.00	>1000 vials prepared
FSCUST-95	Purified respiratory syncytial virus, A2 (FDA)	70048772	953.00	>1000 vials prepared
FSCUST-96	Purified minute virus of mice, prototype (FDA)	70044330	944.00	1000 vials prepared

The 7 viruses will be provided under one BEI catalogue number for their intended use as a panel of viruses for demonstrating the breadth and sensitivity of virus detection by HTS. Once the virus panel is available publicly, the current WHO international reference reagents can be retired and continue to be provided for platform evaluation and establishment of HTS technologies by early users as well as for other studies needed for optimization of HTS virus detection in biological materials from different stages of manufacturing containing different complexities, such as residual host cell nucleic acids.

Collaborative Study design

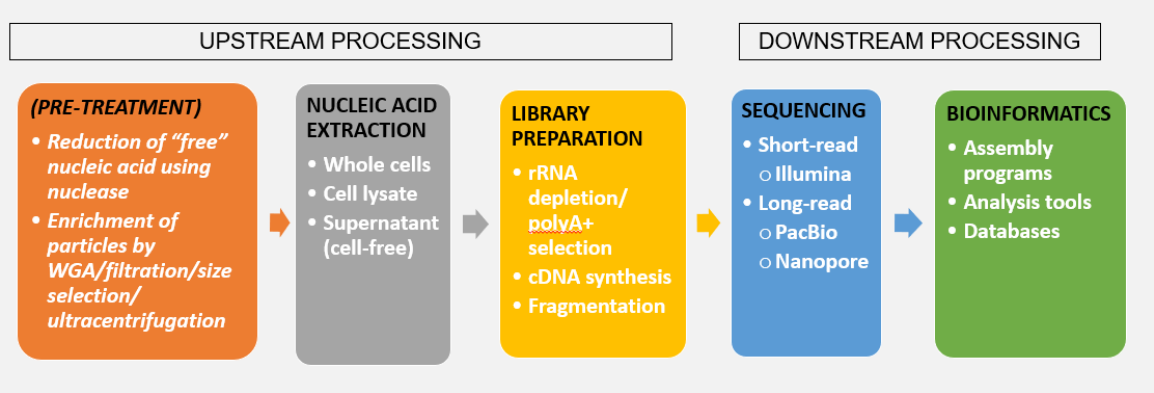
Each laboratory received 1 reference panel containing 1 vial of each of the seven viruses. The study design was developed similar to that previously used to support the establishment of the WHO reference reagents for HTS adventitious virus detection. It was agreed that different spiked levels of the 7 virus stocks would be spiked as a mix into a fixed high-level (10^9 genome copies per mL) of adenovirus 5 (Ad5) to mimic adventitious virus contamination of a high-titer viral vaccine seed or viral vector preparation. The results of the 10^4 spiked level with targeted bioinformatics analysis that were available at the time of this writing this study report are presented to demonstrate representative study results using 2 sequencing platforms (MiSeq and NextSeq) and independent protocols from 1 regulatory agency/2 control labs, 1 CRO, and 1 academic institution. The complete results of the different spike levels and from the other study participants will be presented in the published paper for this study.

The adenovirus 5 (Ad5) used in this study was the same lot as previously used and was the well-characterized Adenovirus Type 5 Reference Material (ARM) made under guidance of the ARM Working Group and US FDA for characterizing adenoviral gene therapy products (ATCC catalogue number VR-1516). The ATCC product information sheet indicated a particle concentration of 5.8×10^{11} particles/mL and infectious titer (HEK 293 cells) of 7×10^{10} NAS IU/mL. The genome copy number of this stock was determined by digital droplet PCR (ddPCR) by one of the collaborating laboratories (3.35×10^{11} copies/ml).

Based on the previous study results, which used low- and high-throughput short-read HTS platforms (Illumina MiSeq and NextSeq, respectively), it was decided that all of the participants would initially test the 10^4 GC/mL level spiked into 10^9 GC/mL Ad5 and could also test other levels of the spiked viruses. It was further decided to use independent protocols and bioinformatics pipelines to demonstrate robustness of HTS for adventitious virus detection under the most variable conditions, which would represent the current real-life scenario since currently there is no consensus for the best protocol for viral detection by HTS. Additionally, detection of the additional retroviruses (SMRV and PERV), which were present in the EBV and PCV1 stocks was also included (data not shown).

Some of the details of the HTS workflow that could influence outcome of the results are shown in Appendix D. These include sample preparation, cDNA synthesis, library preparation, and sequencing platforms. Additionally, the controls to evaluate background signals due to reagents and other materials used in the upstream HTS workflow and the bioinformatics programs and tools used in the downstream workflow are critical for data interpretation regarding the specificity, sensitivity, and breadth of virus detection. The live virus stocks can be used to evaluate the entire workflow (shown in Fig. 1) for detection of a broad range of viruses with diverse properties.

Fig. 1. HTS workflow.



Results

Seven, large-scale virus stocks were prepared and characterized for use in HTS qualification and validation studies. The characterization included: determination of infectious virus titer, genome copy number, determination of full-length virus genomes and variants, and HTS analysis for adventitious virus detection. The results are provided in the ATCC COAs in Appendix C. The HTS analysis indicated no detection of any unexpected viruses: the expected viruses and

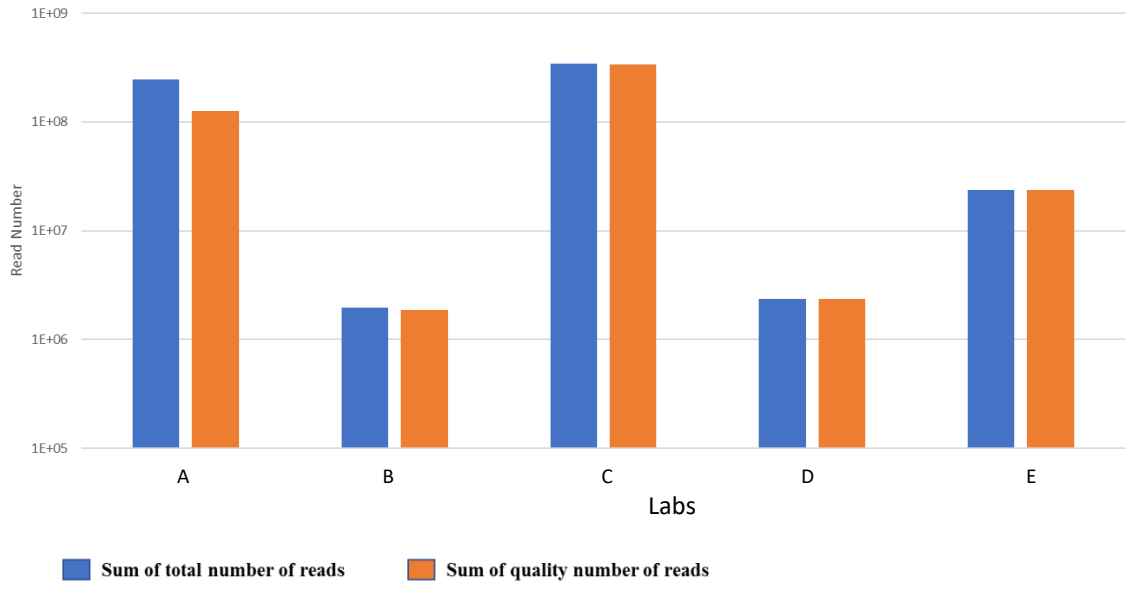
endogenous retroviral sequences due to host cell nucleic acids were detected. Additionally, SMRV and PERV sequences were detected in the EBV and PCV1 virus stocks, respectively, due to the cell lines used in virus propagation.

The 7 virus stocks in the background of high-titer Ad5 were used for conducting spiking studies to evaluate the suitability of the 7 virus stocks as the proposed Reference Virus Panel for HTS validation studies in biologics. The results from 5 participants in the collaborative study for 10^4 genome copies per mL of each of the virus stocks spiked together in the background of 10^9 genome copies per mL of Ad5 are presented in this report. Some of the details of the sample processing are shown in Appendix D. Each lab followed their independent protocols. Controls included medium (with no virus), mix of 7 viruses alone (no Ad5 background), and Ad5 alone.

The results of the critical information for evaluating the suitability of the 7 virus stocks as HTS reference standards for adventitious virus detection are shown in Figs. 2 –7. The results of the spiked sample using Illumina short-read sequencing with targeted bioinformatics analysis are shown.

Different HTS platforms and kits were used for sequencing: three of the laboratories used Illumina NextSeq, a mid-scale sequencing platform, and two used MiSeq, a small-scale sequencing platform.

Fig. 2. Total and QC reads number.

