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PRINCIPLES OF VIRAL CLEARANCE: VIRUS STOCKS PREPARATION AND TITRATION METHODS DEVELOPMENT REQUIRED FOR PHASE III. METHOD VALIDATION ACCORDING TO THE GUIDELINE. SET UP AND OPTIMIZATION OF TITRATION ASSAY EVALUATION

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ACRONIMS

ATCC: American Type Culture Collection

BCA: BiCinchoninic Acid

CPE: Cytopathic Effect

DNA: Deoxyribonucleic acid

dsDNA/RNA: double stranded DNA/RNA

EMEM: Eagle's Minimum Essential Medium

FBS: Fetal Bovine Serum

FDA: US Food and Drug Administration

GMP: Good Manufacturing Practice

HEK-293: Human Embryonic Kidney 293 cells

HPIV3: Human ParaInfluenza Virus type 3

ICH: International Conference on Harmonization

LLC-MK2: Lilly Laboratories Cell-Monkey Kidney 2

LRF: Log Reduction Factor

MCB: Master Cell Bank

MuLV: Murine Leukemia Virus

MVB: Master Virus Bank

MVM: Minute Virus of Mice

O/N: Overnight

PBS: Phosphate Buffered Saline

PES: Polyethersulfone membrane

REO3: Reovirus 3

RNA: Ribonucleic acid

ssDNA/RNA: single stranded DNA/RNA

SuHV-1: Suid Herpes Virus

$TCID_{50}$/mL: 50% Tissue Culture Infectious Dose over mL

USP: United States Pharmacopeia

VPL: Virus Production Lot

WCB: Working Cell Bank

WVB: Working Virus Bank

1 INTRODUCTION

1.1 Viral safety

Viral safety is required for biotechnology drugs manufactured to treat human diseases.

Biotechnology drugs are products derived from characterised cell lines of human or animal sources such as blood products, recombinant products, vaccine, animal or human tissue or fluids derived products.

The risk of viral contamination is a feature common to all biotechnology products derived from cell lines. Such contamination could have serious clinical consequences and can arise from the contamination of the source cell lines themselves (cell substrates) or from adventitious introduction of virus during production [1].

According to the Q5A(R1) Guideline "Viral safety Evaluation of Biotechnology Products Derived from Cell Lines of Human or Animal Origin" defined by ICH International Conference on Harmonization (USA - Japan - Europe), the viral safety of biotechnology products derived from human or animal origin cell lines should be demonstrated before the registration and demand for marketing of these products[1].

Viral contamination can occur through source materials or by the introduction of adventitious viruses during manufacturing. For these reasons three principal barriers preventing the pathogens transmission.

These include: selecting and testing cell lines and other raw materials, including media components, for the absence of undesirable viruses which may be infectious and/or pathogenic for humans; assessing the capacity of the production processes to clear infectious viruses; and testing the product at appropriate steps of production for absence of contaminating infectious viruses[1].

Viral contamination may arise from:

- cell lines used to produce biotechnology products

  cells may have latent or persistent virus infection (e.g., herpesvirus) or endogenous retrovirus which may be transmitted vertically from one cell generation to the next, since the viral genome persists within the cell. Such viruses may be constitutively expressed or may unexpectedly become expressed as an infectious virus. Viruses can be introduced into the Master Cell Bank (MCB)

by several routes such as: 1) derivation of cell lines from infected animals; 2) use of virus to establish the cell line; 3) use of contaminated biological reagents such as animal serum components; 4) contamination during cell handling[1];

- adventitious introduction of virus during manufacturing.
  Adventitious viruses can be introduced into the final product by several routes including, but not limited to, the following: 1) the use of contaminated biological reagents such as animal serum components; 2) the use of a virus for the induction of expression of specific genes encoding a desired protein; 3) the use of a contaminated reagent, such as a monoclonal antibody affinity column; 4) the use of a contaminated excipient during formulation; 5) contamination during cell and medium handling. Monitoring of cell culture parameters can be helpful in the early detection of potential adventitious viral contamination[1].

For this reason cell lines, MCB and Working Cell Bank (WCB), used in the production process must be qualified.

An important part of qualifying a cell line for being used in the production of a biotechnology product is the appropriate testing for the presence or absence of viruses[1]. For the MCB screening for endogenous and non-endogenous viral contamination should be performed.

Testing for non-endogenous viruses should include specific tests, e.g. species-specific tests such as the mouse antibody production (MAP) test. Moreover each WCB as a starting cell substrate for drug production should be tested for adventitious virus either by direct testing or by analysis of cells at the limit of in vitro cell age, initiated from the WCB[1].

Generally, the production cells are obtained by expansion of the WCB; the MCB could also be used to prepare the production cells. Cells at the limit of in vitro cell age should be evaluated once for those endogenous viruses that may have been undetected in the MCB and WCB[1].

Many tests for retroviruses, non-endogenous or adventitious viruses are suggested to be done, using different assays (e.g. antibody production, in vivo and in vitro virus screen, Transmission Electronic Microscope (TEM), Polymerase chain reaction (PCR), retrovirus infectivity), on MCB, Working Cell Bank (WCB) and cells at the limit of in vitro cell age used for production.

Another possibility of adventitious virus contamination can be determined by the unprocessed bulk constitutes one or multiple pooled harvests of cells and culture media.

It is important to design the most relevant and rational protocol for virus tests from the MCB level, through the various steps of drug production, to the final product including evaluation and characterization of viral clearance from unprocessed bulk[1].

The evaluation and characterization of viral clearance plays a critical role in this scheme.

The goal is to obtain the best reasonable assurance that the product is free of virus contamination.

After the production from qualified cell lines, bulk harvest containing biotechnology drugs undergo a purification process (downstream process) to obtain the final drugs to be formulated for the market. This purification process involves different steps such as affinity chromatography and other chromatographic types, pH inactivation and nanofiltration.

It is important to evaluate and to characterize the virus removal or inactivation during this process in order to assure the viral safety of biotechnology products[1].

This is the aim of viral clearance studies.

1.2 viral clearance

A viral clearance study should evaluate the ability of the overall purification process to remove or inactivate a broad spectrum of virus types, including viruses that are known to contaminate or have the potential to contaminate the raw materials, and those that can be introduced during manufacturing[2].

Moreover, the viral clearance studies demonstrate virus removal and/or inactivation in the individual steps during the purification process (e.g. chromatography, nanofiltration, pH, and others) so that each step in the purification scheme may be studied independently of the other steps and is not required to perform all the purification steps in linear order as in the industrial process.

One of the key goals of a manufacturing purification process is to achieve maximal viral clearance without compromising product quality[2]. For this reason, it is important and required by regulatory authorities, the validation of proportionally scale-down to a laboratory scale (downscale process) to the full-scale of the manufacturing process.

In order to not contaminate the manufacturing process, the viral clearance studies should be performed in a segregated facility equipped for virologic work. An example of some steps selected for the down-scale viral clearance study are highlighted in the figure (Fig.1).

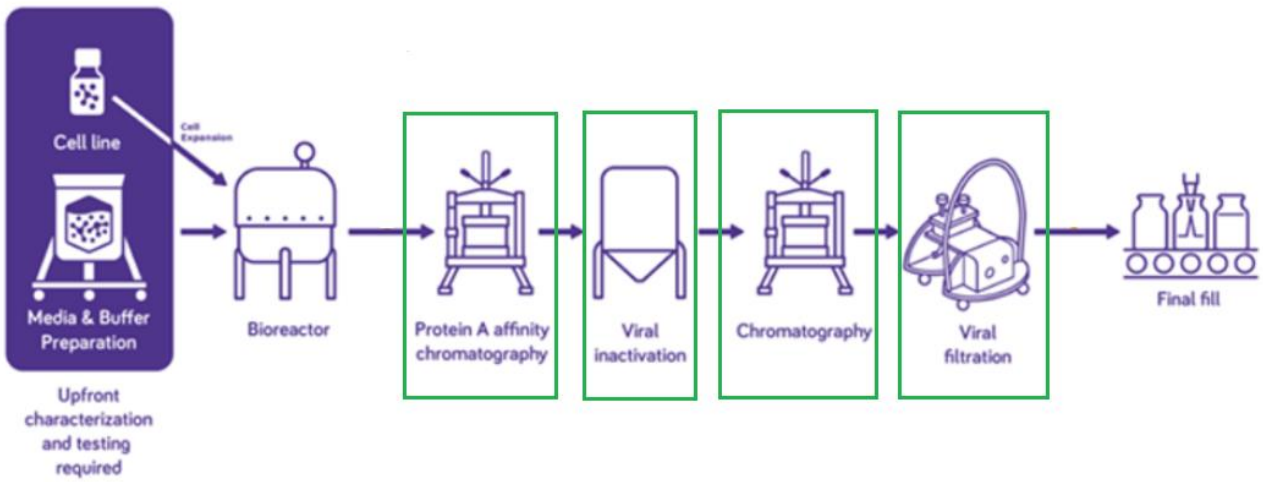


Fig.1

General scheme of manufacturing process: in the green squares the step important for the Viral Clearance studies.

In the Viral Clearance Validation Studies, for example, the parameters of chromatographic steps, should be representative than those used in the manufacturing process (e.g. flow rate, chromatography matrix, buffer composition, ecc).

Scaled-down and manufacturing-scale chromatographic systems should produce similar elution profiles and step yields, and final product analytical profile[2].

The output of the Viral Clearance Validation Studies is the Log Reduction Factor (LRF also known as viral reduction factors VRFs or log reduction values, LRV): it is determined in a logarithmic scale, by calculating the virus titer in the loading and in the product fraction after the purification step. The VRFs derived from specific process steps are used to evaluate the overall capacity of the entire production process to remove or inactivate process-specific or nonspecific viruses[2].

To perform a study, a virus with a defined titre is added (spiking) to intermediate samples (depending on the analysed process phase). Subsequently, the material (samples + virus) is subjected to the purification step that should be evaluated, for measuring the virus inactivation and/or removal grades of the step. At the end, the reduction factor is determined in logarithmic scale, by calculating the ratio of virus titre in the loading and after the purification step.

Anyhow, the virus spike should be as high as possible, in order to determine the capacity of the production step to inactivate and/or remove viruses but the maximum volume of the virus allowed for the spiking is 10% v/v to avoid altering the composition of the production material.

The choice and number of viruses that may be used in a viral clearance study are dictated by the nature and origin of the production cell line, as well as the nature and origin of the animal-derived materials used in production and purification[2].

In general, one specific model virus (e.g. MuLV Murine Leukemia Virus) and one relevant virus (e.g. MVM Minute Virus of Mice) are used for clinical Phase I/II viral clearance studies and for Phase III viral clearance studies additional model viruses should be added.

Selecting model viruses is critical for the design of an appropriate Viral Clearance Validation study.

Viruses are classified in 3 main categories with respect to assessing Viral Clearance:

- "Relevant" viruses: virus used in process evaluation studies which is either the identified virus, or of the same species as the virus that is known, or likely to contaminate the cell substrate or any other reagents or materials used in the production process[1];
- "Specific model" viruses: Virus which is closely related to the known or suspected virus (same genus or family), having similar physical and chemical properties to those of the observed or suspected virus[1];
- "Nonspecific model" viruses: A virus used for characterisation of viral clearance of the process when the purpose is to characterise the capacity of the manufacturing process to remove and/or inactivate viruses in general, i.e., to characterise the robustness of the purification process[1].

CHO (Chinese Hamster Ovary) cell line have frequently been used as substrate for drug production, for example, "Relevant" virus for these cell line could be MVM (Minute Virus of Mice) and a "Specific model" virus MuLV (Murine Leukemia Virus).

In the case of a well-characterized mouse or CHO cell line, Viral Clearance Validation studies for phases I/II should include a murine retroviral model such as MLV and a parvovirus model such as MVM. For pH Inactivation (acid or basic) step MuLV virus model is tested only because MVM is resistant to this kind of treatment.

The selection of the viruses to be used should cover a wide range of physical and chemical properties:

- enveloped and non-enveloped viruses;
- DNA and/or RNA genome;
- high, medium and/or low sensitivity to chemical inactivation;
- size.

The results obtained for such viruses provide useful information about the ability of the production process to remove or inactivate viruses in general[2].

2 AIM

The output of the Viral Clearance Validation Studies, as described previously, is the LRF, that is determined in a logarithmic scale, by calculating the virus titer in the loading and in the product fraction after the purification step.

To reach this output, validated viral titration methods are needed for the different tested viruses.

The first focus of PhD project was on viral titration methods development and validation of three viruses: REO3 (Reovirus 3), SuHV-1(Suid Herpesvirus) and HPIV3 (Human Parainfluenza Virus type 3).

These viruses have been chosen since representative of non-specific model viruses for well characterized CHO cell line.

The validation parameters, according to the International Guideline ICH-Q2 (R1) "Validation of analytical procedures: text and methodology" and GMP (Good Manufacturing Practice), are:

- Robustness;
- Linearity;
- Specificity;
- Precision with repeatability and intermediate precision;
- Accuracy.

Moreover, to performed Viral Clearance Validation Studies high titer and low concentration of impurities virus stocks are needed.

For this reason, the second focus of PhD project is on viral propagation and purification methods development for the three viruses: REO3 (Reovirus 3), SuHV-1 (Suid Herpesvirus) and HPIV3 (Human Parainfluenza Virus type 3).

3 VIRUS

During the PhD project were used viral suspensions of REO3 (Reovirus 3), HPIV3 (Human Parainfluenza virus type 3) and SuHV-1 (Suid Herpes virus).

3.1 REOVIRUS 3 – REO3

Mammalian reoviruses are large, nonenveloped, double-stranded RNA-containing viruses that infect a variety of mammalian species[3]. Reovirus 3 is the most pathogenic reovirus type of laboratory rodents and it causes a syndrome whose symptoms are jaundice, diarrhea, oily hair and neurologic signs[4].

Mammalian *orthoreoviruses* (reoviruses) are members of the *Reoviridae* family[5]. Members of this family have genomes consisting of 10, 11, or 12 segments of double-stranded (ds) RNA. The replication strategy employed by these viruses must take into account that the genome is dsRNA, which is extremely stable, and consequently difficult to dissociate into a form exposing a single-stranded template for RNA-directed mRNA transcription[6].

Reovirus contains 10 dsRNA segments that are packaged into an icosahedral capsid that consists of two protein shells, called outer capsid and core.

A schematic of the virion is shown in Fig. 2.

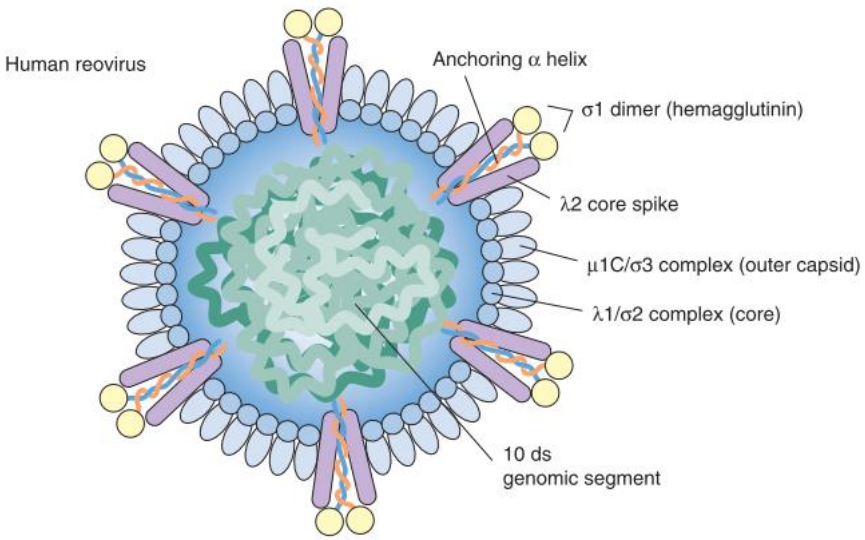


Fig. 2

The 60-nm-diameter human reovirus with its double shell. The 10 segments of the reovirus genome and the proteins encoded are shown[6].

The replication of reovirus in the infected cell are shown in Fig 3. Virus attachment is followed by receptor-mediated endocytosis.

Virion "core" particles are formed by the degradation of the outer shell in the endosome. Viral proteins are translated and structural proteins assemble around newly synthesized viral mRNA.

The complementary strand of the double stranded genomic RNAs is synthesized in the immature capsid while morphogenesis proceeds. Virus release is by cell lysis[6].

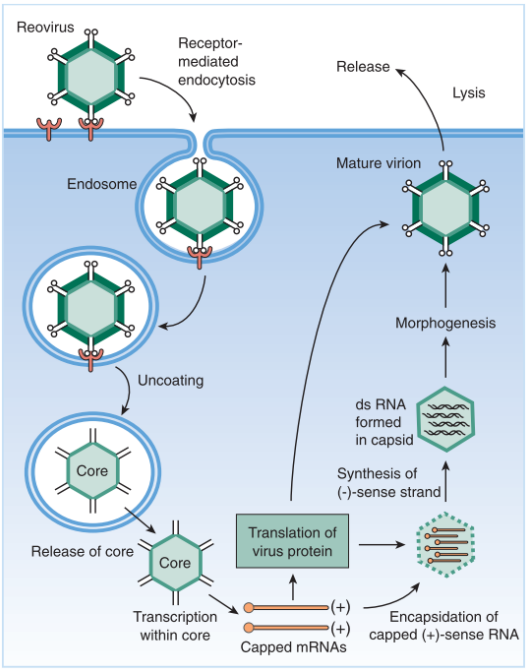


Fig.3

The reovirus replication cycle.

Base on the product sheet by ATCC (American Type Culture Collection) the prefer host for the REO3 virus is the LLC-MK2 cell line. The virus effect on host cell is a Cytopathic Effect (CPE) with refractile rounding and cell sloughing[7].

3.2 Human parainfluenza virus type 3 – HPIV3

These viruses were first discovered in the late 1950s and in 1959 a new taxonomic group was created called "parainfluenza viruses" [8].

HPIV is divided into types 1 to 4 and in particular, HPIV-3 are major causes of lower respiratory infections in infants, young children, the immunocompromised, the chronically ill, and the elderly[9] [10] [11].

These medium-sized viruses are enveloped, and their genomes are organized on a single negative-sense strand of RNA[8] (ssRNA: single strand RNA).

These viruses belong to the *Paramyxoviridae* family divided in two genera of HPIV, *Respirovirus* (HPIV-1 and HPIV-3) and *Rubulavirus* (HPIV-2 and HPIV-4)[8].

HPIV are demonstrated to be pleomorphic enveloped viruses (Fig 4)[12]. The virion attaches by binding to specific receptors on the surface of the cell and this allow the releasing of ssRNA(-) into the cytoplasm. After mRNA syntesis, replication and assembly the HPIVs envelope is derived from the host cell that they last infected.

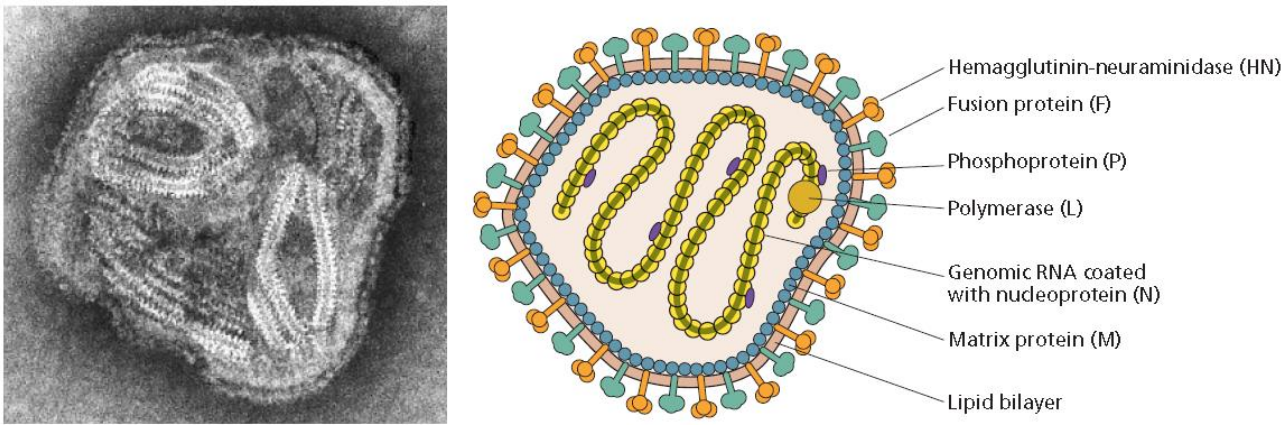


Fig 4

The image show, on the left, a negatively stained paramyxovirus and on the right the schematic description of virus structure[12].

