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ORIGINAL ARTICLE

Evaluating the viral clearance ability of continuous monoclonal antibody purification steps, in order to inactivate and/or remove four model viruses

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ABSTRACT

Background and Objectives: Viral clearance studies are an essential part of a manufacturer's plan to ensure the safety of an injectable biologic product. In this way, viral safety is a critical quality attribute for biologics such as monoclonal antibodies (Mabs). Evaluation of virus purification by downstream processes is a key component of risk mitigation. In this study, the capability of continuous monoclonal antibody purification steps was evaluated in the process of instant monoclonal antibody purification in different stages of purification, and the amount of reduction or inactivation of each step was determined.

Materials and Methods: Four enveloped and non-enveloped viral models VSV, Reovirus, EMCV, and HSV1 were used for spiking in selected samples in the designated tests, to have a comprehensive examination of the ability to clear the virus such as the type of genetic material, chemical resistance, and particle size. A $TCID_{50}$ and qPCR methods were used to measure viral reduction. Two cell lines, Vero (African green monkey kidney) and L929 (Mouse fibroblast) were used for 4 model viruses propagation. The steps that were evaluated included 4 steps monoclonal antibody purification; cation exchange chromatography, acidic pH treatment, affinity chromatography, and nanofiltration.

Results: The nano-filter stage showed the highest viral reduction and cation exchange chromatography showed the lowest reduction. The cumulative decrease using $TCID_{50}$ is equal to 19.27 [log10] for all steps and for the qPCR method is equal to 12.47 [log10] in three steps of nano-filter, affinity chromatography, and ion exchange chromatography.

Conclusion: The overall average reduction coefficient for all four model viruses is significantly high, which indicates the high capacity of the monoclonal antibody production process in inactivating and removing viruses leads to reducing the load of all four model viruses.

Keywords: Viral inactivation; Virus removal; Cation exchange chromatography; Viral clearance

INTRODUCTION

Medicinal proteins such as monoclonal antibodies; recombinant proteins; hormones and coagulation factors have occupied a special place in the pharmaceutical biotechnology industry, subsequently, viral contamination of the production lines of such products, although it is seldom, it is not impossible, which has serious consequences in case of contamination in receivers.

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Also, viral contamination can indirectly endanger the safety of patients through the reduction effect on the amount of active pharmaceutical ingredients due to disinfection activities of the production line (1). Even though new and strict methods have been proposed in the industry to prevent contamination, various endogenous and accidental contaminations have continued to occur over many years, as contamination with viruses such as Porcine Circovirus (PCV1) (2), Cache Valley Virus (CVV) (3, 4), Reovirus (Reo3) (4, 5), Epizootic Hemorrhagic Disease Virus (EHDV) (6), Human Adenovirus (HAdV) (7), Mouse Minute Virus (MMV) (8-10), and Vesivirus 2117 reported (11).

Cell lines derived from humans and rodents such as 33T, CHO, BHK, HeLa, and HepG2, have an important role in the production of medicinal proteins, which the Chinese Hamster Ovary (CHO: Chinese Hamster Ovary) are among the most commonly used cells (12). In this regard, the concern is the contamination of murine hybridoma cells for expression of the drug protein, because this rodent cell line has shown endogenous retrovirus production (at least four types of infectious viruses or "retrovirus-like" particles (13-17). This is because their genomes contain integrated multiple copies of retrovirus-like sequences.

Virus filtration step is commonly utilized in a well-designed recombinant therapeutic protein purification process and is a key component in an overall strategy to minimize the risks of adventitious and endogenous viral particles during the manufacturing of biotechnology products (18).

In an IFN-γ assay, purified protein derivative of *M. bovis* fusion proteins showed equivalent sensibility but better specificity than the same *M. bovis* proteins produced in *E. coli* (19).

The inactivation of viruses is done with the aim of reducing or eliminating viral contamination by chemical or physical methods. In this way, treatment with acidic pH, use of strong solvent/detergent, heat, and radiation are usually used in viral purification of retroviruses. Virus removal employs the physical separation of viral particles from the desired product and mainly includes three methods (i) filtration (ii) chromatography (protein A affinity chromatography; ion exchange chromatography); and (iii) precipitation (Cohen's decomposition, precipitation with ethanol). Ideally, virus removal by filtration is the method of choice because it is not limited by parameters such as pH, ionic strength, and isoelectric point (20-23).

A chosen model viruses are briefly described in below:

- Vesicular stomatitis virus (VSV) has an envelope, single-stranded RNA, and ~200 nm in diameter and belongs to the *Rhabdoviridae* Family. This virus has little resistance to physical and chemical inactivation.

The VSV virus shows high stability in suspension at a temperature of 4 degrees Celsius and above in a period of several weeks as far as the survival rate is considered. It is also widely used as a model for deactivation at pH 4. VSV virus is a zoonosis virus and causes influenza-like illness in infected humans (24).

- Reovirus: This virus is non-enveloped, double-stranded RNA with a diameter of ~60 nm and belongs to the *Reoviridae* Family. This virus has moderate to low resistance to physical and chemical inactivation.

The target organs for infection with this virus are the digestive and respiratory systems. Reovirus infections occur frequently in humans, but most cases are mild or subclinical. The Reovirus dsRNA genome can contain 9, 10, 11, or 12 segments depending on the strain (25).

- Herpes simplex virus 1 (HSV1): This virus is enveloped, double-stranded DNA, with a diameter of ~250 nm, and belongs to the Herpesviridae family. This virus has moderate to low resistance to physical and chemical inactivation.

The characteristic of Herpes viruses is that they may cause latent infections in different types of cells, including nerve, epithelial, and lymphoid cells. HSV1 is a well-accepted model of the *Herpes Simplex* Family (26).