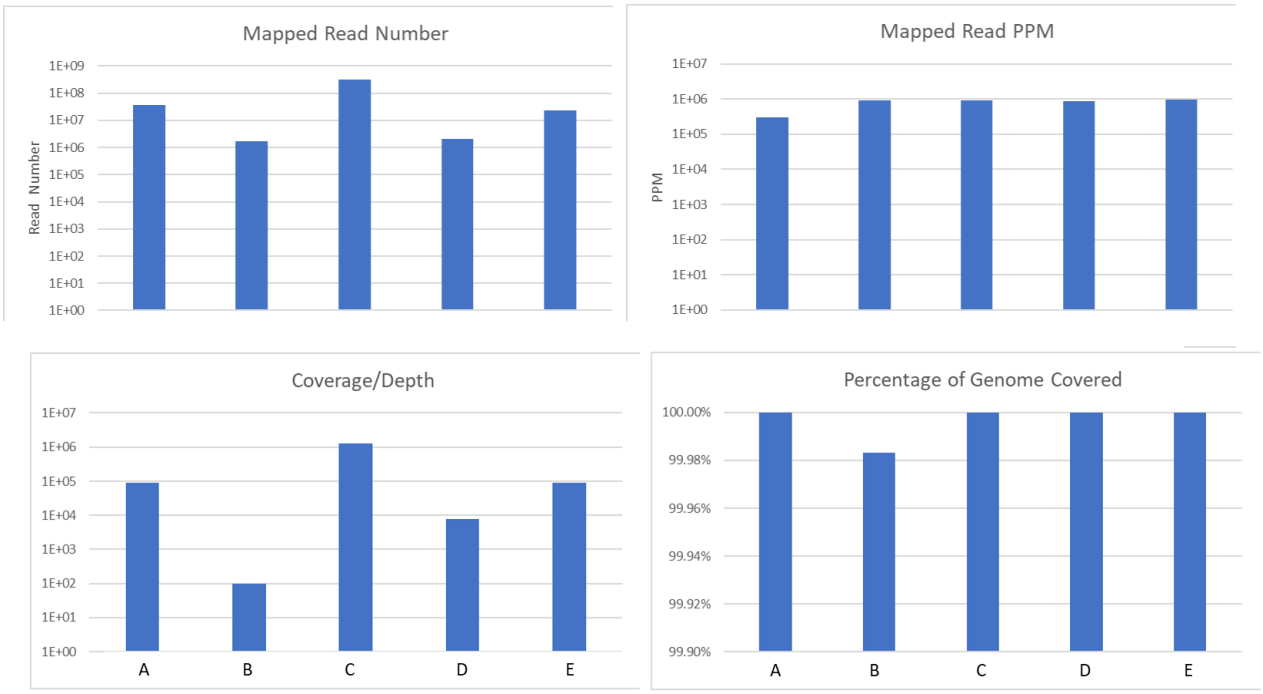


The number of total reads and quality reads are shown in Fig. 2 for each lab. The number of total reads obtained reflects difference in the HTS workflows, particularly the sequencing runs (single sample per flow cell for labs A and C versus multiplex for lab E) and the sequencing platform used (NextSeq for labs A, C, and E, and MiSeq for labs B and D used). The number of quality

reads depends on the sequencing run overall quality and, on the parameters and thresholds used for quality filtering. The quality reads were used for subsequent analysis shown in Figs. 3 – 8. The quality reads (QC) were mapped to the pooled seven virus genomes. The accession numbers are shown in Appendix E. It is noted a low, similar number of reads were obtained by Labs B and D using the MiSeq (2 million) whereas the reads from the NextSeq ranged from 24 M (million) to 345 M for Labs A, C and E. The total and quality number of reads for each lab were within a log.

Reads were mapped to the Ad5 and the reference virus genomes to compare inter-laboratory detection of the different virus standards. In the case of the segmented REO genome, the L3 segment was used since the genome copy number was based on the ddPCR assay targeting this region. The bioinformatics targeted analysis was done with the Ad5 genome, and the 7 reference virus genomes combined into a database as well as genomes of the carryover viruses, SMRV and PERV. The data analysis with the Ad5 background virus genome is shown in Fig. 3.

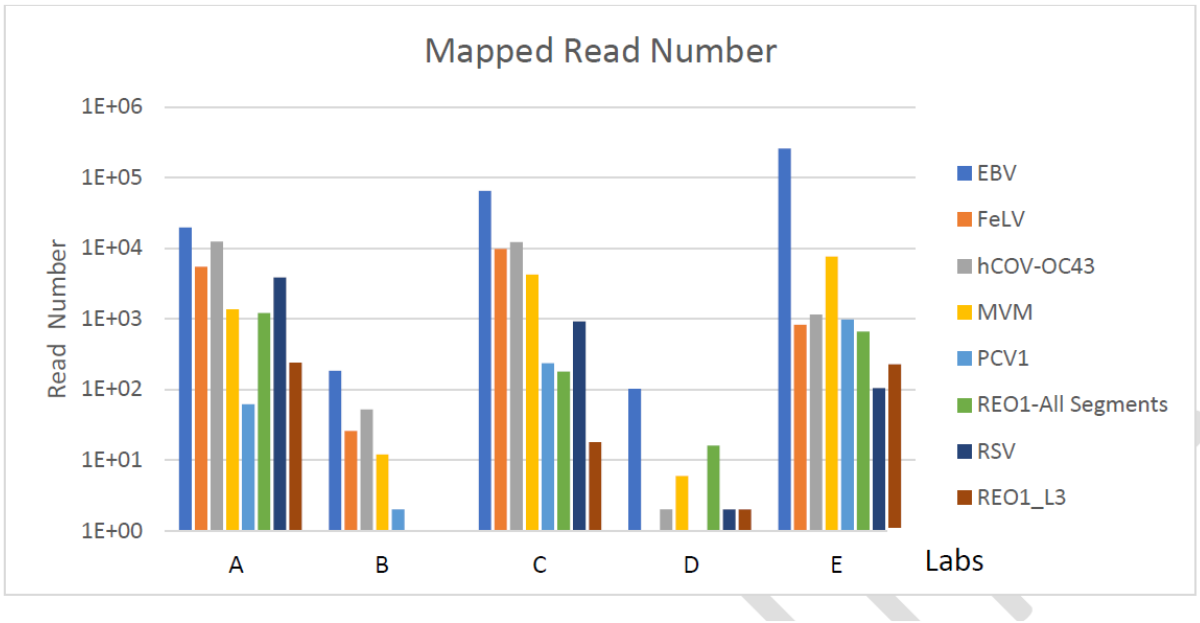
Fig. 3. Data analysis of the Ad5 virus genome.



The results were similar between the labs for detection of Ad5 and the coverage/depth correlated with the difference in the total number of QC reads shown in Fig. 3.

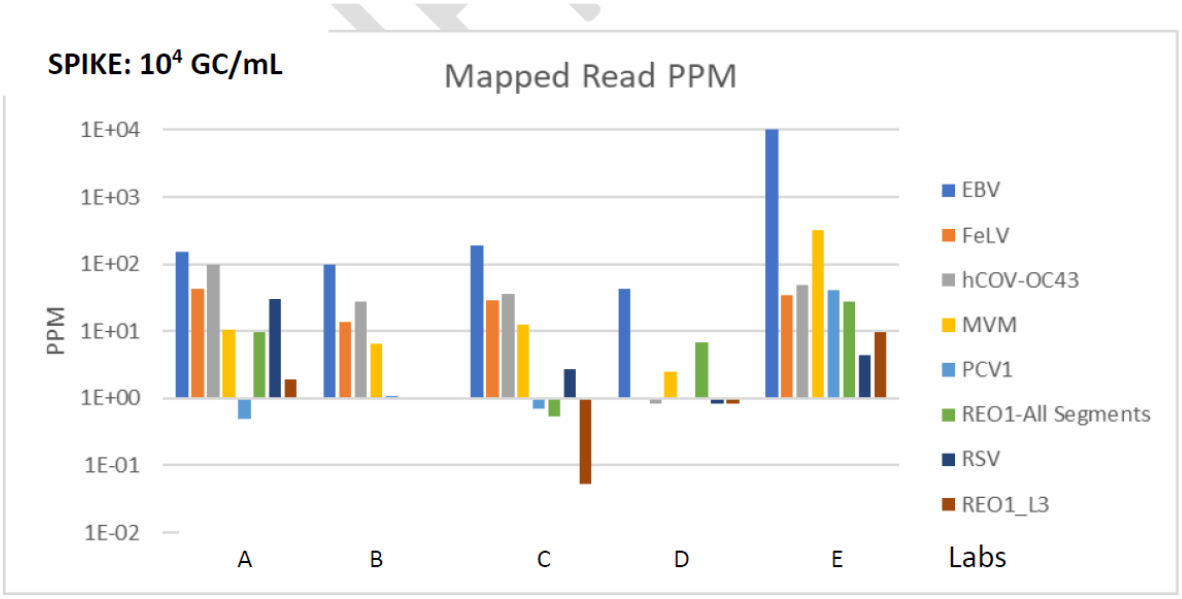
The results for detection of the 7 viruses in the panel using the short-read HTS technologies are shown in Fig. 4. All of the reference viruses were detected at 10^4 GC/mL by the three laboratories using NextSeq with at least 24 M reads and at 10^5 GC/mL by the two laboratories that used the MiSeq with 2 M total reads (Fig. 5), regardless of using independent HTS workflows. The differences seen in the results for detection of different viruses indicates the suitability of the virus panel for developing the expectations for HTS data and for optimizing the HTS workflow for virus detection, as needed.

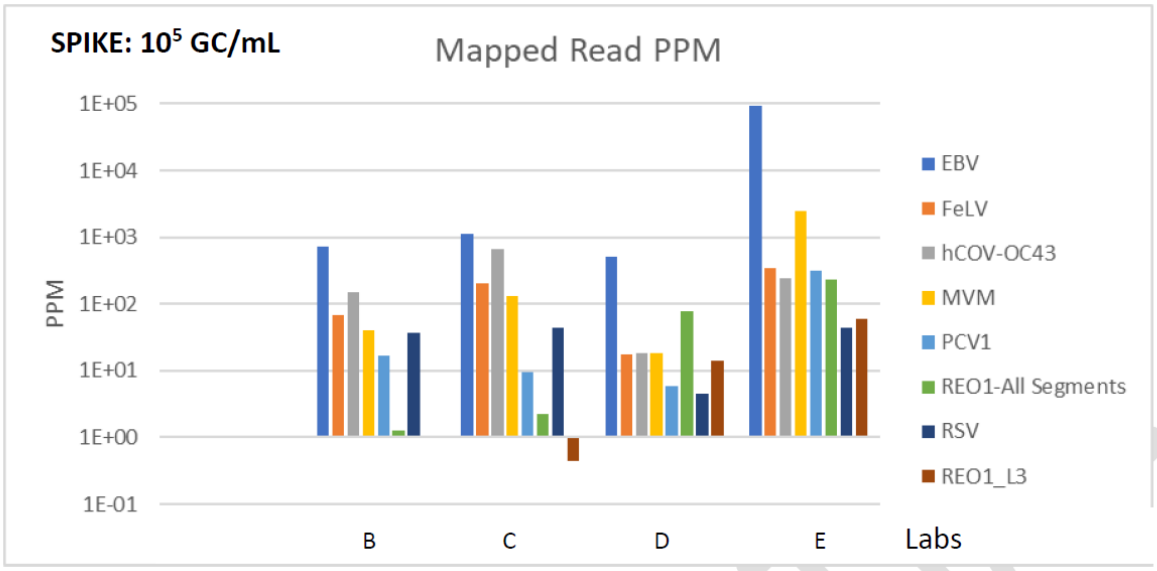
Fig. 4. Read mapping of the 7 reference viruses



Different numbers of quality reads were obtained by the different laboratories. To compare the inter-laboratory results, the mapped reads counts were normalized as parts per million (PPM). The results of the 10^4 GC/mL that were tested by all of the participants are shown in Fig. 5. Additionally, Labs B, C, D, and E also tested the 10^5 GC/mL spike level. The results shown in Fig. 5 indicates all of the viruses were detected at this higher spiked level, even with the lower read number (2M) by Labs B and D. The overall profile of virus detection was similar to Fig. 3.

