World Health Organization

WHO/BS/2020.2394
ENGLISH ONLY

EXPERT COMMITTEE ON BIOLOGICAL STANDARDIZATION
Geneva, 19 to 23 October 2020

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

Laboratory of Retroviruses, Division of Viral Products, Office of Vaccines Research and Review, Center for Biologics Evaluation and Review, U.S. Food and Drug Administration, Silver Spring, Maryland, 20993, U.S.A.

*Study coordinator. T: +1-240-402-9631, E: arifa.khan@fda.hhs.gov

NOTE:
This document has been prepared for the purpose of inviting comments and suggestions on the proposals contained therein, which will then be considered by the Expert Committee on Biological Standardization (ECBS). Comments MUST be received by **5 October 2020** and should be addressed to the World Health Organization, 1211 Geneva 27, Switzerland, attention: Technologies, Standards and Norms (TSN). Comments may also be submitted electronically to the Responsible Officer: **Dr Ivana Knezevic** at email: knezevici@who.int.

Dr Ivana Knezevic, Technologies Standards and Norms, Department of Essential Medicines and Health Products, World Health Organization, CH-1211 Geneva 27, Switzerland. Email: knezevici@who.int.

Summary

Eight laboratories from 6 countries have participated in a collaborative study to evaluate the suitability of 5 reference virus stocks as international virus reference standards for adventitious virus detection by next-generation sequencing (NGS) technologies. The viruses include Epstein-Barr virus (EBV), mammalian orthoreovirus type 1 (Reo), human respiratory syncytial virus (RSV), feline leukemia virus (FeLV) and porcine circovirus type (PCV) and represent virus families with distinct physicochemical properties for broad adventitious virus detection in biologics. The 5 viruses were spiked together at 10^4 genome copies of each virus into a high-titer adenovirus 5 (Ad5) background (10^9 genome copies) to evaluate the breadth of virus detection by NGS in a matrix mimicking a viral vaccine seed. Each laboratory used the same starting virus stocks for the spiking and the background to create their own sample material and then followed their own protocols for sample processing, cDNA synthesis, library preparation, sequencing, and bioinformatics analysis. The results indicated that 10^4 spiked copies of all five viruses were detected by the 8 laboratories, albeit at different levels. The study demonstrates the suitability of the virus stocks for evaluating the inter- and intra-laboratory potential of NGS for broad adventitious virus detection of known and unknown viruses. Furthermore, the use of international virus reference standards can facilitate optimization and potential development of shared NGS protocols and global harmonization of regulatory review of NGS submissions.

Introduction

Next generation sequencing (NGS) has demonstrated capabilities for broad virus detection assay including known and novel viruses [1, 2]. The discovery of porcine circovirus type 1 (PCV1) in a licensed rotavirus vaccine [3] brought NGS to the attention of regulators and industry as a potential new method for adventitious virus detection in biologics. The use of NGS as an alternative method for the conventional virus detection assays is encouraged in guidance documents from the WHO [4] and European Pharmacopeia [5, 6] and there is also flexibility

provided in the FDA guidance [7]. Furthermore, there was general international agreement in the IABS 2nd NGS conference that the use of NGS to substitute the *in vivo* assays would also accomplish the goal of the 3Rs regarding reducing animal use [8] and provide a standardized assay for broad virus detection. This meeting included human biologics and veterinary products and was attended by regulatory agencies, industry (biologics and biotherapeutics), contract research organizations (CROs), and academia [9]. It was noted that NGS can also address some of the limitations of the currently recommended *in vitro* adventitious virus assays, such as when vaccine virus cannot be neutralized in the *in vitro* assay. Furthermore, substitution or replacement of the *in vivo* and *in vitro* assays by NGS can reduce the testing period, which can take 1 month or longer. However, it was noted that standards for NGS were needed for standardization and validation of the method for its applications in biologics. This was also discussed in details at the NIST/FDA workshop in which different types of viral standards were discussed including live viruses which can test the entire NGS workflow as well as other types of standards (non-infectious) that could be used to test various steps in the technology [10].

The need for viral standards for NGS implementation as an alternative assay for adventitious virus detection in biologics was recognized by the FDA/Industry Advanced Virus Detection Interest Group (coordinated by the Parenteral Drug Association, PDA) [11]. An initial collaborative study involving 3 laboratories (FDA/CBER, GSK Vaccines and Sanofi Pasteur) used commercially-available, high-titer, virus stocks to test feasibility of the selected viruses as reference materials for demonstrating broad virus detection by different NGS platforms in different matrices [12]. 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 [13, 14]. The results from this study, which indicated similar virus detection was obtained by the three laboratories regardless of independent protocols and pipelines provided a scientific basis for large scale preparations and well-characterization of the 5 viruses as reference virus stocks for NGS standardization and validation to facilitate its implementation for adventitious virus detection in biologics.

This report includes the results from a larger inter-laboratory study involving 8 laboratories using independent protocols for the entire NGS workflow to evaluate the maximum differences that could be expected for virus detection from different sponsors, without having established shared protocols for the technical and bioinformatics steps in NGS. The data indicates all 5 viruses were detected and supports the use of the five reference virus stocks to demonstrate the capabilities of NGS for broad virus detection for NGS standardization and validation studies.

The adoption of the 5 reference virus stocks would facilitate inter-laboratory comparison and opportunities for NGS optimization for international use of NGS for adventitious virus detection by sponsors and would also facilitate early international harmonization for establishing expectations for NGS by regulators for using NGS for adventitious virus detection in submissions.

Since the 5 reference virus stocks are meant to be used as international standards for NGS, which is a nucleic-acid based technology, the viral genome copy number (VGCN) is the critical unit of measurement for each stock for NGS standardization and validation.

Aims of study

This study was designed to evaluate the suitability of the 5 virus stocks to demonstrate the breadth, specificity, and limit of virus detection by NGS in a sample mimicking a vaccine viral seed. The 5 reference virus stocks were spiked as a mix at 10^4 genome copies in a high-titer background of adenovirus 5 (Ad5). The use of these viruses that represented virus families with distinct physical and chemical properties would demonstrate the capabilities of NGS for broad adventitious virus detection. The study participants tested the worst-case scenario of using the reference virus stocks following their in-house SOPs for the entire NGS workflow, which would be the current situation for sponsors submitting NGS data to regulatory authorities.

Number of laboratories/countries

The characterization of the virus stocks for adventitious viruses and host nucleic acids were done by Illumina NGS HiSeq by the Merck Group lab. Additional NGS data analysis was done by the CBER lab to obtain consensus full-length virus genomes and virus variants. Stability studies at 24-months was done by the ATCC lab. Spiking study participants were collaborators from an ongoing study to evaluate the 5 virus stocks as potential NGS standards for adventitious virus detection in material mimicking a vaccine viral seed. The study was initiated with members of the FDA/Industry led Advanced Virus Detection Technologies Interest Group (AVDTIG) coordinated by the Parenteral Drug Association (PDA). The study included manufacturers of vaccines, gene therapies, and therapeutics, contract research organizations, and regulatory agencies. The individual participants, laboratories and countries are listed in Appendix A.

Materials

Viruses and cell lines

The 5 viruses were selected to represent different virus families of potential safety concern in vaccines and potentially in other biologics and consisted of distinct physical and chemical properties to evaluate the entire NGS workflow including virus extraction, cDNA synthesis, sequencing, and bioinformatics pipelines (Table 1). The viruses used in the original study were obtained by commercial laboratory from the American Type Culture Collection (ATCC, Herndon, VA, U.S.A.). These same five virus strains were prepared under a contract from the

FDA/CBER at large scale using cell lines available at ATCC: Epstein-Barr virus strain B95-8 (EBV), mammalian othoreovirus type 1 strain Lang (Reo), human respiratory syncytial virus strain A2 (RSV), feline leukemia virus strain KT-FeLV-UCD-1 (FeLV)].

Table 1. 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

Virus preparation

Virus preparation protocols were established with ATCC. Virus specifications 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.

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	B95-8 (persistently-infected cell line: CRL-1612)	B95-8 (CRL-1612)

Viruses were grown in cells using culture conditions to maximize virus yield and were harvested, clarified to remove cell debris, and further processed using TFF. Optimum TFF conditions were determined for each virus for filter size, volumes and flow rates. Viruses (except FeLV) were also nuclease-treated to reduce host cellular nucleic acids. In some cases, batches were pooled to obtain final specifications for infectious titer and genome copy number. The final viruses are in a liquid final formulation of 10 mM Tris-HCl, 135 mM NaCl, 0.5% BSA, 5% Trehalose. Automated filling was done with Essen Pipeline. All of the work was performed at ATCC with CBER oversight.

Some of the critical dates for the manufacturing and testing of the virus stocks are indicated in Table 3.