A number of primary and secondary cell lines support the growth of HPIV and the most common are LLC-MK2, Vero,[8] ecc. In particular HPIV3 grows to high titer in LLC-MK2 cells with serum-free medium[8].

3.3 Suid herpesvirus – SuHV-1

Aujeszky's disease (AD, pseudorabies) is a notifiable disease that causes substantial economic losses to the swine industry in countries, where the disease is present.

The causative agent is Suid herpesvirus 1 (SuHV1, syn. Aujeszky's disease virus [ADV] or pseudorabies virus [PrV]), which belongs to the family *Herpesviridae*, subfamily *Alphaherpesvirinae*, genus *Varicellovirus*[13].

Members of the family *Suidae* (true pigs) are the only natural hosts for SuHV-1, although the virus can infect numerous other mammals including ruminants, carnivores and rodents[14].

Indeed *Alphaherpesviruses* have a broad host range in the laboratory and will infect a wide variety of cultured cells or experimental animals[15]. The SuHV-1 genome is linear double-strand DNA (ds-DNA) with internal and terminal repeats.

Herpesviruses are enveloped and approximately spherical, with a diameter of 100–300 nm and the nucleocapsid is surrounded by or embedded within a structure known as the tegument [15] (see fig. 5)

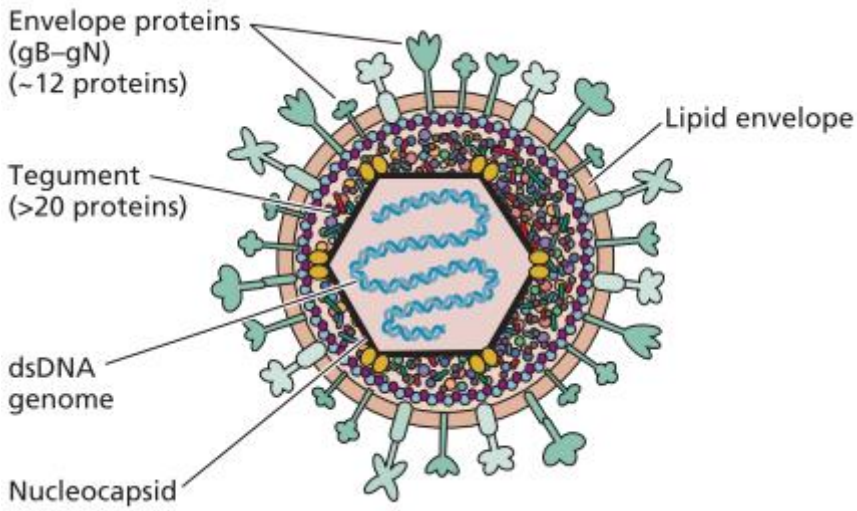


Fig. 5

Virion structure of *Alphaherpesviruses*

Virus entry can involve fusion of the viral membrane at the cell's surface (Fig.6) and transit of nucleocapsids through the nuclear membrane by budding through the inner leaflet of the nuclear membrane followed by de-envelopment and release of nucleocapsids into the cytoplasm.

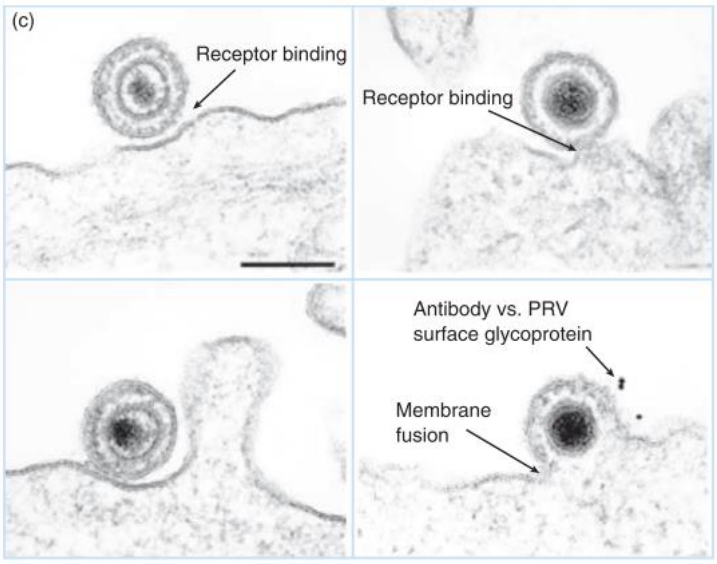


Fig. 6

The fusion of pseudorabies virus with the plasma membrane of an infected cultured cell. Initial association between viral envelope glycoproteins and the cellular receptor on the plasma membrane through the final fusion event is shown[6 12 16].

4 CELL LINES

Viruses, as mentioned before, are biological entities with characteristics of obligate parasites. They do not have the biochemical structures necessary for the replication except through cells substrate.

Cells are used for the detection, for growing and for quantification of viruses. Furthermore, each virus has an elective cellular substrate for its propagation and titration.

During the PhD project were tested different cell lines for the set-up and development of the titration, propagation and purification methods for the three viruses:

- LLC-MK2 DERIVATIVE (ATCC® CCL-7.1™)
- HEK-293 (ATCC® CRL-1573™)
- VERO (ATCC® CCL-81™)
- ST (ATCC® CRL-1746™)
- MDBK (NBL-1) (ATCC® CCL-22™)

4.1 LLC-MK2 DERIVATIVE

Lilly Laboratories Cell-Monkey Kidney 2 Derivative (LLC-MK2 Derivative) is an epithelial adherent line that was immortalized in the 1950s.

As reported in LLC-MK2 Derivative (ATCC® CCL7.1™) product sheet[17], cells were cultivated in Eagle's Minimum Essential Medium (EMEM) and fetal bovine serum (FBS) to a final concentration of 10%.

In the images below (Fig.7) an example of low density and high density of this cells line.

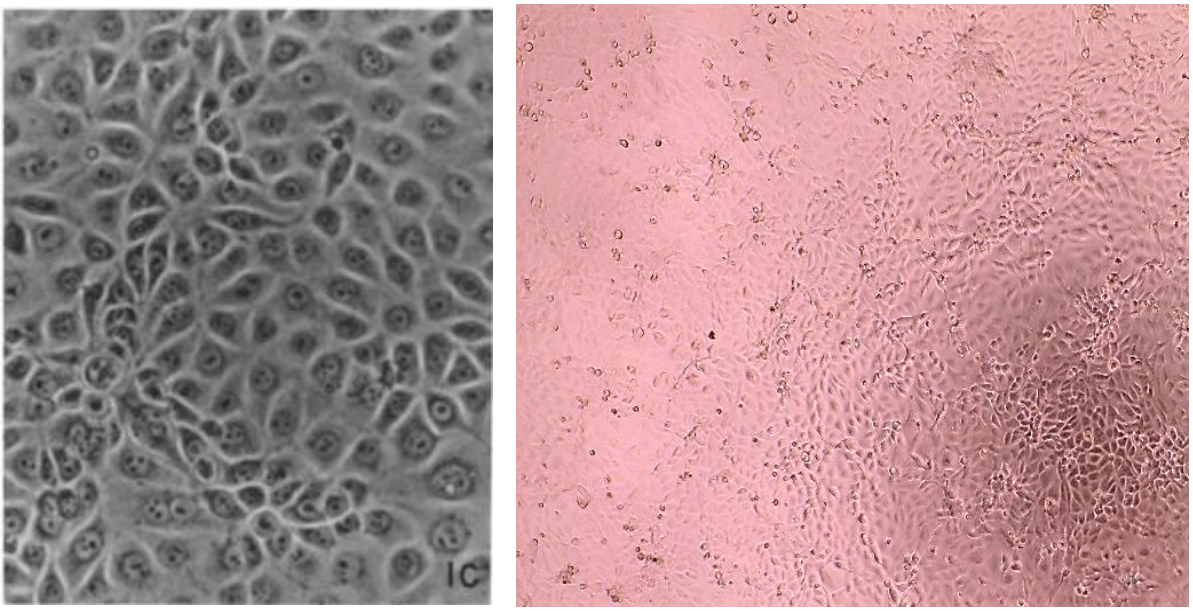


Fig.7

Image of LLC-MK2 Derivative:

left from Hull et al.: Kidney cell strains; right image taken with EVOS microscope.

4.2 HEK-293

Human Embryonic Kidney 293 is an epithelial adherent line that was created by a transfection with Adenovirus 5 DNA in the 1970s.

As reported in HEK-293 (ATCC® CRL-1573™) product sheet[18], cells were cultivated in Eagle's Minimum Essential Medium (EMEM) and fetal bovine serum (FBS) to a final concentration of 10%.

In the images below (Fig.8) an example of low density and high density of this cells line.

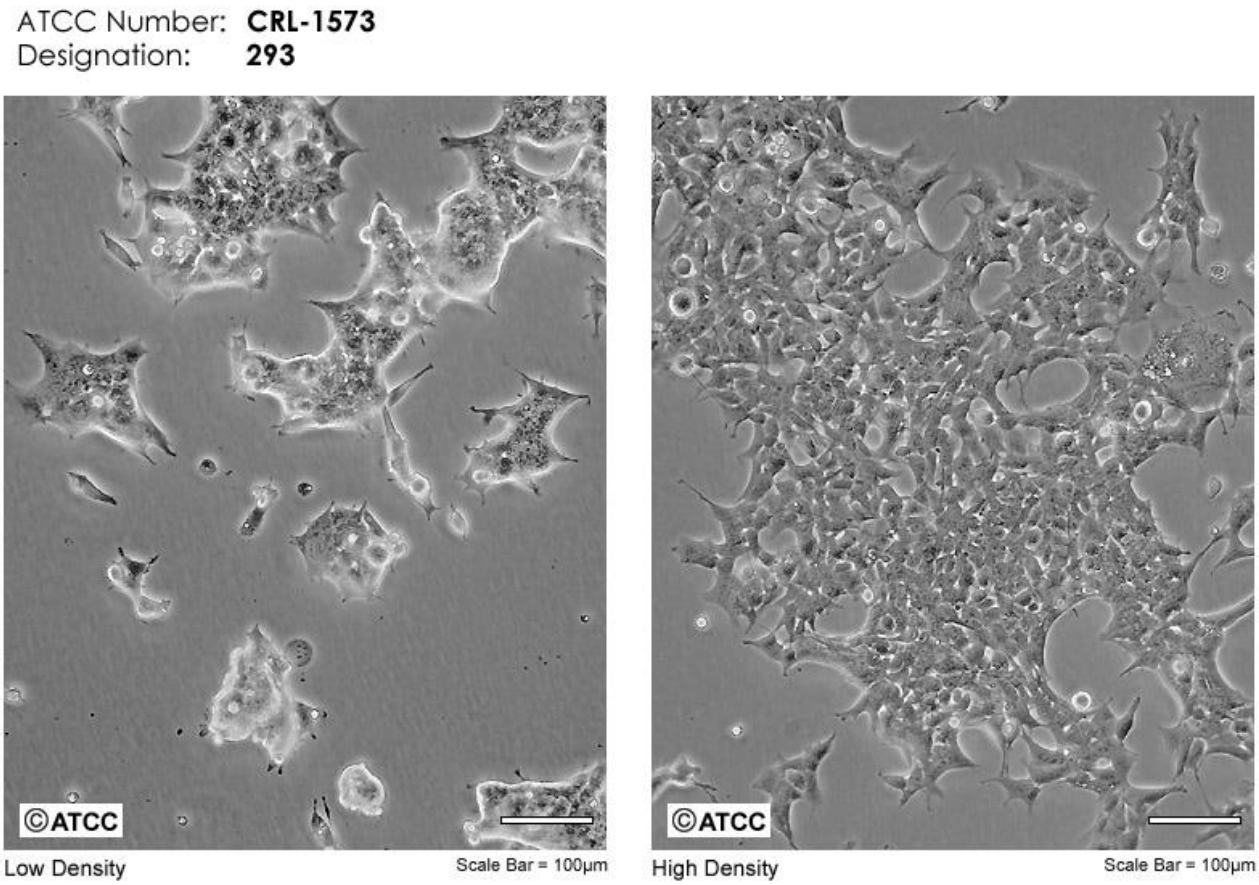


Fig.8

Cell micrograph of HEK-293[19].

4.3 VERO

VERO is an adherent epithelial cell line from kidney of an adult *Cercopithecus aethiops.*

As reported in VERO (ATCC® CCL-81™) product sheet[20], cells were cultivated in Eagle's Minimum Essential Medium (EMEM) and fetal bovine serum (FBS) to a final concentration of 10%.

In the images below (Fig.9) an example of low density and high density of this cells line.

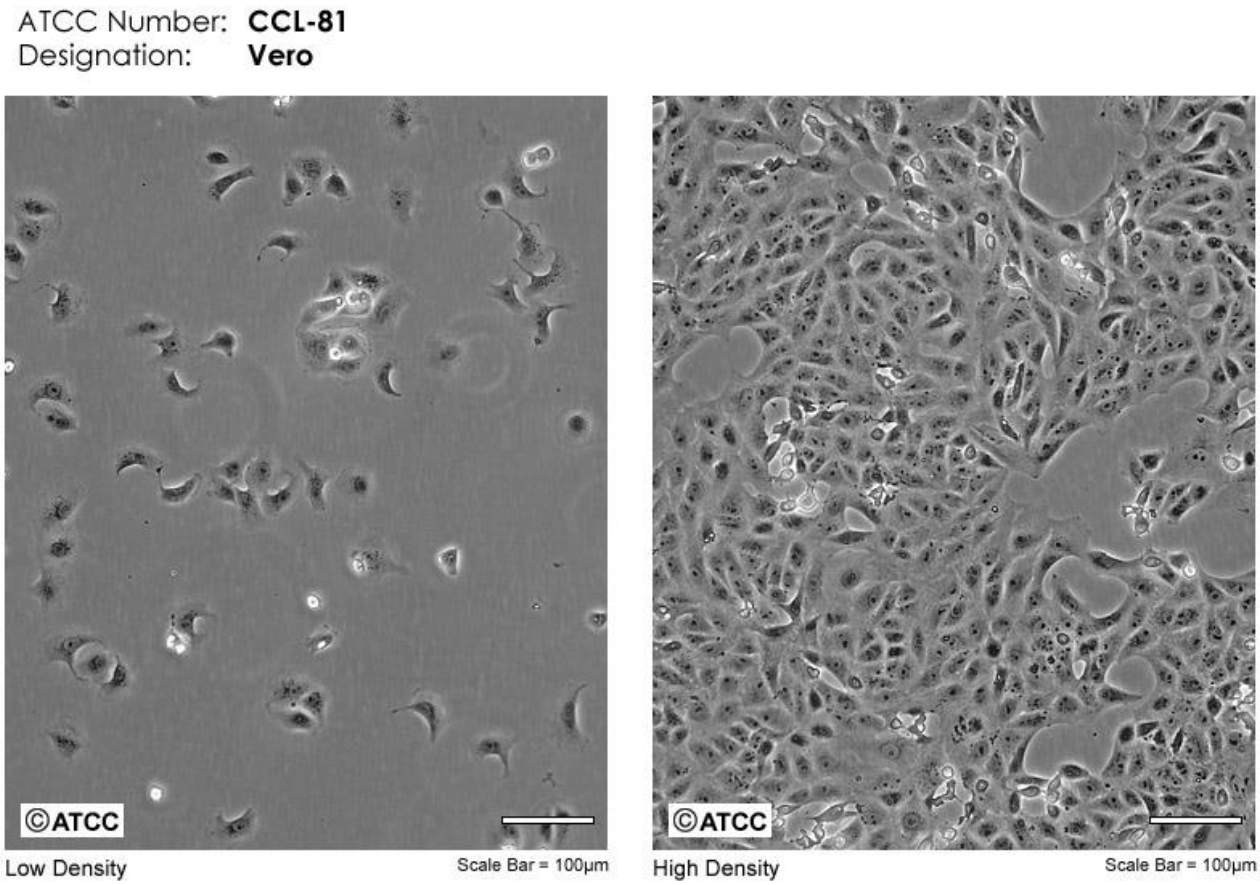


Fig.9

Cell micrograph of VERO[19].

4.4 ST

ST is an adherent fibroblast cell line from testis of a male Sus scrofa (pig).

As reported ST (ATCC® CRL-1746™) in product sheet[21], cells were cultivated in Eagle's Minimum Essential Medium (EMEM) and fetal bovine serum (FBS) to a final concentration of 10%.

In the images below (Fig.10) an example of low density and high density of this cells line.

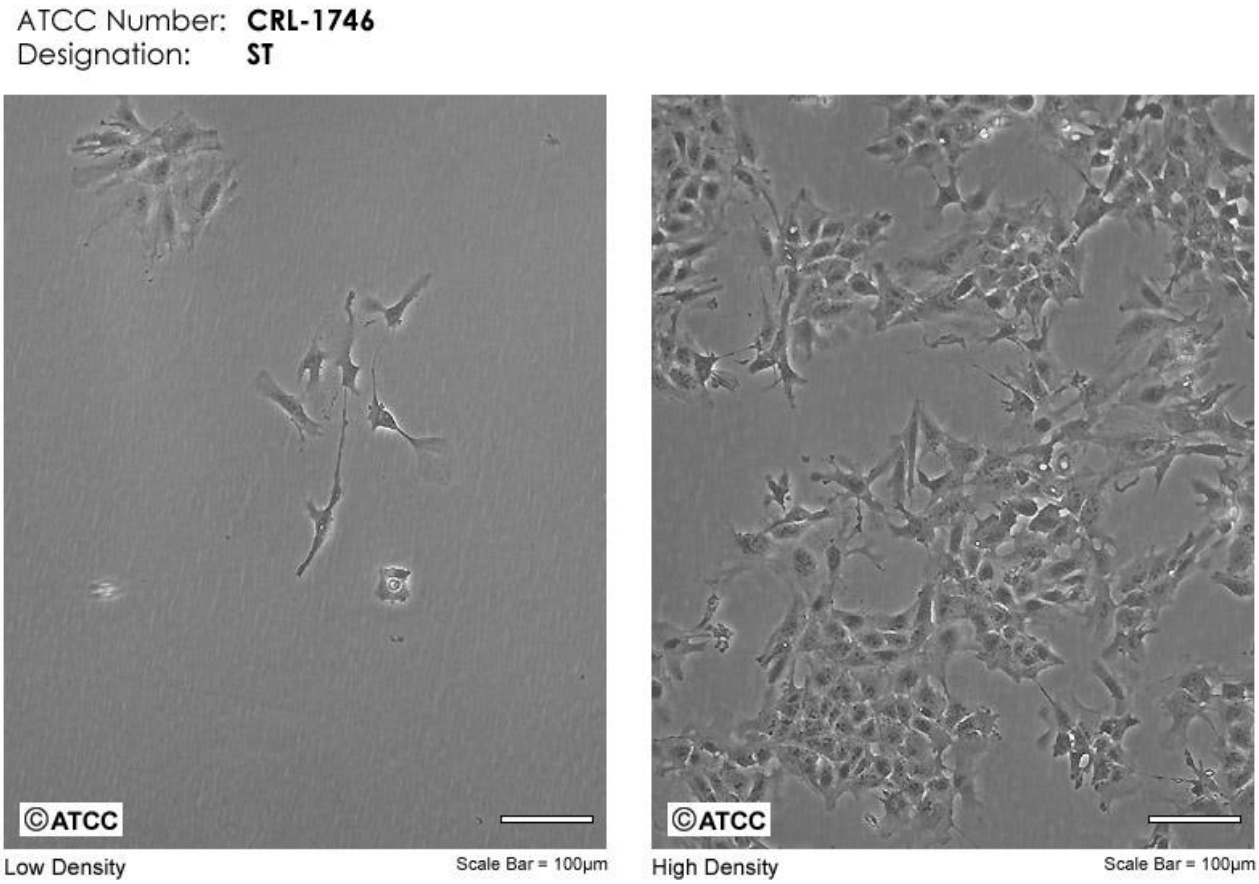


Fig.10

Cell micrograph of ST[19].

4.5 MDBK (NBL-1)

MDBK (NBL-1) is an adherent epithelial cell line from kidney of a male *Bos taurus* (cow).

As reported MDBK (NBL-1) (ATCC® CCL-22™) in product sheet[22], cells were cultivated in Eagle's Minimum Essential Medium (EMEM) and fetal bovine serum (FBS) to a final concentration of 10%.

In the images below (Fig.11) an example of 24hr and 48hr post seeding of this cells line[23] [24].