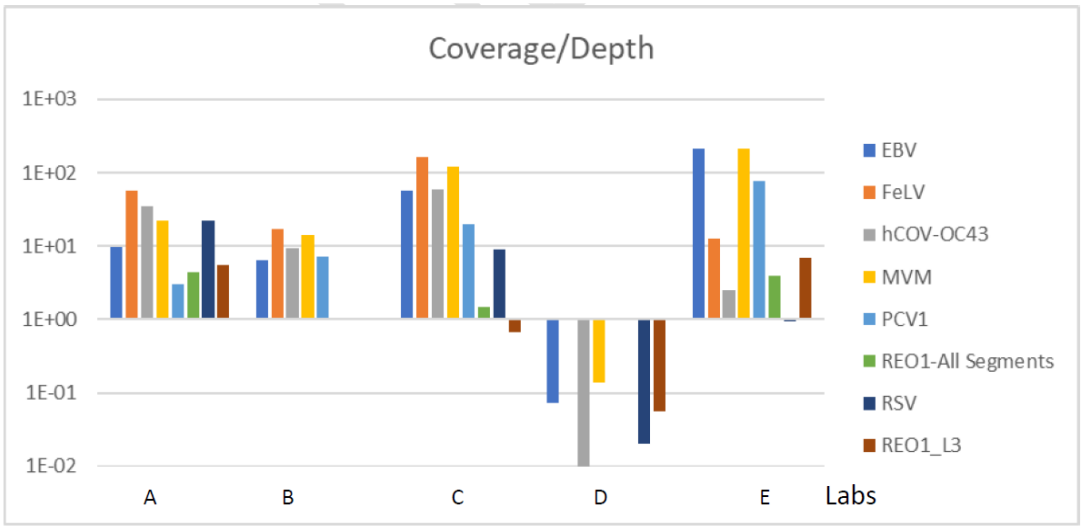
Fig. 5. Parts per million read counts (of mapped reads) for two spike levels.





The coverage depth is the average number of times a given nucleotide in the genome is sequenced (i.e., covered by a read) and provides information regarding the quality and reliability of the sequencing data. The results shown in Fig. 6 reflect differences in the HTS protocols, and not due to differences in the number of QC reads analyzed (shown in Fig. 2).

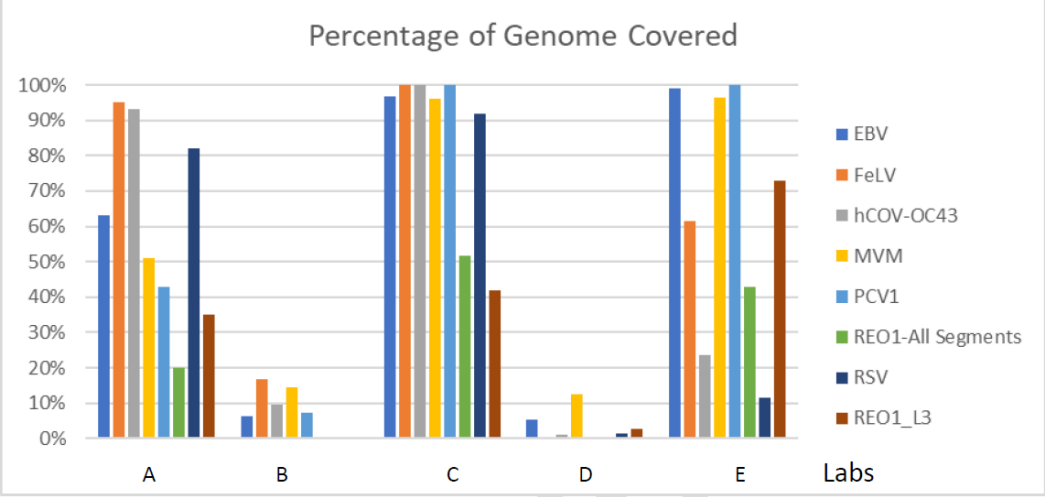
Fig. 6. Coverage Depth.



The coverage length indicates confidence in virus detection since more coverage of the reference virus genome indicates the potential for a full-length virus genome to be present in the sample. The coverage length depends on several parameters including the sequencing depth (total

number of reads). The results in Fig. 7 shows different coverage of virus genome lengths were observed and although better coverage was seen with higher number of total reads, optimization of the HTS workflow is still needed especially for the double-strand, segmented REO genome.

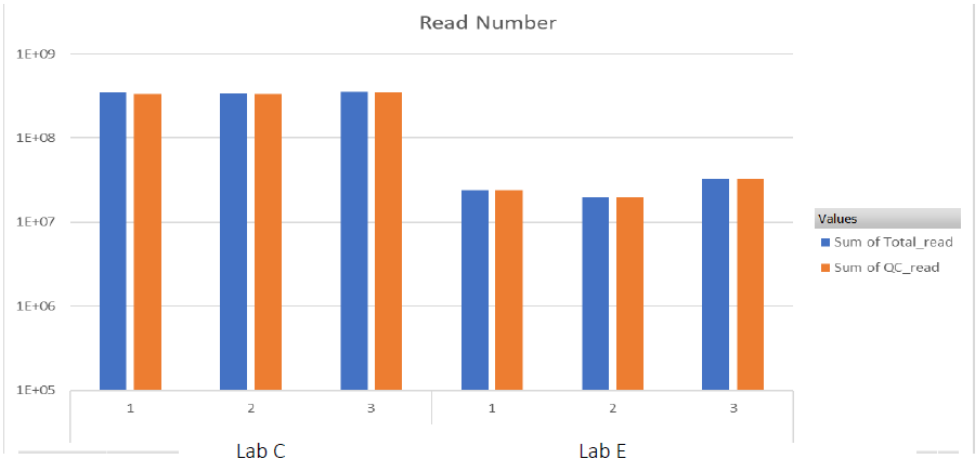
Fig. 7. Coverage length.



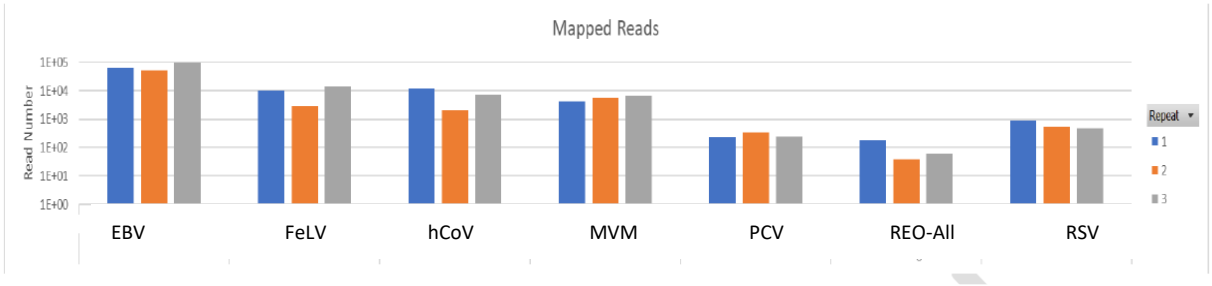
While Labs A, B, and D performed one HTS run, Labs C and E performed HTS in three independent spiking experiments. The results are shown in Fig. 8 demonstrating reproducibility of using the virus panel for evaluating the performance of HTS using independent sequencing protocols and platforms.

Fig. 8. Intra-lab reproducibility of HTS results

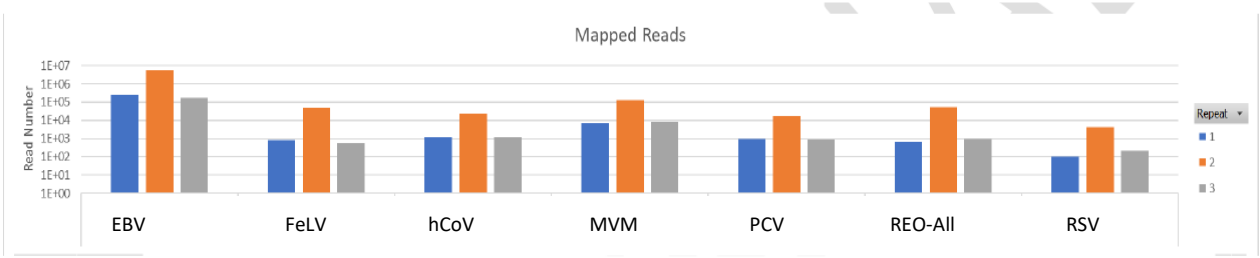
Labs C and E: Total and QC reads



Lab C: mapped reads to reference viruses



Lab E mapped reads to reference viruses



Conclusions

The proposed WHO virus reference panel contains 7 viruses representing families of potential concern in vaccines and other biologics. The viruses were selected based on distinct physicochemical and genome properties to demonstrate capabilities for broad virus detection: they include DNA and RNA genomes of large and small size, double and single strand, linear and circular; the viruses are enveloped and non-enveloped with large and small particle size. The results demonstrated that the proposed WHO reference virus panel is suitable for evaluating the performance of the entire HTS workflow. All of the 7 viruses were detected at 10^4 GC/mL in a high titer (10^9 GC/mL Ad5) virus matrix by analyzing $\geq$ 24 M reads (Labs A, C, and E), whereas 5 viruses were detected using 2 M reads (Labs B and D); however, all 7 viruses were detected at the 10^5 GC/mL spike level. The results are based on testing in a sample mimicking a low complexity matrix, with reduced host cell background e.g., a vaccine seed or viral vector preparation. Virus detection was based on the number of reads mapped, the coverage depth and the length of genome covered using the criteria established by each lab for a positive result. Difference in virus detection between the different labs reflects difference in the protocols used in the HTS workflow.