Table 3. Dates of Manufacturing Processes and Testing

Virus	**Code ID**	**Start date**	**Date of Harvest**	**TFF**	**Date of Vialing (-70°C to -80 °C storage)**	**Testing**			
						Sterility	**Mycoplasma (PCR)**	**Genome copy number (ddPCR)**	**Infectious titer**
PCV1	SC-VR-6000P	Dec 1 2015	Dec 8 2015	Feb 24 2015	Feb 24 2016	Mar 10 2016	Mar 7 2016	March 7 2016	May 19 2016
Reo	SC-VR-6001P	Aug 7 2015 Sept 5 2015	Aug 11 2015 (P2) Sept 29 2015 (P3)	Feb 10 2016 (pooled P2 and P3)	Feb 10 2016	Feb 29 2016	Feb 24 2016	Feb 12 2016	Mar 12 2016
FeLV	SC-VR-6002P	Apr 8 2016	Apr 18 2016	June 15 2016 (run 1) June 17 2016 (run 2)	June 17 2016	July 5 2016	Jul 29 2016	July 11 2016	July 22 2016

RSV	SC-VR-6003P	July 10 2015	July 14 2015	Feb 3 2016	Feb 4 2016	Feb 22 2016	Feb 24 2016	Feb 18 2016	March 12 2016
EBV	SC-VR-6004P	Aug 18 2015 Nov 23 2015 Mar 25 2016	Sept 3 2015 Dec 23 2015 Apr 21 2016 April 25 2016 Apr 28 2016	May 17, 19, 21 2016	May 24 2016	June 10 2016	Sept 6 2016	May 31 2016	Sept 26 2016

Vialing and storage

Viruses were vialed individually in aliquots of about 0.45 mL and stored at -80 °C. The material was stored in polypropylene tubes with screw caps to withstand long-term storage at low temperature and the caps contained a rubber gasket to reduce moisture. The storage site is ATCC located at 10801 University Boulevard, Manassas, VA 20110 USA. with Dr. Heather Couch as the Program Manager (ATCC Federal Solutions). ATCC is the largest international repository and distributor of cells and viruses, with temperature control and monitoring facilities. ATCC is also the current repository of the WHO Vero cell line and other FDA cell lines used for vaccine production. The code IDs for each virus stocks and the total number of vials prepared are indicated in Table 4.

Table 4. Virus vials prepared.

Virus Name	Code ID	Total number of vials prepared
Porcine circovirus type 1	SC-VR-6000P	413
Mammalian orthoreovirus type 1	SC-VR-6001P	425
Feline leukemia virus	SC-VR-6002P	514
Human respiratory syncytial virus	SC-VR-6003P	410
Epstein-Barr virus	SC-VR-6004P	512

Testing

An ATCC Certificate of Analysis is provided for each virus, which includes sterility and mycoplasma testing, infectious titer, genome copy number and two-year stability results. These

are attached in the Appendix B. It should be noted that the cell lines used for EBV and PCV1 also produced squirrel monkey retrovirus (SMRV) [15] and porcine endogenous retrovirus (PERV) [16], respectively. The genome copy number of these viruses were determined in the CBER lab and information will be included in updated COAs. The viruses were tested for adventitious viruses and host residual sequences by NGS at Millipore Sigma/Merck Group (the datasets will be submitted to NCBI/NIH with publication of a paper on virus characterization). Additionally, the NGS data was used to obtain consensus virus genome sequences and virus variants by the CBER lab. The genome of FeLV strain Kawakami-Theilen (KT) was recently published [17] since it was not previously available in GenBank.

The viral genome copy number was determined as the post-vial unitage using ddPCR assays, which were established at ATCC for the 5 viruses. The genes targeted for PCR were: PCV1 replicase; orthoreovirus core protein (L2); FeLV gag polyprotein; RSV N protein; and EBV EBER1 non-coding RNA. Viral nucleic acid was extracted using the QiaAmp Viral RNA Mini Kit (280 uL used for 2 x extraction) A dilution series of each virus was tested in triplicate by ddPCR assays. The final calculations were obtained from 9 samples (3 dilutions) on the BioRad QX200 Droplet Digital PCR (ddPCR) system.

The infectious titer was determined in cell lines suitable for each virus with the appropriate read-out assay. PCV1 titer at 16 days post-infection in ST cells (ATCC CRL-1746) at 37 °C with 5% CO2, was determined by endpoint PCR with PCV1 specific primers. Reo titer was determined by CPE at 9 days in LLC-MK2 cells (ATCC CCL-7.1) at 37 °C with 5% CO2 and humidity. FeLV titer was determined at day 7 in MYA-1 cells (ATCC CRL-2417) at 37 °C with 5% CO2 and humidity by endpoint PCR with FeLV specific primers. RSV titer was determined at 8 days in Hep-2 cells (ATCC CCL-23) at 37 °C with 5% CO2 and humidity, using immunofluorescence Light Diagnostics™, RSV FITC Reagent (Millipore catalog #5022). EBV titer was determined based on cell transformation at 60 days in irradiated human lung fibroblast cells (ATCC 55-X) at 37 °C with 5% CO2 and humidity.

Mycoplasma testing was peformed by DNA PCR using the Universal Mycoplasma Detection Kit (ATCC 30-1012K™).

Sterility testing (BacT/ALERT 3D) was done using IAST bottle at 32 °C, 14-day incubation for aerobes and INST bottle at 32 °C, 14-day incubation for anaerobes.

Stability studies on the product in the final container

The 5 virus stocks are vialed individually and stored under temperature-controlled conditions at -80°C for long-term storage. The 24-month stability studies were done at ATCC using the same ddPCR assays and conditions except for orthoreovirus where high-temperature was replaced with DMSO for denaturation of the double-strand RNA. Futhermore, the ATCC ddPCR assays

for the 5 viruses were transferred to the CBER lab, which conducted the studies at around 36 months, in the same manner as in ATCC, except that the virus extractions were done using phenol/chloroform/isoamyl. Similar results were obtained for the 24-month testing; the slightly higher number for the Reo in the CBER study could be due to the difference in the extraction method or subtleties in the denaturation of the double stranded RNA genome.

The results of the real-time stability studies are shown in Table 5. The assays are described above under the Testing section. These studies support long-term stability of the virus stocks under the -80°C storage conditions, since with no remarkable change in genome copy number (unitage) was seen at 24 - 36 months. Additionally, 24-month stability study was also done on the infectious titer, which also indicated no change under the storage conditions (Table 6). The materials will continue to be stored at -80 °C until vials have been used, except those retained as archival samples.

Table 5. Stability studies: Virus genome copy number (Unitage of vials)

Virus	**Virus Genome Copies**		
	ATCC	**ATCC**	**CBER**
	2016	**2018**	**2019**
PCV1	1.2×10^{11} (*March 7 2016: 9 rep.*)	2.7×10^{11} *(April 22 2018: 8 rep)*	3.0×10^{11} *(Feb. 28, 2019: 9 rep)*
Reo	3.17×10^{9} *(Feb 12 2016: 9 rep)*	2.4×10^{9} *(May 23 2018: 9 rep)*	1.4×10^{10} *(March 7 2019: 9 rep)*
FeLV	9.86×10^{10} *(July 11 2016: 9 rep)*	5.3×10^{10} *(Aug 1 2018: 8 rep)*	5.3×10^{10} *(March 7, 2019: 9 rep)*
RSV	3.02×10^{9} *(Feb 18 2016: 9 rep)*	1.04×10^{9} *(April 19 2018: 9 rep)*	1.0×10^{9} *(March 7, 2019: 9 rep)*
EBV	4.24×10^{8} *(May 31, 2016: 9 rep)*	3.7×10^{8} *(May 16 2018: 9 rep)*	3.8×10^{8} *(March 5, 2019: 9 rep)*

Table 6. Stability testing: Infectious titer

Virus	**Infectious Titer**	
	ATCC	**ATCC**
	2016	**2018**
PCV1	1.6×10^{7} *(May 19 2016)*	1.19×10^{7} *(April 26 2018)*
Reo	1.1×10^{10} (March 12 2016)	1.08×10^{10} (April 12 2018)

FeLV	1.6×10^7 *(July 22 2016)*	2.32×10^7 *(Aug 2 2018)*
RSV	1.16×10^6 *(March 12 2016)*	1.08×10^6 *(April 13 2018)*
EBV	1.05×10^7 *(Sept 26 2016)*	1.08×10^7 *(May 11 2018)*

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.

Distribution and current inventory (March, 2020)

The primary purpose of these virus stocks was to facilitate collaborative studies to evaluate the suitability of these viruses as NGS standards and to demonstrate specificity, sensitivity and breadth of virus detection for NGS method standardization. This information would facilitate further use of the viruses for validation studies and implementation of NGS for adventitious virus testing. So far, generally, only 2-3 vials of each virus have been provided under an FDA MTA for NGS spiking studies, conducted as a part of the Advanced Virus Detection Technologies Interest Group (AVDTIG) and recently also to other sponsors and CROs. Since Nov. 2017, these have included 11 laboratories in the US and 7 in Europe, including the 8 participating in the study reported here. The number of requests have now increased and for a greater number of vials due to the wider adoption of NGS. The number of requests is expected to continue to increase due to the current use of NGS to shorten the adventitious virus testing time for accelerate SARS-Cov-2 vaccine development. Once the viruses are approved as WHO IS, they can be distributed similarly as the WHO-Vero cell line, which is by request to FDA/CBER project officer (currently Dr. Arifa Khan) and shipment through ATCC (or another repository).