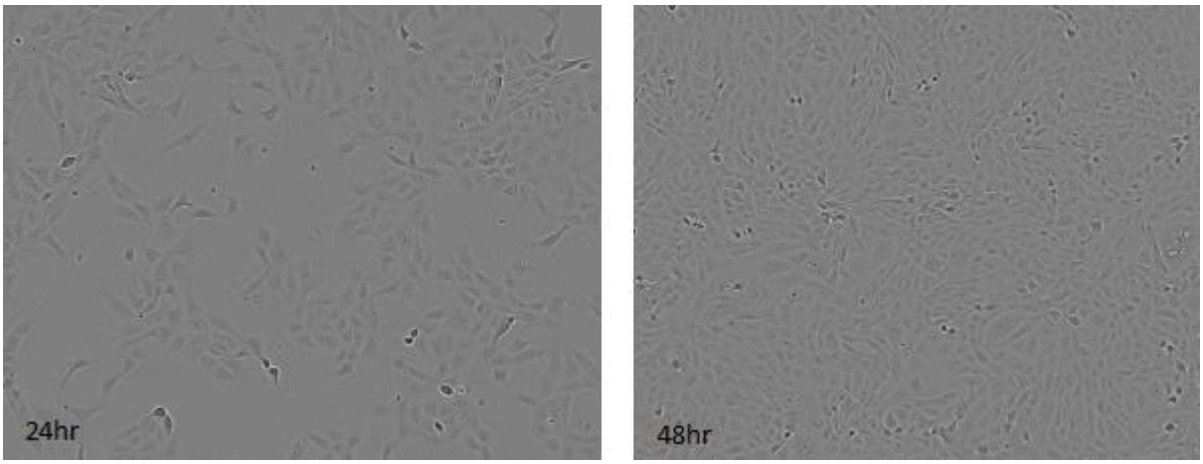


Fig.11

Cell micrograph of MDBK (NBL-1) 24hr and 48hr post seeding from ECACC site.

5 TITRATION METHOD VALIDATION

This part describes the titration methods validation for REO3, HPIV3 and SuHV-1.

The objective of the performed activities was to validate, in accordance with the ICH Q2(R1) guidelines "Validation of Analytical Procedures: Text and Methodology" [25] and FDA Guidance for Industry "Analytical Procedures and Methods Validation for Drugs and Biologics"[26] , methods for determining the viral titer of REO3, HPIV3 and SuHV-1 suspensions.

The methods are used to determine the viral titer of REO3, HPIV3 and SuHV-1 suspensions which induces a cytopathic effect (CPE) on cells, expressed in $Log_{10}TCID_{50}$/ml or $TCID_{50}$/ml.

The methods are considered a quantitative/quantal assay (Appendix 3)[1] that allows the dilution required to infect 50% of the cultured cells, meaning the $TCID_{50}$ (*tissue culture infective dose*) of the viral suspension, to be determined.

In particular, the parameters checked in validation were:

- robustness
- precision (repeatability and intermediate precision)
- specificity
- accuracy
- linearity

These methods are based on the evaluation of CPE that different scalar dilutions of the viral suspension induce on the cell monolayer.

5.1 Materials

As part of the method, the test substance and cells, equipment/materials reported below, were used during method validation.

5.1.1 standard

Viral suspensions of REO3 virus obtained following the propagation of an original VR-232™ ATCC® vial (Lot 58395507) on LLC-MK2 Derivative cells ATCC® (Lot 3633198).

The titrations were performed on HEK- 293 cells (Lot 61714301).

Viral suspensions of HPIV3 virus obtained following the propagation of an original Cat.n°VR-93™ ATCC® vial (Lot No. 59380357 (Reference Lot 19W)) on LLC-MK2 Derivative cells ATCC® (Lot 3633198).

The titrations were performed on VERO cells ATCC® (Lot 60150897).

Viral suspensions of SuHV-1 virus obtained following the propagation of an original vial of Suides Herpes Virus (VIRUS BANK SUIDES HERPES VIRUS (SuHV-1) STAMM KAPLAN RVB-574 –Lot #15/280211/300311) on MDBK (NBL-1) cells ATCC® vial (Lot 60627700) or ST cells ATCC® vial.

The titrations were performed on VERO cells ATCC® (Lot:60150897).

5.1.2 Materials & reagents

- deep-well polypropylene plates
- 96-well flat bottom plates with low evaporation
- Reservoir
- Single-channel and multi-channel pipettes
- Pipette tips
- Sterile screws
- Falcon tubes
- EMEM 10% Fetal Bovine Serum (FBS) medium
- EMEM 2% Fetal Bovine Serum (FBS)
- EMEM 2% Fetal Bovine Serum (FBS) medium with Penicillin-Streptomycin 1% and Amphotericin B 1%
- EMEM 1% Fetal Bovine Serum (FBS)
- EMEM 1% Fetal Bovine Serum (FBS) medium with Penicillin-Streptomycin 1% and Amphotericin B 1%
- EMEM (0%) medium
- Phosphate Buffer Saline (PBS)
- TNE buffer

5.1.3 equipment

- CO_2 incubators
- Optical Microscopes
- EVOS Microscopes
- Refrigerators
- Freezers -80°C
- Biological Hoods
- Nucleocounter
- Ultrasound bath

5.2 calculation & validity of results

The calculation of results is done through visual analysis using an optical microscope by assessing the integrity of the cell monolayer. Through observation, the operator defines in which wells is detectable a CPE on the cell monolayer and check the wells as positive or negative to obtain by "*Spearman Karber Formula*" the $Log_{10}TCID_{50}$ value. The formula applied is:

$$Log_{10}TCID_{50} = X_o - (d/2) + d\Sigma pi$$

X_0= Log_{10} of the reciprocal of the highest dilution which infects 100% of the wells
d = Log_{10} of the dilution factor
pi = proportion of positive results with respect to the number of wells infected at each individual dilution.
Σ pi = sum of pi starting with the highest dilution with 100% infected wells.

Following the ICH "Viral Safety Evaluation of Biotechnology Products Derived from Cell Lines of Human or Animal Origin Q5A(R1)-Appendix 3", the 95% confidence limits for results of within-assay variation normally should be on the order of ± 0.5 log_{10} of the mean [1]. For this reason, the Standard Deviation (Sd) is also obtained, with which it is possible to obtain the Maximum (max) and Minimum (min) Titer.
The calculation to obtain max and min is the following:

$$max = Log_{10}TCID_{50} - (2* Sd)$$
$$min = Log_{10}TCID_{50} + (2* Sd)$$

The test is considered valid if:

- The difference between max and $Log_{10}TCID_{50}$ is ≤ 0.5 Log and if
- The difference between $Log_{10}TCID_{50}$ and min is ≤ 0.5 Log

In addition, the Negative Control present in each plate should not present any anomalies (e.g. detachment of the cell monolayer or uneven cell monolayer).

5.3 REO3 titration Method validation

5.3.1 Method description

The method is used to determine the titer expressed as $Log_{10}TCID_{50}$/mL of a REO3 viral suspension through the evaluation of the cytopathic effect induced by the virus on the monolayer of HEK-293 cells in adhesion.

5.3.2 Analitical procedure

The method is based on the evaluation of the cytopathic effect that different scalar dilutions of the viral suspension induce on the cell monolayer.

5.3.2.1 Preparation of scalar dilution of REO3

A pool of the REO3 viral suspension was used for the preparation of the samples for Robustness, specificity, precision, accuracy and linearity. Subsequently, each sample was diluted with 1:3 scalar dilutions prepared as indicated below.

Each titration consists of 3 plates of HEK-293 cells (96-well flat bottom plates with low evaporation):

- Plate 1 → infection with dilutions from 3^{-1} to 3^{-11}
- Plate 2 → infection with dilutions from 3^{-12} to 3^{-22}
- Plate 3 → infection with dilutions from 3^{-23} to 3^{-33}

For each titration (Plate 1, 2 and 3) the dilutions are prepared in a deep-well polypropylene plates as described below:

- Fill the first lines of the deep-well polypropylene plates with EMEM 2% FBS medium with antibiotic and antifungal (Penicillin-Streptomycin 1% and Amphotericin B 1 %).

→ rows A and B were used for the infection of Plate 1 (dilutions from 3^{-1} to 3^{-11} and negative control)

→ rows C and D will be used for the infection of Plate 2 (dilutions from 3^{-12} to 3^{-22} and negative control)
→ rows E and F will be used for the infection of Plate 3 (dilutions from 3^{-23} to 3^{-33} and negative control)

- Add REO3 viral suspension in row A column 1 and row B column 1 to have a 1:3 diluted suspension.

- Take the selected volume from row A and B column 1 (WELL A1 and WELL B1) and add in row A and B column 2 (WELL A2 and WELL B2), continue in this way until column 11 (skip column 12 → negative control) → dilutions from 3^{-1} to 3^{-11}

- Take the selected volume from row A and B column 11 (WELL A11 and WELL B11) and add in row C and D, continue the dilutions in this way until column 11 (skip column 12 → negative control) → dilutions from 3^{-12} to 3^{-22}

- Take the selected volume from row C and D column 11(WELL C11 and WELL D11) and add in row E and F, continue the dilutions in this way until column 11 (skip column 12 → negative control) → dilutions from 3^{-23} to 3^{-33}

For the preparation of the DILUTION PLATE follow the diagram below:

	1	2	3	4	5	6	7	8	9	10	11	12	
A	EMEM 2%FBS + **REO-3 (3^{-1})**	EMEM 2%FBS + **WELL A1 (3^{-2})**	EMEM 2%FBS + **WELL A2 (3^{-3})**	EMEM 2%FBS + **WELL A3 (3^{-4})**	EMEM 2%FBS + **WELL A4 (3^{-5})**	EMEM 2%FBS + **WELL A5 (3^{-6})**	EMEM 2%FBS + **WELL A6 (3^{-7})**	EMEM 2%FBS + **WELL A7 (3^{-8})**	EMEM 2%FBS + **WELL A8 (3^{-9})**	EMEM 2%FBS + **WELL A9 (3^{-10})**	EMEM 2%FBS + **WELL A10 (3^{-11})**	EMEM 2%FBS **C-**	**1st PLATE**
B	EMEM 2%FBS + **REO-3 (3^{-1})**	EMEM 2%FBS + **WELL B1 (3^{-2})**	EMEM 2%FBS + **WELL B2 (3^{-3})**	EMEM 2%FBS + **WELL B3 (3^{-4})**	EMEM 2%FBS + **WELL B4 (3^{-5})**	EMEM 2%FBS + **WELL B5 (3^{-6})**	EMEM 2%FBS + **WELL B6 (3^{-7})**	EMEM 2%FBS + **WELL B7 (3^{-8})**	EMEM 2%FBS + **WELL B8 (3^{-9})**	EMEM 2%FBS + **WELL B9 (3^{-10})**	EMEM 2%FBS + **WELL B10 (3^{-11})**	EMEM 2%FBS **C-**	
C	EMEM 2%FBS + **WELL A11 (3^{-12})**	EMEM 2%FBS + **WELL C1 (3^{-13})**	EMEM 2%FBS + **WELL C2 (3^{-14})**	EMEM 2%FBS + **WELL C3 (3^{-15})**	EMEM 2%FBS + **WELL C4 (3^{-16})**	EMEM 2%FBS + **WELL C5 (3^{-17})**	EMEM 2%FBS + **WELL C6 (3^{-18})**	EMEM 2%FBS + **WELL C7 (3^{-19})**	EMEM 2%FBS + **WELL C8 (3^{-20})**	EMEM 2%FBS + **WELL C9 (3^{-21})**	EMEM 2%FBS + **WELL C10 (3^{-22})**	EMEM 2%FBS **C-**	**2nd PLATE**
D	EMEM 2%FBS + **WELL B11 (3^{-12})**	EMEM 2%FBS + **WELL D1 (3^{-13})**	EMEM 2%FBS + **WELL D2 (3^{-14})**	EMEM 2%FBS + **WELL D3 (3^{-15})**	EMEM 2%FBS + **WELL D4 (3^{-16})**	EMEM 2%FBS + **WELL D5 (3^{-17})**	EMEM 2%FBS + **WELL D6 (3^{-18})**	EMEM 2%FBS + **WELL D7 (3^{-19})**	EMEM 2%FBS + **WELL D8 (3^{-20})**	EMEM 2%FBS + **WELL D9 (3^{-21})**	EMEM 2%FBS + **WELL D10 (3^{-22})**	EMEM 2%FBS **C-**	
E	EMEM 2%FBS + **WELL C11 (3^{-23})**	EMEM 2%FBS + **WELL E1 (3^{-24})**	EMEM 2%FBS + **WELL E2 (3^{-25})**	EMEM 2%FBS + **WELL E3 (3^{-26})**	EMEM 2%FBS + **WELL E4 (3^{-27})**	EMEM 2%FBS + **WELL E5 (3^{-28})**	EMEM 2%FBS + **WELL E6 (3^{-29})**	EMEM 2%FBS + **WELL E7 (3^{-30})**	EMEM 2%FBS + **WELL E8 (3^{-31})**	EMEM 2%FBS + **WELL E9 (3^{-32})**	EMEM 2%FBS + **WELL E10 (3^{-33})**	EMEM 2%FBS **C-**	**3rd PLATE**
F	EMEM 2%FBS + **WELL D11 (3^{-23})**	EMEM 2%FBS + **WELL F1 (3^{-24})**	EMEM 2%FBS + **WELL F2 (3^{-25})**	EMEM 2%FBS + **WELL F3 (3^{-26})**	EMEM 2%FBS + **WELL F4 (3^{-27})**	EMEM 2%FBS + **WELL F5 (3^{-28})**	EMEM 2%FBS + **WELL F6 (3^{-29})**	EMEM 2%FBS + **WELL F7 (3^{-30})**	EMEM 2%FBS + **WELL F8 (3^{-31})**	EMEM 2%FBS + **WELL F9 (3^{-32})**	EMEM 2%FBS + **WELL F10 (3^{-33})**	EMEM 2%FBS **C-**	
G													
H													

5.3.2.2 Titration procedure

The day before the titration assay, the plates were prepared as described below. Bring HEK-293 cells, grown in EMEM 10 % FBS medium, to a concentration of:

- $2.0*10^5$ cell/ml
- $2.5*10^5$ cell/ml (TARGET)
- $3.0*10^5$ cell/ml

Then take three plates 96-well flat bottom low evaporation plates (1st, 2nd, and 3rd Plate) for each titration to be performed; dispensing the cell suspension obtained in each well for each plate as shown below.

	1	2	3	4	5	6	7	8	9	10	11	12
A	HEK-293 + EMEM 10%FBS	HEK-293 + EMEM 10%FBS	HEK-293 + EMEM 10%FBS	HEK-293 + EMEM 10%FBS	HEK-293 + EMEM 10%FBS	HEK-293 + EMEM 10%FBS	HEK-293 + EMEM 10%FBS	HEK-293 + EMEM 10%FBS	HEK-293 + EMEM 10%FBS	HEK-293 + EMEM 10%FBS	HEK-293 + EMEM 10%FBS	HEK-293 + EMEM 10%FBS C-
B	HEK-293 + EMEM 10%FBS	HEK-293 + EMEM 10%FBS	HEK-293 + EMEM 10%FBS	HEK-293 + EMEM 10%FBS	HEK-293 + EMEM 10%FBS	HEK-293 + EMEM 10%FBS	HEK-293 + EMEM 10%FBS	HEK-293 + EMEM 10%FBS	HEK-293 + EMEM 10%FBS	HEK-293 + EMEM 10%FBS	HEK-293 + EMEM 10%FBS	HEK-293 + EMEM 10%FBS C-
C	HEK-293 + EMEM 10%FBS	HEK-293 + EMEM 10%FBS	HEK-293 + EMEM 10%FBS	HEK-293 + EMEM 10%FBS	HEK-293 + EMEM 10%FBS	HEK-293 + EMEM 10%FBS	HEK-293 + EMEM 10%FBS	HEK-293 + EMEM 10%FBS	HEK-293 + EMEM 10%FBS	HEK-293 + EMEM 10%FBS	HEK-293 + EMEM 10%FBS	HEK-293 + EMEM 10%FBS C-
D	HEK-293 + EMEM 10%FBS	HEK-293 + EMEM 10%FBS	HEK-293 + EMEM 10%FBS	HEK-293 + EMEM 10%FBS	HEK-293 + EMEM 10%FBS	HEK-293 + EMEM 10%FBS	HEK-293 + EMEM 10%FBS	HEK-293 + EMEM 10%FBS	HEK-293 + EMEM 10%FBS	HEK-293 + EMEM 10%FBS	HEK-293 + EMEM 10%FBS	HEK-293 + EMEM 10%FBS C-
E	HEK-293 + EMEM 10%FBS	HEK-293 + EMEM 10%FBS	HEK-293 + EMEM 10%FBS	HEK-293 + EMEM 10%FBS	HEK-293 + EMEM 10%FBS	HEK-293 + EMEM 10%FBS	HEK-293 + EMEM 10%FBS	HEK-293 + EMEM 10%FBS	HEK-293 + EMEM 10%FBS	HEK-293 + EMEM 10%FBS	HEK-293 + EMEM 10%FBS	HEK-293 + EMEM 10%FBS C-
F	HEK-293 + EMEM 10%FBS	HEK-293 + EMEM 10%FBS	HEK-293 + EMEM 10%FBS	HEK-293 + EMEM 10%FBS	HEK-293 + EMEM 10%FBS	HEK-293 + EMEM 10%FBS	HEK-293 + EMEM 10%FBS	HEK-293 + EMEM 10%FBS	HEK-293 + EMEM 10%FBS	HEK-293 + EMEM 10%FBS	HEK-293 + EMEM 10%FBS	HEK-293 + EMEM 10%FBS C-
G	HEK-293 + EMEM 10%FBS	HEK-293 + EMEM 10%FBS	HEK-293 + EMEM 10%FBS	HEK-293 + EMEM 10%FBS	HEK-293 + EMEM 10%FBS	HEK-293 + EMEM 10%FBS	HEK-293 + EMEM 10%FBS	HEK-293 + EMEM 10%FBS	HEK-293 + EMEM 10%FBS	HEK-293 + EMEM 10%FBS	HEK-293 + EMEM 10%FBS	HEK-293 + EMEM 10%FBS C-
H	HEK-293 + EMEM 10%FBS	HEK-293 + EMEM 10%FBS	HEK-293 + EMEM 10%FBS	HEK-293 + EMEM 10%FBS	HEK-293 + EMEM 10%FBS	HEK-293 + EMEM 10%FBS	HEK-293 + EMEM 10%FBS	HEK-293 + EMEM 10%FBS	HEK-293 + EMEM 10%FBS	HEK-293 + EMEM 10%FBS	HEK-293 + EMEM 10%FBS	HEK-293 + EMEM 10%FBS C-

The plates were placed in an incubator at 37.0 °C ± 1.0 °C and 5.0± 1.0 % CO_2 for 24 hours.

After this time, the cell monolayer was washed with PBS to remove any serum or dead cell residues and is was proceeded with infection of the 1st, 2nd, and 3rd Plates as indicated below.

Infection of the 1st Plate:

With a multichannel pipette, the defined volume was taken from row A of the deep-well polypropylene plates and dispense it into row A of the 1st Plate. Action was repeated 3 more times, respectively into rows B, C and D of the 1st Plate.

Follow the same process with row B of the deep-well polypropylene plates and dispense it into row E, F, G and H of the 1st Plate.