The results in this study from 5 laboratories, which includes two different HTS platforms (low throughput MiSeq and high throughput NextSeq) and 5 independent HTS workflow, sufficiently presents a spectrum of results and demonstrates the value of using the virus panel for evaluation of different HTS platforms and the need for optimization of protocols for performing HTS. The study results show the suitability of using the proposed WHO virus reference panel for validation studies (with an optimized protocol) to evaluate broad virus detection for supporting the use of HTS for replacement of the animal-based adventitious virus detection assays and to replace or supplement the *in vitro* cell culture assays. The data pending from the other labs will provide additional support to use HTS for adventitious virus detection.

Ongoing work

HTS data is pending from three collaborating labs using the NextSeq platform. Additionallyh, two labs will evaluate the long-read Nanopore sequencing technology. Additionally, the analysis of results from other spiked levels will be completed to determine LOD of the 7 viruses in the panel. These data strengthen the results of this report for publication. This information will provide users with the expected results for sensitivity and breadth of virus detection for using the WHO reference virus panel for HTS implementation for adventitious virus detection in biologics.

The details of the HTS protocols will be further reviewed between the different laboratories to determine the critical parameters that contributed to the difference in the results and help develop conditions to optimize detection of all 7 viruses with the short-read and long-read technologies.

Proposal (preparation code number and proposed potency)

The individual vials of the virus stocks are labelled as below and accompanied with the ATCC COA that includes infectious titer and genome copy number and other relevant information (included in Appendix C). The label on each vial is unique for each virus (copied below).

CBER-FSCUST-90 Purified human betacoronavirus 1 (hCOV), strain OC43 Lot: 70047896	CBER-FSCUST-91 Purified porcine circovirus type 1 Lot: 70048776	CBER-FSCUST-92 Purified mammalian orthoreovirus, strain Lang Lot: 70048775

CBER-FSCUST-93
Purified feline leukemia virus, strain Thielen
Lot: 70048774

CBER-FSCUST-94
Purified Epstein-Barr virus (HHV-4), strain b95-8
Lot: 70048773

CBER-FSCUST-96
Purified minute virus of mice, strain Prototype (p)
Lot 7004430

CBER-FSCUST-95
Purified human respiratory syncytial virus, strain A2
Lot: 70048772

The 7 viruses are meant to be used as a panel for demonstrating breadth and sensitivity of broad virus detection and therefore will be designated as the WHO International Reference Panel for Adventitious Virus Detection by HTS, which will be provided through the BEI resources repository under catalogue number NR-59630. For the purposes of HTS, the unitage of each is indicated as genome copy number per mL (Table 7).

Table 7. Virus panel and unitage

Box Label and ID	**Vial Code Number**	**Vial Unitage (genome copy number per mL)**
WHO International Reference Panel for Adventitious Virus Detection by HTS	CBER-FSCUST-90	2.6×10^{10}
	CBER-FSCUST-91	8.1×10^{9}
	CBER-FSCUST-92	1.5×10^{10}
	CBER-FSCUST-93	4.0×10^{10}
	CBER-FSCUST-94	2.8×10^{7}
	CBER-FSCUST-95	5.5×10^{10}

NR-7370	CBER-FSCUST-96	1.2×10^{10}

Comments from Participants

- There was no disagreement expressed in the comments received. There were some questions for clarification and edits and these have been addressed.
- One laboratory expressed: "We are surprised to see that the results varied widely at each facility, regardless of analyzing the same samples. So, we think it's an informative paper and providing the virus reference standards is very important."
- One laboratory needed clarification about why the RSV and PCV1 virus preparation did not include a filtration step. *Response: The text now includes that these viruses were not filtered to avoid loss in titer of these viruses.*

Acknowledgements

Funding for production and testing of the virus stocks was provided by the U.S. Food and Drug Organization, Center for Biologics Evaluation and Research (CBER) under contract 75F40120P00690. We are thankful for the important contributions of the study group participants listed in Appendix A.

Appendix A. Study group

Country	Affiliation	Names	Study status
Belgium	GSK, Rixensart	Christophe Lambert Stephanie Nguyen Anne-Sophie Colinet Noémie Deneyer Olivier Vandeputte Jean-Pol Cassart	*Data pending*

France / U.S.	Pathoquest, Paris / Wayne, PA	Marc Eloit Jose-Crispin Zavala Alvarado Colette Cote Joey Norikane Sandrine Moreira Rousseau	*Data pending*
Germany	Paul- Ehrlich-Institut (PEI), Langen	Gibran Horemheb Rubio Quintanares Janice Brückmann Johannes Blümel Markus Braun	Data provided
Italy	Merck, Colleretto Giacosa	Simone Olgiati Valeria Zanda	*Data pending*
Japan	Kobe University, Kobe	Kazuhisa Uchida Keisuke Yusa Yuan Yuzhe Noriko Hashiba	Data provided
	National Institute of Health Sciences, Kanagawa	Ken Kono Takamasa Hirai Kiyoko Kataoka	Data provided
United Kingdom	Medicines and Healthcare products Regulatory Agency (MHRA), South Mimms, Hertfordshire	Vinoy Ramachandran Ryan Mate Preni Sinnakandu Dammy Shittu Edward Mee	*Data pending*
United States of America	Center for Biologics Evaluation and Research, U.S. Food and Drug Administration (CBER/FDA), Silver Spring, MD	Arifa S. Khan Pei-Ju Chin Sandra M. Fuentes Jen-Hui Tsou	Data provided
	Massachusetts Institute of Technology, Cambridge, MA	Charley Swofford Paul Barone Stacey Springs	Data analysis pending
	Millipore Sigma, Rockville, MD	Bradley Hasson Scott Eubank Rebecca Bova Alison Armstrong	Data provided

Appendix B-1. Virus manufacturing

Virus	Code ID	Start date	Date of Harvest	Ultracentrifugation/Pelling	Step sucrose gradient	Date of Vialing
hCOV	CBER-FSCUST-90	7/15/21	**Bulk Clarified + Filtered** **#1**: 4L (7/20/21) **#2:** 2.8L (9/20/21)	w/o sucrose	**20/60%** **#1:** 10/8/21 **#2:** 10/22/21	11/23/21
PCV1	CBER-FSCUST-91	11/29/21	**Bulk Clarified** #1: 1.26L (12/3/2021) **#2:** 1.1L **(**1/19/2022) **#3:** 3.3L (2/2/2022) **# 4**: 2.8L (5/2/2022)	w/o sucrose	**20/70%** **#1:** 2/15/22 **#2:** 4/1/22 **#3.1:** 4/5/22 **#3.2:** 4/15/22 **#4:** 5/20/22	5/31/22
REO	CBER-FSCUST-92	3/24/21	**Bulk Clarified + Filtered** P1, 3/26/21 **#1:** P2, 1.769L (4/27/21) **#2**: P2**,** 2.35L **(**9/2/21)	Pelleted w/o sucrose	**20/60%** **#1:** 4/22/22 **#2:** 5/2022	5/24/22
FELV	CBER-FSCUST-93	7/23/21	**Bulk Clarified + Filtered** **#1:** Harvest 1, 4.9L (8/10/21) Harvest 2, 5.2L (8/13/21) **#2:** Harvest 1, 5.1L **(**9/7/21) Harvest 2, 5.1L (9/10/21)	Pelleted through 20% sucrose cushion.	**20/60%** **#1:** Harvest 1, 2/10/22 Harvest 2, 2/18/22 **#2:** Harvest 1**,** 3/11/22 Harvest 2, 4/29/22	6/8/22
EBV	CBER-FSCUST-94	4/21/22	**Bulk Clarified + Filtered** #1: 6/21/21 #2: 6/29/21 #3: 7/30/21 #4: 8/2/21 #5: 8/6/21 #6: 8/23/21	10% PEG precipitation	**20/70%** **Bulk 1**: #1: 12/10/21 **#2:** 12/23/21 #3: * #4: 2/1/22 #5: * #6: 2/7/22; 2/22/22; 3/8/22	4/21/22
RSV	CBER-FSCUST-95	7/27/21	**Bulk Clarified** **#1:** ~1.8L (8/2/21) **#2:** ~1.8L **(**8/4/21) **#3:** 3.2L **(**9/27/21) **#4:** 1.9L **(**10/4/21) **#5:** 2L **(**10/13/21)	Pelleted through 20% sucrose	**30/60%** **#1:** 9/15/21**;** 9/17/21 **#2:** 8/27/21; 9/17/21 **#3:** 10/15/21; **1**0/21/21 **#4:** 10/27/21 **#5:** 10/29/21	11/16/21
MVM	CBER-FSCUST-96	4/8/21	**Bulk Clarified + Filtered** p1 4/12/21 **# 1:** p2, 1.5L; 5/12/21 **#2:** p2, 3.6L, 5/21/21 **#3:** p2, 3.9L**,** 6/8/21	Pelleted w/o sucrose	**20/70%** **#1:** 6/24/21 **#2:** 7/14/21 **#3:** 8/3/21	9/8/21