- Encephalomyocarditis virus (EMCV): This virus is non-enveloped, single-stranded RNA with a diameter of ~30 nm and belongs to the *Picornaviridae* Family. This virus has moderate to high resistance to physical and chemical inactivation.

A genome consisting of single-stranded positive-sense RNA with an approximate weight of 7.8 kilobytes, allows the direct translation of RNA into a polyprotein. EMC virus is not only the cause of myocarditis and encephalitis but also the cause of neurological diseases, reproductive disorders, and diabetes in many mammalian species (27).

The purpose of this study is to the capability of continuous monoclonal antibody purification steps

in the physical removal and inactivation of model viruses.

MATERIALS AND METHODS

Selected viral models and their characteristics 4 viruses were selected based on the ICH (International Council for Harmonisation) standard) (28, 29): model viruses (Table 1) represent potential viral contaminants of examined samples that exhibit a range of physicochemical characteristics of viruses to test the ability of the purification process to inactivate, eliminate, or reduce virus titer. All four model viruses were standard strains and generously supplied by LIVOGEN Co., with cell culture amplification characterization, and complementary identification tests including molecular specifications, were considered as certificate of analysis (COA) issued for them.

Cell lines used for virus propagation. For virus propagation, Vero (African green monkey kidney) and L929 (mouse fibroblast) cell lines obtained from the cell bank of Pasteur Institute of Iran (certificate number: Vero IBRCC10001/L929 IBRCC10102) were used.

Titration of model viruses using the (Tissue Culture Infectious Dose) $TCID_{50}$ method. Virus titers are calculated using the Reed and Muench method. The method of Reed and Muench is widely used to calculate the 50% endpoint. By accumulating the infected and non-infected test units over the whole dilution range, the effective test population is enlarged beyond the actual number of test units on either side of the 50% endpoint. The dilution that would correspond to the 50% endpoint lies somewhere between the $10^{-6.0}$ (66.7% infected) and $10^{-7.0}$ (14.3% infected) dilutions.

The proportionate distance between these two dilutions is calculated in the following manner: (% pos. above 50%) - 50% / (% pos. above 50%)- (% pos. below 50%) = Proportionate Distance 66.7% - 50.0% / 66.7% - 14.3% = 0.3 = Proportionate Distance Given that the log of the dilution above 50% is -6.0, the proportionate distance, as calculated previously, is 0.3, and the log of the dilution factor is -1 (serial 10-fold dilutions were used), the 50% endpoint is now calculated in the following way: (log dilution above 50%) + (proportionate distance × log dilution factor) = log ID; So, (-6.0) + (0.3 × -1.0) = -6.3, $TCID_{50} = 10^{-6.3}$

This is the end-point dilution, namely the dilution that will infect 50% of the test units inoculated. The reciprocal of this number yields the titer in terms of infectious dose per unit volume. If the inoculum added to an individual test unit was 0.1 mL, the titer of the virus suspension would therefore be: $10^{6.3}$ $TCID_{50}$/0.1mL = 10 × $10^{6.3}$ $TCID_{50}$/mL = $10^{7.3}$ $TCID_{50}$/mL. Infected test units would be wells exhibiting obvious CPE in a $TCID_{50}$, dead animals in an LD_{50}, or infected eggs in the(Embryo infectious dose 50) EID50. Five test units were inoculated at each dilution. The cumulative infected column is calculated based on the assumption that the 4-test unit that was infected at the 10^{-7} dilution of the virus would also have been infected at a 10^{-6} dilution. Therefore, at $10^{-6.0}$ there would be 13 (7 at $10^{-7.0}$+ 6 at $10^{-6.0}$) cumulative infected units. Similarly, at $10^{-5.0}$ the cumulative infected units would be 4 (at 10^{-7}) + 6 (at 10^{-6}) + 8 (at 10^{-5}) + 3 (at 10^{-4}) = 21.

Titration was done on all 4 model viruses before spiking, by the $TCID_{50}$ method. Titration was done by preparing serial dilutions of 0.1 log10 of each sample. Then, 100 µL of each dilution was inoculated in 8 replicates on a 96-well cell culture plate previously coated with the respective cell line. After incubation at 37 ± 5°C, 5 ± 1% CO_2, and 90 ± 5% humidity for 3 ± 1 days for all model viruses, cells were then evaluated

Table 1. Model viruses' specification

Model Virus	Taxonomy (Family)	Genome	Structure	Size (nm)	Chemical Resistance	Cell Line
Vesicular stomatitis virus (VSV)	*Rhabdoviridae*	ssRNA	Enveloped	50-200	Low	Vero
Encephalomyocarditis virus (EMCV)	*Picornaviridae*	ssRNA	Non- enveloped	25-30	Medium to High	L929
Herpes simplex virus 1 (HSV1)	*Herpesviridae*	dsDNA	Enveloped	155-250	Low to Medium	Vero
Reovirus	*Reoviridae*	dsRNA	Nonenveloped	60-80	Low to Medium	L929

for evidence of CPE originating from the model virus.

Negative control wells inoculated with the same cell culture medium and positive control wells inoculated with a control virus with a specific titer, whose titer was within the titer range, were determined and investigated. This strategy was used to ensure the validity of each set of titrations.

In order to accurately determine the titration of model viruses, the unstained monolayer cell culture was employed and also the infected unstained cells were observed for CPE after 3 ± 1 day of incubation. Then all the plates were stained with Giemsa dye and the $TCID_{50}$ titer of the viruses was calculated and expressed as $TCID_{50}$ units/mL based on log10.