	1	2	3	4	5	6	7	8	9	10	11	12	
A	EMEM 2%FBS + **REO-3 (3^{-1})**	EMEM 2%FBS + **WELL A1 (3^{-2})**	EMEM 2%FBS + **WELL A2 (3^{-3})**	EMEM 2%FBS + **WELL A3 (3^{-4})**	EMEM 2%FBS + **WELL A4 (3^{-5})**	EMEM 2%FBS + **WELL A5 (3^{-6})**	EMEM 2%FBS + **WELL A6 (3^{-7})**	EMEM 2%FBS + **WELL A7 (3^{-8})**	EMEM 2%FBS + **WELL A8 (3^{-9})**	EMEM 2%FBS + **WELL A9 (3^{-10})**	EMEM 2%FBS + **WELL A10 (3^{-11})**	EMEM 2%FBS **C-**	**1st PLATE**
B	EMEM 2%FBS + **REO-3 (3^{-1})**	EMEM 2%FBS + **WELL B1 (3^{-2})**	EMEM 2%FBS + **WELL B2 (3^{-3})**	EMEM 2%FBS + **WELL B3 (3^{-4})**	EMEM 2%FBS + **WELL B4 (3^{-5})**	EMEM 2%FBS + **WELL B5 (3^{-6})**	EMEM 2%FBS + **WELL B6 (3^{-7})**	EMEM 2%FBS + **WELL B7 (3^{-8})**	EMEM 2%FBS + **WELL B8 (3^{-9})**	EMEM 2%FBS + **WELL B9 (3^{-10})**	EMEM 2%FBS + **WELL B10 (3^{-11})**	EMEM 2%FBS **C-**	
C	EMEM 2%FBS + **WELL A11 (3^{-12})**	EMEM 2%FBS + **WELL C1 (3^{-13})**	EMEM 2%FBS + **WELL C2 (3^{-14})**	EMEM 2%FBS + **WELL C3 (3^{-15})**	EMEM 2%FBS + **WELL C4 (3^{-16})**	EMEM 2%FBS + **WELL C5 (3^{-17})**	EMEM 2%FBS + **WELL C6 (3^{-18})**	EMEM 2%FBS + **WELL C7 (3^{-19})**	EMEM 2%FBS + **WELL C8 (3^{-20})**	EMEM 2%FBS + **WELL C9 (3^{-21})**	EMEM 2%FBS + **WELL C10 (3^{-22})**	EMEM 2%FBS **C-**	**2nd PLATE**
D	EMEM 2%FBS + **WELL B11 (3^{-12})**	EMEM 2%FBS + **WELL D1 (3^{-13})**	EMEM 2%FBS + **WELL D2 (3^{-14})**	EMEM 2%FBS + **WELL D3 (3^{-15})**	EMEM 2%FBS + **WELL D4 (3^{-16})**	EMEM 2%FBS + **WELL D5 (3^{-17})**	EMEM 2%FBS + **WELL D6 (3^{-18})**	EMEM 2%FBS + **WELL D7 (3^{-19})**	EMEM 2%FBS + **WELL D8 (3^{-20})**	EMEM 2%FBS + **WELL D9 (3^{-21})**	EMEM 2%FBS + **WELL D10 (3^{-22})**	EMEM 2%FBS **C-**	
E	EMEM 2%FBS + **WELL C11 (3^{-23})**	EMEM 2%FBS + **WELL E1 (3^{-24})**	EMEM 2%FBS + **WELL E2 (3^{-25})**	EMEM 2%FBS + **WELL E3 (3^{-26})**	EMEM 2%FBS + **WELL E4 (3^{-27})**	EMEM 2%FBS + **WELL E5 (3^{-28})**	EMEM 2%FBS + **WELL E6 (3^{-29})**	EMEM 2%FBS + **WELL E7 (3^{-30})**	EMEM 2%FBS + **WELL E8 (3^{-31})**	EMEM 2%FBS + **WELL E9 (3^{-32})**	EMEM 2%FBS + **WELL E10 (3^{-33})**	EMEM 2%FBS **C-**	**3rd PLATE**
F	EMEM 2%FBS + **WELL D11 (3^{-23})**	EMEM 2%FBS + **WELL F1 (3^{-24})**	EMEM 2%FBS + **WELL F2 (3^{-25})**	EMEM 2%FBS + **WELL F3 (3^{-26})**	EMEM 2%FBS + **WELL F4 (3^{-27})**	EMEM 2%FBS + **WELL F5 (3^{-28})**	EMEM 2%FBS + **WELL F6 (3^{-29})**	EMEM 2%FBS + **WELL F7 (3^{-30})**	EMEM 2%FBS + **WELL F8 (3^{-31})**	EMEM 2%FBS + **WELL F9 (3^{-32})**	EMEM 2%FBS + **WELL F10 (3^{-33})**	EMEM 2%FBS **C-**	
G													
H													

1st PLATE: from 3^{-1} to 3^{-11}

	1	2	3	4	5	6	7	8	9	10	11	12
A	HEK-293 EMEM 2%FBS + REO-3 (3^{-1})	HEK-293 EMEM 2%FBS + REO-3 (3^{-2})	HEK-293 EMEM 2%FBS + REO-3 (3^{-3})	HEK-293 EMEM 2%FBS + REO-3 (3^{-4})	HEK-293 EMEM 2%FBS + REO-3 (3^{-5})	HEK-293 EMEM 2%FBS + REO-3 (3^{-6})	HEK-293 EMEM 2%FBS + REO-3 (3^{-7})	HEK-293 EMEM 2%FBS + REO-3 (3^{-8})	HEK-293 EMEM 2%FBS + REO-3 (3^{-9})	HEK-293 EMEM 2%FBS + REO-3 (3^{-10})	HEK-293 EMEM 2%FBS + REO-3 (3^{-11})	HEK-293 EMEM 2%FBS
B	HEK-293 EMEM 2%FBS + REO-3 (3^{-1})	HEK-293 EMEM 2%FBS + REO-3 (3^{-2})	HEK-293 EMEM 2%FBS + REO-3 (3^{-3})	HEK-293 EMEM 2%FBS + REO-3 (3^{-4})	HEK-293 EMEM 2%FBS + REO-3 (3^{-5})	HEK-293 EMEM 2%FBS + REO-3 (3^{-6})	HEK-293 EMEM 2%FBS + REO-3 (3^{-7})	HEK-293 EMEM 2%FBS + REO-3 (3^{-8})	HEK-293 EMEM 2%FBS + REO-3 (3^{-9})	HEK-293 EMEM 2%FBS + REO-3 (3^{-10})	HEK-293 EMEM 2%FBS + REO-3 (3^{-11})	HEK-293 EMEM 2%FBS
C	HEK-293 EMEM 2%FBS + REO-3 (3^{-1})	HEK-293 EMEM 2%FBS + REO-3 (3^{-2})	HEK-293 EMEM 2%FBS + REO-3 (3^{-3})	HEK-293 EMEM 2%FBS + REO-3 (3^{-4})	HEK-293 EMEM 2%FBS + REO-3 (3^{-5})	HEK-293 EMEM 2%FBS + REO-3 (3^{-6})	HEK-293 EMEM 2%FBS + REO-3 (3^{-7})	HEK-293 EMEM 2%FBS + REO-3 (3^{-8})	HEK-293 EMEM 2%FBS + REO-3 (3^{-9})	HEK-293 EMEM 2%FBS + REO-3 (3^{-10})	HEK-293 EMEM 2%FBS + REO-3 (3^{-11})	HEK-293 EMEM 2%FBS
D	HEK-293 EMEM 2%FBS + REO-3 (3^{-1})	HEK-293 EMEM 2%FBS + REO-3 (3^{-2})	HEK-293 EMEM 2%FBS + REO-3 (3^{-3})	HEK-293 EMEM 2%FBS + REO-3 (3^{-4})	HEK-293 EMEM 2%FBS + REO-3 (3^{-5})	HEK-293 EMEM 2%FBS + REO-3 (3^{-6})	HEK-293 EMEM 2%FBS + REO-3 (3^{-7})	HEK-293 EMEM 2%FBS + REO-3 (3^{-8})	HEK-293 EMEM 2%FBS + REO-3 (3^{-9})	HEK-293 EMEM 2%FBS + REO-3 (3^{-10})	HEK-293 EMEM 2%FBS + REO-3 (3^{-11})	HEK-293 EMEM 2%FBS
E	HEK-293 EMEM 2%FBS + REO-3 (3^{-1})	HEK-293 EMEM 2%FBS + REO-3 (3^{-2})	HEK-293 EMEM 2%FBS + REO-3 (3^{-3})	HEK-293 EMEM 2%FBS + REO-3 (3^{-4})	HEK-293 EMEM 2%FBS + REO-3 (3^{-5})	HEK-293 EMEM 2%FBS + REO-3 (3^{-6})	HEK-293 EMEM 2%FBS + REO-3 (3^{-7})	HEK-293 EMEM 2%FBS + REO-3 (3^{-8})	HEK-293 EMEM 2%FBS + REO-3 (3^{-9})	HEK-293 EMEM 2%FBS + REO-3 (3^{-10})	HEK-293 EMEM 2%FBS + REO-3 (3^{-11})	HEK-293 EMEM 2%FBS
F	HEK-293 EMEM 2%FBS + REO-3 (3^{-1})	HEK-293 EMEM 2%FBS + REO-3 (3^{-2})	HEK-293 EMEM 2%FBS + REO-3 (3^{-3})	HEK-293 EMEM 2%FBS + REO-3 (3^{-4})	HEK-293 EMEM 2%FBS + REO-3 (3^{-5})	HEK-293 EMEM 2%FBS + REO-3 (3^{-6})	HEK-293 EMEM 2%FBS + REO-3 (3^{-7})	HEK-293 EMEM 2%FBS + REO-3 (3^{-8})	HEK-293 EMEM 2%FBS + REO-3 (3^{-9})	HEK-293 EMEM 2%FBS + REO-3 (3^{-10})	HEK-293 EMEM 2%FBS + REO-3 (3^{-11})	HEK-293 EMEM 2%FBS
G	HEK-293 EMEM 2%FBS + REO-3 (3^{-1})	HEK-293 EMEM 2%FBS + REO-3 (3^{-2})	HEK-293 EMEM 2%FBS + REO-3 (3^{-3})	HEK-293 EMEM 2%FBS + REO-3 (3^{-4})	HEK-293 EMEM 2%FBS + REO-3 (3^{-5})	HEK-293 EMEM 2%FBS + REO-3 (3^{-6})	HEK-293 EMEM 2%FBS + REO-3 (3^{-7})	HEK-293 EMEM 2%FBS + REO-3 (3^{-8})	HEK-293 EMEM 2%FBS + REO-3 (3^{-9})	HEK-293 EMEM 2%FBS + REO-3 (3^{-10})	HEK-293 EMEM 2%FBS + REO-3 (3^{-11})	HEK-293 EMEM 2%FBS
H	HEK-293 EMEM 2%FBS + REO-3 (3^{-1})	HEK-293 EMEM 2%FBS + REO-3 (3^{-2})	HEK-293 EMEM 2%FBS + REO-3 (3^{-3})	HEK-293 EMEM 2%FBS + REO-3 (3^{-4})	HEK-293 EMEM 2%FBS + REO-3 (3^{-5})	HEK-293 EMEM 2%FBS + REO-3 (3^{-6})	HEK-293 EMEM 2%FBS + REO-3 (3^{-7})	HEK-293 EMEM 2%FBS + REO-3 (3^{-8})	HEK-293 EMEM 2%FBS + REO-3 (3^{-9})	HEK-293 EMEM 2%FBS + REO-3 (3^{-10})	HEK-293 EMEM 2%FBS + REO-3 (3^{-11})	HEK-293 EMEM 2%FBS

Infection of the 2nd Plate:

With a multichannel pipette, the defined volume was taken from row C of the deep-well polypropylene plates and dispense it into row A of the 1st Plate. Action was repeated 3 more times, respectively into rows B, C and D of the 2nd Plate.

Follow the same process with row D of the deep-well polypropylene plates and dispense it into row E, F, G and H of the 2nd Plate.

	1	2	3	4	5	6	7	8	9	10	11	12	
A	EMEM 2%FBS + **REO-3 (3^{-1})**	EMEM 2%FBS + **WELL A1 (3^{-2})**	EMEM 2%FBS + **WELL A2 (3^{-3})**	EMEM 2%FBS + **WELL A3 (3^{-4})**	EMEM 2%FBS + **WELL A4 (3^{-5})**	EMEM 2%FBS + **WELL A5 (3^{-6})**	EMEM 2%FBS + **WELL A6 (3^{-7})**	EMEM 2%FBS + **WELL A7 (3^{-8})**	EMEM 2%FBS + **WELL A8 (3^{-9})**	EMEM 2%FBS + **WELL A9 (3^{-10})**	EMEM 2%FBS + **WELL A10 (3^{-11})**	EMEM 2%FBS **C-**	**1st PLATE**
B	EMEM 2%FBS + **REO-3 (3^{-1})**	EMEM 2%FBS + **WELL B1 (3^{-2})**	EMEM 2%FBS + **WELL B2 (3^{-3})**	EMEM 2%FBS + **WELL B3 (3^{-4})**	EMEM 2%FBS + **WELL B4 (3^{-5})**	EMEM 2%FBS + **WELL B5 (3^{-6})**	EMEM 2%FBS + **WELL B6 (3^{-7})**	EMEM 2%FBS + **WELL B7 (3^{-8})**	EMEM 2%FBS + **WELL B8 (3^{-9})**	EMEM 2%FBS + **WELL B9 (3^{-10})**	EMEM 2%FBS + **WELL B10 (3^{-11})**	EMEM 2%FBS **C-**	
C	EMEM 2%FBS + **WELL A11 (3^{-12})**	EMEM 2%FBS + **WELL C1 (3^{-13})**	EMEM 2%FBS + **WELL C2 (3^{-14})**	EMEM 2%FBS + **WELL C3 (3^{-15})**	EMEM 2%FBS + **WELL C4 (3^{-16})**	EMEM 2%FBS + **WELL C5 (3^{-17})**	EMEM 2%FBS + **WELL C6 (3^{-18})**	EMEM 2%FBS + **WELL C7 (3^{-19})**	EMEM 2%FBS + **WELL C8 (3^{-20})**	EMEM 2%FBS + **WELL C9 (3^{-21})**	EMEM 2%FBS + **WELL C10 (3^{-22})**	EMEM 2%FBS **C-**	**2nd PLATE**
D	EMEM 2%FBS + **WELL B11 (3^{-12})**	EMEM 2%FBS + **WELL D1 (3^{-13})**	EMEM 2%FBS + **WELL D2 (3^{-14})**	EMEM 2%FBS + **WELL D3 (3^{-15})**	EMEM 2%FBS + **WELL D4 (3^{-16})**	EMEM 2%FBS + **WELL D5 (3^{-17})**	EMEM 2%FBS + **WELL D6 (3^{-18})**	EMEM 2%FBS + **WELL D7 (3^{-19})**	EMEM 2%FBS + **WELL D8 (3^{-20})**	EMEM 2%FBS + **WELL D9 (3^{-21})**	EMEM 2%FBS + **WELL D10 (3^{-22})**	EMEM 2%FBS **C-**	
E	EMEM 2%FBS + **WELL C11 (3^{-23})**	EMEM 2%FBS + **WELL E1 (3^{-24})**	EMEM 2%FBS + **WELL E2 (3^{-25})**	EMEM 2%FBS + **WELL E3 (3^{-26})**	EMEM 2%FBS + **WELL E4 (3^{-27})**	EMEM 2%FBS + **WELL E5 (3^{-28})**	EMEM 2%FBS + **WELL E6 (3^{-29})**	EMEM 2%FBS + **WELL E7 (3^{-30})**	EMEM 2%FBS + **WELL E8 (3^{-31})**	EMEM 2%FBS + **WELL E9 (3^{-32})**	EMEM 2%FBS + **WELL E10 (3^{-33})**	EMEM 2%FBS **C-**	**3rd PLATE**
F	EMEM 2%FBS + **WELL D11 (3^{-23})**	EMEM 2%FBS + **WELL F1 (3^{-24})**	EMEM 2%FBS + **WELL F2 (3^{-25})**	EMEM 2%FBS + **WELL F3 (3^{-26})**	EMEM 2%FBS + **WELL F4 (3^{-27})**	EMEM 2%FBS + **WELL F5 (3^{-28})**	EMEM 2%FBS + **WELL F6 (3^{-29})**	EMEM 2%FBS + **WELL F7 (3^{-30})**	EMEM 2%FBS + **WELL F8 (3^{-31})**	EMEM 2%FBS + **WELL F9 (3^{-32})**	EMEM 2%FBS + **WELL F10 (3^{-33})**	EMEM 2%FBS **C-**	
G													
H													

2nd PLATE: from 3^{-12} to 3^{-22}

	1	2	3	4	5	6	7	8	9	10	11	12
A	HEK-293 EMEM 2%FBS + REO-3 (3^{-12})	HEK-293 EMEM 2%FBS + REO-3 (3^{-13})	HEK-293 EMEM 2%FBS + REO-3 (3^{-14})	HEK-293 EMEM 2%FBS + REO-3 (3^{-15})	HEK-293 EMEM 2%FBS + REO-3 (3^{-16})	HEK-293 EMEM 2%FBS + REO-3 (3^{-17})	HEK-293 EMEM 2%FBS + REO-3 (3^{-18})	HEK-293 EMEM 2%FBS + REO-3 (3^{-19})	HEK-293 EMEM 2%FBS + REO-3 (3^{-20})	HEK-293 EMEM 2%FBS + REO-3 (3^{-21})	HEK-293 EMEM 2%FBS + REO-3 (3^{-22})	HEK-293 + EMEM 2%FBS
B	HEK-293 EMEM 2%FBS + REO-3 (3^{-12})	HEK-293 EMEM 2%FBS + REO-3 (3^{-13})	HEK-293 EMEM 2%FBS + REO-3 (3^{-14})	HEK-293 EMEM 2%FBS + REO-3 (3^{-15})	HEK-293 EMEM 2%FBS + REO-3 (3^{-16})	HEK-293 EMEM 2%FBS + REO-3 (3^{-17})	HEK-293 EMEM 2%FBS + REO-3 (3^{-18})	HEK-293 EMEM 2%FBS + REO-3 (3^{-19})	HEK-293 EMEM 2%FBS + REO-3 (3^{-20})	HEK-293 EMEM 2%FBS + REO-3 (3^{-21})	HEK-293 EMEM 2%FBS + REO-3 (3^{-22})	HEK-293 + EMEM 2%FBS
C	HEK-293 EMEM 2%FBS + REO-3 (3^{-12})	HEK-293 EMEM 2%FBS + REO-3 (3^{-13})	HEK-293 EMEM 2%FBS + REO-3 (3^{-14})	HEK-293 EMEM 2%FBS + REO-3 (3^{-15})	HEK-293 EMEM 2%FBS + REO-3 (3^{-16})	HEK-293 EMEM 2%FBS + REO-3 (3^{-17})	HEK-293 EMEM 2%FBS + REO-3 (3^{-18})	HEK-293 EMEM 2%FBS + REO-3 (3^{-19})	HEK-293 EMEM 2%FBS + REO-3 (3^{-20})	HEK-293 EMEM 2%FBS + REO-3 (3^{-21})	HEK-293 EMEM 2%FBS + REO-3 (3^{-22})	HEK-293 + EMEM 2%FBS
D	HEK-293 EMEM 2%FBS + REO-3 (3^{-12})	HEK-293 EMEM 2%FBS + REO-3 (3^{-13})	HEK-293 EMEM 2%FBS + REO-3 (3^{-14})	HEK-293 EMEM 2%FBS + REO-3 (3^{-15})	HEK-293 EMEM 2%FBS + REO-3 (3^{-16})	HEK-293 EMEM 2%FBS + REO-3 (3^{-17})	HEK-293 EMEM 2%FBS + REO-3 (3^{-18})	HEK-293 EMEM 2%FBS + REO-3 (3^{-19})	HEK-293 EMEM 2%FBS + REO-3 (3^{-20})	HEK-293 EMEM 2%FBS + REO-3 (3^{-21})	HEK-293 EMEM 2%FBS + REO-3 (3^{-22})	HEK-293 + EMEM 2%FBS
E	HEK-293 EMEM 2%FBS + REO-3 (3^{-12})	HEK-293 EMEM 2%FBS + REO-3 (3^{-13})	HEK-293 EMEM 2%FBS + REO-3 (3^{-14})	HEK-293 EMEM 2%FBS + REO-3 (3^{-15})	HEK-293 EMEM 2%FBS + REO-3 (3^{-16})	HEK-293 EMEM 2%FBS + REO-3 (3^{-17})	HEK-293 EMEM 2%FBS + REO-3 (3^{-18})	HEK-293 EMEM 2%FBS + REO-3 (3^{-19})	HEK-293 EMEM 2%FBS + REO-3 (3^{-20})	HEK-293 EMEM 2%FBS + REO-3 (3^{-21})	HEK-293 EMEM 2%FBS + REO-3 (3^{-22})	HEK-293 + EMEM 2%FBS
F	HEK-293 EMEM 2%FBS + REO-3 (3^{-12})	HEK-293 EMEM 2%FBS + REO-3 (3^{-13})	HEK-293 EMEM 2%FBS + REO-3 (3^{-14})	HEK-293 EMEM 2%FBS + REO-3 (3^{-15})	HEK-293 EMEM 2%FBS + REO-3 (3^{-16})	HEK-293 EMEM 2%FBS + REO-3 (3^{-17})	HEK-293 EMEM 2%FBS + REO-3 (3^{-18})	HEK-293 EMEM 2%FBS + REO-3 (3^{-19})	HEK-293 EMEM 2%FBS + REO-3 (3^{-20})	HEK-293 EMEM 2%FBS + REO-3 (3^{-21})	HEK-293 EMEM 2%FBS + REO-3 (3^{-22})	HEK-293 + EMEM 2%FBS
G	HEK-293 EMEM 2%FBS + REO-3 (3^{-12})	HEK-293 EMEM 2%FBS + REO-3 (3^{-13})	HEK-293 EMEM 2%FBS + REO-3 (3^{-14})	HEK-293 EMEM 2%FBS + REO-3 (3^{-15})	HEK-293 EMEM 2%FBS + REO-3 (3^{-16})	HEK-293 EMEM 2%FBS + REO-3 (3^{-17})	HEK-293 EMEM 2%FBS + REO-3 (3^{-18})	HEK-293 EMEM 2%FBS + REO-3 (3^{-19})	HEK-293 EMEM 2%FBS + REO-3 (3^{-20})	HEK-293 EMEM 2%FBS + REO-3 (3^{-21})	HEK-293 EMEM 2%FBS + REO-3 (3^{-22})	HEK-293 + EMEM 2%FBS
H	HEK-293 EMEM 2%FBS + REO-3 (3^{-12})	HEK-293 EMEM 2%FBS + REO-3 (3^{-13})	HEK-293 EMEM 2%FBS + REO-3 (3^{-14})	HEK-293 EMEM 2%FBS + REO-3 (3^{-15})	HEK-293 EMEM 2%FBS + REO-3 (3^{-16})	HEK-293 EMEM 2%FBS + REO-3 (3^{-17})	HEK-293 EMEM 2%FBS + REO-3 (3^{-18})	HEK-293 EMEM 2%FBS + REO-3 (3^{-19})	HEK-293 EMEM 2%FBS + REO-3 (3^{-20})	HEK-293 EMEM 2%FBS + REO-3 (3^{-21})	HEK-293 EMEM 2%FBS + REO-3 (3^{-22})	HEK-293 + EMEM 2%FBS

Infection of the 3rd Plate:

With a multichannel pipette, the defined volume was taken from row E of the deep-well polypropylene plates and dispense it into row A of the 1st Plate. Action was repeated 3 more times, respectively into rows B, C and D of the 3rd Plate.