Study design

Each study laboratory received 2 vials of the 5 viruses for spiking studies to evaluate suitability of the viruses as WHO IS for adventitious virus detection by NGS. The study design was developed through extensive discussions within the AVDTIG Spiking Study subgroup 2B under a Confidentiality Data Agreement (CDA). It was agreed that different spiked levels of the 5 virus stocks would be spiked as 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.

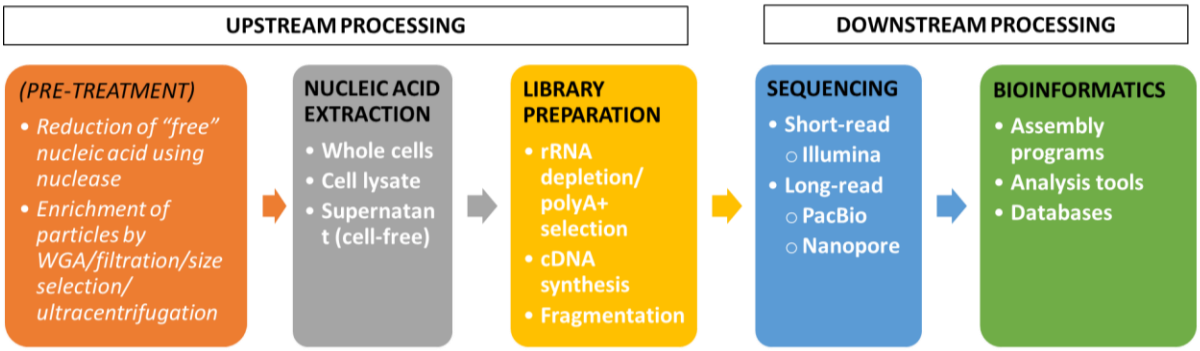
The adenovirus 5 (Ad5) used in this study was the well-characterized Adenovirus Type 5 Reference Material (ARM) made under guidance of the ARM Working Group and US FDA for characterizing adenoviral gene therapy products (ATCC catalogue number VR-1516). The ATCC product information sheet indicated a particle concentration of 5.8×10^{11} particles/mL

and infectious titer (HEK 293 cells) of 7 x 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 x 10^{11} copies/ml).

It was uncertain whether all of the laboratories would detect spike levels lower than 10^4 genome copies. Therefore, to enable comparison of the NGS data across different laboratories, it was decided that all of the participants would initially test the 10^4 genome copies/mL level and could add another or more levels. It was further decided to use independent protocols and bioinformatics pipelines to demonstrate robustness of NGS for adventitious virus detection under the most variable conditions, which would represent the current real-life scenario since there are no common protocols or pipelines used for viral detection by NGS. Additionally, there was flexibility in the bioinformatics analysis to include detection of the additional retroviruses (SMRV and PERV), which were present in the EBV and PCV1 stocks.

Some of the details of the NGS workflow that could influence outcome of the results are shown in Appendix C. These include steps for sample preparation, cDNA synthesis, library preparation, and NGS platforms used. Additionally, the controls to evaluate background signals due to reagents and other materials used in the upstream NGS workflow, the bioinformatics programs, and tools used in the downstream workflow are critical for the outcome 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. Overview of an NGS workflow.



Assay methods

The virus stocks were tested for sterility and infectious titer, genome copy number, whole virus genome sequence along with viral variants were determined.

Sterility testing, infectious titer, and genome copy number were determined by ATCC. 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. Virus-specific ddPCR assays were established at

ATCC for quantification of virus genome copy number. CBER laboratory established the same assays using the ATCC primer/probes and additional ddPCR assays for quantification of the SMRV and PERV genomes, which were found due to the host cell lines used for propagation of EBV and PCV1 preparations, respectively.

Illumina HiSeq was used for NGS analysis of each virus stocks for adventitious viruses by the Merck Group. Furthermore, using this data, the consensus sequence of each virus genome was obtained and variants determined by the CBER lab using CLC Genomics Workbench V11. The sequences of FeLV strain KT were not available in GenBank and were published from this study [17]. The datasets will be submitted to NCBI along with the paper for publication (in preparation).

Results

Five, large-scale virus stocks were prepared and characterized for NGS standardization and validation studies. The characterization included: determination of infectious virus titer, genome copy number, determination of full-length virus genomes and variants, and NGS analysis for adventitious virus detection. The results are provided in the ATCC COAs in Appendix B. The NGS 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.

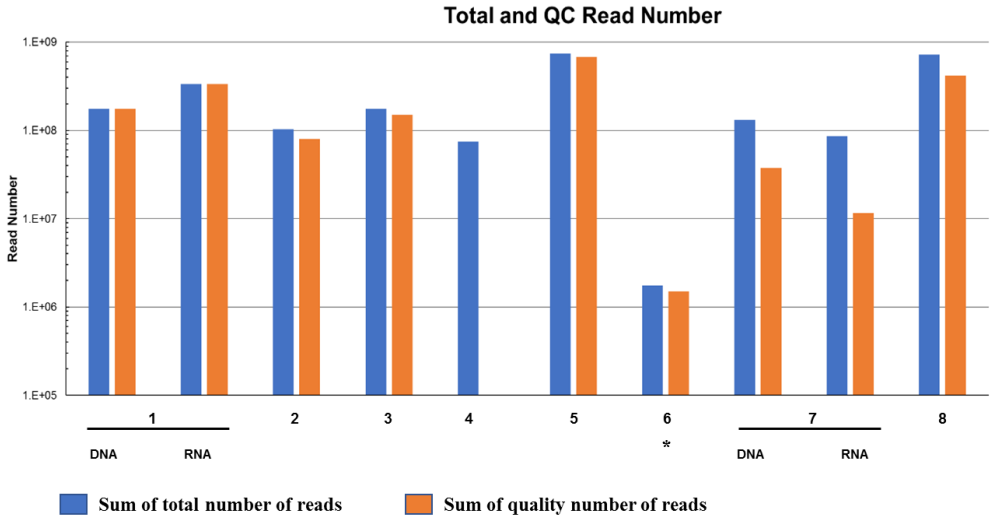
The 5 virus stocks were used for conducting spiking studies to evaluate their suitability as NGS reference standards. The results from the collaborative study of the 10^4 genome copies per mL of each of the virus stocks spiked together in the background of 10^9 genome copies per mL of Ad5 are presented in this report. Some of the details of the sample processing are shown in Appendix C. Labs #1 and #7 extracted and sequenced DNA and RNA separately and results from both are shown in Fig. 2 – 5. One of the laboratories (#7) pelleted the virus before further processing in order to sequence viral sequences associated with virus particles and exclude viral sequences in the cell such as mRNAs or endogenous retroviruses. The inclusion of controls was optional.

Different NGS platforms and kits were used for sequencing: five of the laboratories used Illumina NextSeq, two used HiSeq (labs 3 and 4), and one (lab 6) used MiSeq, a smaller-scale sequencing platform. In all cases the number of reads generated were 80M – 175 M except for MiSeq, where about 2M reads were generated. Majority of the hits were due to expected viruses or endogenous retroviral sequences that are expected to be present in residual, host cellular nucleic acids.

The critical information for evaluating suitability of the 5 virus stocks as NGS reference standards for adventitious virus detection are shown in Figs. 2 – 4. Data from the targeted analysis is included where the raw reads were mapped to the pooled 5 virus reference genomes

or to individual reference genomes (lab 3, who did not find a difference in the results doing it either way). The accession numbers are shown in Appendix D. Also, it is noted that results from lab 6 is using MiSeq (asterisk).