Viral genome titration using qPCR. Measuring the genome titer level of all model viruses in different samples was done using the SYBR Green qPCR method. For viral nucleic acid extraction of model viruses, a viral nucleic acid extraction kit (Favorgen, FavorPrep™ Cat. No. FA YNKOO I) was used. The sequence of the primers used for qPCR is as follows (Table 2) (sequences of 5 to 3): (30).

Table 2. The sequence of the primers used for qPCR is as follows

Primer	Sequence (5' to 3')
EMCV-F	CTGAAAACACAAACGCAACTG
EMCV-R	CACTGAGTTCGGGCAAGT
VSV-F	AGCAGACGGTTGGATGTGT
VSV-R	TGAAGGATCGGATGGACTGT
HSV1-F	CACCCGCCAGTAAGTCATC
HSV1-R	CAACAAAAAGCCACGGAAG
Reovirus-F	TTCTCTCAAGACCACACGC
Reovirus-R	GATCAAACCGTCCAACCC

The process step is performed at a laboratory scale and qPCR is used to determine the virus titre of viral particles in the various collected fractions. This way it can be determined how much of the virus is physically removed from the product sample.

Using qPCR, it is possible to distinguish whether the virus has been removed from the product sample or whether it is still present but inactivated.

Test repeatability. Each study designated repeats two times for each step and the sum of the result of each step is used as required data to be reported in the result section.

Reduction factor evaluation using titration. Two methods of qPCR and $TCID_{50}$ were used to check the removal steps and also to check the inactivation and removal steps, accordingly.

Spike material. The spike materials for this step of the study were designated to be 2 mL of VSV=$10^{8.0}$ Titre ($TCID_{50}$/mL), 2 mL of Reovirus=$10^{7.0}$ Titre ($TCID_{50}$/mL), 2 mL of HSV1=$10^{7.0}$ Titre ($TCID_{50}$/mL) and 2 mL of VSV=$10^{9.0}$ Titre ($TCID_{50}$/mL).

Calculation of reduction factors. Reduction factors (RF) are calculated as detailed in the CPMP Note for Guidance on the Performance of Virus Clearance Studies (2): RF=(V1×T1)/(V2×T2)

Where: V1 and T1 are the volume and titer of the starting material respectively, and V2 and T2 are the volume and titer of the product fraction respectively in logarithmic terms, this equation can be expressed as log10[RF]=[log10 (V1) + log10 (T1)]-[log10 (V2) + log10 (T2)]

Reduction factors were rounded to two decimal digits but all calculations prior to calculation of the reduction factors were performed using numbers to at least three decimal digits when possible.

The 95% confidence limits of the reduction factors were calculated as detailed in the Committee for Proprietary Medicinal Products(CPMP) Note for Guidance on the Performance of Virus Clearance Studies. 95%CI:±√s 2+a 2 Where: ± s is the 95% confidence limit of the titer of the starting material, and ± a is the 95% confidence limit of the titer of the product fraction Log reductions of the order of 4 logs or more are indicative of a clear effect with the particular test virus under investigation (30, 31).

Chemical and physical treatment methods. Ion exchange chromatography, acidic pH treatment, affinity chromatography, and viral nanofiltration were used.

Ion exchange chromatography. The eluate obtained from monoclonal antibody purification was used as the starting material for this study. The resin used in the ion exchange chromatography step was SP Sepharose FF and one molar hydrochloric acid and two molar Tris were employed to adjust the pH.

The buffers and reagents used included: Maxima SYBER Green qPCR Master mix (Thermo Scientific, Cat. No. K0222), Random Hexame (Sinaclon, Cat. No. PS409), Sample buffer, and Equilibration Buffer.

Immediately after spiking, the model viruses were divided and loaded in the samples like the flow chart drawn in Fig. 1.

Sample number one is the initial sample containing separately spiked model viruses. Sample number two is a control sample containing the virus that is not subjected to any treatment or process. The third sample is related to the output of the column after passing one sample through the column and then passing the resin-balanced buffer. The fourth sample is related to Eluate Monoclonal Antibodies and the fifth sample is related to the column cleaning process.

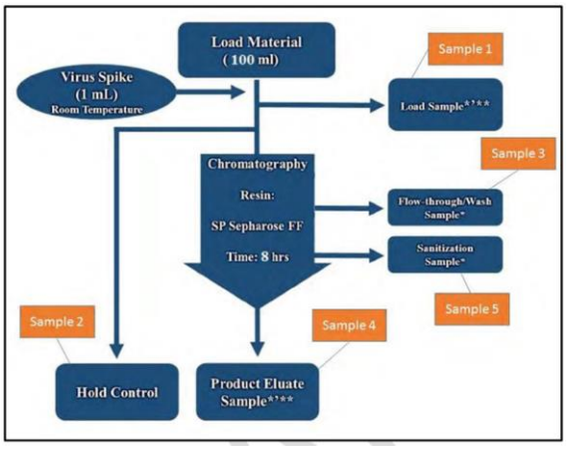

Fig. 1. Cation exchange chromatography step; procedure and sample

Acidic pH treatment. The titer of the virus used in this step includes the following, which were prepared in 5 ml volumes: VSV with a concentration of $10^{8.0}$, Reovirus with a concentration of $10^{7.0}$, HSV1 with a concentration of $10^{7.0}$ and EMCV with a concentration of $10^{9.0}$ $TCID_{50}$ were prepared.

Immediately after spiking, the model viruses were divided and loaded in the samples like the flow chart drawn in Fig. 2.

Sample number one is the initial sample containing separately spiked model viruses. Sample number two is the sample taken at minute zero after virus inoculation and acid treatment. The third sample corresponds to 60 minutes after virus inoculation and acid treatment. The fourth sample is a control sample with the virus without any treatment.