Follow the same process with row F of the deep-well polypropylene plates and dispense it into row E, F, G and H of the 3rd Plate.

	1	2	3	4	5	6	7	8	9	10	11	12	
A	EMEM 2%FBS + **REO-3 (3^{-1})**	EMEM 2%FBS + **WELL A1 (3^{-2})**	EMEM 2%FBS + **WELL A2 (3^{-3})**	EMEM 2%FBS + **WELL A3 (3^{-4})**	EMEM 2%FBS + **WELL A4 (3^{-5})**	EMEM 2%FBS + **WELL A5 (3^{-6})**	EMEM 2%FBS + **WELL A6 (3^{-7})**	EMEM 2%FBS + **WELL A7 (3^{-8})**	EMEM 2%FBS + **WELL A8 (3^{-9})**	EMEM 2%FBS + **WELL A9 (3^{-10})**	EMEM 2%FBS + **WELL A10 (3^{-11})**	EMEM 2%FBS **C-**	**1st PLATE**
B	EMEM 2%FBS + **REO-3 (3^{-1})**	EMEM 2%FBS + **WELL B1 (3^{-2})**	EMEM 2%FBS + **WELL B2 (3^{-3})**	EMEM 2%FBS + **WELL B3 (3^{-4})**	EMEM 2%FBS + **WELL B4 (3^{-5})**	EMEM 2%FBS + **WELL B5 (3^{-6})**	EMEM 2%FBS + **WELL B6 (3^{-7})**	EMEM 2%FBS + **WELL B7 (3^{-8})**	EMEM 2%FBS + **WELL B8 (3^{-9})**	EMEM 2%FBS + **WELL B9 (3^{-10})**	EMEM 2%FBS + **WELL B10 (3^{-11})**	EMEM 2%FBS **C-**	
C	EMEM 2%FBS + **WELL A11 (3^{-12})**	EMEM 2%FBS + **WELL C1 (3^{-13})**	EMEM 2%FBS + **WELL C2 (3^{-14})**	EMEM 2%FBS + **WELL C3 (3^{-15})**	EMEM 2%FBS + **WELL C4 (3^{-16})**	EMEM 2%FBS + **WELL C5 (3^{-17})**	EMEM 2%FBS + **WELL C6 (3^{-18})**	EMEM 2%FBS + **WELL C7 (3^{-19})**	EMEM 2%FBS + **WELL C8 (3^{-20})**	EMEM 2%FBS + **WELL C9 (3^{-21})**	EMEM 2%FBS + **WELL C10 (3^{-22})**	EMEM 2%FBS **C-**	**2nd PLATE**
D	EMEM 2%FBS + **WELL B11 (3^{-12})**	EMEM 2%FBS + **WELL D1 (3^{-13})**	EMEM 2%FBS + **WELL D2 (3^{-14})**	EMEM 2%FBS + **WELL D3 (3^{-15})**	EMEM 2%FBS + **WELL D4 (3^{-16})**	EMEM 2%FBS + **WELL D5 (3^{-17})**	EMEM 2%FBS + **WELL D6 (3^{-18})**	EMEM 2%FBS + **WELL D7 (3^{-19})**	EMEM 2%FBS + **WELL D8 (3^{-20})**	EMEM 2%FBS + **WELL D9 (3^{-21})**	EMEM 2%FBS + **WELL D10 (3^{-22})**	EMEM 2%FBS **C-**	
E	EMEM 2%FBS + **WELL C11 (3^{-23})**	EMEM 2%FBS + **WELL E1 (3^{-24})**	EMEM 2%FBS + **WELL E2 (3^{-25})**	EMEM 2%FBS + **WELL E3 (3^{-26})**	EMEM 2%FBS + **WELL E4 (3^{-27})**	EMEM 2%FBS + **WELL E5 (3^{-28})**	EMEM 2%FBS + **WELL E6 (3^{-29})**	EMEM 2%FBS + **WELL E7 (3^{-30})**	EMEM 2%FBS + **WELL E8 (3^{-31})**	EMEM 2%FBS + **WELL E9 (3^{-32})**	EMEM 2%FBS + **WELL E10 (3^{-33})**	EMEM 2%FBS **C-**	**3rd PLATE**
F	EMEM 2%FBS + **WELL D11 (3^{-23})**	EMEM 2%FBS + **WELL F1 (3^{-24})**	EMEM 2%FBS + **WELL F2 (3^{-25})**	EMEM 2%FBS + **WELL F3 (3^{-26})**	EMEM 2%FBS + **WELL F4 (3^{-27})**	EMEM 2%FBS + **WELL F5 (3^{-28})**	EMEM 2%FBS + **WELL F6 (3^{-29})**	EMEM 2%FBS + **WELL F7 (3^{-30})**	EMEM 2%FBS + **WELL F8 (3^{-31})**	EMEM 2%FBS + **WELL F9 (3^{-32})**	EMEM 2%FBS + **WELL F10 (3^{-33})**	EMEM 2%FBS **C-**	
G													
H													

3rd PLATE: from 3^{-23} to 3^{-33}

	1	2	3	4	5	6	7	8	9	10	11	12
A	HEK-293 EMEM 2%FBS + REO-3 (3^{-23})	HEK-293 EMEM 2%FBS + REO-3 (3^{-24})	HEK-293 EMEM 2%FBS + REO-3 (3^{-25})	HEK-293 EMEM 2%FBS + REO-3 (3^{-26})	HEK-293 EMEM 2%FBS + REO-3 (3^{-27})	HEK-293 EMEM 2%FBS + REO-3 (3^{-28})	HEK-293 EMEM 2%FBS + REO-3 (3^{-29})	HEK-293 EMEM 2%FBS + REO-3 (3^{-30})	HEK-293 EMEM 2%FBS + REO-3 (3^{-31})	HEK-293 EMEM 2%FBS + REO-3 (3^{-32})	HEK-293 EMEM 2%FBS + REO-3 (3^{-33})	HEK-293 + EMEM 2%FBS
B	HEK-293 EMEM 2%FBS + REO-3 (3^{-23})	HEK-293 EMEM 2%FBS + REO-3 (3^{-24})	HEK-293 EMEM 2%FBS + REO-3 (3^{-25})	HEK-293 EMEM 2%FBS + REO-3 (3^{-26})	HEK-293 EMEM 2%FBS + REO-3 (3^{-27})	HEK-293 EMEM 2%FBS + REO-3 (3^{-28})	HEK-293 EMEM 2%FBS + REO-3 (3^{-29})	HEK-293 EMEM 2%FBS + REO-3 (3^{-30})	HEK-293 EMEM 2%FBS + REO-3 (3^{-31})	HEK-293 EMEM 2%FBS + REO-3 (3^{-32})	HEK-293 EMEM 2%FBS + REO-3 (3^{-33})	HEK-293 + EMEM 2%FBS
C	HEK-293 EMEM 2%FBS + REO-3 (3^{-23})	HEK-293 EMEM 2%FBS + REO-3 (3^{-24})	HEK-293 EMEM 2%FBS + REO-3 (3^{-25})	HEK-293 EMEM 2%FBS + REO-3 (3^{-26})	HEK-293 EMEM 2%FBS + REO-3 (3^{-27})	HEK-293 EMEM 2%FBS + REO-3 (3^{-28})	HEK-293 EMEM 2%FBS + REO-3 (3^{-29})	HEK-293 EMEM 2%FBS + REO-3 (3^{-30})	HEK-293 EMEM 2%FBS + REO-3 (3^{-31})	HEK-293 EMEM 2%FBS + REO-3 (3^{-32})	HEK-293 EMEM 2%FBS + REO-3 (3^{-33})	HEK-293 + EMEM 2%FBS
D	HEK-293 EMEM 2%FBS + REO-3 (3^{-23})	HEK-293 EMEM 2%FBS + REO-3 (3^{-24})	HEK-293 EMEM 2%FBS + REO-3 (3^{-25})	HEK-293 EMEM 2%FBS + REO-3 (3^{-26})	HEK-293 EMEM 2%FBS + REO-3 (3^{-27})	HEK-293 EMEM 2%FBS + REO-3 (3^{-28})	HEK-293 EMEM 2%FBS + REO-3 (3^{-29})	HEK-293 EMEM 2%FBS + REO-3 (3^{-30})	HEK-293 EMEM 2%FBS + REO-3 (3^{-31})	HEK-293 EMEM 2%FBS + REO-3 (3^{-32})	HEK-293 EMEM 2%FBS + REO-3 (3^{-33})	HEK-293 + EMEM 2%FBS
E	HEK-293 EMEM 2%FBS + REO-3 (3^{-23})	HEK-293 EMEM 2%FBS + REO-3 (3^{-24})	HEK-293 EMEM 2%FBS + REO-3 (3^{-25})	HEK-293 EMEM 2%FBS + REO-3 (3^{-26})	HEK-293 EMEM 2%FBS + REO-3 (3^{-27})	HEK-293 EMEM 2%FBS + REO-3 (3^{-28})	HEK-293 EMEM 2%FBS + REO-3 (3^{-29})	HEK-293 EMEM 2%FBS + REO-3 (3^{-30})	HEK-293 EMEM 2%FBS + REO-3 (3^{-31})	HEK-293 EMEM 2%FBS + REO-3 (3^{-32})	HEK-293 EMEM 2%FBS + REO-3 (3^{-33})	HEK-293 + EMEM 2%FBS
F	HEK-293 EMEM 2%FBS + REO-3 (3^{-23})	HEK-293 EMEM 2%FBS + REO-3 (3^{-24})	HEK-293 EMEM 2%FBS + REO-3 (3^{-25})	HEK-293 EMEM 2%FBS + REO-3 (3^{-26})	HEK-293 EMEM 2%FBS + REO-3 (3^{-27})	HEK-293 EMEM 2%FBS + REO-3 (3^{-28})	HEK-293 EMEM 2%FBS + REO-3 (3^{-29})	HEK-293 EMEM 2%FBS + REO-3 (3^{-30})	HEK-293 EMEM 2%FBS + REO-3 (3^{-31})	HEK-293 EMEM 2%FBS + REO-3 (3^{-32})	HEK-293 EMEM 2%FBS + REO-3 (3^{-33})	HEK-293 + EMEM 2%FBS
G	HEK-293 EMEM 2%FBS + REO-3 (3^{-23})	HEK-293 EMEM 2%FBS + REO-3 (3^{-24})	HEK-293 EMEM 2%FBS + REO-3 (3^{-25})	HEK-293 EMEM 2%FBS + REO-3 (3^{-26})	HEK-293 EMEM 2%FBS + REO-3 (3^{-27})	HEK-293 EMEM 2%FBS + REO-3 (3^{-28})	HEK-293 EMEM 2%FBS + REO-3 (3^{-29})	HEK-293 EMEM 2%FBS + REO-3 (3^{-30})	HEK-293 EMEM 2%FBS + REO-3 (3^{-31})	HEK-293 EMEM 2%FBS + REO-3 (3^{-32})	HEK-293 EMEM 2%FBS + REO-3 (3^{-33})	HEK-293 + EMEM 2%FBS
H	HEK-293 EMEM 2%FBS + REO-3 (3^{-23})	HEK-293 EMEM 2%FBS + REO-3 (3^{-24})	HEK-293 EMEM 2%FBS + REO-3 (3^{-25})	HEK-293 EMEM 2%FBS + REO-3 (3^{-26})	HEK-293 EMEM 2%FBS + REO-3 (3^{-27})	HEK-293 EMEM 2%FBS + REO-3 (3^{-28})	HEK-293 EMEM 2%FBS + REO-3 (3^{-29})	HEK-293 EMEM 2%FBS + REO-3 (3^{-30})	HEK-293 EMEM 2%FBS + REO-3 (3^{-31})	HEK-293 EMEM 2%FBS + REO-3 (3^{-32})	HEK-293 EMEM 2%FBS + REO-3 (3^{-33})	HEK-293 + EMEM 2%FBS

At the end of the infection, the 3 Plates were placed in an incubator at 37.0 °C ± 1.0 °C and 5.0± 1.0 % CO_2 for 4-6 days. After 4-6 days, the plates were evaluated with an optical microscope to detect the presence/absence of a CPE on the cell monolayer.

5.3.3 Results

5.3.3.1 Robustness

The aim of robustness is verifying that the performance of the analytical method is not influenced by deliberate changes that can be introduced during the execution of some of the phases of the test, evaluated as potentially critical in the risk assessment phase.

The following tests were performed provided by the Design of Experiment (DoE) and the Risk Assessment, established using the JMP software, to verify the robustness of the analytical method. The order of tests was randomized by the JMP software.

Following the Risk Assessment, the parameters that were classified as medium or high-risk were:

- Cell concentration for the titration test
- Different culture medium
- Different incubation times

Lower and higher values of the set-point:

Parameter	Lower value	Set-point	Higher value
Cell concentration for the titration test	$2*10^5$ cell/ml	$2.5*10^5$ cell/ml	$3*10^5$ cell/ml
Different incubation times	4 days	5 days	6 days

With regards to the parameter "Different culture medium", the following conditions were defined:

- EMEM 2% FBS
- EMEM 2% FBS with antibiotic and antifungal

After the execution of all the programmed analytical sessions, using the JMP software, starting from the results, the p-values relating to each critical parameter and the parameter interactions were calculated in addition to calculating the "Lack of Fit".

The robustness of the method was verified since all the values were greater than the significance level set at 0.05.

Test number	Post infection incubation days	cell concentration (cell/mL)	cell culture media[(1)]	Virus Titer ($Log_{10}TCID_{50}$/ml)
1	5	250000	A	10.423
2	4	300000	A	10,423
3	5	250000	B	10.602
4	6	200000	B	10.841
5	6	200000	A	10.721
6	6	300000	A	10.781
7	6	300000	B	10.841
8	4	300000	B	10.364
9	4	200000	A	10.721
10	5	250000	A	10.542
11	4	200000	B	10.364

Notes:

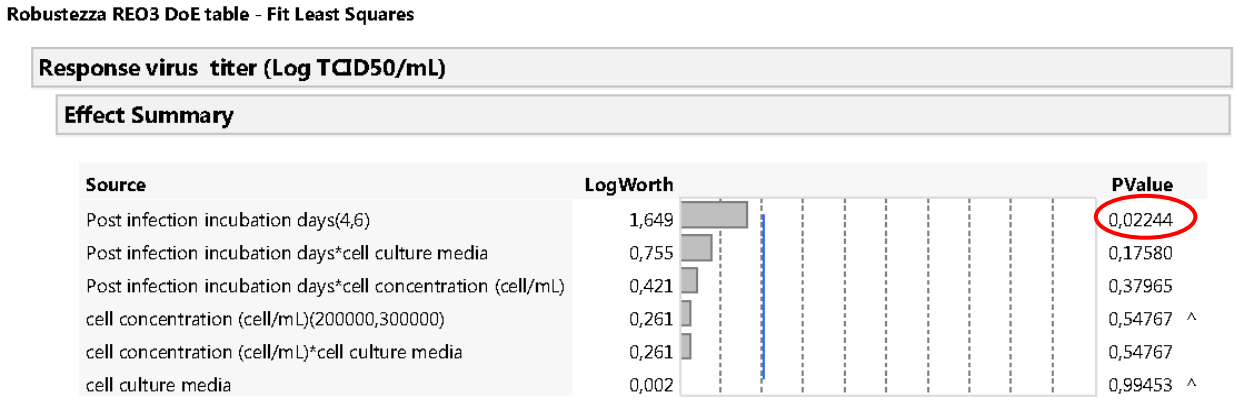
(1) Cell culture media:

A = EMEM 2% FBS

B = EMEM 2% FBS + antibiotic and antifungal

The "p-Value" in the "Effect Summary" section (*Fig.12*) summarizes the significance of each parameter and each interaction. The p-value, highlighted in red, was lower than the level of significance, than we proceed to the evaluation of the global variance.

Fig.12



The value "Prob> F" (**0.1584**), Fig.13 , in the "Analysis of Variance" section (global p-value of the ANOVA analysis for the total variance of the model) was not significant because it was higher than the significance level set at 0.05 (> 0.05), so no parameters or interactions should to be considered significant.

Fig.13

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio
Model	6	0,28975238	0,048292	2,9347
Error	4	0,06582253	0,016456	**Prob > F**
C. Total	10	0,35557491		0,1584

5.3.3.2 Intermediate precision

To test the intermediate precision parameter, the five samples obtained from the initial pool were analyzed in duplicate as:

- Pure (undiluted)
- Diluted 1:10
- Diluted 1:100
- Diluted 1:1000
- Diluted 1:10000

The test was performed (two different titrations for each sample) by two different operators during two independent analytical sessions.

The difference between the Mean Value of each analysis (expressed as $Log_{10}TCID_{50}$/ml) performed during the entire validation phase (two different operators performed two independent analytical sessions) should be within ± 0.5 Log_{10}.
This criterion was established based on what is required by ICH Q5A (R1)[1].

The table below shows the obtained results:

	Titration	Measured titer ($Log_{10}TCID_{50}/ml$)	Difference between measured titers ($Log_{10}TCID_{50}/ml$)
Initial titer	*Op.1/T.1*	11.02	0.18
	Op.2/T.1	10.84	
	Op.1/T.1	11.02	0.24
	Op.2/T.2	10.78	
	Op.1/T.2	10.96	0.12
	Op.2/T.1	10.84	
	Op.1/T.2	10.96	0.18
	Op.2/T.2	10.78	
1:10	*Op.1/T.1*	9.77	0.12
	Op.2/T.1	9.89	
	Op.1/T.1	9.77	0.06
	Op.2/T.2	9.71	
	Op.1/T.2	10.19	0.30
	Op.2/T.1	9.89	
	Op.1/T.2	10.19	0.48
	Op.2/T.2	9.71	
1:100	*Op.1/T.1*	8.52	0.12
	Op.2/T.1	8.40	
	Op.1/T.1	8.52	0.11
	Op.2/T.2	8.63	
	Op.1/T.2	8.87	0.47
	Op.2/T.1	8.40	
	Op.1/T.2	8.87	0.24
	Op.2/T.2	8.63	
1:1000	*Op.1/T.1*	7.56	0.12
	Op.2/T.1	7.68	
	Op.1/T.1	7.56	0
	Op.2/T.2	7.56	
	Op.1/T.2	7.86	0.18
	Op.2/T.1	7.68	
	Op.1/T.2	7.86	0.30
	Op.2/T.2	7.56	
1:10000	*Op.1/T.1*	6.79	0.17
	Op.2/T.1	6.96	
	Op.1/T.1	6.79	0.12
	Op.2/T.2	6.67	
	Op.1/T.2	6.55	0.41
	Op.2/T.1	6.96	
	Op.1/T.2	6.55	0.12
	Op.2/T.2	6.67	

5.3.3.3 Repeatability

To test the repeatability parameter, the five samples prepared from the initial pool were analyzed in duplicate:

- Pure (undiluted)
- Diluted 1:10
- Diluted 1:100
- Diluted 1:1000
- Diluted 1:10000

The test was performed (two different titrations for each sample) by two different operators during two independent analytical sessions.