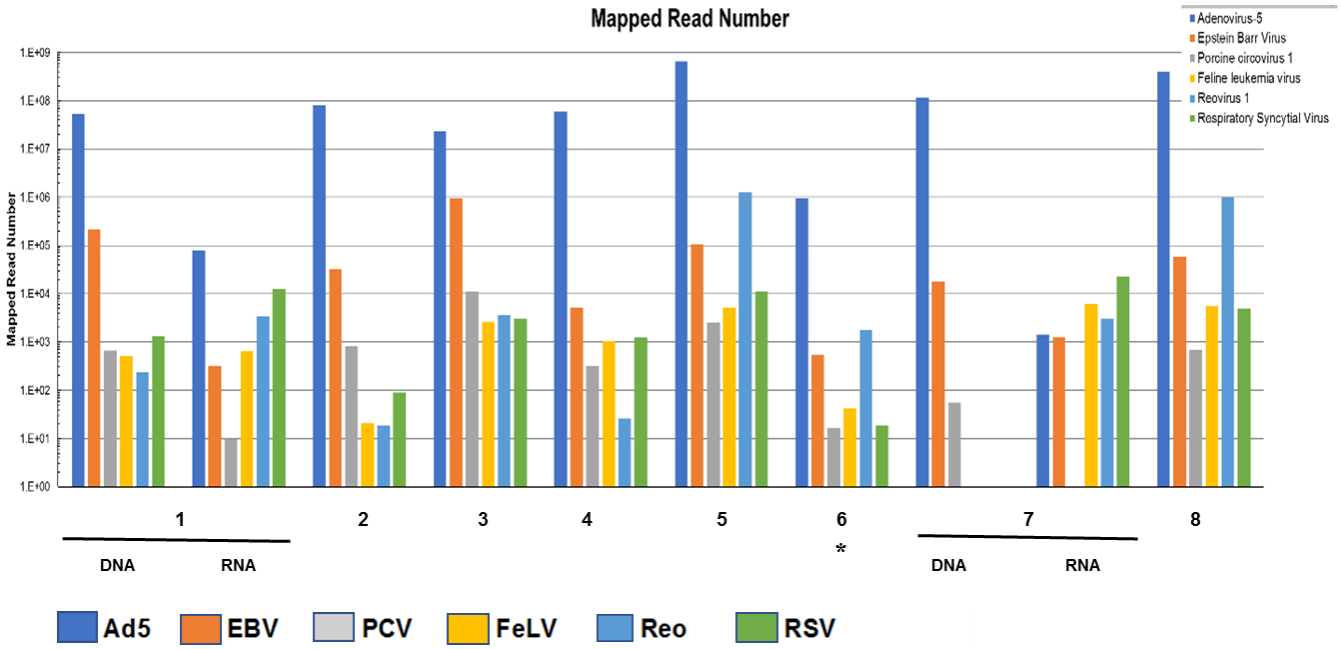
Fig. 2. Reads counts.



The number of total reads and quality reads are shown in Fig. 2 for each lab. The number of quality reads depends on the sequencing run overall quality and on the parameters and thresholds used for quality filtering. Labs 1 and 7 performed NGS on separate DNA and RNA extracted nucleic acids whereas the other labs extracted total nucleic acid containing DNA and RNA. Lab 4's bioinformatics pipeline did not filter for quality reads. The low number of reads for lab 6 is due to using the smaller-scale MiSeq sequencer. The number of total reads obtained reflects the efficiency of the different steps in sample processing and the sequencing platform and number of quality reads depends on the bioinformatics pipeline. For lab 1, there was no difference in the two numbers. In general, the total and quality reads obtained were within a log for all of the labs, except lab 6, which used the MiSeq. Also, the quality reads for lab 7 were somewhat lower than other labs but this may reflect differences in the parameters and thresholds used for the quality filtering, which needs consideration for inter-laboratory studies.

The quality reads (or the total reads in case of lab 4) were used for subsequent analysis shown in Figs. 3 – 5. Reads were mapped to the reference virus genomes to compare inter-laboratory detection of the different virus standards. The number of reads mapped to the reference virus genomes is shown in Fig. 3. Reference virus genomes were combined into a database for the NGS analysis (except for lab 3, which did it individually and combined, without a difference in the results, and included the results of the individual analysis in the figure).

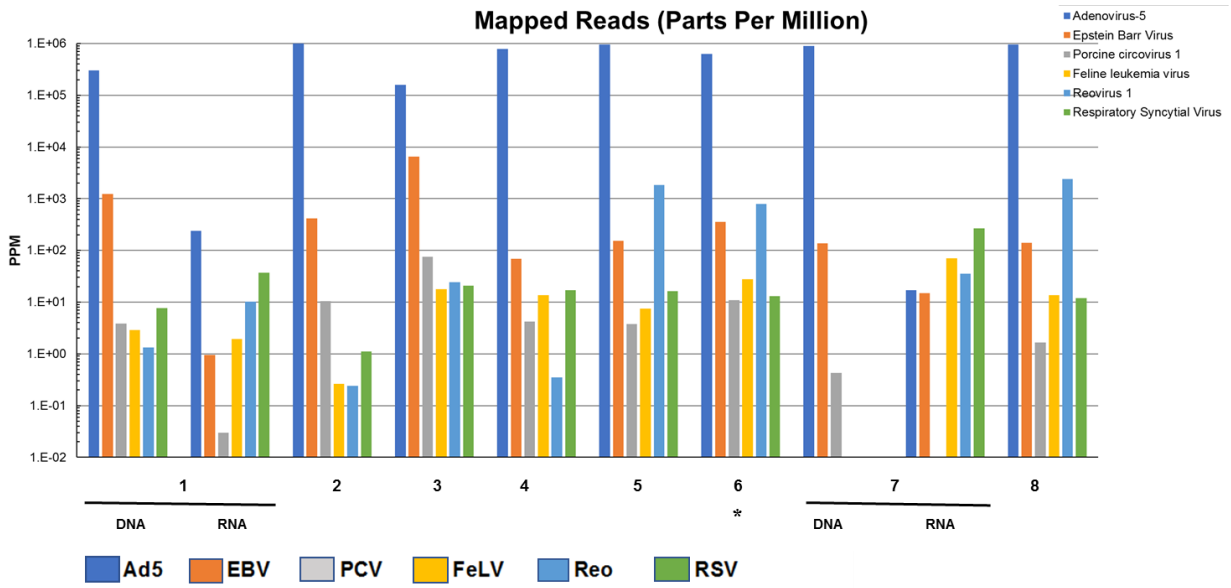
Fig. 3. Number of reads mapped to reference virus genomes.



The results in Fig. 3 indicates that all 5 viruses were detected by all of the laboratories, regardless of the sequencing platform and whether DNA and RNA were extracted separately or as total nucleic acid. Interestingly, the results of the virus pelleting was similar to others, indicating that the virus stocks contained intact virions. It was noted there were more mapped reads for the double-stranded DNA viruses (Ad5 and EBV), which could reflect their larger genome size and most for Ad5, which was present in high titer (10^9 genome copies). In general, similar results were seen for the two single-stranded RNA viruses (FeLV and RSV), whereas detection of single-stranded DNA virus (PCV1) and double stranded RNA virus (Reo) was more variable between the different laboratories, reflecting their high chemical resistance in the sample processing step. The differences seen in the results for detection of different viruses indicates the suitability of the 5 viruses for developing expectations for NGS data and for optimizing the NGS workflow for virus detection, as needed.

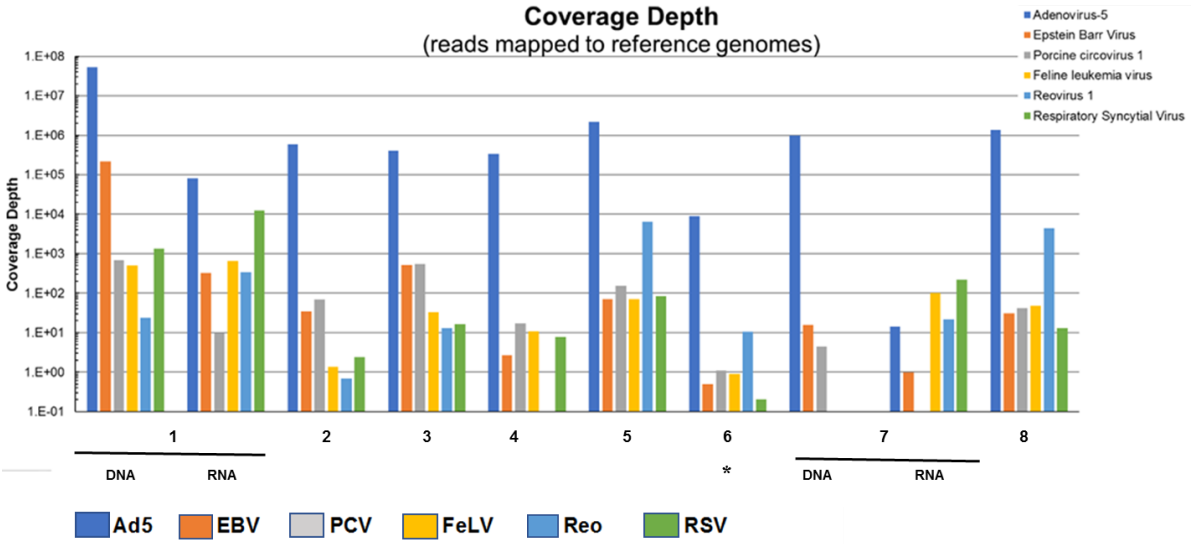
Different number of quality reads were obtained in the different laboratories. To compare the inter-laboratory results, the mapped reads counts were normalized as parts per million. The results are shown in Fig. 4. The overall profile of virus detection was similar to Fig. 3.

Fig. 4. Parts per million (PPM) read counts (of mapped reads).