The amount of virus used in this step includes the following, which were prepared in 1.8 ml volumes: VSV with a concentration of $10^{8.0}$, Reovirus with a concentration of $10^{7.0}$, HSV1 with a concentration of $10^{7.0}$ and EMCV with a concentration of $10^{9.0}$ $TCID_{50}$

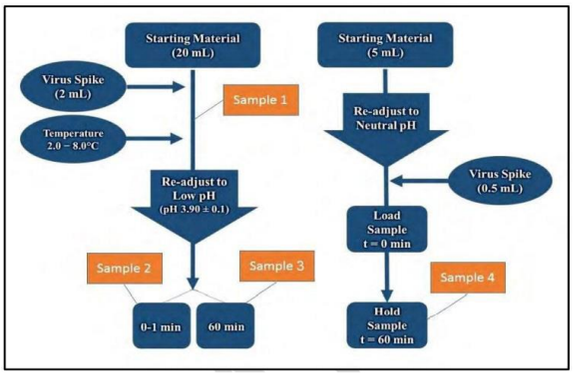

Fig. 2. pH treatment step; procedure and sample

were prepared.

Immediately after spiking, the model viruses were divided and loaded in the samples like the flow chart drawn in Fig. 3.

Sample number one is the starting sample containing individually spiked model viruses. Sample number two is a control sample containing the virus, which is not subjected to any treatment or process. The third sample is related to the output of the column after passing one sample through the column and then passing the resin-balanced buffer. The fourth sample is related to the monoclonal antibody eluate, and the number five sample is related to the column cleaning process.

Viral nanofiltration. The amount of virus which was prepared in 2 ml volume including: VSV with a concentration of $10^{8.0}$, Reovirus with a concentration of $10^{7.0}$, HSV1 with a concentration of $10^{7.0}$ and EMCV with a concentration of $10^{9.0}$ $TCID_{50}$ were formulated.

Immediately after spiking, the model viruses were divided and loaded in the samples like the flow chart drawn in Fig. 4.

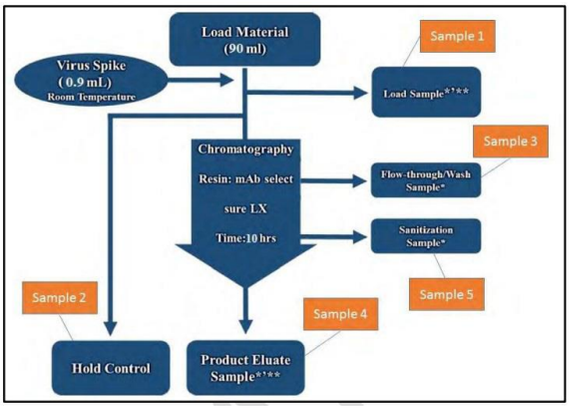

Fig. 3. pH treatment step; procedure and sample

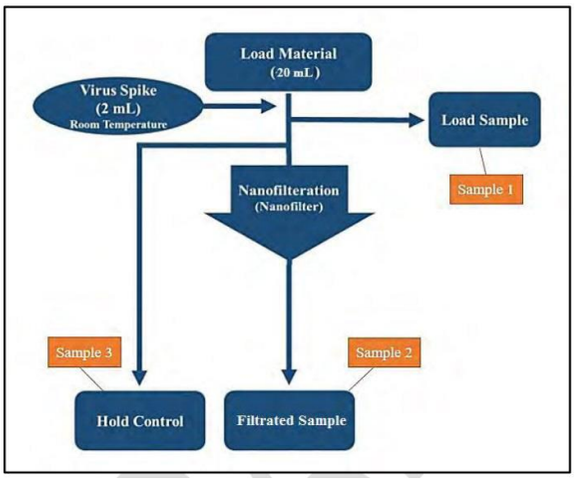

Fig. 4. Nano Viral-filtration step; procedure and sample

Sample number one was the initial sample containing spiked model viruses separately and before the filtration step. Sample number two is a filtered sample. The third sample is a control sample containing the virus that has not been subjected to any treatment or process.

RESULTS

qPCR and $TCID_{50}$ results for cation exchange chromatography treatment step. The results of controlling the presence of the virus and its genome using $TCID_{50}$ and q-PCR in the treatment stage of cation exchange chromatography, the reduction factors obtained for the treatment step of cation exchange chromatography are presented in the Table 3.

qPCR analysis. The qPCR results are summarized in Fig. 5.

$TCID_{50}$ results for the acidic pH treatment step. The reduction factors obtained for the acidic pH treatment step are presented in Table 4 below.

qPCR and $TCID_{50}$ results for affinity chromatography treatment step. The reduction factors obtained for the affinity chromatography treatment step are presented in Table 5.

Table 4. Summary of calculated virus reduction factors for low pH treatment step. (Reduction factors [log10] ± 95% confidence index). Reduction factor of samples vs spiked starting material (Sample 1). Note that two replicates were used for each sample that demonstrated as Run 1 & Run 2.

	Sample 2	Sample 3	Sample 4
Reovirus	Run. 1: 1.08	Run 1: 3.30	Run 1: 1.00
	Run 2: 1.10	Run 2:3.75	Run 2: 0.75
	Average: 1.09	Average: 3.52	Average: 0.87
VSV	Run 1: 1.43	Run 1: 4.75	Run 1: 0.75
	Run 2: 2.50	Run 2: 4.00	Run 2: 0.50
	Average: 1.96	Average: 4.37	Average: 0.63
HSV1	Run 1: 1.06	Run 1: 5.33	Run 1: 0.25
	Run 2: 1.21	Run 2: 5.66	Run 2: 0.50
	Average: 1.13	Average: 5.49	Average: 0.37
EMCV	Run 1: 1.80	Run 1: 5.33	Run 1: 0.25
	Run 2: 2.16	Run 2: 5.66	Run 2: 0.50
	Average: 1.98	Average: 5.49	Average: 0.37

Table 3. Reduction factor of samples vs spiked starting material (Sample 1). Note that two replicates were used for each sample that demonstrated as Run 1 & Run 2.