The difference between the titers of each analysis of the same sample, expressed as $Log_{10}TCID_{50}$/ml and measured through the two titrations performed by the same operator, should fall within the interval ± 0.5 Log_{10}.

This criterion was established based on what is required by ICH Q5A (R1)[1].

The table below shows the obtained results:

		Titration	Measured titer ($Log_{10}TCID_{50}/ml$)	Difference between measured titers ($Log_{10}TCID_{50}/ml$)
OPERATOR 1	INITIAL TITER	1	11.02	0.06
		2	10.96	
	1:10	1	9.77	0.42
		2	10.19	
	1:100	1	8.52	0.35
		2	8.87	
	1:1000	1	7.56	0.30
		2	7.86	
	1:10000	1	6.79	0.24
		2	6.55	
OPERATOR 2	INITIAL TITER	1	10.84	0.06
		2	10.78	
	1:10	1	9.89	0.18
		2	9.71	
	1:100	1	8.40	0.23
		2	8.63	
	1:1000	1	7.68	0.12
		2	7.56	
	1:10000	1	6.96	0.29
		2	6.67	

5.3.3.4 specificity

The specificity of the method was evaluated comparing the CPE with the negative control present on each plate, using an optical microscope.

5.3.3.5 Accuracy

To test the accuracy parameter, the five samples obtained from the initial pool were analyzed in duplicate:

- Pure (undiluted)
- Diluted 1:10
- Diluted 1:100
- Diluted 1:1000
- Diluted 1:10000

The test was performed (two different titrations for each sample) by two different operators during two independent analytical sessions.

The difference between the expected titer of the sample and the titer obtained using this method (average value $Log_{10}TCID_{50}$/ml) should be within the interval of ± 0.5 $Log_{10}TCID_{50}$.

This criterion was established based on what is required by ICH Q5A (R1)[1].

The table below shows the obtained results:

		Expected titer ($Log_{10}TCID_{50}$/ml)	Titration	Measured titer ($Log_{10}TCID_{50}$/ml)	Difference between the measured titer ($Log_{10}TCID_{50}$/ml) and the expected titer ($Log_{10}TCID_{50}$/ml)
Operator 1	***INITIAL TITER***	Mean of Initial Titer of titration 1 and 2 = 10.99	1	11.02	N.A. To be used as a reference value
			2	10.96	N.A. To be used as a reference value
	1:10	9.99	1	9.77	0.22
			2	10.19	0.20
	1:100	8.99	1	8.52	0.47
			2	8.87	0.12
	1:1000	7.99	1	7.56	0.43
			2	7.86	0.13
	1:10000	6.99	1	6.79	0.20
			2	6.55	0.44
Operator 2	***INITIAL TITER***	Mean of Initial Titer of titration 1 and 2 = 10.81	1	10.84	N.A. To be used as a reference value
			2	10.78	N.A. To be used as a reference value
	1:10	9.81	1	9.89	0.08
			2	9.71	0.10
	1:100	8.81	1	8.40	0.41
			2	8.63	0.18
	1:1000	7.81	1	7.68	0.13
			2	7.56	0.25
	1:10000	6.81	1	6.96	0.15
			2	6.67	0.14

5.3.3.6 Linearity

To test the linearity parameter, the five samples obtained from the initial pool were analyzed in duplicate:

- Pure (undiluted)
- Diluted 1:10
- Diluted 1:100
- Diluted 1:1000
- Diluted 1:10000

The test was performed (two different titrations for each sample) by two different operators during two independent analytical sessions.

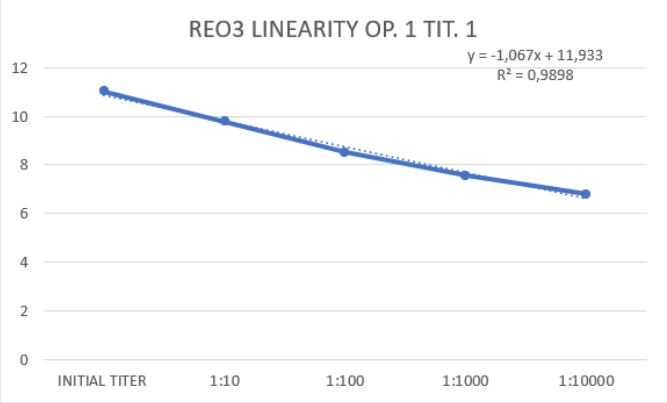
The coefficient of determination (R^2) of the line (described by the linear equation $y=ax+b$) obtained from a graph where the expected titers of the samples are represented on the x axis (INITIAL TITER (A), dilution 1:10 (B), dilution 1:100 (C), dilution 1:1000 (D), dilution 1:10000 (E)) and the titer obtained using this method on the y axis, should be greater than or equal to 0.98.

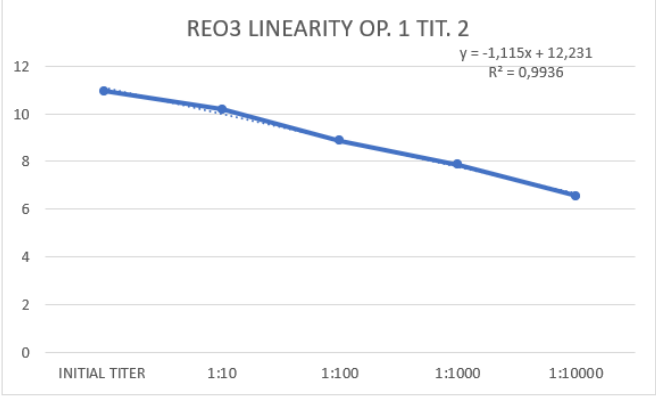
The table below show the obtained results:

LINEARITY		R^2
Operator 1	Titration 1	0.99
	Titration 2	0.99
Operator 2	Titration 1	0.98
	Titration 2	1.00

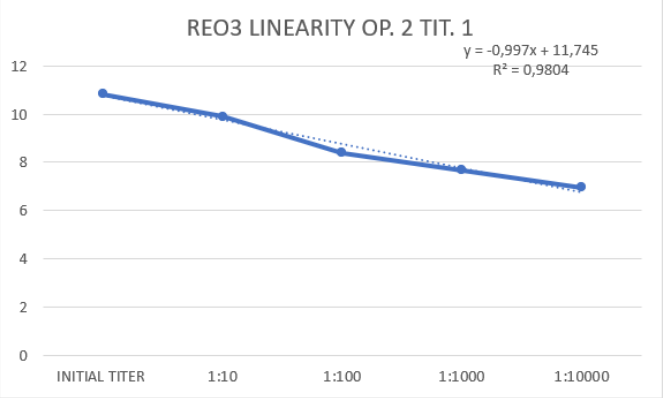
The obtained graphs are shown below:

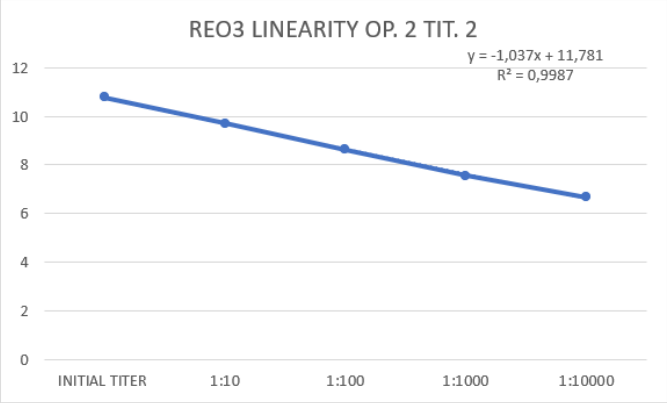
OPERATOR 1:





OPERATOR 2:





5.3.4 titration Method conclusions

The Robustness of the method was verified: all parameters were in the respective proposed ranges at the end of all the tests, with the support provided by the JMP statistical software.

Cell concentration for titration test:

- $2.0 * 10^5$ cell / ml;
- $2.5 * 10^5$ cells / ml;
- $3.0 * 10^5$ cells / ml.

Different culture media:

- EMEM 2% FBS;
- EMEM 2% FBS with antibiotic and antifungal

Different incubation times:

- 4 days;
- 5 days;
- 6 days.

For Validation the precision, specificity, accuracy and linearity parameters were verified during the method validation process and were compliant with the acceptance criteria.

Following the activities performed during the validation process the method was considered Validated.

5.4 HPIV3 titration Method validation

5.4.1 Method description

The method is used to determine the titer expressed as $Log_{10}TCID_{50}$/mL of a HPIV3 viral suspension through the evaluation of the cytopathic effect induced by the virus on the monolayer of VERO cells in adhesion.

5.4.2 Analitical procedure

The method is based on the evaluation of the cytopathic effect that different scalar dilutions of the viral suspension induce on the cell monolayer.

5.4.2.1 preparation of scalar dilution of HPIV3

A pool of the HPIV3 viral suspension was used for the preparation of the samples for robustness, specificity, precision, accuracy and linearity. Subsequently, each sample was diluted with 1:3 scalar dilutions prepared as indicated below.

Each titration consists of three plates of VERO cells (96-well flat bottom plates with low evaporation):

- Plate 1 → infection with dilutions from 3^{-1} to 3^{-11}
- Plate 2 → infection with dilutions from 3^{-12} to 3^{-22}
- Plate 3 → infection with dilutions from 3^{-23} to 3^{-33}

For each titration (Plate 1, 2 and 3) the dilutions were prepared in a deep-well polypropylene plates as described below:

- Fill the first lines of the deep-well polypropylene plates with 600 µl of EMEM 1% FBS medium with antibiotic and antifungal (Penicillin-Streptomycin 1% and Amphotericin B 1 %).

→ rows A and B will be used for the infection of Plate 1 (dilutions from 3^{-1} to 3^{-11} and negative control)

→ rows C and D will be used for the infection of Plate 2 (dilutions from 3^{-12} to 3^{-22} and negative control)
→ rows E and F will be used for the infection of Plate 3 (dilutions from 3^{-23} to 3^{-33} and negative control)

- Add HPIV3 viral suspension in row A column 1 and in row B column 1 to have a 1:3 diluted suspension.

- Take the selected volume from row A and B column 1 (WELL A1 and WELL B1) and add in row A and B column 2 (WELL A2 and WELL B2), continue in this way until column 11 (skip column 12 → negative control) → dilutions from 3^{-1} to 3^{-11}

- Take the selected volume from row A and B column 11 (WELL A11 and WELL B11) and add in row C and D, continue the dilutions in this way until column 11 (skip column 12 → negative control) → dilutions from 3^{-12} to 3^{-22}

- Take the selected volume from row C and D column 11(WELL C11 and WELL D11) and add in row E and F, continue the dilutions in this way until column 11 (skip column 12 → negative control) → dilutions from 3^{-23} to 3^{-33}

For the preparation of the dilution plate follow the diagram below:

	1	2	3	4	5	6	7	8	9	10	11	12	
A	EMEM 1%FBS + **HPIV3 (3^{-1})**	EMEM 1%FBS + **WELL A1 (3^{-2})**	EMEM 1%FBS + **WELL A2 (3^{-3})**	EMEM 1%FBS + **WELL A3 (3^{-4})**	EMEM 1%FBS + **WELL A4 (3^{-5})**	EMEM 1%FBS + **WELL A5 (3^{-6})**	EMEM 1%FBS + **WELL A6 (3^{-7})**	EMEM 1%FBS + **WELL A7 (3^{-8})**	EMEM 1%FBS + **WELL A8 (3^{-9})**	EMEM 1%FBS + **WELL A9 (3^{-10})**	EMEM 1%FBS + **WELL A10 (3^{-11})**	EMEM 1%FBS **C-**	**1st PLATE**
B	EMEM 1%FBS + **HPIV3 (3^{-1})**	EMEM 1%FBS + **WELL B1 (3^{-2})**	EMEM 1%FBS + **WELL B2 (3^{-3})**	EMEM 1%FBS + **WELL B3 (3^{-4})**	EMEM 1%FBS + **WELL B4 (3^{-5})**	EMEM 1%FBS + **WELL B5 (3^{-6})**	EMEM 1%FBS + **WELL B6 (3^{-7})**	EMEM 1%FBS + **WELL B7 (3^{-8})**	EMEM 1%FBS + **WELL B8 (3^{-9})**	EMEM 1%FBS + **WELL B9 (3^{-10})**	EMEM 1%FBS + **WELL B10 (3^{-11})**	EMEM 1%FBS **C-**	
C	EMEM 1%FBS + **WELL A11 (3^{-12})**	EMEM 1%FBS + **WELL C1 (3^{-13})**	EMEM 1%FBS + **WELL C2 (3^{-14})**	EMEM 1%FBS + **WELL C3 (3^{-15})**	EMEM 1%FBS + **WELL C4 (3^{-16})**	EMEM 1%FBS + **WELL C5 (3^{-17})**	EMEM 1%FBS + **WELL C6 (3^{-18})**	EMEM 1%FBS + **WELL C7 (3^{-19})**	EMEM 1%FBS + **WELL C8 (3^{-20})**	EMEM 1%FBS + **WELL C9 (3^{-21})**	EMEM 1%FBS + **WELL C10 (3^{-22})**	EMEM 1%FBS **C-**	**2nd PLATE**
D	EMEM 1%FBS + **WELL B11 (3^{-12})**	EMEM 1%FBS + **WELL D1 (3^{-13})**	EMEM 1%FBS + **WELL D2 (3^{-14})**	EMEM 1%FBS + **WELL D3 (3^{-15})**	EMEM 1%FBS + **WELL D4 (3^{-16})**	EMEM 1%FBS + **WELL D5 (3^{-17})**	EMEM 1%FBS + **WELL D6 (3^{-18})**	EMEM 1%FBS + **WELL D7 (3^{-19})**	EMEM 1%FBS + **WELL D8 (3^{-20})**	EMEM 1%FBS + **WELL D9 (3^{-21})**	EMEM 1%FBS + **WELL D10 (3^{-22})**	EMEM 1%FBS **C-**	
E	EMEM 1%FBS + **WELL C11 (3^{-23})**	EMEM 1%FBS + **WELL E1 (3^{-24})**	EMEM 1%FBS + **WELL E2 (3^{-25})**	EMEM 1%FBS + **WELL E3 (3^{-26})**	EMEM 1%FBS + **WELL E4 (3^{-27})**	EMEM 1%FBS + **WELL E5 (3^{-28})**	EMEM 1%FBS + **WELL E6 (3^{-29})**	EMEM 1%FBS + **WELL E7 (3^{-30})**	EMEM 1%FBS + **WELL E8 (3^{-31})**	EMEM 1%FBS + **WELL E9 (3^{-32})**	EMEM 1%FBS + **WELL E10 (3^{-33})**	EMEM 1%FBS **C-**	**3rd PLATE**
F	EMEM 1%FBS + **WELL D11 (3^{-23})**	EMEM 1%FBS + **WELL F1 (3^{-24})**	EMEM 1%FBS + **WELL F2 (3^{-25})**	EMEM 1%FBS + **WELL F3 (3^{-26})**	EMEM 1%FBS + **WELL F4 (3^{-27})**	EMEM 1%FBS + **WELL F5 (3^{-28})**	EMEM 1%FBS + **WELL F6 (3^{-29})**	EMEM 1%FBS + **WELL F7 (3^{-30})**	EMEM 1%FBS + **WELL F8 (3^{-31})**	EMEM 1%FBS + **WELL F9 (3^{-32})**	EMEM 1%FBS + **WELL F10 (3^{-33})**	EMEM 1%FBS **C-**	
G													
H													

5.4.2.2 Titration procedure

The day before the titration assay, the plates were prepared as described below. Bring VERO cells, grown in EMEM 10 % FBS medium, to a concentration of:

-$0.5*10^5$ cell/ml
-$1.0*10^5$ cell/ml (TARGET)
-$1.5*10^5$ cell/ml

Then take three plates 96-well flat bottom, low evaporation plates (1st, 2nd and 3rd Plate) for each titration to be performed; dispensing the cell suspension obtained in each well for each plate as shown below.

	1	2	3	4	5	6	7	8	9	10	11	12
A	VERO + EMEM 10% FBS	VERO + EMEM 10% FBS	VERO + EMEM 10% FBS	VERO + EMEM 10% FBS	VERO + EMEM 10% FBS	VERO + EMEM 10% FBS	VERO + EMEM 10% FBS	VERO + EMEM 10% FBS	VERO + EMEM 10% FBS	VERO + EMEM 10% FBS	VERO + EMEM 10% FBS	VERO + EMEM 10% FBS **C-**
B	VERO + EMEM 10% FBS	VERO + EMEM 10% FBS	VERO + EMEM 10% FBS	VERO + EMEM 10% FBS	VERO + EMEM 10% FBS	VERO + EMEM 10% FBS	VERO + EMEM 10% FBS	VERO + EMEM 10% FBS	VERO + EMEM 10% FBS	VERO + EMEM 10% FBS	VERO + EMEM 10% FBS	VERO + EMEM 10% FBS **C-**
C	VERO + EMEM 10% FBS	VERO + EMEM 10% FBS	VERO + EMEM 10% FBS	VERO + EMEM 10% FBS	VERO + EMEM 10% FBS	VERO + EMEM 10% FBS	VERO + EMEM 10% FBS	VERO + EMEM 10% FBS	VERO + EMEM 10% FBS	VERO + EMEM 10% FBS	VERO + EMEM 10% FBS	VERO + EMEM 10% FBS **C-**
D	VERO + EMEM 10% FBS	VERO + EMEM 10% FBS	VERO + EMEM 10% FBS	VERO + EMEM 10% FBS	VERO + EMEM 10% FBS	VERO + EMEM 10% FBS	VERO + EMEM 10% FBS	VERO + EMEM 10% FBS	VERO + EMEM 10% FBS	VERO + EMEM 10% FBS	VERO + EMEM 10% FBS	VERO + EMEM 10% FBS **C-**
E	VERO + EMEM 10% FBS	VERO + EMEM 10% FBS	VERO + EMEM 10% FBS	VERO + EMEM 10% FBS	VERO + EMEM 10% FBS	VERO + EMEM 10% FBS	VERO + EMEM 10% FBS	VERO + EMEM 10% FBS	VERO + EMEM 10% FBS	VERO + EMEM 10% FBS	VERO + EMEM 10% FBS	VERO + EMEM 10% FBS **C-**
F	VERO + EMEM 10% FBS	VERO + EMEM 10% FBS	VERO + EMEM 10% FBS	VERO + EMEM 10% FBS	VERO + EMEM 10% FBS	VERO + EMEM 10% FBS	VERO + EMEM 10% FBS	VERO + EMEM 10% FBS	VERO + EMEM 10% FBS	VERO + EMEM 10% FBS	VERO + EMEM 10% FBS	VERO + EMEM 10% FBS **C-**
G	VERO + EMEM 10% FBS	VERO + EMEM 10% FBS	VERO + EMEM 10% FBS	VERO + EMEM 10% FBS	VERO + EMEM 10% FBS	VERO + EMEM 10% FBS	VERO + EMEM 10% FBS	VERO + EMEM 10% FBS	VERO + EMEM 10% FBS	VERO + EMEM 10% FBS	VERO + EMEM 10% FBS	VERO + EMEM 10% FBS **C-**
H	VERO + EMEM 10% FBS	VERO + EMEM 10% FBS	VERO + EMEM 10% FBS	VERO + EMEM 10% FBS	VERO + EMEM 10% FBS	VERO + EMEM 10% FBS	VERO + EMEM 10% FBS	VERO + EMEM 10% FBS	VERO + EMEM 10% FBS	VERO + EMEM 10% FBS	VERO + EMEM 10% FBS	VERO + EMEM 10% FBS **C-**

The plates were placed in an incubator at 37.0 ± 1.0 °C and 5.0± 1.0 CO_2 for 24 hours.

After this time wash the cell monolayer with PBS to remove any serum or dead cell residues and proceeded with infection of the 1st, 2nd, and 3rd Plates as indicated below.