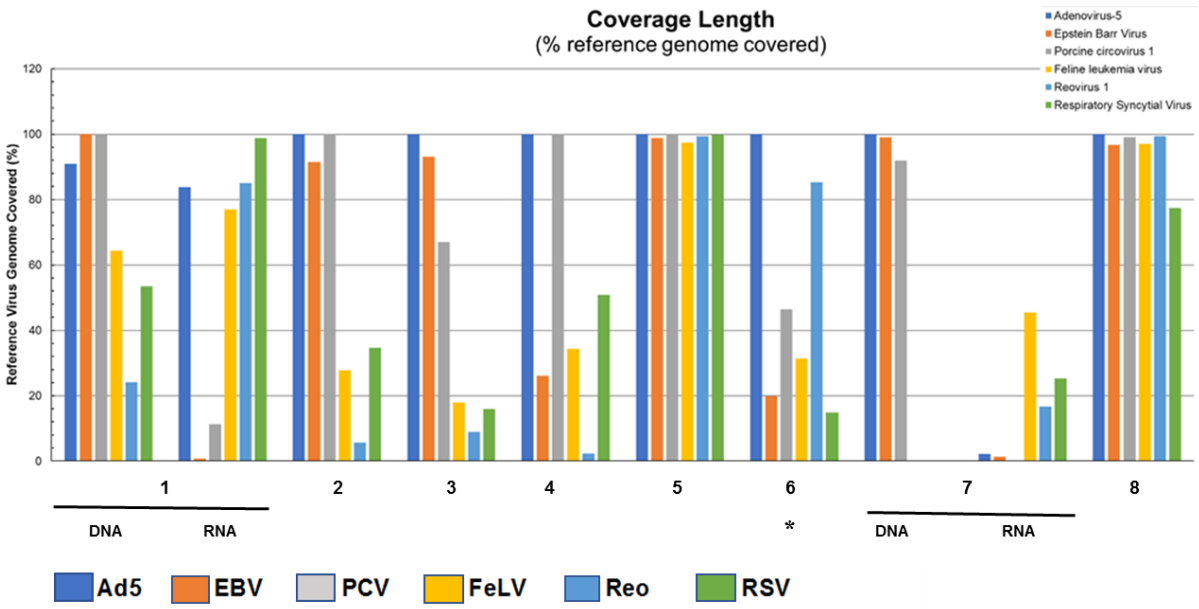
The coverage depth indicates reliability of the results for virus detection. The results shown in Fig. 5 indicates all 5 viruses were detected by all of the laboratories except for Reo by lab 4.

Fig. 5. 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. 6 shows different coverage of virus genome lengths were observed and for some labs (labs 1, 5, and 8) all 5 viruses had nearly 80% or greater genome length covered. It should be noted that labs 5 and 8 also had higher number of total reads. Generally, DNA viruses gave better results than RNA viruses, regardless of the genome size or structure.

Fig. 6. Coverage length.



Conclusions

The results demonstrated that the 5 reference viruses are suitable for NGS standardization by evaluating the NGS workflow for sensitivity and specificity of detection of different types of viruses (double and single stranded DNA and RNA viruses, linear and circular genomes; enveloped and non-enveloped; large and small particle size).

The 5 viruses represent distinct physicochemical properties of different virus families of potential concern in vaccines and other biologics and can be used for validation studies to demonstrate NGS capabilities for broad virus detection and support use of NGS for substitution or replacement of current adventitious virus detection assays.

Ongoing work

NGS targeted data analysis has been completed and presented in this report using the known reference viruses to demonstrate breadth of virus detection. NGS data analysis is continuing to test the bioinformatics pipeline for agnostic analysis using BLASTN against the NCBI nr/nt and RVDB databases to determine the specificity of virus detection using the reference virus stocks. This information will provide users with the expected true hits for determining sensitivity of virus detection using the reference virus standards.

The details of the NGS protocols will be further reviewed between the different laboratories to determine the critical parameters that affected difference in the results and develop conditions to optimize detection of all 5 viruses.

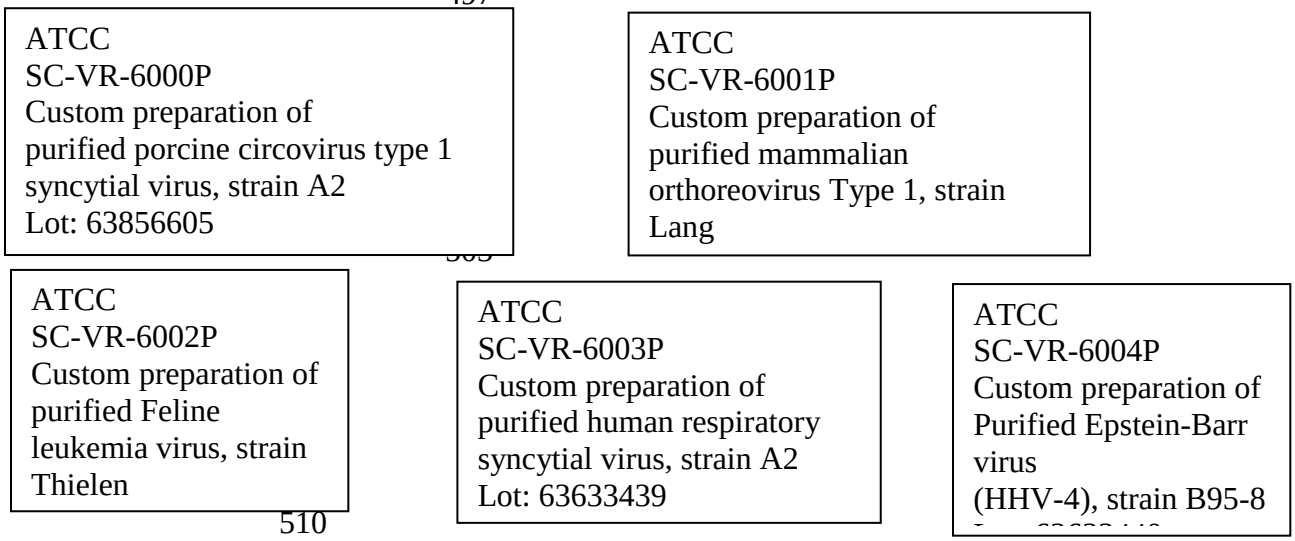
Proposal (preparation code number and proposed potency)

It is proposed that the candidate preparations in vials coded SC-VR-6000P, SC-VR-6001P, SC-VR-6002P, SC-VR-6003P, and SC-VR-6003P are established as the 1st International Virus Standards for Adventitious Virus Detection in Biological Products by NGS, with assigned unitage of genome copies per mL, 2.7×10^{11}, 1.4×10^{10}, 5.3×10^{10}, 1.04×10^{9}, and 3.7×10^{8}, respectively, be. The proposed standards are recommended to be used as a panel of 5 viruses to demonstrate breadth and sensitivity of NGS but can be used individually for virus/family-specific investigations. The proposed standards are intended to be used directly from the vial by manufacturers of biologics, regulatory and reference laboratories and contract research organizations to evaluate the NGS workflow for establishment, standardization and validation of NGS, and also for NGS evaluation to supplement, substitute or replace the currently recommended assays for adventitious virus detection (such as PCR, cell-based assays and animal assays).

The virus stocks are currently labelled as below and accompanied with the ATCC COA that includes infectious titer and genome copy number (included in Appendix B). The label on each vial is unique for each virus type. To avoid any changes in temperature that may occur in re-labelling the viruses with WHO codes, the viruses will be shipped with the WHO IS package insert that will combine the critical information for the virus characterization from the ATCC COA and new information about the adoption by WHO as IS.

Current labels

Current labels on each vial indicates, manufacturing facility, virus code number, virus name and strain, and lot number (it should be noted there is only one lot of each virus).



Proposed label and potency

The 5 viruses would be packaged in a box labelled with the 5 virus code numbers, which uniquely identifies each virus as shown in Table 7. Additionally, the unitage of each is indicated as genome copy per mL.

Table 7. Labels and vial unitage.

Box Label	Vial Label (code number)	Vial Unitage (genome copy number per mL
WHO-IS SC-VR-6000P SC-VR-6001P SC-VR-6002P SC-VR-6003P SC-VR-6004P	SC-VR-6000P	2.7×10^{11}
	SC-VR-6001P	1.4×10^{10}
	SC-VR-6002P	5.3×10^{10}
	SC-VR-6003P	1.04×10^{9}
	SC-VR-6004P	3.7×10^{8}

Comments from participants

Pros

- The five viruses broadly represent the different type of viral genome and physical properties of different viral families.
- The characterization package of the five viruses as a reference stock is also very thorough (GEQ, Titer, NGS sequence data for each sample, genomic sequence of the virus, COA, associated publications) are not found from other source.
- The reference standard stocks as separate purified viral stocks and in high titer is also highly desirable to allow for the most flexibility in NGS experiments and validations.