	Sample 2	Sample 3	Sample 4 ($TCID_{50}$)	Sample 4 (qPCR)	Sample 5
Reovirus	Run 1: 0.75	Run 1:2.66	Run 1: 3.66	Run 1: 2.76	Run 1: 4.00
	Run 2: 1.00	Run 2: 1.75	Run 2: 3.50	Run 2: 3.02	Run 2: 3.50
	Average: 0.87	Average: 2.20	Average: 3.58	Average: 2.89	Average: 3.75
VSV	Run 1: 0.50	Run 1: 2.20	Run 1: 3.75	Run 1: 2.14	Run 1: 5.50
	Run 2: 0.75	Run 2: 1.50	Run 2: 3.66	Run 2: 2.83	Run 2: 5.00
	Average: 0.62	Average: 1.85	Average: 3.70	Average: 2.48	Average: 5.25
HSV1	Run 1: 0.50	Run 1: 2.25	Run 1: 4.00	Run 1: 2.53	Run 1: 3.50
	Run 2: 0.25	Run 2: 3.00	Run 2: 4.25	Run 2: 2.55	Run 2: 3.25
	Average: 0.37	Average: 2.62	Average: 4.12	Average: 2.54	Average: 3.37
EMCV	Run 1: 0.25	Run 1: 4.25	Run 1: 5.00	Run 1: 2.40	Run 1: 5.00
	Run 2: 0.75	Run 2: 3.75	Run 2: 4.50	Run 2: 3.03	Run 2: 4.75
	Average: 0.50	Average: 4.00	Average: 4.75	Average: 2.71	Average: 4.87

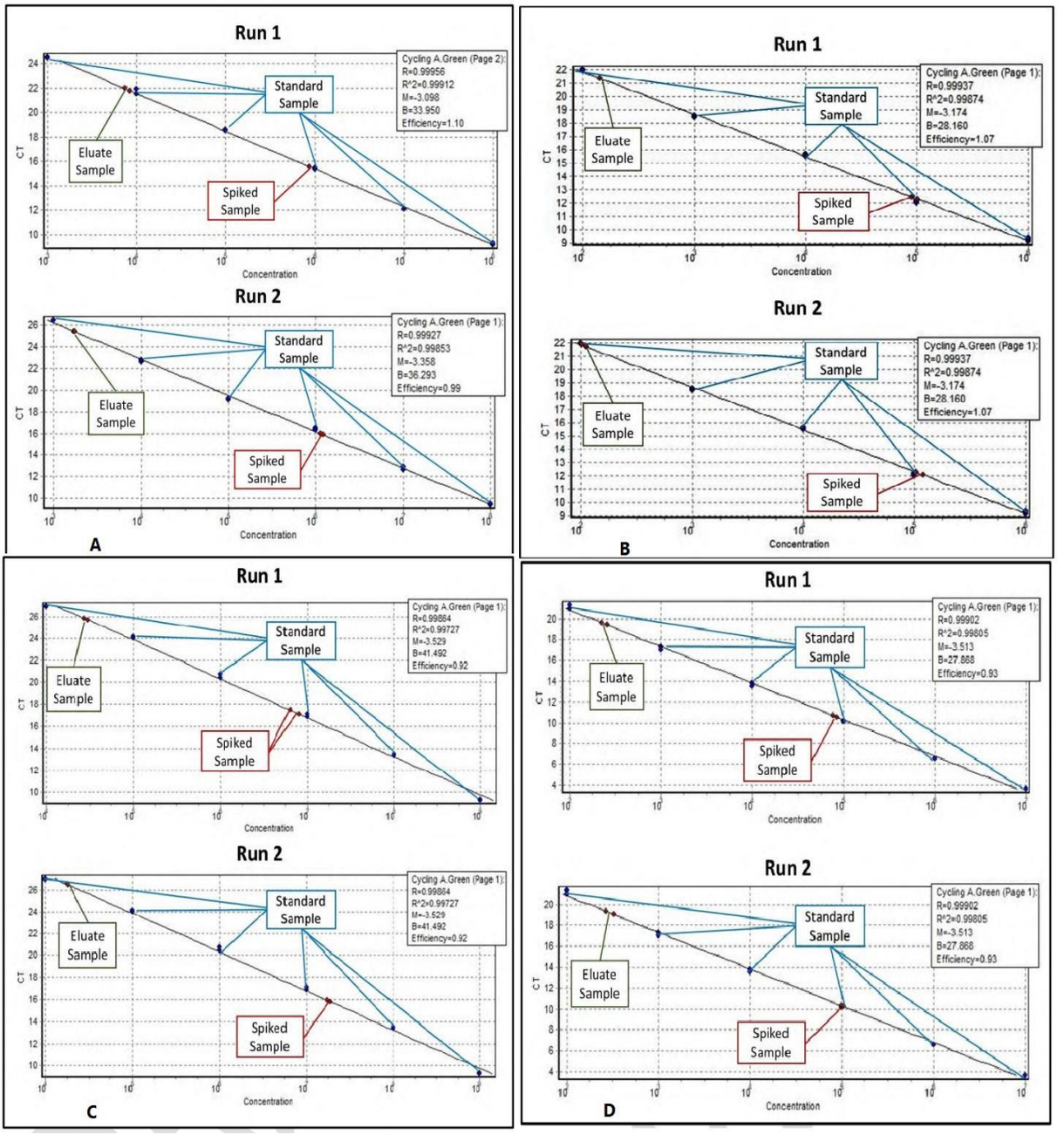

Fig. 5. A. The standard curve and results of VSV qPCR. The standard cure was created using $10^{8.0}$-$10^{3.0}$ serial dilution of the VSV virus.