Infection of the 1st Plate:

With a multichannel pipette, the defined volume was taken from row A of the deep-well polypropylene plates and dispense it into row A of the 1st Plate. Action was repeated 3 more times, respectively into rows B, C and D of the 1st Plate.

Follow the same process with row B of the deep-well polypropylene plates and dispense it into row E, F, G and H of the 1st Plate.

	1	2	3	4	5	6	7	8	9	10	11	12	
A	EMEM 1%FBS + **HPIV3 (3^{-1})**	EMEM 1%FBS + **WELL A1 (3^{-2})**	EMEM 1%FBS + **WELL A2 (3^{-3})**	EMEM 1%FBS + **WELL A3 (3^{-4})**	EMEM 1%FBS + **WELL A4 (3^{-5})**	EMEM 1%FBS + **WELL A5 (3^{-6})**	EMEM 1%FBS + **WELL A6 (3^{-7})**	EMEM 1%FBS + **WELL A7 (3^{-8})**	EMEM 1%FBS + **WELL A8 (3^{-9})**	EMEM 1%FBS + **WELL A9 (3^{-10})**	EMEM 1%FBS + **WELL A10 (3^{-11})**	EMEM 1%FBS **C-**	**1st PLATE**
B	EMEM 1%FBS + **HPIV3 (3^{-1})**	EMEM 1%FBS + **WELL B1 (3^{-2})**	EMEM 1%FBS + **WELL B2 (3^{-3})**	EMEM 1%FBS + **WELL B3 (3^{-4})**	EMEM 1%FBS + **WELL B4 (3^{-5})**	EMEM 1%FBS + **WELL B5 (3^{-6})**	EMEM 1%FBS + **WELL B6 (3^{-7})**	EMEM 1%FBS + **WELL B7 (3^{-8})**	EMEM 1%FBS + **WELL B8 (3^{-9})**	EMEM 1%FBS + **WELL B9 (3^{-10})**	EMEM 1%FBS + **WELL B10 (3^{-11})**	EMEM 1%FBS **C-**	
C	EMEM 1%FBS + **WELL A11 (3^{-12})**	EMEM 1%FBS + **WELL C1 (3^{-13})**	EMEM 1%FBS + **WELL C2 (3^{-14})**	EMEM 1%FBS + **WELL C3 (3^{-15})**	EMEM 1%FBS + **WELL C4 (3^{-16})**	EMEM 1%FBS + **WELL C5 (3^{-17})**	EMEM 1%FBS + **WELL C6 (3^{-18})**	EMEM 1%FBS + **WELL C7 (3^{-19})**	EMEM 1%FBS + **WELL C8 (3^{-20})**	EMEM 1%FBS + **WELL C9 (3^{-21})**	EMEM 1%FBS + **WELL C10 (3^{-22})**	EMEM 1%FBS **C-**	**2nd PLATE**
D	EMEM 1%FBS + **WELL B11 (3^{-12})**	EMEM 1%FBS + **WELL D1 (3^{-13})**	EMEM 1%FBS + **WELL D2 (3^{-14})**	EMEM 1%FBS + **WELL D3 (3^{-15})**	EMEM 1%FBS + **WELL D4 (3^{-16})**	EMEM 1%FBS + **WELL D5 (3^{-17})**	EMEM 1%FBS + **WELL D6 (3^{-18})**	EMEM 1%FBS + **WELL D7 (3^{-19})**	EMEM 1%FBS + **WELL D8 (3^{-20})**	EMEM 1%FBS + **WELL D9 (3^{-21})**	EMEM 1%FBS + **WELL D10 (3^{-22})**	EMEM 1%FBS **C-**	
E	EMEM 1%FBS + **WELL C11 (3^{-23})**	EMEM 1%FBS + **WELL E1 (3^{-24})**	EMEM 1%FBS + **WELL E2 (3^{-25})**	EMEM 1%FBS + **WELL E3 (3^{-26})**	EMEM 1%FBS + **WELL E4 (3^{-27})**	EMEM 1%FBS + **WELL E5 (3^{-28})**	EMEM 1%FBS + **WELL E6 (3^{-29})**	EMEM 1%FBS + **WELL E7 (3^{-30})**	EMEM 1%FBS + **WELL E8 (3^{-31})**	EMEM 1%FBS + **WELL E9 (3^{-32})**	EMEM 1%FBS + **WELL E10 (3^{-33})**	EMEM 1%FBS **C-**	**3rd PLATE**
F	EMEM 1%FBS + **WELL D11 (3^{-23})**	EMEM 1%FBS + **WELL F1 (3^{-24})**	EMEM 1%FBS + **WELL F2 (3^{-25})**	EMEM 1%FBS + **WELL F3 (3^{-26})**	EMEM 1%FBS + **WELL F4 (3^{-27})**	EMEM 1%FBS + **WELL F5 (3^{-28})**	EMEM 1%FBS + **WELL F6 (3^{-29})**	EMEM 1%FBS + **WELL F7 (3^{-30})**	EMEM 1%FBS + **WELL F8 (3^{-31})**	EMEM 1%FBS + **WELL F9 (3^{-32})**	EMEM 1%FBS + **WELL F10 (3^{-33})**	EMEM 1%FBS **C-**	
G													
H													

1st PLATE: from 3^{-1} to 3^{-11}

	1	2	3	4	5	6	7	8	9	10	11	12
A	VERO EMEM 1% FBS + HPIV-3 (3^{-1})	VERO EMEM 1% FBS + HPIV-3 (3^{-2})	VERO EMEM 1% FBS + HPIV-3 (3^{-3})	VERO EMEM 1% FBS + HPIV-3 (3^{-4})	VERO EMEM 1% FBS + HPIV-3 (3^{-5})	VERO EMEM 1% FBS + HPIV-3 (3^{-6})	VERO EMEM 1% FBS + HPIV-3 (3^{-7})	VERO EMEM 1% FBS + HPIV-3 (3^{-8})	VERO EMEM 1% FBS + HPIV-3 (3^{-9})	VERO EMEM 1% FBS + HPIV-3 (3^{-10})	VERO EMEM 1% FBS + HPIV-3 (3^{-11})	VERO EMEM 1% FBS
B	VERO EMEM 1% FBS + HPIV-3 (3^{-1})	VERO EMEM 1% FBS + HPIV-3 (3^{-2})	VERO EMEM 1% FBS + HPIV-3 (3^{-3})	VERO EMEM 1% FBS + HPIV-3 (3^{-4})	VERO EMEM 1% FBS + HPIV-3 (3^{-5})	VERO EMEM 1% FBS + HPIV-3 (3^{-6})	VERO EMEM 1% FBS + HPIV-3 (3^{-7})	VERO EMEM 1% FBS + HPIV-3 (3^{-8})	VERO EMEM 1% FBS + HPIV-3 (3^{-9})	VERO EMEM 1% FBS + HPIV-3 (3^{-10})	VERO EMEM 1% FBS + HPIV-3 (3^{-11})	VERO EMEM 1% FBS
C	VERO EMEM 1% FBS + HPIV-3 (3^{-1})	VERO EMEM 1% FBS + HPIV-3 (3^{-2})	VERO EMEM 1% FBS + HPIV-3 (3^{-3})	VERO EMEM 1% FBS + HPIV-3 (3^{-4})	VERO EMEM 1% FBS + HPIV-3 (3^{-5})	VERO EMEM 1% FBS + HPIV-3 (3^{-6})	VERO EMEM 1% FBS + HPIV-3 (3^{-7})	VERO EMEM 1% FBS + HPIV-3 (3^{-8})	VERO EMEM 1% FBS + HPIV-3 (3^{-9})	VERO EMEM 1% FBS + HPIV-3 (3^{-10})	VERO EMEM 1% FBS + HPIV-3 (3^{-11})	VERO EMEM 1% FBS
D	VERO EMEM 1% FBS + HPIV-3 (3^{-1})	VERO EMEM 1% FBS + HPIV-3 (3^{-2})	VERO EMEM 1% FBS + HPIV-3 (3^{-3})	VERO EMEM 1% FBS + HPIV-3 (3^{-4})	VERO EMEM 1% FBS + HPIV-3 (3^{-5})	VERO EMEM 1% FBS + HPIV-3 (3^{-6})	VERO EMEM 1% FBS + HPIV-3 (3^{-7})	VERO EMEM 1% FBS + HPIV-3 (3^{-8})	VERO EMEM 1% FBS + HPIV-3 (3^{-9})	VERO EMEM 1% FBS + HPIV-3 (3^{-10})	VERO EMEM 1% FBS + HPIV-3 (3^{-11})	VERO EMEM 1% FBS
E	VERO EMEM 1% FBS + HPIV-3 (3^{-1})	VERO EMEM 1% FBS + HPIV-3 (3^{-2})	VERO EMEM 1% FBS + HPIV-3 (3^{-3})	VERO EMEM 1% FBS + HPIV-3 (3^{-4})	VERO EMEM 1% FBS + HPIV-3 (3^{-5})	VERO EMEM 1% FBS + HPIV-3 (3^{-6})	VERO EMEM 1% FBS + HPIV-3 (3^{-7})	VERO EMEM 1% FBS + HPIV-3 (3^{-8})	VERO EMEM 1% FBS + HPIV-3 (3^{-9})	VERO EMEM 1% FBS + HPIV-3 (3^{-10})	VERO EMEM 1% FBS + HPIV-3 (3^{-11})	VERO EMEM 1% FBS
F	VERO EMEM 1% FBS + HPIV-3 (3^{-1})	VERO EMEM 1% FBS + HPIV-3 (3^{-2})	VERO EMEM 1% FBS + HPIV-3 (3^{-3})	VERO EMEM 1% FBS + HPIV-3 (3^{-4})	VERO EMEM 1% FBS + HPIV-3 (3^{-5})	VERO EMEM 1% FBS + HPIV-3 (3^{-6})	VERO EMEM 1% FBS + HPIV-3 (3^{-7})	VERO EMEM 1% FBS + HPIV-3 (3^{-8})	VERO EMEM 1% FBS + HPIV-3 (3^{-9})	VERO EMEM 1% FBS + HPIV-3 (3^{-10})	VERO EMEM 1% FBS + HPIV-3 (3^{-11})	VERO EMEM 1% FBS
G	VERO EMEM 1% FBS + HPIV-3 (3^{-1})	VERO EMEM 1% FBS + HPIV-3 (3^{-2})	VERO EMEM 1% FBS + HPIV-3 (3^{-3})	VERO EMEM 1% FBS + HPIV-3 (3^{-4})	VERO EMEM 1% FBS + HPIV-3 (3^{-5})	VERO EMEM 1% FBS + HPIV-3 (3^{-6})	VERO EMEM 1% FBS + HPIV-3 (3^{-7})	VERO EMEM 1% FBS + HPIV-3 (3^{-8})	VERO EMEM 1% FBS + HPIV-3 (3^{-9})	VERO EMEM 1% FBS + HPIV-3 (3^{-10})	VERO EMEM 1% FBS + HPIV-3 (3^{-11})	VERO EMEM 1% FBS
H	VERO EMEM 1% FBS + HPIV-3 (3^{-1})	VERO EMEM 1% FBS + HPIV-3 (3^{-2})	VERO EMEM 1% FBS + HPIV-3 (3^{-3})	VERO EMEM 1% FBS + HPIV-3 (3^{-4})	VERO EMEM 1% FBS + HPIV-3 (3^{-5})	VERO EMEM 1% FBS + HPIV-3 (3^{-6})	VERO EMEM 1% FBS + HPIV-3 (3^{-7})	VERO EMEM 1% FBS + HPIV-3 (3^{-8})	VERO EMEM 1% FBS + HPIV-3 (3^{-9})	VERO EMEM 1% FBS + HPIV-3 (3^{-10})	VERO EMEM 1% FBS + HPIV-3 (3^{-11})	VERO EMEM 1% FBS

Infection of the 2nd Plate:

With a multichannel pipette, the defined volume was taken from row C of the deep-well polypropylene plates and dispense it into row A of the 1st Plate. Action was repeated 3 more times, respectively into rows B, C and D of the 2nd Plate.

Follow the same process with row D of the deep-well polypropylene plates and dispense it into row E, F, G and H of the 2nd Plate.

	1	2	3	4	5	6	7	8	9	10	11	12	
A	EMEM 1%FBS + **HPIV3 (3^{-1})**	EMEM 1%FBS + **WELL A1 (3^{-2})**	EMEM 1%FBS + **WELL A2 (3^{-3})**	EMEM 1%FBS + **WELL A3 (3^{-4})**	EMEM 1%FBS + **WELL A4 (3^{-5})**	EMEM 1%FBS + **WELL A5 (3^{-6})**	EMEM 1%FBS + **WELL A6 (3^{-7})**	EMEM 1%FBS + **WELL A7 (3^{-8})**	EMEM 1%FBS + **WELL A8 (3^{-9})**	EMEM 1%FBS + **WELL A9 (3^{-10})**	EMEM 1%FBS + **WELL A10 (3^{-11})**	EMEM 1%FBS **C-**	**1st PLATE**
B	EMEM 1%FBS + **HPIV3 (3^{-1})**	EMEM 1%FBS + **WELL B1 (3^{-2})**	EMEM 1%FBS + **WELL B2 (3^{-3})**	EMEM 1%FBS + **WELL B3 (3^{-4})**	EMEM 1%FBS + **WELL B4 (3^{-5})**	EMEM 1%FBS + **WELL B5 (3^{-6})**	EMEM 1%FBS + **WELL B6 (3^{-7})**	EMEM 1%FBS + **WELL B7 (3^{-8})**	EMEM 1%FBS + **WELL B8 (3^{-9})**	EMEM 1%FBS + **WELL B9 (3^{-10})**	EMEM 1%FBS + **WELL B10 (3^{-11})**	EMEM 1%FBS **C-**	
C	EMEM 1%FBS + **WELL A11 (3^{-12})**	EMEM 1%FBS + **WELL C1 (3^{-13})**	EMEM 1%FBS + **WELL C2 (3^{-14})**	EMEM 1%FBS + **WELL C3 (3^{-15})**	EMEM 1%FBS + **WELL C4 (3^{-16})**	EMEM 1%FBS + **WELL C5 (3^{-17})**	EMEM 1%FBS + **WELL C6 (3^{-18})**	EMEM 1%FBS + **WELL C7 (3^{-19})**	EMEM 1%FBS + **WELL C8 (3^{-20})**	EMEM 1%FBS + **WELL C9 (3^{-21})**	EMEM 1%FBS + **WELL C10 (3^{-22})**	EMEM 1%FBS **C-**	**2nd PLATE**
D	EMEM 1%FBS + **WELL B11 (3^{-12})**	EMEM 1%FBS + **WELL D1 (3^{-13})**	EMEM 1%FBS + **WELL D2 (3^{-14})**	EMEM 1%FBS + **WELL D3 (3^{-15})**	EMEM 1%FBS + **WELL D4 (3^{-16})**	EMEM 1%FBS + **WELL D5 (3^{-17})**	EMEM 1%FBS + **WELL D6 (3^{-18})**	EMEM 1%FBS + **WELL D7 (3^{-19})**	EMEM 1%FBS + **WELL D8 (3^{-20})**	EMEM 1%FBS + **WELL D9 (3^{-21})**	EMEM 1%FBS + **WELL D10 (3^{-22})**	EMEM 1%FBS **C-**	
E	EMEM 1%FBS + **WELL C11 (3^{-23})**	EMEM 1%FBS + **WELL E1 (3^{-24})**	EMEM 1%FBS + **WELL E2 (3^{-25})**	EMEM 1%FBS + **WELL E3 (3^{-26})**	EMEM 1%FBS + **WELL E4 (3^{-27})**	EMEM 1%FBS + **WELL E5 (3^{-28})**	EMEM 1%FBS + **WELL E6 (3^{-29})**	EMEM 1%FBS + **WELL E7 (3^{-30})**	EMEM 1%FBS + **WELL E8 (3^{-31})**	EMEM 1%FBS + **WELL E9 (3^{-32})**	EMEM 1%FBS + **WELL E10 (3^{-33})**	EMEM 1%FBS **C-**	**3rd PLATE**
F	EMEM 1%FBS + **WELL D11 (3^{-23})**	EMEM 1%FBS + **WELL F1 (3^{-24})**	EMEM 1%FBS + **WELL F2 (3^{-25})**	EMEM 1%FBS + **WELL F3 (3^{-26})**	EMEM 1%FBS + **WELL F4 (3^{-27})**	EMEM 1%FBS + **WELL F5 (3^{-28})**	EMEM 1%FBS + **WELL F6 (3^{-29})**	EMEM 1%FBS + **WELL F7 (3^{-30})**	EMEM 1%FBS + **WELL F8 (3^{-31})**	EMEM 1%FBS + **WELL F9 (3^{-32})**	EMEM 1%FBS + **WELL F10 (3^{-33})**	EMEM 1%FBS **C-**	
G													
H													

2nd PLATE: from 3^{-12} to 3^{-22}

	1	2	3	4	5	6	7	8	9	10	11	12
A	VERO EMEM 1% FBS + HPIV-3 (3^{-12})	VERO EMEM 1% FBS + HPIV-3 (3^{-13})	VERO EMEM 1% FBS + HPIV-3 (3^{-14})	VERO EMEM 1% FBS + HPIV-3 (3^{-15})	VERO EMEM 1% FBS + HPIV-3 (3^{-16})	VERO EMEM 1% FBS + HPIV-3 (3^{-17})	VERO EMEM 1% FBS + HPIV-3 (3^{-18})	VERO EMEM 1% FBS + HPIV-3 (3^{-19})	VERO EMEM 1% FBS + HPIV-3 (3^{-20})	VERO EMEM 1% FBS + HPIV-3 (3^{-21})	VERO EMEM 1% FBS + HPIV-3 (3^{-22})	VERO EMEM 1% FBS
B	VERO EMEM 1% FBS + HPIV-3 (3^{-12})	VERO EMEM 1% FBS + HPIV-3 (3^{-13})	VERO EMEM 1% FBS + HPIV-3 (3^{-14})	VERO EMEM 1% FBS + HPIV-3 (3^{-15})	VERO EMEM 1% FBS + HPIV-3 (3^{-16})	VERO EMEM 1% FBS + HPIV-3 (3^{-17})	VERO EMEM 1% FBS + HPIV-3 (3^{-18})	VERO EMEM 1% FBS + HPIV-3 (3^{-19})	VERO EMEM 1% FBS + HPIV-3 (3^{-20})	VERO EMEM 1% FBS + HPIV-3 (3^{-21})	VERO EMEM 1% FBS + HPIV-3 (3^{-22})	VERO EMEM 1% FBS
C	VERO EMEM 1% FBS + HPIV-3 (3^{-12})	VERO EMEM 1% FBS + HPIV-3 (3^{-13})	VERO EMEM 1% FBS + HPIV-3 (3^{-14})	VERO EMEM 1% FBS + HPIV-3 (3^{-15})	VERO EMEM 1% FBS + HPIV-3 (3^{-16})	VERO EMEM 1% FBS + HPIV-3 (3^{-17})	VERO EMEM 1% FBS + HPIV-3 (3^{-18})	VERO EMEM 1% FBS + HPIV-3 (3^{-19})	VERO EMEM 1% FBS + HPIV-3 (3^{-20})	VERO EMEM 1% FBS + HPIV-3 (3^{-21})	VERO EMEM 1% FBS + HPIV-3 (3^{-22})	VERO EMEM 1% FBS
D	VERO EMEM 1% FBS + HPIV-3 (3^{-12})	VERO EMEM 1% FBS + HPIV-3 (3^{-13})	VERO EMEM 1% FBS + HPIV-3 (3^{-14})	VERO EMEM 1% FBS + HPIV-3 (3^{-15})	VERO EMEM 1% FBS + HPIV-3 (3^{-16})	VERO EMEM 1% FBS + HPIV-3 (3^{-17})	VERO EMEM 1% FBS + HPIV-3 (3^{-18})	VERO EMEM 1% FBS + HPIV-3 (3^{-19})	VERO EMEM 1% FBS + HPIV-3 (3^{-20})	VERO EMEM 1% FBS + HPIV-3 (3^{-21})	VERO EMEM 1% FBS + HPIV-3 (3^{-22})	VERO EMEM 1% FBS
E	VERO EMEM 1% FBS + HPIV-3 (3^{-12})	VERO EMEM 1% FBS + HPIV-3 (3^{-13})	VERO EMEM 1% FBS + HPIV-3 (3^{-14})	VERO EMEM 1% FBS + HPIV-3 (3^{-15})	VERO EMEM 1% FBS + HPIV-3 (3^{-16})	VERO EMEM 1% FBS + HPIV-3 (3^{-17})	VERO EMEM 1% FBS + HPIV-3 (3^{-18})	VERO EMEM 1% FBS + HPIV-3 (3^{-19})	VERO EMEM 1% FBS + HPIV-3 (3^{-20})	VERO EMEM 1% FBS + HPIV-3 (3^{-21})	VERO EMEM 1% FBS + HPIV-3 (3^{-22})	VERO EMEM 1% FBS
F	VERO EMEM 1% FBS + HPIV-3 (3^{-12})	VERO EMEM 1% FBS + HPIV-3 (3^{-13})	VERO EMEM 1% FBS + HPIV-3 (3^{-14})	VERO EMEM 1% FBS + HPIV-3 (3^{-15})	VERO EMEM 1% FBS + HPIV-3 (3^{-16})	VERO EMEM 1% FBS + HPIV-3 (3^{-17})	VERO EMEM 1% FBS + HPIV-3 (3^{-18})	VERO EMEM 1% FBS + HPIV-3 (3^{-19})	VERO EMEM 1% FBS + HPIV-3 (3^{-20})	VERO EMEM 1% FBS + HPIV-3 (3^{-21})	VERO EMEM 1% FBS + HPIV-3 (3^{-22})	VERO EMEM 1% FBS
G	VERO EMEM 1% FBS + HPIV-3 (3^{-12})	VERO EMEM 1% FBS + HPIV-3 (3^{-13})	VERO EMEM 1% FBS + HPIV-3 (3^{-14})	VERO EMEM 1% FBS + HPIV-3 (3^{-15})	VERO EMEM 1% FBS + HPIV-3 (3^{-16})	VERO EMEM 1% FBS + HPIV-3 (3^{-17})	VERO EMEM 1% FBS + HPIV-3 (3^{-18})	VERO EMEM 1% FBS + HPIV-3 (3^{-19})	VERO EMEM 1% FBS + HPIV-3 (3^{-20})	VERO EMEM 1% FBS + HPIV-3 (3^{-21})	VERO EMEM 1% FBS + HPIV-3 (3^{-22})	VERO EMEM 1% FBS
H	VERO EMEM 1% FBS + HPIV-3 (3^{-12})	VERO EMEM 1% FBS + HPIV-3 (3^{-13})	VERO EMEM 1% FBS + HPIV-3 (3^{-14})	VERO EMEM 1% FBS + HPIV-3 (3^{-15})	VERO EMEM 1% FBS + HPIV-3 (3^{-16})	VERO EMEM 1% FBS + HPIV-3 (3^{-17})	VERO EMEM 1% FBS + HPIV-3 (3^{-18})	VERO EMEM 1% FBS + HPIV-3 (3^{-19})	VERO EMEM 1% FBS + HPIV-3 (3^{-20})	VERO EMEM 1% FBS + HPIV-3 (3^{-21})	VERO EMEM 1% FBS + HPIV-3 (3^{-22})	VERO EMEM 1% FBS

Infection of the 3rd Plate:

With a multichannel pipette, the defined volume was taken from row E of the deep-well polypropylene plates and dispense it into row A of the 1st Plate. Action was repeated 3 more times, respectively into rows B, C and D of the 3rd Plate.