*Information for dates pending

Appendix B-2. Virus testing

Virus	Code ID	Date of Vialing	Testing			
			Sterility	Mycoplasma PCR	Genome Copy Number (ddPCR)	Infectious titer
hCOV	CBER-FSCUST-90	11/23/21	12/15/21	12/6/21	12/23/21	12/8/21
PCV1	CBER-FSCUST-91	5/31/22	6/20/22	6/14/22	6/16/22	7/26/22
REO	CBER-FSCUST-92	5/24/22	6/9/22	5/26/22	6/2/22	5/26/22
FeLV	CBER-FSCUST-93	6/8/22	6/23/2022	6/20/22	6/15/22	6/16/22
EBV	CBER-FSCUST-94	4/21/22	4/22/22	5/23/22	5/16/22	5/5/22
RSV	CBER-FSCUST-95	11/16/21	12/1/23	12/6/21	11/18/21	12/7/21
MVM	CBER-FSCUST-96	9/8/21	10/19/21	9/21/21	10/13/21	9/17/21

Appendix C. ATCC Certificates of Analysis

- Betacoronavirus, OC43 (FSCUST-90)
- Porcine Circovirus type 1 (FSCUST-91)
- Mammalian (human) Orthoreovirus type 1, strain Lang (FSCUST-92)
- Feline Leukemia Virus, strain Theilen (FSCUST-93)
- Epstein-Barr Virus (HHV-4, strain B95-8) (FSCUST-94)
- Human Respiratory Syncytial Virus, strain A2 (FSCUST-95)
- Minute Virus of Mice, strain Prototype (p) (FSCUST-96)

BETACORONAVIRUS OC43

ATCC®

CERTIFICATE OF ANALYSIS

ATCC® Number:	**FSCUST-90™**
Lot Number:	**70047896**
Product Name:	**Purified betacoronavirus, OC43[1,2]**
Volume:	**≥ 0.5 mL per vial**
Product Format:	**Frozen**
Date of Vialing[3]:	**23 NOV 2021**
Storage Conditions:	**-70°C or colder**

Test / Method	Specification	Result
Titer (Post-vial)[4]	≥ 1×10^6 $TCID_{50}$/mL	1.58×10^7 $TCID_{50}$/mL
Genome Copy Number by ddPCR (Post-vial)[5,6]	≥ 1×10^{10} genome copies/mL	2.64×10^{10} genome copies/mL
Test for Mycoplasma Contamination DNA detection by PCR of test article nucleic acid [Universal Mycoplasma Detection Kit (ATCC® 30-1012K™)]	None detected	None detected
Sterility Test (BacT/ALERT 3D) iAST bottle (aerobic) at 32°C, 14-day incubation iNST bottle (anaerobic) at 32°C, 14-day incubation	 No growth No growth	 No growth No growth

[1]Purified Betacoronavirus (hCoV), OC43 (ATCC® VR-1558™)was grown in HRT-18G cells (ATCC® CRL-11663™) at 33°C with humidity and 5% CO_2.
[2]Preparation was vialed in 10 mM Tris-HCl, 135 mM NaCl, 0.5% BSA and 5% trehalose and may contain residual cellular nucleic acids.
[3]The product was vialed from pooled bulk material that was harvested on different dates. Dates of harvest for bulk materials were 20JUL2021 and 20SEP2021. All bulks were frozen following harvest.
[4]8 days in HRT-18G cells (ATCC® CRL-11663™) at 33°C with 5% CO_2, as determined by CPE.
[5]ddPCR data was obtained post-vial from 18 replicates on the BioRad QX200 Droplet Digital PCR (ddPCR™) System; gene assayed was replicase polyprotein Orf1ab.
[6]ddPCR samples contain virus genomes and residual mRNAs.

Technical Manager or designee; ATCC Federal Solutions

The preparation of the virus stock was directed by Dr. Arifa Khan at the U.S. Food and Drug Administration (FDA), Center for Biologics Evaluation and Research (CBER); funding for their production and testing was provided under contract 75F40120P00690. For additional information on characterization of the virus stocks please contact Dr. Arifa Khan (Arifa.Khan@fda.hhs.gov).

DISCLAIMER: This material is not intended to be used as a regulatory standard.

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PORCINE CIRCOVIRUS TYPE 1

ATCC®

CERTIFICATE OF ANALYSIS

ATCC® Number: **FSCUST-91™**
Lot Number: **70048776**

Product Name: **Purified porcine circovirus, type 1[1,2]**
Volume: **≥ 0.5 mL per vial**
Product Format: **Frozen**
Date of Vialing[3]: **31 MAY 2022**
Storage Conditions: **-70°C or colder**

Test / Method	Specification	Result
Titer (Post-vial)[4]	≥ 1 × 10^6 $TCID_{50}$/mL	2.32 × 10^6 $TCID_{50}$/mL
Genome Copy Number by ddPCR (Post-vial)[5,6]	≥ 1 × 10^{10} genome copies/mL	8.07× 10^9 genome copies/mL
Test for Mycoplasma Contamination DNA detection by PCR of test article nucleic acid [Universal Mycoplasma Detection Kit (ATCC® 30-1012K™)]	None detected	None detected
Sterility Test (BacT/ALERT 3D) iAST bottle (aerobic) at 32°C, 14-day incubation iNST bottle (anaerobic) at 32°C, 14-day incubation	 No growth No growth	 No growth No growth

[1]Purified porcine circovirus, type 1 was grown in PK(15) cells (ATCC® CCL-33™) at 37°C with humidity and 5% CO_2. PK(15) cells are known to contain porcine endogenous retrovirus [Pol. J. Microbiol. (2012), 61: 211-215. PubMed: 29334069].
[2]Preparation was vialed in 10 mM Tris-HCl, 135 mM NaCl, 0.5% BSA and 5% trehalose and may contain residual cellular nucleic acids.
[3]The product was vialed from pooled bulk material that was harvested on different dates. Dates of harvest for bulk materials were 03DEC2021, 19JAN2022, 02FEB2022 and 02MAY2022. All bulks were frozen following harvest.
[4]16 days in ST cells (ATCC® CRL-1746™) at 37°C with 5% CO_2, as determined by PCR.
[5]ddPCR data was obtained post-vial from 9 replicates on the BioRad QX200 Droplet Digital PCR (ddPCR™) System; gene assayed was replication associated protein.
[6]ddPCR samples contain virus genomes and residual mRNAs.

Technical Manager or designee; ATCC Federal Solutions

The preparation of the virus stock was directed by Dr. Arifa Khan at the U.S. Food and Drug Administration (FDA), Center for Biologics Evaluation and Research (CBER); funding for their production and testing was provided under contract 75F40120P00690. For additional information on characterization of the virus stocks please contact Dr. Arifa Khan (Arifa.Khan@fda.hhs.gov).

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MAMMALIAN ORTHOREOVIRUS

ATCC®

CERTIFICATE OF ANALYSIS

ATCC® Number: FSCUST-92™
Lot Number: 70048775

Product Name: Purified mammalian orthoreovirus, Lang[1,2]
Volume: ≥ 0.5 mL per vial
Product Format: Frozen
Date of Vialing[3]: 24 MAY 2022
Storage Conditions: -70°C or colder

Test / Method	Specification	Result
Titer (Post-vial)[4]	≥ 1 × 10^6 $TCID_{50}$/mL	8.89 × 10^8 $TCID_{50}$/mL
Genome Copy Number by ddPCR (Post-vial)[5,6]	≥ 1 × 10^{10} genome copies/mL	1.50 × 10^{10} genome copies/mL
Test for Mycoplasma Contamination DNA detection by PCR of test article nucleic acid [Universal Mycoplasma Detection Kit (ATCC® 30-1012K™)]	None detected	None detected
Sterility Test (BacT/ALERT 3D) iAST bottle (aerobic) at 32°C, 14-day incubation iNST bottle (anaerobic) at 32°C, 14-day incubation	 No growth No growth	 No growth No growth

[1]Purified mammalian orthoreovirus (MRV), Lang (ATCC® VR-230™) was grown in LLC-MK2 derivative cells (ATCC® CCL-7.1™) at 37°C with humidity and 5% CO_2.
[2]Preparation was vialed in 10 mM Tris-HCl, 135 mM NaCl, 0.5% BSA and 5% trehalose and may contain residual cellular nucleic acids.
[3]The product was vialed from pooled bulk material that was harvested on different dates. Dates of harvest for bulk materials were 27APR2021 and 02SEP2021. All bulks were frozen following harvest.
[4]8 days in LLC-MK2 derivative cells (ATCC® CCL-7.1™) at 37°C with 5% CO_2, as determined by CPE.
[5]ddPCR data was obtained post-vial from 9 replicates on the BioRad QX200 Droplet Digital PCR (ddPCR™) System; gene assayed was core protein L3.
[6]ddPCR samples contain virus genomes and residual mRNAs.

Sonia Bjorum Brower Digitally signed by Sonia Bjorum Brower Date: 2023.08.15 12:53:38 -04'00'

Technical Manager or designee; ATCC Federal Solutions

The preparation of the virus stock was directed by Dr. Arifa Khan at the U.S. Food and Drug Administration (FDA), Center for Biologics Evaluation and Research (CBER); funding for their production and testing was provided under contract 75F40120P00690. For additional information on characterization of the virus stocks please contact Dr. Arifa Khan (Arifa.Khan@fda.hhs.gov).

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Template Doc ID: 31194 Template Revision: 6 Template Effective Date: 02/21/2020

FELINE LEUKEMIA VIRUS

CERTIFICATE OF ANALYSIS

ATCC® Number: FSCUST-93™
Lot Number: 70048774

Product Name: Purified feline leukemia virus, Theilen[1,2]
Volume: ≥ 0.5 mL per vial
Product Format: Frozen
Date of Vialing[3]: 08 JUN 2022
Storage Conditions: -70°C or colder

Test / Method	Specification	Result
Titer (Post-vial)[4]	≥ 1×10^6 $TCID_{50}$/mL	1.99×10^7 $TCID_{50}$/mL
Genome Copy Number by ddPCR (Post-vial)[5,6]	≥ 1×10^{10} genome copies/mL	4.01×10^{10} genome copies/mL
Test for Mycoplasma Contamination DNA detection by PCR of test article nucleic acid [Universal Mycoplasma Detection Kit (ATCC® 30-1012K™)]	None detected	None detected
Sterility Test (BacT/ALERT 3D) iAST bottle (aerobic) at 32°C, 14-day incubation iNST bottle (anaerobic) at 32°C, 14-day incubation	 No growth No growth	 No growth No growth

[1]Purified feline leukemia virus, Theilen was grown in FL74-UCD-1 cells (ATCC® CRL-8012™) at 37°C with humidity and 5% CO_2.
[2]Preparation was vialed in 10 mM Tris-HCl, 135 mM NaCl, 0.5% BSA and 5% trehalose and may contain residual cellular nucleic acids.
[3]The product was vialed from pooled bulk material that was harvested on different dates. Dates of harvest for bulk materials were 10AUG2021, 13AUG2021, 07SEP2021 and 10SEP2021. All bulks were frozen following harvest.
[4]7 days in CRFK cells (ATCC® CCL-94™) at 37°C with 5% CO_2, as determined by PCR.
[5]ddPCR data was obtained post-vial from 9 replicates on the BioRad QX200 Droplet Digital PCR (ddPCR™) System; gene assayed was envelope polyprotein.
[6]ddPCR samples contain virus genomes and residual mRNAs.