B. The standard curve and results of Reovirus qPCR. The standard cure was created using $10^{6.0}$-$10^{2.0}$ serial dilution of the Reovirus.

C. The standard curve and results of EMCV qPCR. The standard cure was created using $10^{9.0}$-$10^{4.0}$ serial dilution of the EMCV virus.

D. The standard curve and results of HSV1 qPCR. The standard cure was created using $10^{7.0}$-$10^{2.0}$ serial dilution of the HSV1 virus.

Table 5. Summary of calculated virus reduction factors for affinity chromatography treatment step. (Reduction factors [log10] ± 95% confidence index). Note that two replicates were used for each sample that demonstrated as Run 1 & Run 2.

	Sample 2	Sample 3	Sample 4 ($TCID_{50}$)	Sample 4 (qPCR)	Sample 5
Reovirus	Run 1: 0.25	Run 1: 1.50	Run 1: 3.75	Run 1: 2.81	Run 1: 3.50
	Run 2: 0.50	Run 2: 1.00	Run 2: 4.25	Run 2: 2.80	Run 2: 3.25
	Average: 0.37	Average: 1.25	Average: 4.00	Average: 2.81	Average: 3.38
VSV	Run 1: 0.75	Run 1: 1.25	Run 1: 5.00	Run 1: 2.82	Run 1: 4.00
	Run 2: 0.50	Run 2: 1.00	Run 2: 4.50	Run 2: 3.06	Run 2: 4.25
	Average: 0.62	Average: 1.12	Average: 4.75	Average: 2.94	Average: 4.12
HSV1	Run 1: 0.50	Run 1: 0.75	Run 1: 4.50	Run 1: 3.05	Run 1: 5.00
	Run 2: 0.50	Run 2: 1.25	Run 2: 4.00	Run 2: 2.90	Run 2: 4.50
	Average: 0.50	Average: 1.00	Average: 4.25	Average: 2.97	Average: 4.75
EMCV	Run 1: 0.25	Run 1: 1.00	Run 1: 5.00	Run 1: 3.03	Run 1: 4.75
	Run 2: 0.75	Run 2: 0.75	Run 2: 5.50	Run 2: 2.80	Run 2: 5.25
	Average: 0.50	Average: 0.87	Average: 5.25	Average: 2.91	Average: 5.00

qPCR analysis. The qPCR results are summarized in Fig. 6.

qPCR and $TCID_{50}$ results for viral nanofiltration step. The reduction factors obtained for the nanofiltration step are presented in Table 6.

qPCR analysis. The qPCR results are summarized in Fig. 7.

Table 6. Summary of calculated virus reduction factors for nanofiltration step. (Reduction factors [log10] ± 95% confidence index). Reduction factor of samples vs spiked starting material (Sample 1).

	Sample 2 ($TCID_{50}$)	Sample 2 (qPCR)	Sample 3 ($TCID_{50}$)	Sample 3 (qPCR)
Reovirus	Run 1: 6.00	Run 1: 6.08	Run 1: 0.75	Run 1: 0.07
	Run 2: 6.00	Run 2: 6.11	Run 2: 0.50	Run 2: 0.12
	Average: 6.00	Average: 6.09	Average: 0.50	Average: 0.09
VSV	Run 1: 7.00	Run 1: 6.97	Run 1: 1.00	Run 1: 0.11
	Run 2: 7.00	Run 2: 7.04	Run 2: 0.50	Run 2: 0.18
	Average: 7.00	Average: 7.00	Average: 0.75	Average: 0.14
HSV1	Run 1: 6.00	Run 1: 6.16	Run 1: 0.25	Run 1: 0.08
	Run 2: 6.00	Run 2: 6.21	Run 2: 1.00	Run 2: 0.02
	Average: 6.00	Average: 6.18	Average: 0.62	Average: 0.05
EMCV	Run 1: 8.00	Run 1: 8.12	Run 1: 0.50	Run 1: 0.22
	Run 2: 8.00	Run 2: 7.99	Run 2: 0.25	Run 2: 0.12
	Average: 8.00	Average: 8.05	Average: 0.37	Average: 0.17

DISCUSSION

Viral purification studies are a necessary part and one of the requirements for registering biopharmaceutical products such as monoclonal antibodies and their approval by regulatory bodies (31).

These studies are conducted to assess the capacity of the manufacturing process to remove or inactivate viruses that could potentially contaminate biological raw materials. These studies are a key component of risk reduction to reduce known or unknown (new) viruses that May be present in the product (32).

Viral purification studies are carried out at least on two stages of the product production process, and at least with one viral sample and one virus-like particle employed (32).

Goussen et al. (33) used a type of model virus called MMV and a step of the production process called Protein-A chromatography. M. Asper et al. (34) have used a virus model called xenotropic murine leukemia virus and a stage of the production process called nanofiltration in their study, while in this study we have used 4 virus models and 4 stages of the production process.