Cons

- They are stored in fairly large aliquots compared to what you might actually need, which could result in multiple freeze thaws or a need to set up all expected experiments at once.
    - *Response: it is expected to make smaller aliquots at the time of the initial thaw, re-freeze, and then use the aliquots for NGS studies. In this way all of the aliquots will have the same thaw and freeze conditions. Each aliquot can be used for 4-5 spiking studies.*

Acknowledgements

Funding for preparation and characterization of the virus stocks was provided by U.S. Food and Drug Organization. The 24-month virus stability study was kindly performed by ATCC. Jacek Remani and Lauren Rodrigues are thanked for oversight of the maintenance of the Illumina sequencer at Sanofi Pasteur.

Appendix A.

List of Participants and role in the study

Role in study	Names	Affiliation	Country
AVDTIG Spiking Study 2B	Dr. Maria M. Bednar Dr. Aurash Mohaimani	Biogen, Cambridge, MA	United States of America
	Dr. Arifa S. Khan Dr. Pei-Ju Chin	Center for Biologics Evaluation and Research, Food and Drug Administration, Silver Spring, MD	United States of America
	Dr. Andreas Leimbach Dr. Jacqueline Weber-Lehmann Dr. Daniel Richter Dr. Arman Tehrani Dr. Tobias Paprotka Dr. Weihong Wang Dr. Katherine Bergman Dr. Jeri Ann Boose	Eurofins Genomics, Konstanz Eurofins Lancaster Laboratories, Lancaster, PA	Germany United States of America
	Dr. Anne-Sophie Colinet Dr. Christophe Lambert Dr. Olivier Vandeputte Muriel Lahaye Dr. Jean-Pol Cassart	GSK Vaccines, Rixensart	Belgium

	Dr. Colette Cote	Millipore Sigma	United States of America
	Dr. Edward Mee	National Institute for Biological Standards and Control, South Mimms, Hertfordshire	United Kingdom
	Prof. Marc Eloit	Pathoquest, Paris Institut Pasteur, Paris	France
	Dr. Siemon H.S. Ng Dr. Robert Charlebois Dr. Artus Pedyczak Dr. Sara hashemi Ms. Briolange Martinho	Sanofi Pasteur Ltd., Toronto, Ontario	Canada
Virus stocks characterization: NGS	Dr. Fabio Le Neve Dr. Valeria Zanda Dr. Antonio Lembo	Merck Group, Giacosa	Italy
	Dr. Arifa S. Khan Dr. Pei-Ju Chin	Center for Biologics Evaluation and Research, Food and Drug Administration, Silver Spring, MD	United States of America
Virus stocks characterization: ddPCR	Dr. Arifa S. Khan Dr. Pei-Ju Chin	Center for Biologics Evaluation and Research, Food and Drug Administration, Silver Spring, MD	United States of America
Ad5 characterization: ddPCR	Dr. Anne-Sophie Colinet	GSK Vaccines, Rixensart	Belgium

	Dr. Olivier Vandeputte Muriel Lahaye		
Virus stocks stability: 24 months	Dr. Sujatha Rashid Dr. Teresa Towle	American Type Culture Collection, Herndon, VA	United States of America

547

Appendix B.

ATCC Certificates of Analysis

- Porcine Circovirus Type 1
- Mammalian Orthoreovirus
- Feline Leukemia Virus
- Respiratory Syncytial Virus
- Epstein-Barr Virus

PROCINE CIRCOVIRUS TYPE 1

CERTIFICATE OF ANALYSIS

ATCC® Number: SC-VR-6000P™

Lot Number: 63856605

Description: Custom preparation of porcine circovirus type 1[1,2]

Volume: Approximately 0.45 mL per vial

Product Format: Frozen

Expiration Date: Not applicable

Storage Conditions - 70°C or colder

Test / Method	Specification	Result	
		MAR/APR 2016	APR 2018
Titer (Post-vial)[3]	≥ 1 x 10^6 $TCID_{50}$/mL	1.6 x 10^7 $TCID_{50}$/mL	1.2 x 10^7 $TCID_{50}$/mL
Genome Copy Number by ddPCR (Post-vial)[4]	≥ 1 x 10^{10} genome copies/mL	1.2 x 10^{11} genome copies/mL	2.7 x 10^{11} 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	Test not repeated
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	 Test not repeated Test not repeated

[1]Porcine circovirus type 1 (PCV1) was grown in PK(15) porcine kidney cells (ATCC® CCL-33™) at 37°C with 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 DNA.
[3]16 days in ST cells (ATCC® CRL-1746™) at 37°C with 5% CO_2, as determined by endpoint PCR with PCV1 specific primers.
[4]ddPCR data was obtained post-vial from 9 replicates on the BioRad QX200 Droplet Digital PCR (ddPCR™) System.

Heather Couch Digitally signed by Heather Couch Date: 2018.10.18 09:35:27 -04'00' 18OCT2018

Heather Couch
Program Manager, Microbiology and Government 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 HHSF223201510136A. For additional information on characterization of the virus stocks (transmission electron microscopy and next generation sequencing), please contact Dr. Arifa Khan (Arifa.Khan@fda.hhs.gov).

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

ATCC hereby represents and warrants that the material provided under this certificate is pure and has been subjected to the tests and procedures specified and that the results described, along with any other data provided in this certificate, are true and correct to the best of the company's knowledge and belief. This certificate does not extend to the growth and/or passage of any living organism or cell line beyond what is supplied within the container received from ATCC.

This product is intended to be used for laboratory research use only. It is not intended for use in humans, animals, or for diagnostics. 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.

ATCC products may not be resold, modified for resale, used to provide commercial services, or to manufacture commercial products without prior written agreement from ATCC.

ATCC
10801 University Boulevard
Manassas, VA 20110-2209 USA
www.atcc.org

800-638-6597 or 703-365-2700
Fax: 703-365-2750
E-mail: tech@atcc.org
or contact your local distributor

MAMMALIAN ORTHOREOVIRUS

ATCC®

CERTIFICATE OF ANALYSIS

ATCC® Number: SC-VR-6001P™

Lot Number: 63633442

Description: Custom preparation of mammalian orthoreovirus type 1, strain Lang[1,2]

Volume: Approximately 0.45 mL per vial

Product Format: Frozen

Expiration Date: Not applicable

Storage Conditions - 70°C or colder

Test / Method	Specification	Result	
		FEB/MAR 2016	APR/MAY 2018
Titer (Post-vial)[3]	≥ 1 x 10^6 $TCID_{50}$/mL	1.1 x 10^{10} $TCID_{50}$/mL	1.1 x 10^{10} $TCID_{50}$/mL
Genome Copy Number by ddPCR (Post-vial)[4]	≥ 1 x 10^{10} genome copies/mL	3.2 x 10^9 genome copies/mL	2.4 x 10^9 genome copies/mL[5]
Test for Mycoplasma Contamination			
DNA detection by PCR of test article nucleic acid [Universal Mycoplasma Detection Kit (ATCC® 30-1012K™)]	None detected	None detected	Test not repeated
Sterility Test (BacT/ALERT 3D)			
iAST bottle (aerobic) at 32°C, 14-day incubation	No growth	No growth	Test not repeated
iNST bottle (anaerobic) at 32°C, 14-day incubation	No growth	No growth	Test not repeated

[1]Mammalian orthoreovirus type 1, strain Lang, was grown in LLC-MK2 derivative Rhesus monkey kidney cells (ATCC® CCL-7.1™) at 37°C with 5% CO_2 and humidity.
[2]Preparation was vialed in 10 mM Tris-HCl, 135 mM NaCl, 0.5% BSA and 5% trehalose and may contain residual cellular DNA.
[3]9 days in LLC-MK2 cells (ATCC® CCL-7.1™) at 37°C with 5% CO_2 and humidity, as determined by CPE.
[4]ddPCR data was obtained post-vial from 9 replicates on the BioRad QX200 Droplet Digital PCR (ddPCR™) System.
[5]Prior to dilution, RNA was treated with DMSO.

Heather Couch
Digitally signed by Heather Couch
Date: 2018.10.18 09:39:02 -04'00'
18OCT2018

Heather Couch
Program Manager, Microbiology and Government 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 HHSF223201510136A. For additional information on characterization of the virus stocks (transmission electron microscopy and next generation sequencing), please contact Dr. Arifa Khan (Arifa.Khan@fda.hhs.gov).