Technical Manager or designee; ATCC Federal Solutions

The preparation of the virus stock was directed by Dr. Arifa Khan at the U.S. Food and Drug Administration (FDA), Center for Biologics Evaluation and Research (CBER); funding for their production and testing was provided under contract 75F40120P00690. For additional information on characterization of the virus stocks please contact Dr. Arifa Khan (Arifa.Khan@fda.hhs.gov).

DISCLAIMER: This material is not intended to be used as a regulatory standard.

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EPSTEIN-BARR VIRUS (HHV4)

ATCC®

CERTIFICATE OF ANALYSIS

ATCC® Number: FSCUST-94™
Lot Number: 70048773

Product Name: Purified Epstein-Barr virus (human herpes virus 4), B95-8[1,2]
Volume: ≥ 0.5 mL per vial
Product Format: Frozen
Date of Vialing[3]: 21 APR 2022
Storage Conditions: -70°C or colder

Test / Method	Specification	Result
Titer (Post-vial)[4]	≥ 1×10^5 $TCID_{50}$/mL	1.12×10^5 $TCID_{50}$/mL
Genome Copy Number by ddPCR (Post-vial)[5,6]	≥ 1×10^{10} genome copies/mL	2.82×10^7 genome copies/mL
Test for Mycoplasma Contamination DNA detection by PCR of test article nucleic acid [Universal Mycoplasma Detection Kit (ATCC® 30-1012K™)]	None detected	None detected
Sterility Test (BacT/ALERT 3D) iAST bottle (aerobic) at 32°C, 14-day incubation iNST bottle (anaerobic) at 32°C, 14-day incubation	 No growth No growth	 No growth No growth

[1]Purified Epstein-Barr virus (human herpes virus 4), B95-8 was isolated from B95-8 Leukocyte Marmoset culture (ATCC® CRL-1612™) grown at 37°C with humidity with 5% CO_2. The B95-8 marmoset cell line is known to contain squirrel monkey retrovirus [Virology. (1995), 209: 374-383. PubMed: 7778272].
[2]Preparation was vialed in 10 mM Tris-HCl, 135 mM NaCl, 0.5% BSA and 5% trehalose and may contain residual cellular nucleic acids.
[3]The product was vialed from pooled bulk material that was harvested on different dates. Dates of harvest for bulk materials were 21JUN2021, 29JUN2021, 30JUL2021, 02AUG2021, 06AUG2021 and 23AUG2021. All bulks were frozen following harvest.
[4]46 days in IRR-MRC-5 Irradiated Fibroblast Human cells (ATCC® 55-X™) at 37°C with 5% CO_2, as determined by transformation.
[5]ddPCR data was obtained post-vial from 9 replicates on the BioRad QX200 Droplet Digital PCR (ddPCR™) System; gene assayed was EBER1 noncoding RNA.
[6]ddPCR samples contain virus genomes and residual mRNAs.

Technical Manager or designee; ATCC Federal Solutions

The preparation of the virus stock was directed by Dr. Arifa Khan at the U.S. Food and Drug Administration (FDA), Center for Biologics Evaluation and Research (CBER); funding for their production and testing was provided under contract 75F40120P00620. For additional information on characterization of the virus stocks please contact Dr. Arifa Khan (Arifa.Khan@fda.hhs.gov).

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RESPIRATORY SYNCYTIAL VIRUS

ATCC®

CERTIFICATE OF ANALYSIS

ATCC® Number: FSCUST-95™
Lot Number: 70048772

Product Name: Purified respiratory syncytial virus, A2[1,2]
Volume: ≥ 0.5 mL per vial
Product Format: Frozen
Date of Vialing[3]: 16 NOV 2021
Storage Conditions: -70°C or colder

Test / Method	Specification	Result
Titer (Post-vial)[4]	≥ 1 × 10^6 $TCID_{50}$/mL	5.00 × 10^6 $TCID_{50}$/mL
Genome Copy Number by ddPCR (Post-vial)[5,6]	≥ 1 × 10^{10} genome copies/mL	5.53 × 10^{10} genome copies/mL
Test for Mycoplasma Contamination DNA detection by PCR of test article nucleic acid [Universal Mycoplasma Detection Kit (ATCC® 30-1012K™)]	None detected	None detected
Sterility Test (BacT/ALERT 3D) iAST bottle (aerobic) at 32°C, 14-day incubation iNST bottle (anaerobic) at 32°C, 14-day incubation	 No growth No growth	 No growth No growth

[1]Human respiratory syncytial virus, A2 (ATCC® VR-1540™) was grown in HEp-2 cells (ATCC® CCL-23™) at 37°C with humidity and 5% CO_2.
[2]Preparation was vialed in 10 mM Tris-HCl, 135 mM NaCl, 0.5% BSA and 5% trehalose and may contain residual cellular nucleic acids.
[3]The product was vialed from pooled bulk material that was harvested on different dates. Dates of harvest for bulk materials were 02AUG2021, 04AUG2021, 27SEP2021, 04OCT2021 and 13OCT2021. All bulks were frozen following harvest.
[4]7 days in HEp-2 cells (ATCC® CCL-23™) at 37°C with 5% CO_2, as determined by CPE and DFA with LIGHT DIAGNOSTICS™ Respiratory Syncytial Virus FITC Reagent (Millipore Sigma 5022).
[5]ddPCR data was obtained post-vial from 11 replicates on the BioRad QX200 Droplet Digital PCR (ddPCR™) System; gene assayed was N protein.
[6]ddPCR samples contain virus genomes and residual mRNAs.

Technical Manager or designee; ATCC Federal Solutions

The preparation of the virus stock was directed by Dr. Arifa Khan at the U.S. Food and Drug Administration (FDA), Center for Biologics Evaluation and Research (CBER); funding for their production and testing was provided under contract 75F40120P00690. For additional information on characterization of the virus stocks please contact Dr. Arifa Khan (Arifa.Khan@fda.hhs.gov).

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MINUTE VIRUS OF MICE

ATCC®

CERTIFICATE OF ANALYSIS

ATCC® Number: FSCUST-96™
Lot Number: 70044330

Product Name: Purified minute virus of mice, strain Prototype (p)[1,2]
Volume: ≥ 0.5 mL per vial
Product Format: Frozen
Date of Vialing[3]: 08 SEP 2021
Storage Conditions: -70°C or colder

Test / Method	Specification	Result
Titer (Post-vial)[4]	≥ 1×10^6 $TCID_{50}$/mL	1.58×10^7 $TCID_{50}$/mL
Genome Copy Number by ddPCR (Post-vial)[5,6]	≥ 1×10^{10} genome copies/mL	1.18×10^{10} genome copies/mL
Test for Mycoplasma Contamination DNA detection by PCR of test article nucleic acid [Universal Mycoplasma Detection Kit (ATCC® 30-1012K™)]	None detected	None detected
Sterility Test (BacT/ALERT 3D) iAST bottle (aerobic) at 32°C, 14-day incubation iNST bottle (anaerobic) at 32°C, 14-day incubation	 No growth No growth	 No growth No growth

[1]Minute virus of mice, Prototype (p) (ATCC® VR-1346™) was grown in A9 cells (ATCC® CCL-1.4™) at 37°C with humidity and 5% CO_2.
[2]Preparation was vialed in 10 mM Tris-HCl, 135 mM NaCl, 0.5% BSA and 5% trehalose and may contain residual cellular nucleic acids.
[3]The product was vialed from pooled bulk material that were harvested on different dates. Dates of harvest for bulk materials were 12APR2021, 12MAY2021, 21MAY2021, 08JUN2021. All bulks were frozen following harvest.
[4]8 days in A9 cells (ATCC® CCL-1.4™) at 37°C with 5% CO_2, as determined by CPE and IFA with MVMVP21-S antibody at 1:200 dilution.
[5]ddPCR data was obtained post-vial from 9 replicates on the BioRad QX200 Droplet Digital PCR (ddPCR™) System; gene assayed was nonstructural protein NS-1.
[6]ddPCR samples contain virus genomes and residual mRNAs.

Technical Manager or designee; ATCC Federal Solutions

The preparation of the virus stock was directed by Dr. Arifa S. Khan at the U.S. Food and Drug Administration (FDA), Center for Biologics Evaluation and Research (CBER); funding for their production and testing was provided under contract 75F40120P00620. For additional information on characterization of the virus stocks please contact Dr. Arifa Khan (Arifa.Khan@fda.hhs.gov).