In an overall comparison of the 4 viruses together, low pH inactivation affected all four model viruses with an average reduction factor of 4.06. The affinity chromatography step showed more logarithmic reduction than the cation exchange chromatography step. In both chromatography, the amount of reduction factor obtained by the method from $TCID_{50}$ indicates the total power of inactivation and removal at this stage, while the qPCR results showed that the

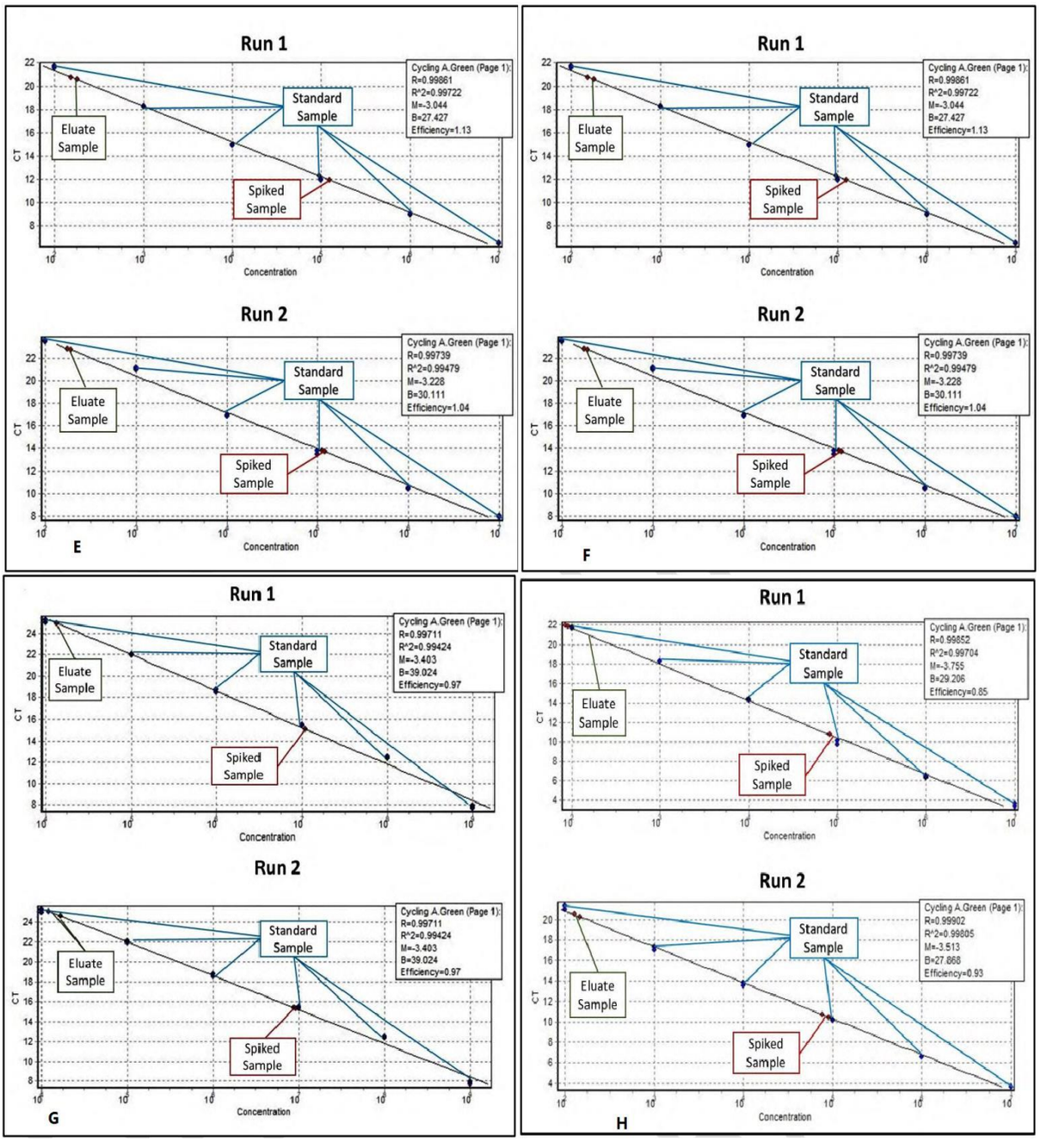

Fig. 6. E. The standard curve and results of VSV qPCR. The standard cure was created using $10^{8.0}$ -$10^{3.0}$ serial dilution of the VSV virus.

F. The standard curve and results of Reovirus qPCR. The standard cure was created using $10^{7.0}$ -$10^{2.0}$ serial dilution of the Reovirus virus.

G. The standard curve and results of EMCV qPCR. The standard cure was created using $10^{9.0}$ -$10^{4.0}$ serial dilution of the EMCV virus.

H. The standard curve and results of HSV1 qPCR. The standard cure was created using $10^{7.0}$ -$10^{2.0}$ serial dilution of the HSV1 virus.