ATCC
10801 University Boulevard
Manassas, VA 20110-2209 USA
www.atcc.org

800-638-6597 or 703-365-2700
Fax: 703-365-2750
E-mail: tech@atcc.org
or contact your local distributor

563 **FELINE LEUKEMIA VIRUS**

ATCC®

CERTIFICATE OF ANALYSIS

ATCC® Number: SC-VR-6002P™

Lot Number: 63856597

Description: Custom preparation of feline leukemia virus, strain Thielen[1,2]

Volume: Approximately 0.45 mL per vial

Product Format: Frozen

Expiration Date: Not applicable

Storage Conditions - 70°C or colder

Test / Method	Specification	Result	
		JUL 2016	AUG 2018
Titer (Post-vial)[3]	≥ 1 x 10^6 $TCID_{50}$/mL	1.6 x 10^7 $TCID_{50}$/mL	2.3 x 10^7 $TCID_{50}$/mL
Genome Copy Number by ddPCR (Post-vial)[4]	≥ 1 x 10^{10} genome copies/mL	9.9 x 10^{10} genome copies/mL	5.3 x 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	Test not repeated
Sterility Test (BacT/ALERT 3D)			
iAST bottle (aerobic) at 32°C, 14-day incubation	No growth	No growth	Test not repeated
iNST bottle (anaerobic) at 32°C, 14-day incubation	No growth	No growth	Test not repeated

[1]Feline leukemia virus (FLV), strain Thielen, was grown in FL74-UCD-1 cat lymphoblast cells (ATCC® CRL-8012™) at 36°C.
[2]Preparation was vialed in 10 mM Tris-HCl, 135 mM NaCl, 0.5% BSA and 5% trehalose and may contain residual cellular DNA.
[3]7 days in MYA-1 cells (ATCC® CRL-2417™) at 37°C with 5% CO_2 and humidity, as determined by endpoint PCR with FLV specific primers.
[4]ddPCR data was obtained post-vial from 9 replicates on the BioRad QX200 Droplet Digital PCR (ddPCR™) System.

Heather Couch

Digitally signed by Heather Couch
Date: 2018.10.18 09:40:14 -04'00'

18OCT2018

Heather Couch
Program Manager, Microbiology and Government 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 HHSF223201510136A. For additional information on characterization of the virus stocks (transmission electron microscopy and next generation sequencing), please contact Dr. Arifa Khan (Arifa.Khan@fda.hhs.gov).

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

ATCC hereby represents and warrants that the material provided under this certificate is pure and has been subjected to the tests and procedures specified and that the results described, along with any other data provided in this certificate, are true and correct to the best of the company's knowledge and belief. This certificate does not extend to the growth and/or passage of any living organism or cell line beyond what is supplied within the container received from ATCC.

This product is intended to be used for laboratory research use only. It is not intended for use in humans, animals, or for diagnostics. 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.

ATCC products may not be resold, modified for resale, used to provide commercial services, or to manufacture commercial products without prior written agreement from ATCC.

ATCC
10801 University Boulevard
Manassas, VA 20110-2209 USA
www.atcc.org

800-638-6597 or 703-365-2700
Fax: 703-365-2750
E-mail: tech@atcc.org
or contact your local distributor

564
565

566 **RESPIRATORY SYNCYTIAL VIRUS**

ATCC®

CERTIFICATE OF ANALYSIS

ATCC® Number:	**SC-VR-6003P™**
Lot Number:	**63633439**
Description:	**Custom preparation of human respiratory syncytial virus, strain A2[1,2]**
Volume:	**Approximately 0.45 mL per vial**
Product Format:	**Frozen**
Expiration Date:	**Not applicable**
Storage Conditions	**- 70°C or colder**

Test / Method	Specification	Result	
		APR 2016	APR 2018
Titer (Post-vial)[3]	≥ 1 x 10^6 $TCID_{50}$/mL	1.6 x 10^6 $TCID_{50}$/mL	1.1 x 10^6 $TCID_{50}$/mL
Genome Copy Number by ddPCR (Post-vial)[4]	≥ 1 x 10^{10} genome copies/mL	3.0 x 10^9 genome copies/mL	1.0 x 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	Test not repeated
Sterility Test (BacT/ALERT 3D)			
iAST bottle (aerobic) at 32°C, 14-day incubation	No growth	No growth	Test not repeated
iNST bottle (anaerobic) at 32°C, 14-day incubation	No growth	No growth	Test not repeated

[1]Human respiratory syncytial virus, strain A2, was grown in HEp-2 cells (ATCC® CCL-23™) at 37°C with 5% CO_2 and humidity.
[2]Preparation was vialed in 10 mM Tris-HCl, 135 mM NaCl, 0.5% BSA and 5% trehalose and may contain residual cellular DNA.
[3]8 days in HEp-2 cells (ATCC® CCL-23™) at 37°C with 5% CO_2 and humidity, as determined by Immunofluorescence Light Diagnostics™ Respiratory Syncytial Virus FITC Reagent (Millipore catalog # 5022).
[4]ddPCR data was obtained post-vial from 9 replicates on the BioRad QX200 Droplet Digital PCR (ddPCR™) System.

Heather Couch
Digitally signed by Heather Couch
Date: 2018.10.18 09:41:22 -04'00'
18OCT2018

Heather Couch
Program Manager, Microbiology and Government 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 HHSF223201510136A. For additional information on characterization of the virus stocks (transmission electron microscopy and next generation sequencing), please contact Dr. Arifa Khan (Arifa.Khan@fda.hhs.gov).

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

ATCC hereby represents and warrants that the material provided under this certificate is pure and has been subjected to the tests and procedures specified and that the results described, along with any other data provided in this certificate, are true and correct to the best of the company's knowledge and belief. This certificate does not extend to the growth and/or passage of any living organism or cell line beyond what is supplied within the container received from ATCC.

This product is intended to be used for laboratory research use only. It is not intended for use in humans, animals, or for diagnostics. 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.

ATCC products may not be resold, modified for resale, used to provide commercial services, or to manufacture commercial products without prior written agreement from ATCC.

ATCC
10801 University Boulevard
Manassas, VA 20110-2209 USA
www.atcc.org

800-638-6597 or 703-365-2700
Fax: 703-365-2750
E-mail: tech@atcc.org
or contact your local distributor

Template Doc ID: 31194 Template Revision: 5 Template Effective Date: 10/16/2017

567

EPSTEIN-BARR VIRUS

ATCC®

CERTIFICATE OF ANALYSIS

ATCC® Number: SC-VR-6004P™

Lot Number: 63633440

Description: Custom preparation of Epstein-Barr virus (HHV-4), strain B95-8[1,2]

Volume: Approximately 0.45 mL per vial

Product Format: Frozen

Expiration Date: Not applicable

Storage Conditions - 70°C or colder

Test / Method	Specification	Result	
		JUN/SEP 2016	MAY/JUN 2018
Titer (Post-vial)[3]	≥ 1 x 10^6 $TCID_{50}$/mL	1.6 x 10^7 $TCID_{50}$/mL	1.1 x 10^7 $TCID_{50}$/mL
Genome Copy Number by ddPCR (Post-vial)[4]	≥ 1 x 10^{10} genome copies/mL	4.2 x 10^8 genome copies/mL	3.7 x 10^8 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	Test not repeated
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	 Test not repeated Test not repeated

[1]Epstein-Barr virus (HHV-4), strain B95-8, was grown in B95-8 Marmoset leukocyte cells (ATCC® CRL-1612™) at 37°C with 5% CO_2 and humidity.
[2]Preparation was vialed in 10 mM Tris-HCl, 135 mM NaCl, 0.5% BSA and 5% trehalose and may contain residual cellular DNA.
[3]60 days in irradiated human lung fibroblast cells (ATCC® 55-X™) at 37°C with 5% CO_2 and humidity, as determined by transformation.
[4]ddPCR data was obtained post-vial from 9 replicates on the BioRad QX200 Droplet Digital PCR (ddPCR™) System.

Heather Couch
Digitally signed by Heather Couch
Date: 2018.10.18 09:46:55 -04'00'
18OCT2018

Heather Couch
Program Manager, Microbiology and Government 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 HHSF223201510136A. For additional information on characterization of the virus stocks (transmission electron microscopy and next generation sequencing), please contact Dr. Arifa Khan (Arifa.Khan@fda.hhs.gov).

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

ATCC hereby represents and warrants that the material provided under this certificate is pure and has been subjected to the tests and procedures specified and that the results described, along with any other data provided in this certificate, are true and correct to the best of the company's knowledge and belief. This certificate does not extend to the growth and/or passage of any living organism or cell line beyond what is supplied within the container received from ATCC.

This product is intended to be used for laboratory research use only. It is not intended for use in humans, animals, or for diagnostics. 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.

ATCC products may not be resold, modified for resale, used to provide commercial services, or to manufacture commercial products without prior written agreement from ATCC.