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Template Doc ID: 31194 Template Revision: 6 Template Effective Date: 02/21/2020

Appendix D. Sample preparation and Sequencing

Sample Prep	A	B	C	D	E
Volume used for spiking	1 mL	1 mL	1 mL	1 mL	1.0 mL
Volume used for Ext	1 mL (5 x 200 µL)	0.1 mL	0.2 mL	0.2 mL	0.4 mL
Pre-extraction Treatment	No	No	No	No	Proteinase K
Extraction kit (DNA+RNA)	QIAamp MinElute Virus Spin Kit (w/o carrier RNA) (2 columns)	Quick-DNA/RNA Viral MagBead (Zymo Research, R2140)	QIAamp MinElute Virus Spin Kit (w/o carrier RNA)	QIAamp MinElute Virus Spin Kit (w/o carrier RNA)	MagMax Viral/Pathogen in Automatic Kingfisher Duo Prime equipment
Extracted volume	50 µL	30 µL	30 µL	50 µL	50 µL
Pre-amplification	-	13 µL was used for ds-cDNA synthesis using Maxima H Minus Double-Stranded cDNA Synthesis Kit (Thermo Scientific)	-	13 µL was used for ds-cDNA synthesis using Maxima H Minus Double-Stranded cDNA Synthesis Kit (Thermo Scientific)	-
cDNA purification	-	AMPure XP beads v[beads]:v[DNA]=1:1, ds-cDNA eluted in 20 µL ddH2O	SuperScript IV VILO Master Mix, NEB Next Ultra II Non-Directional Second Strand Synthesis, Zymo DNA Clean & Concentrator Kit, AMPure Clean-up	AMPure XP beads v[beads]:v[DNA]=1.8:1, ds-cDNA eluted in 50 µL ddH2O	-
Amount for library prep	10 µL	5 µL	5 µL	30 µL	40 µL
Cellular RNA depletion	Illumina Ribo-Zero Gold (Beads)	No	No	No	No
Library amplification	cDNA	cDNA	cDNA	cDNA	cDNA

Sequencing library preparation	Nextera XT DNA Library Prep	Nextera XT DNA Library Prep Kit	Nextera XT DNA Library Preparation Kit	Illumina DNA Prep	40 µL of the extracted nucleic acid were divided in 3 equal parts. Retrotranscription: two parts were used for RT. One was denatured at 95°C and the other one at 75°C (combined with random hexamers). After denaturation, both parts were mixed and retrotrascribed at 50°C using Maxima H- enzyme. After RT, the third aliquot was mixed with the cDNA generated and Second Strand DNA generated using NEBNext Ultra II Non directional RNA. Clean up was done using magnetic beads (1.8x), followed by library preparation using Twist Library

					Preparation EF Kit 2.0 (the last amplification step was done by 11 cycles). Hybridization /capture was done on the library prep using VirCapSeq-VERT probes, with a final amplification of 16 cycles and clean up.
Sequencing platform	NextSeq Mid Output 500/550	MiSeq	NextSeq2000	MiSeq	NextSeq 2000
CONTROLS: medium (no viruses)	Yes	Yes	Yes	Yes	Yes
CONTROLS: 7-viruses mix	Yes	Yes	Yes	Yes	Yes
CONTROLS: Ad5	Yes	Yes	No	Yes	Yes
CONTROLS	No	0.4 pM of PhiX174 spike-in	No	0.125 pM of PhiX174 spike-in	No

APPENDIX E. Genome accession numbers for mapping reads in the study

Virus	Accession number
Epstein Barr Virus	V01555.2
Respiratory Syncytial Virus	JF920069.1
Reovirus 1 (individual segments and assembled segments for complete genome were used)	M24734.1 (REO1LAM3P) AF378003.1 (L2) AF129820.1 AF461682.1 AF490617.1 AF174382.1 M10260.1 (REO1S1A) L19774.1 (REO1MCPS2A) M18389.1 (REOS3NSA) X61586.1
Feline leukemia virus	MT129531.1
Porcine circovirus 1	NC_001792.2
Human betacoronavirus OC43	NC_006213.1
Minute virus of mice	J02275.1
Adenovirus-5*	AY339865.1
Squirrel monkey retrovirus (data not shown)	NC_001514.1
Porcine endogenous retrovirus (data not shown)	AF038600.1

*****Not provided. Can be substituted with another suitable virus.

APPENDIX F. DRAFT INSTRUCTIONS FOR USE

Custodian

Center for Biologics Evaluation and Research
U.S. Food and Drug Administration
10903 New Hampshire Ave
Silver Spring, MD 20993-0002, USA
Contact: Dr. Arifa S. Khan (arifa.khan@fda.hhs.gov)

Distributor

BEI Resources Repository
10801 University Boulevard
Manassas, VA 20110-2209 USA
Contact: BEI Customer Care: Contact@BEIResources.org; (800) 359-7370

Material Name, Status, Identifying Code, and Year of Establishment

First WHO International Reference Panel for adventitious virus detection in biological products using high-throughput sequencing technologies

Identifying Code	Establishment Date
FSCUST-90	November 23, 2021
FSCUST-91	May 31, 2022
FSCUST-92	May 24, 2022
FSCUST-93	June 8, 2022
FSCUST-94	April 21, 2022
FSCUST-95	November 16, 2021
FSCUST-96	September 8, 2021

1. INTENDED USE OF THE MATERIAL

The primary use of the material is to support qualification and validation of high-throughput sequencing technologies for adventitious virus detection in biologics. The material is intended to be used as a panel of the 7 viruses to demonstrate broad virus detection and not for using individual viruses.

The secondary use of the material can be to generate data in support of the replacement of current adventitious virus detection assays.

2. CAUTION

THIS MATERIAL IS NOT INTENDED FOR USE IN HUMANS, ANIMALS, OR FOR DIAGNOSTICS.

The material contains viruses sourced from human and animal origin, and residual host cell nucleic acids (see details in Appendix G). The viruses are in a liquid final formulation of 10 mM Tris-HCl, 135 mM NaCl, 0.5% BSA, 5% Trehalose.

Identifying Code	**Reference Virus**	**Other Viruses** (expected due to the cell line used for propagation)
FSCUST-90	Betacoronavirus, OC43	
FSCUST-91	Porcine circovirus, type 1	Endogenous porcine retrovirus (PERV)
FSCUST-92	Mammalian orthoreovirus, Lang strain	
FSCUST-93	Feline leukemia virus, (Kawakami-) Thielen strain (KT)	
FSCUST-94	Epstein-Barr Virus (human herpes virus 4), strain B95-8	Squirrel monkey retrovirus (SMRV)
FSCUST-95	Human respiratory syncytial virus, A2	
FSCUST-96	Minute virus of mice, Prototype (p) strain	

The material contains live, infectious virus and some may be pathogenic in some species. Therefore, appropriate Biosafety Level (BSL) practices should always be used with this material. Refer to the Product Information Sheet for instructions on the correct use of this product. It should be used according to your own laboratory's safety procedures for using personal protective equipment (PPE) and avoid generation of aerosols and use of sharps (needles, scalpels) to avoid cuts and skin punctures. In case of any exposure to infectious material, steps should be followed according to your own laboratory's safety manual.

Irradiated fetal bovine serum used in the growth of the viruses (Hyclone; catalogue number SH30070.03) was manufactured in the United States in cGMP (21 CFR 820) compliant and ISO9001 certified facilities. It does not require a Material Safety Data Sheet under the Occupational Safety and Health Administration standard entitled "Hazard Communication" 29 CFR 1910.1200 for the United States.

3. DIRECTIONS FOR OPENING THE SCREW CAP VIALS

The material is stored in polypropylene for stability at low temperatures (-70 °C or colder). The vials have a screw cap with a rubber gasket. Vials should be opened in an appropriate biosafety cabinet. The cap should be removed by turning anti-clockwise. Care should be taken on removal of the cap to prevent the contents spilling (such as placement in an appropriate holder).

4. DIRECTIONS FOR STORAGE AND HANDLING

The material is shipped in dry ice and should be stored immediately on arrival under controlled temperature monitoring at -70 °C or colder and avoiding any changes in temperature during transfer to vials.

When handling the virus, use safety procedures according to your laboratory guideline: for example, work in a suitable biosafety cabinet, wear lab coat, gloves, inactivate all surfaces exposed to virus using appropriate virocides before autoclaving or incinerating disposal items. Used vials and all materials exposed to the virus during the handling or in performing the studies should disposed according to laboratory's guidelines for infectious agents.

5. PREPARATIONOF VIRUS STOCK ALIQUOTS

- Remove virus from storage and transfer to lab in dry or wet ice
- Thaw virus by gently swirling in 37 °C water bath until only a tiny piece of ice remains (should be less than 1 min; each vial contains $\geq$ 0.5 mL). Transfer to wet ice to continue complete thawing.
- In biosafety cabinet work with one virus at a time, clean surfaces and allow 45 mins in between different viruses with biosafety blower on, to avoid cross-contamination of the stock viruses. Make appropriate single-use aliquots of each virus based on future work. Use sterile screw capped cryogenic vials with a rubber gasket in the cap (RNase and DNase free) and store at -70 °C or colder. Aliquots should not be less than 10 uL for long-term storage. It is best to store undiluted virus in serum-free medium for long-term stability.
- This initial freeze-thaw step will ensure that the virus used in all of the studies had undergone 1 thaw and freeze after being received in the laboratory and no change in the potency of virus due to difference in sample handling.

6. A SCHEMA FOR PREPARATION OF VIRUS DILUTION FOR A SPIKING STUDY

- All reagents and materials used must be sterile, RNAse and DNAse free.
- To make a dilution, remove volume from one tube, deliver to a new tube, change to a new pipette tip or pipette and mix up and down 3 times (avoid rigorousness and bubbles), then use a new tip or pipette to remove volume and continue till last dilution where final volume is discarded.

- Store each dilution in aliquots to be used for nucleic acid extraction in screw cap tubes at -70 °C or colder. (volumes in table are rounded to the nearest whole number). Avoid further freeze-thaw.