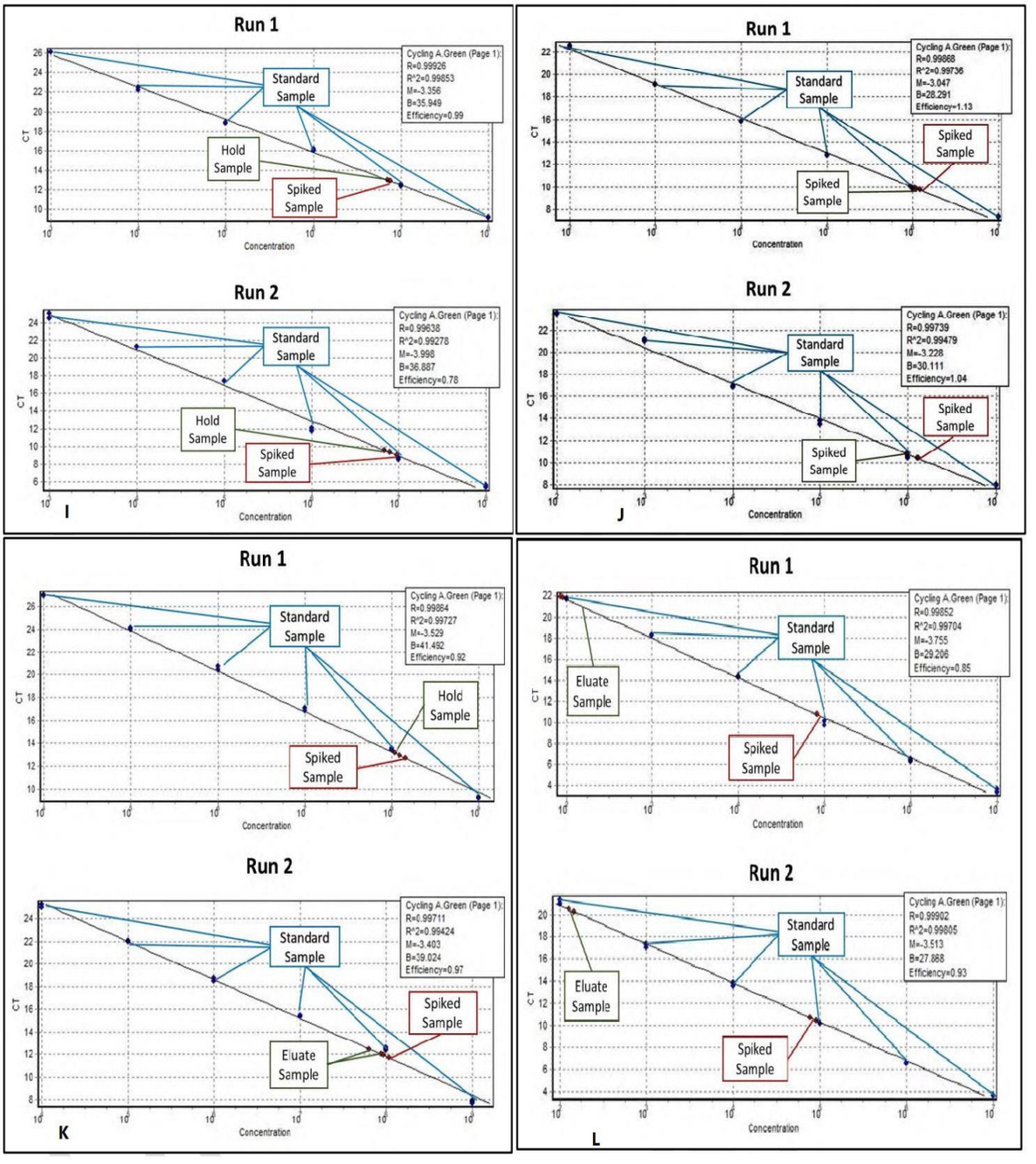

Fig. 7. I. The standard curve and results of VSV qPCR. The standard cure was created using $10^{8.0}$ -$10^{3.0}$ serial dilution of the VSV virus.

J. The standard curve and results of Reovirus qPCR. The standard cure was created using $10^{7.0}$ -$10^{2.0}$ serial dilution of the Reovirus virus.

K. The standard curve and results of EMCV qPCR. The standard cure was created using $10^{9.0}$ -$10^{4.0}$ serial dilution of the EMCV virus.

L. The standard curve and results of HSV1 qPCR. The standard cure was created using $10^{7.0}$ -$10^{2.0}$ serial dilution of the HSV1 virus.

reduction in entry into the system was achieved due to the removal of viruses. According to the results obtained in the nanofiltration step, it is obvious that this step was the most efficient step to remove the virus, which led to a 6-8 logarithmic viral reduction, even in the case of the smallest virus (EMCV).

CONCLUSION

For each virus, separately, e.g., in the case of HSV1, the treatment step with acidic pH was more effective compared to the other two steps (cation exchange and affinity chromatography). In the case of VSV, the affinity chromatography step was more effective than the cation exchange step and similar to the results of the low pH treatment. EMCV, as the most stringent in size and resistance, was completely affected by the process steps and showed that the process and all its steps were efficient in inactivating and removing the virus. Also, Reovirus had a similar rate of reduction of the identified particles from the test material entering the system through the low pH treatment steps and chromatography steps. Overall, the most efficient process to clean it up was nanofiltration, which removed almost all of it.

As Table 7 shows, the overall average reduction coefficient for all four viruses is significantly high, which indicates the high capacity of the production process in deactivating and removing viruses and reducing the load of all four model viruses.

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Table 7. The overall reduction factor of the manufacturing process

	Reduction factor of model viruses in the viral clearance process				**Average**
	HSV1	VSV	EMCV	Reovirus	**Reduction Factors**
	5.49	4.37	3.12	3.52	4.06
Low pH Treatment	$TCID_{50}$: 3.58	$TCID_{50}$: 3.70	$TCID_{50}$: 4.75	$TCID_{50}$: 3.58	$TCID_{50}$: 3.90
Chromatography	qPCR: 2.89	qPCR: 2.48	qPCR: 2.71	qPCR: 2.89	qPCR: 2.74
(Cation Exchange)	$TCID_{50}$: 4.25	$TCID_{50}$: 4.75	$TCID_{50}$: 5.25	$TCID_{50}$: 4.00	$TCID_{50}$: 4.56
Chromatography (Affinity)	qPCR: 2.97	qPCR: 2.94	qPCR: 2.91	qPCR: 2.81	qPCR: 2.90
Nanofiltration	$TCID_{50}$: 6.00	$TCID_{50}$: 7.00	$TCID_{50}$: 8.00	$TCID_{50}$: 6.00	$TCID_{50}$: 6.75
Cumulative Reduction Factors	qPCR: 6.18	qPCR: 7.00	qPCR: 8.05	qPCR: 6.09	qPCR: 6.83
	$TCID_{50}$: 19.32	$TCID_{50}$: 19.82	$TCID_{50}$: 21.12	$TCID_{50}$:17.10	N.A.
	qPCR: 12.04	qPCR: 12.42	qPCR: 13.67	qPCR: 11.79	

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