ATCC
10801 University Boulevard
Manassas, VA 20110-2209 USA
www.atcc.org

800-638-6597 or 703-365-2700
Fax: 703-365-2750
E-mail: tech@atcc.org
or contact your local distributor

Template Doc ID: 31194 Template Revision: 5 Template Effective Date: 10/16/2017

Appendix C. Sample preparation and Sequencing

Sample Prep	Lab 1	Lab 2	Lab 3	Lab 4	Lab 5	Lab 6	Lab 7	Lab 8
Volume used for spiking	1 mL (ea)	1 mL	400 uL	200 uL	1 mL	140 uL	1 mL	1 mL
Volume used for Ext	140 uL (RNA) 200 uL (DNA)	1 mL	400 uL	200 uL	1 mL (5x200 uL)		(virus pellet)	900 uL
Extracted volume	30 uL (RNA) 35 uL (DNA	150 uL	50 uL	100 uL	100 uL (5 x 20 uL)	60 uL	50 uL	50 uL
Pre-extraction Treatment	DNase (RNA)	Low speed-clarification			no		RNase/DNase Ultracentrifug (=particles)	
Extraction (DNA+RNA)		MinElute Virus spin kits	Phenol/ Chloroform	Silica column	MinElute Virus spin kits	MinElute Virus spin kits	Pathogen cador mini kit (on column extraction)	PureLink DNA/ RNA
DNA	Qiagen Viral MinElute							WAKO
RNA	Qiagen Viral RNA							
Post-extraction treatment	rRNA depletion: Kappa kit (RNA)				MinElute PCR purification (=10 uL)		rRNA-sp probes	ribominus
Amount for library prep	10 uL (RNA) 11.5 uL (DNA)				No			30 uL of cDNA (= 662.4 uL of starting)
Library ampliication	cDNA (DNA)	cDNA with amp	cDNA syn	cDNA with amp	No	RepliG Whole Transcriptome Amplification		
Sequencing library preparation	Nextera DNA Flex (DNA) Hyper KapaPrep with RiboErase (RNA)	Nextera XT	SMAR-Seq Stranded	TruSeq DNA Nano	Nextera XT	DNA Flex	Accel NGS-1S + (for DNA viruses) SMARTER stranded total RNA-Seq kit (RNA viruses)	Nextera XT
Sequencing platform	NextSeq 500 v2.5	NextSeq 550 v2.5	HiSeq 2500 cBot PE rapid v2.0	HiSeq 2500 v4.0	NextSeq 500 v2.5	MiSeq	NextSeq 500/550 (hi V2)	NextSeq 500 high output cell
CONTROLS: Sequencing					PhiX			
CONTROLS: Spike Sample-positive	-	Ad5	Ad5	Ad5		Ad5	Ad5	-

CONTROLS: Spike Sample-negative	-	water	DMEM no serum	water	TE	Water (from the WTA step)	Ramos cell lysate	-

572

573

APPENDIX D. Reference genome accession numbers for mapping reads

Virus	Accession number
Epstein Barr Virus	V01555.2
Respiratory Syncytial Virus	JF920069.1
Reovirus 1	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	NC_001940.1
Porcine circovirus 1	NC_001792.2
Adenovirus-5	AY339865.1

APPENDIX E. 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

American Type Culture Collection
10801 University Boulevard
Manassas, VA 20110-2209 USA
Contact: Heather Couch (hcouch@atcc.org)

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

1ST International Virus Reference Standards for Adventitious Virus Detection in Biological Products by Next-Generation Sequencing Technologies (CBER)

SC-VR-6000P 08Dec2015
SC-VR-6001P 28Jul2015
SC-VR-6002P 18Apr2016
SC-VR-6003P 14Jul2015
SC-VR-6004P 03Sept2015

1. INTENDED USE OF THE MATERIAL

The primary use of the material is to support standardization and validation of next-generation sequencing technologies for adventitious virus detection of biologics

The secondary use of the material can be to support 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, which have been sourced from human and animal origin. The viruses are in a liquid final formulation of 10 mM Tris-HCl, 135 mM NaCl, 0.5% BSA, 5% Trehalose:

Porcine circovirus type 1 (SC-VR-6000P)
Mammalian orthoreovirus Type 1, strain Land (SC-VR-6001P)
Feline leukemia virus strain Thielen (SC-VR-6002P)
Human respiratory syncytial virus strain A2 (SC-VR-6003P)
Epstein Barr virus (HHV-4) strain B95-8 (SC-VR-6004P)

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 discarded 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.

The fetal bovine serum used in the growth of the viruses (ATCC 30-2020) was manufactured from fetal bovine blood collected in USDA inspected abattoirs located in the United States. 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. The vials have a screw cap with a rubber gasket. 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 -80 °C and avoiding any changes in temperature during transfer to vials.

At time of use, standard procedures to retain intactness of live viruses should be used. For example, the vial should be thawed on wet ice or quickly by swirling at 37 °C until a small ice crystal remains and immediately placed on wet ice. Each vial contains about 0.45 ml virus. Upon first time thaw, small aliquots of the virus should be made based on intended use per study and all should be again stored at -80 °C prior to first use in a study. If not sure, then it is best to make aliquot of small volume, which can be combined later, however, the volume should be sufficient to avoid loss by evaporation and stored in cryogenic vials with a rubber gasket in the cap. This 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.

Used vials and all materials exposed to the virus during the handling or in performing the studies should disposed according to laboratory's practices for infectious agents.

5. CITATION

If the material used by the Recipient, it's Affiliates or 3rd party contract service providers provides information that is included in a part of a public presentation or publication, it is important to correctly cite CBER's materials with the title of the preparation, code number, and CBER's name and address.

6. LIABILITY AND LOSS

This material is being provided as a service to the scientific community. It is being supplied with no warranties, express or implied.

Recipient agrees that the material is being provided for not-for-profit purposes only and further agrees not to claim, infer, or imply endorsement by the Government of the United States of America, the Department of Health and Human Services, the FDA, or any employee (Referred to as the Agency in below).

Further, the Agency shall under no circumstances whatsoever be liable to the Recipient, whether in contract, tort (including negligence), breach of statutory duty, or otherwise, for any loss of data, loss of profit, loss of business or goodwill, or any indirect or consequential loss or damage suffered or incurred by the Recipient arising in relation to the supply of the Materials or the use, keeping, production or disposal of the Materials or any waste products arising from the use thereof by the Recipient or by any other person.

The Recipient shall indemnify and hold the Agency, its officers, employees and agents harmless against any loss, claim, damage or liability including reasonable legal costs and fees (of whatsoever kind or nature) made against the Agency which may arise as a result of the wilful act, omission or negligence of the Recipient or its employees, or the use, keeping, production or disposal of the Materials or any waste products arising from the use thereof by the Recipient or on its behalf.

APPENDIX F. 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: American Type Culture Collection 10801 University Boulevard Manassas, VA 20110-2209, USA	Name: Heather Couch Email: hcouch@atcc.org

Status of Material		
1ST International Virus Reference Standards for Adventitious Virus Detection in Biological Products by Next-Generation Sequencing Technologies (CBER)		
Identifying Code	Establishment Date (final vials)	Potency (genome copies per mL): May/June 2018
SC-VR-6000P	Mar 2 2016	2.7×10^{11}
SC-VR-6001P	Feb 12 2016	$2.4 \times 10^{9}2$
SC-VR-6002P	June 20 2016	5.3×10^{10}
SC-VR-6003P	Feb 5 2016	1×10^{9}
SC-VR-6004P	May 25, 2016	3.7×10^{8}

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 orgin		
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.

705
706

APPENDIX G. 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.

Identifying Code	**Reference Virus**	**Other Viruses** (due to the cell line used for propagation)	**Other nucleic acids**
SC-VR-6000P	Porcine circovirus, type 1	Endogenous porcine retrovirus (PERV)	Host cell sequences with porcine ERVs*
SC-VR-6001P	Mammalian orthoreovirus, Lang strain		Host cell sequences with human ERVs
SC-VR-6002P	Feline leukemia virus, (Kawakami-) Thielen strain (KT)		Host cell sequences with feline ERVs
SC-VR-6003P	Human respiratory syncytial virus, A2		Host cell sequences with human ERVs
SC-VR-6004P	Epstein-Barr Virus (HHV4), strain B95-8	Squirrel monkey retrovirus (SMRV)	Host cell sequences with simian 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 animal species.

LITERATURE REFERENCE 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. J Virol. 2010;84:6033-40.

[4] 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].

[5] 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.

[6] 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.

[7] 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.

[8] European Medicines Agency. 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.

[9] 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.

[10] 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.

[11] 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.

[12] 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.

[13] 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.

765 [14] Lambert C, Braxton C, Charlebois RL, Deyati A, Duncan P, La Neve F, et al. Considerations for
766 Optimization of High-Throughput Sequencing Bioinformatics Pipelines for Virus Detection. Viruses.
767 2018;10.
768 [15] Sun R, Grogan E, Shedd D, Bykovsky AF, Kushnaryov VM, Grossberg SE, et al. Transmissible
769 retrovirus in Epstein-Barr virus-producer B95-8 cells. Virology. 1995;209:374-83.
770 [16] Armstrong JA, Porterfield JS, De Madrid AT. C-type virus particles in pig kidney cell lines. J Gen Virol.
771 1971;10:195-8.
772 [17] Chin PJ, La Neve F, Zanda V, Khan AS. Complete Genome Sequence of Feline Leukemia Virus
773 Kawakami-Theilen Strain KT-FeLV-UCD-1. Microbiol Resour Announc. 2020;9.

774