Virus Stock	GC/mL in virus stock	Dil. A: $1x10^9$/mL		Dil. B: $1x10^8$/mL		Dil.C: $1x10^7$/mL		Dil. D: $1x10^6$/mL etc…..	
		Virus Stock (uL)	*Diluent: No-serum Medium (uL)*	*Virus Dil A (uL)*	*Diluent: No-serum Medium (uL)*	*Virus Dil B (uL)*	*Diluent: No-serum Medium (uL)*	*Virus Dil C (uL)*	*Diluent: No-serum Medium (uL)*
REO	1.50×10^{10}	67	933	100	900	100	900	100	900
RSV	5.53×10^{10}	18	982	100	900	100	900	100	900
FELV	4.01×10^{10}	25	975	100	900	100	900	100	900
PCV	8.07×10^{09}	124	876	100	900	100	900	100	900
EBV	2.80×10^{07}	-	-	-	-	357	643	100	900
OC43	2.64×10^{10}	38	962	100	900	100	900	100	900
MVM	1.18×10^{10}	85	915	100	900	100	900	100	900

7. CITATION

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APPENDIX G. MATERIAL SAFETY SHEET

Contact information		
Custodian Laboratory	Address: Center for Biologics Evaluation and Research U.S. Food and Drug Administration 10903 New Hampshire Avenue Silver Spring, MD 20993-0002, USA	Name: Dr. Arifa S. Khan Email: arifa.khan@fda.hhs.gov
Distributing Laboratory	Address: BEI Resources Repository 10801 University Boulevard Manassas, VA 20110-2209, USA	Name: BEI customer care Contact@BEIResources.org (800)359-7370

Status of Material
First WHO International Reference Panel for adventitious virus detection in biological products using high-throughput sequencing technologies

Identifying Code	Establishment Date	Potency (genome copies per mL):
FSCUST-90	November 23, 2021	2.64×10^{10} (Dec. 2021)
FSCUST-91	May 31, 2022	8.07×10^{9} (June 2022)
FSCUST-92	May 24, 2022	1.50×10^{10} (May 2022)
FSCUST-93	June 8, 2022	4.01×10^{10} (June 2022)
FSCUST-94	April 21, 2022	2.82×10^{7} (April 2022)
FSCUST-95	November 16, 2021	5.53×10^{10} (Dec 2021)
FSCUST-96	September 8, 2021	1.18×10^{10} (Oct 2021)

Physical Properties	
Appearance (-80 °C)	Frozen
Appearance (at 4 °C and room temperature)	Clear, liquid
Fire hazard	None

Chemical Properties			
Stable:	Yes	Corrosive:	No
Hygroscopic:	No	Oxidizing:	No
Flammable:	No	Irritant:	No

Other (specify): Contains material of human and animal origin
Handling: This material contains infectious Biosafety Level 2 viruses as described above. See Appendix E, section 2 Caution
Toxicological Properties
Effects of inhalation: Not established, avoid inhalation
Effects of ingestion: Not established, avoid ingestion
Effects of skin absorption: Not established, avoid skin contact
Suggested First Aid
Inhalation: Follow laboratory's safety and exposure procedures. Seek medical advice
Ingestion: Follow laboratory's safety and exposure procedures. Seek medical advice
Injection: Follow laboratory's safety and exposure procedures. Seek medical advice
Contact with eyes: Follow laboratory's safety and exposure procedures. Seek medical advice
Contact with skin: Follow laboratory's safety and exposure procedures. Seek medical advice
Action on Spillage and Method of Disposal
Spillage of vial contents should be taken up with absorbent material wetted with a virucidal agent. Rinse area with a virucidal agent followed by water.
Absorbent materials used to treat spillage should be treated as biologically hazardous waste and autoclaved.

APPENDIX H. REFERENCE

The viruses and other nucleic acid sequences, which were detected in individual vials by ddPCR and next generation sequencing are indicated below and may be used for reference. The 7 viruses will be provided as a panel (catalogue number NR-59630) from the BEI Resources Repository (https://www.niaid.nih.gov/research/bei-resources-repository).

Identifying Code	**Lot No**	**Reference Virus**	**Other Viruses** (due to the cell line used for propagation)	**Other nucleic acids**
FSCUST-90	70047896	Betacoronavirus, OC43		Host cell sequences with human ERVs
FSCUST-91	70048776	Porcine circovirus, type 1	Endogenous porcine retrovirus (PERV)	Host cell sequences with porcine ERVs*
FSCUST-92	70048775	Mammalian orthoreovirus, Lang strain		Host cell sequences with human ERVs
FSCUST-93	70048774	Feline leukemia virus, (Kawakami-) Thielen strain (KT)		Host cell sequences with feline ERVs
FSCUST-94	70048773	Epstein-Barr Virus (human herpes virus 4), strain B95-8	Squirrel monkey retrovirus (SMRV)	Host cell sequences with simian ERVs
FSCUST-95	70048772	Human respiratory syncytial virus, A2		Host cell sequences with human ERVs
FSCUST-96	70044330	Minute virus of mice, Prototype (p) strain		Host cell sequences with murine ERVs

* ERVs are endogenous retroviral sequences that are a normal component of the host cell DNA and are not known to be associated with an infectious virus in humans but can be associated with an infectious virus in certain animal species.

LITERATURE LIST

[1] Khan AS, King, K.E. Brack, K., Cassart, J-P, Chiu, C., Dehghani, H., Duncan, P., Jaing, C., Kolman, J., Munroe, D., Palermo, A., Plavsic, M., Sampath, R., Slezak, T., Takle, G., Taliaferro, L.P., Toso, E., Vacante, D., and Willkommen, H. Emerging methods for virus detection. PDA Technical Report. 2015;71.
[2] Scientific principles for regulatory risk evaluation on finding an adventitious agent in a marketed vaccine. In: WHO expert committee on biological standardization: sixty-fifth report. Geneva: World Health Organization; 2015. Annex 2 (WHO Technical Report Series, No. 993, https://apps.who.int/iris/bitstream/handle/10665/173739/9789240694095_eng.pdf;jsessionid=A55EA3492466DE356E42C29FDEB8E93F?sequence=1. [Accessed 29 April 2020].
[3] Victoria JG, Wang C, Jones MS, Jaing C, McLoughlin K, Gardner S, et al. Viral nucleic acids in live-attenuated vaccines: detection of minority variants and an adventitious virus. Journal of virology. 2010;84:6033-40.
[4] Ma H, Galvin TA, Glasner DR, Shaheduzzaman S, Khan AS. Identification of a novel rhabdovirus in Spodoptera frugiperda cell lines. Journal of virology. 2014;88:6576-85.
[5] Recommendations for the evaluation of animal cell cultures as substrates for the manufacture of biological medicinal products and for the characterization of cell banks. In: WHO expert committee on biological standardization: sixtyfirst report. Geneva: World Health Organization; 2013. Annex 3 (WHO Technical Report Series, No. 978.
https://www.who.int/biologicals/expert_committee/TRS_978_61st_report.pdf?ua=1. [Accessed 29 April 2020].
[6] Substitution of in vivo methods by in vitro methods for the quality control of vaccines. General chapter 5.2.14. Ph. Eur. tenth ed. Strasbourgh, France. Council of Europe; 2020.

[7] Tests for extraneous agents in viral vaccines for human use. General chaper 2.6.16. Ph. Eur. tenth ed. Strasbourg, France: Council of Europe; 2020.
[8] US Food and Drug Adminstration. Guidance for Industry: Characterizatoin and qualification of cell substrates and other biological materials used in the production of viral vaccines for infectious disease indications.
http://wwwfdagov/downloads/biologicsbloodvaccines/guidancecomplianceregulatoryinformation/guidances/vaccines/ucm202439pdf. 2010.
[9] Hamonisation IC. ICH Q5A(R2): Viral Safety Evaluation of Biotechnology Products Derived from Cell Lines of Human or Animal Origin. 2023.
[10] Agency. EM. Guideline on the principles of regulatory acceptance of 3Rs (Replacement R, Refinement) testing approaches. 2016 (EMA/CHMP/CVMP/JEG-3Rs/450091/2012), http://www.ema.europa.eu/docs/
en_GB/document_library/Scientificguideline/2017/03/WC500224306.pdf.
[11] Khan AS, Blumel, J., Deforce, D., Gruber, M.F., Jungback, C., Knezevic, I., Mallet, L., Mackay, D., Matthijnssens, J., O'Leary, M., Theuns, S., Victoria, J., Neels, P. Report of the second international conference on next generation sequencing for adventitous virus detection in biologics for humans and animals. Biologicals. 2020;in press.
[12] Khan AS, Mallet L, Blumel J, Cassart JP, Knezevic I, Ng SHS, et al. Report of the third conference on next-generation sequencing for adventitious virus detection in biologics for humans and animals. Biologicals : journal of the International Association of Biological Standardization. 2023;83:101696.
[13] Cleveland MH, Anekella B, Brewer M, Chin PJ, Couch H, Delwart E, et al. Report of the 2019 NIST-FDA workshop on standards for next generation sequencing detection of viral adventitious agents in biologics and biomanufacturing. Biologicals. 2020;64:76-82.

[14] Khan AS, Vacante DA, Cassart JP, Ng SH, Lambert C, Charlebois RL, et al. Advanced Virus Detection Technologies Interest Group (AVDTIG): Efforts for High Throughput Sequencing (HTS) for Virus Detection. PDA J Pharm Sci Technol. 2016.
[15] Khan AS, Ng SHS, Vandeputte O, Aljanahi A, Deyati A, Cassart JP, et al. A Multicenter Study To Evaluate the Performance of High-Throughput Sequencing for Virus Detection. mSphere. 2017;2.
[16] Ng SH, Braxton C, Eloit M, Feng SF, Fragnoud R, Mallet L, et al. Current Perspectives on High-Throughput Sequencing (HTS) for Adventitious Virus Detection: Upstream Sample Processing and Library Preparation. Viruses. 2018;10.
[17] Lambert C, Braxton C, Charlebois RL, Deyati A, Duncan P, La Neve F, et al. Considerations for Optimization of High-Throughput Sequencing Bioinformatics Pipelines for Virus Detection. Viruses. 2018;10.
[18] Sun R, Grogan E, Shedd D, Bykovsky AF, Kushnaryov VM, Grossberg SE, et al. Transmissible retrovirus in Epstein-Barr virus-producer B95-8 cells. Virology. 1995;209:374-83.
[19] Armstrong JA, Porterfield JS, De Madrid AT. C-type virus particles in pig kidney cell lines. J Gen Virol. 1971;10:195-8.