Follow the same process with row F of the deep-well polypropylene plates and dispense it into row E, F, G and H of the 3rd Plate.

	1	2	3	4	5	6	7	8	9	10	11	12	
A	EMEM 1%FBS + **HPIV3 (3^{-1})**	EMEM 1%FBS + **WELL A1 (3^{-2})**	EMEM 1%FBS + **WELL A2 (3^{-3})**	EMEM 1%FBS + **WELL A3 (3^{-4})**	EMEM 1%FBS + **WELL A4 (3^{-5})**	EMEM 1%FBS + **WELL A5 (3^{-6})**	EMEM 1%FBS + **WELL A6 (3^{-7})**	EMEM 1%FBS + **WELL A7 (3^{-8})**	EMEM 1%FBS + **WELL A8 (3^{-9})**	EMEM 1%FBS + **WELL A9 (3^{-10})**	EMEM 1%FBS + **WELL A10 (3^{-11})**	EMEM 1%FBS **C-**	**1st PLATE**
B	EMEM 1%FBS + **HPIV3 (3^{-1})**	EMEM 1%FBS + **WELL B1 (3^{-2})**	EMEM 1%FBS + **WELL B2 (3^{-3})**	EMEM 1%FBS + **WELL B3 (3^{-4})**	EMEM 1%FBS + **WELL B4 (3^{-5})**	EMEM 1%FBS + **WELL B5 (3^{-6})**	EMEM 1%FBS + **WELL B6 (3^{-7})**	EMEM 1%FBS + **WELL B7 (3^{-8})**	EMEM 1%FBS + **WELL B8 (3^{-9})**	EMEM 1%FBS + **WELL B9 (3^{-10})**	EMEM 1%FBS + **WELL B10 (3^{-11})**	EMEM 1%FBS **C-**	
C	EMEM 1%FBS + **WELL A11 (3^{-12})**	EMEM 1%FBS + **WELL C1 (3^{-13})**	EMEM 1%FBS + **WELL C2 (3^{-14})**	EMEM 1%FBS + **WELL C3 (3^{-15})**	EMEM 1%FBS + **WELL C4 (3^{-16})**	EMEM 1%FBS + **WELL C5 (3^{-17})**	EMEM 1%FBS + **WELL C6 (3^{-18})**	EMEM 1%FBS + **WELL C7 (3^{-19})**	EMEM 1%FBS + **WELL C8 (3^{-20})**	EMEM 1%FBS + **WELL C9 (3^{-21})**	EMEM 1%FBS + **WELL C10 (3^{-22})**	EMEM 1%FBS **C-**	**2nd PLATE**
D	EMEM 1%FBS + **WELL B11 (3^{-12})**	EMEM 1%FBS + **WELL D1 (3^{-13})**	EMEM 1%FBS + **WELL D2 (3^{-14})**	EMEM 1%FBS + **WELL D3 (3^{-15})**	EMEM 1%FBS + **WELL D4 (3^{-16})**	EMEM 1%FBS + **WELL D5 (3^{-17})**	EMEM 1%FBS + **WELL D6 (3^{-18})**	EMEM 1%FBS + **WELL D7 (3^{-19})**	EMEM 1%FBS + **WELL D8 (3^{-20})**	EMEM 1%FBS + **WELL D9 (3^{-21})**	EMEM 1%FBS + **WELL D10 (3^{-22})**	EMEM 1%FBS **C-**	
E	EMEM 1%FBS + **WELL C11 (3^{-23})**	EMEM 1%FBS + **WELL E1 (3^{-24})**	EMEM 1%FBS + **WELL E2 (3^{-25})**	EMEM 1%FBS + **WELL E3 (3^{-26})**	EMEM 1%FBS + **WELL E4 (3^{-27})**	EMEM 1%FBS + **WELL E5 (3^{-28})**	EMEM 1%FBS + **WELL E6 (3^{-29})**	EMEM 1%FBS + **WELL E7 (3^{-30})**	EMEM 1%FBS + **WELL E8 (3^{-31})**	EMEM 1%FBS + **WELL E9 (3^{-32})**	EMEM 1%FBS + **WELL E10 (3^{-33})**	EMEM 1%FBS **C-**	**3rd PLATE**
F	EMEM 1%FBS + **WELL D11 (3^{-23})**	EMEM 1%FBS + **WELL F1 (3^{-24})**	EMEM 1%FBS + **WELL F2 (3^{-25})**	EMEM 1%FBS + **WELL F3 (3^{-26})**	EMEM 1%FBS + **WELL F4 (3^{-27})**	EMEM 1%FBS + **WELL F5 (3^{-28})**	EMEM 1%FBS + **WELL F6 (3^{-29})**	EMEM 1%FBS + **WELL F7 (3^{-30})**	EMEM 1%FBS + **WELL F8 (3^{-31})**	EMEM 1%FBS + **WELL F9 (3^{-32})**	EMEM 1%FBS + **WELL F10 (3^{-33})**	EMEM 1%FBS **C-**	
G													
H													

3rd PLATE: from 3^{-23} to 3^{-33}

	1	2	3	4	5	6	7	8	9	10	11	12
A	VERO EMEM 1% FBS + HPIV-3 (3^{-23})	VERO EMEM 1% FBS + HPIV-3 (3^{-24})	VERO EMEM 1% FBS + HPIV-3 (3^{-25})	VERO EMEM 1% FBS + HPIV-3 (3^{-26})	VERO EMEM 1% FBS + HPIV-3 (3^{-27})	VERO EMEM 1% FBS + HPIV-3 (3^{-28})	VERO EMEM 1% FBS + HPIV-3 (3^{-29})	VERO EMEM 1% FBS + HPIV-3 (3^{-30})	VERO EMEM 1% FBS + HPIV-3 (3^{-31})	VERO EMEM 1% FBS + HPIV-3 (3^{-32})	VERO EMEM 1% FBS + HPIV-3 (3^{-33})	VERO EMEM 1% FBS
B	VERO EMEM 1% FBS + HPIV-3 (3^{-23})	VERO EMEM 1% FBS + HPIV-3 (3^{-24})	VERO EMEM 1% FBS + HPIV-3 (3^{-25})	VERO EMEM 1% FBS + HPIV-3 (3^{-26})	VERO EMEM 1% FBS + HPIV-3 (3^{-27})	VERO EMEM 1% FBS + HPIV-3 (3^{-28})	VERO EMEM 1% FBS + HPIV-3 (3^{-29})	VERO EMEM 1% FBS + HPIV-3 (3^{-30})	VERO EMEM 1% FBS + HPIV-3 (3^{-31})	VERO EMEM 1% FBS + HPIV-3 (3^{-32})	VERO EMEM 1% FBS + HPIV-3 (3^{-33})	VERO EMEM 1% FBS
C	VERO EMEM 1% FBS + HPIV-3 (3^{-23})	VERO EMEM 1% FBS + HPIV-3 (3^{-24})	VERO EMEM 1% FBS + HPIV-3 (3^{-25})	VERO EMEM 1% FBS + HPIV-3 (3^{-26})	VERO EMEM 1% FBS + HPIV-3 (3^{-27})	VERO EMEM 1% FBS + HPIV-3 (3^{-28})	VERO EMEM 1% FBS + HPIV-3 (3^{-29})	VERO EMEM 1% FBS + HPIV-3 (3^{-30})	VERO EMEM 1% FBS + HPIV-3 (3^{-31})	VERO EMEM 1% FBS + HPIV-3 (3^{-32})	VERO EMEM 1% FBS + HPIV-3 (3^{-33})	VERO EMEM 1% FBS
D	VERO EMEM 1% FBS + HPIV-3 (3^{-23})	VERO EMEM 1% FBS + HPIV-3 (3^{-24})	VERO EMEM 1% FBS + HPIV-3 (3^{-25})	VERO EMEM 1% FBS + HPIV-3 (3^{-26})	VERO EMEM 1% FBS + HPIV-3 (3^{-27})	VERO EMEM 1% FBS + HPIV-3 (3^{-28})	VERO EMEM 1% FBS + HPIV-3 (3^{-29})	VERO EMEM 1% FBS + HPIV-3 (3^{-30})	VERO EMEM 1% FBS + HPIV-3 (3^{-31})	VERO EMEM 1% FBS + HPIV-3 (3^{-32})	VERO EMEM 1% FBS + HPIV-3 (3^{-33})	VERO EMEM 1% FBS
E	VERO EMEM 1% FBS + HPIV-3 (3^{-23})	VERO EMEM 1% FBS + HPIV-3 (3^{-24})	VERO EMEM 1% FBS + HPIV-3 (3^{-25})	VERO EMEM 1% FBS + HPIV-3 (3^{-26})	VERO EMEM 1% FBS + HPIV-3 (3^{-27})	VERO EMEM 1% FBS + HPIV-3 (3^{-28})	VERO EMEM 1% FBS + HPIV-3 (3^{-29})	VERO EMEM 1% FBS + HPIV-3 (3^{-30})	VERO EMEM 1% FBS + HPIV-3 (3^{-31})	VERO EMEM 1% FBS + HPIV-3 (3^{-32})	VERO EMEM 1% FBS + HPIV-3 (3^{-33})	VERO EMEM 1% FBS
F	VERO EMEM 1% FBS + HPIV-3 (3^{-23})	VERO EMEM 1% FBS + HPIV-3 (3^{-24})	VERO EMEM 1% FBS + HPIV-3 (3^{-25})	VERO EMEM 1% FBS + HPIV-3 (3^{-26})	VERO EMEM 1% FBS + HPIV-3 (3^{-27})	VERO EMEM 1% FBS + HPIV-3 (3^{-28})	VERO EMEM 1% FBS + HPIV-3 (3^{-29})	VERO EMEM 1% FBS + HPIV-3 (3^{-30})	VERO EMEM 1% FBS + HPIV-3 (3^{-31})	VERO EMEM 1% FBS + HPIV-3 (3^{-32})	VERO EMEM 1% FBS + HPIV-3 (3^{-33})	VERO EMEM 1% FBS
G	VERO EMEM 1% FBS + HPIV-3 (3^{-23})	VERO EMEM 1% FBS + HPIV-3 (3^{-24})	VERO EMEM 1% FBS + HPIV-3 (3^{-25})	VERO EMEM 1% FBS + HPIV-3 (3^{-26})	VERO EMEM 1% FBS + HPIV-3 (3^{-27})	VERO EMEM 1% FBS + HPIV-3 (3^{-28})	VERO EMEM 1% FBS + HPIV-3 (3^{-29})	VERO EMEM 1% FBS + HPIV-3 (3^{-30})	VERO EMEM 1% FBS + HPIV-3 (3^{-31})	VERO EMEM 1% FBS + HPIV-3 (3^{-32})	VERO EMEM 1% FBS + HPIV-3 (3^{-33})	VERO EMEM 1% FBS
H	VERO EMEM 1% FBS + HPIV-3 (3^{-23})	VERO EMEM 1% FBS + HPIV-3 (3^{-24})	VERO EMEM 1% FBS + HPIV-3 (3^{-25})	VERO EMEM 1% FBS + HPIV-3 (3^{-26})	VERO EMEM 1% FBS + HPIV-3 (3^{-27})	VERO EMEM 1% FBS + HPIV-3 (3^{-28})	VERO EMEM 1% FBS + HPIV-3 (3^{-29})	VERO EMEM 1% FBS + HPIV-3 (3^{-30})	VERO EMEM 1% FBS + HPIV-3 (3^{-31})	VERO EMEM 1% FBS + HPIV-3 (3^{-32})	VERO EMEM 1% FBS + HPIV-3 (3^{-33})	VERO EMEM 1% FBS

At the end of the infection, the three Plates were placed in an incubator at 37.0± 1.0 °C and 5.0± 1.0 % CO_2 for 6-8 days. After 6-8 days, the titration was stopped with Methanol and stained with Crystal Violet 1%; then the plates were wash with water and let dry. The plates were evaluated with an optical microscope to detect the presence/absence of a CPE on the cell monolayer.

5.4.3 Results

5.4.3.1 Robustness

The aim of robustness was verifying that the performance of the analytical method is not influenced by deliberate changes that can be introduced during the execution of some of the phases of the test, evaluated as potentially critical in the risk assessment phase.

The following tests were performed provided by the Design of Experiment (DoE) and the Risk Assessment, established using the JMP software, to verify the robustness of the analytical method. The order of tests was randomized by the JMP software.

Following the Risk Assessment, the parameters that were classified as medium or high-risk were:

- Cell concentration for the titration test
- Different culture medium
- Different incubation times

Lower and higher values of the set-point:

Parameter	Lower value	Set-point	Higher value
Cell concentration for the titration test	$0.5*10^5$ cell/ml	$1.0*10^5$ cell/ml	$1.5*10^5$ cell/ml
Different incubation times	6 days	7 days	8 days

With regards to the parameter "Different culture medium", the following conditions were used:

- EMEM 1% FBS
- EMEM 1% FBS with antibiotic and antifungal

After executing all the programmed analytical sessions, using the JMP software, starting from the results, the p-values relating to each critical parameter and the parameter interactions were calculated in addition to calculating the "Lack of Fit".

The robustness of the method was verified: all the values were greater than the significance level set at 0.05.

Test number	Cell Culture Medium (1)	Cell Conc. (cell/mL)	Days after infection	Viral Titer ($Log_{10}TCID_{50}$/mL)
1	Ab	150000	6	7.80
2	No Ab	150000	8	8.10
3	No Ab	100000	7	7.98
4	No Ab	50000	6	8.10
5	No Ab	150000	6	8.16
6	No Ab	100000	7	8.22
7	No Ab	50000	8	8.10
8	Ab	150000	8	7.92
9	Ab	50000	6	8.22
10	No Ab	100000	7	8.16
11	Ab	50000	8	7.86

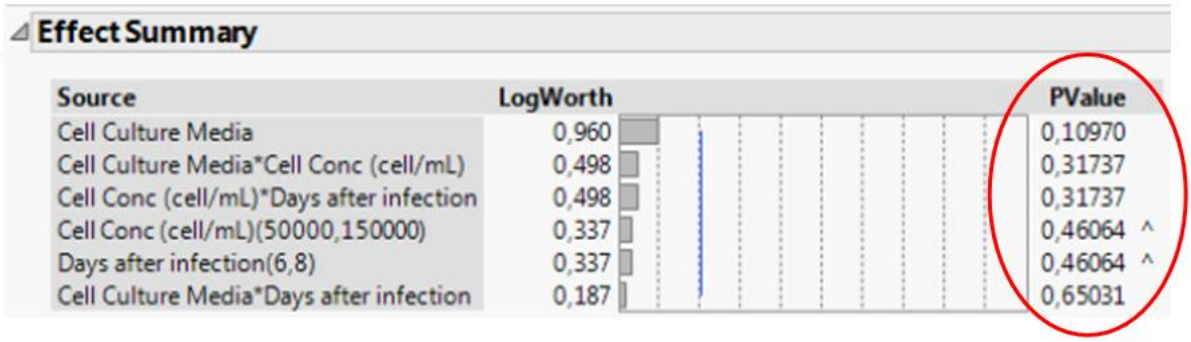
Note:

(1) Cell culture medium: No AB= EMEM 1% FBS

AB= EMEM 1% FBS + antibiotic

After executing all the tests listed above, the evaluation of results was performed using the JMP 12.0 software. The results are shown below (*Fig.14)*

The "P-Value" in the "Effect Summary" section (*Fig.14*) summarizes the significance of each parameter and each interaction. The "P-Values", circled in red, were all greater than the significance level set at 0.05 (> 0.05).



Effect Summary

Source	LogWorth	PValue
Cell Culture Media	0,960	0,10970
Cell Culture Media*Cell Conc (cell/mL)	0,498	0,31737
Cell Conc (cell/mL)*Days after infection	0,498	0,31737
Cell Conc (cell/mL)(50000,150000)	0,337	0,46064
Days after infection(6,8)	0,337	0,46064
Cell Culture Media*Days after infection	0,187	0,65031

Fig.14

Furthermore, in the "Lack of Fit" section, it is calculated whether the linear model used by the software is able to predict how a response varies as the values set for each parameter vary. This is verified if the "Prob>F" value is greater than the significance level. The "Prob>F" value (*Fig.15*), circled in blue, was greater than the significance level set at 0.05 (> 0.05).

Lack Of Fit

Source	DF	Sum of Squares	Mean Square	F Ratio
Lack Of Fit	2	0,03649286	0,018246	1,1696
Pure Error	2	0,03120000	0,015600	Prob > F
Total Error	4	0,06769286		0,4609
				Max RSq
				0,8510

Fig.15

5.4.3.2 INTERMEDIATE PRECISION

To test the intermediate precision parameter, the five samples obtained from the initial pool were analyzed in duplicate as:

- Pure (undiluted)
- Diluted 1:10
- Diluted 1:100
- Diluted 1:1000
- Diluted 1:10000

The test was performed (two different titrations for each sample) by two different operators during two independent analytical sessions.

The difference between the Mean Value of each analysis (expressed as $Log_{10}TCID_{50}$/ml) performed during the entire validation phase (two different operators perform two independent analytical sessions) should be within ± 0.5 Log_{10}. This criterion was established based on what is required by ICH Q5A (R1)[1].

The table below shows the obtained results:

	Titration	Measured titer ($Log_{10}TCID_{50}/ml$)	Difference between measured titers ($Log_{10}TCID_{50}/ml$)
Initial Titer	*Op.1/T.1*	8.10	0.06
	Op.2/T.1	8.16	
	Op.1/T.1	8.10	0.12
	Op.2/T.2	7.98	
	Op.1/T.2	8.16	0
	Op.2/T.1	8.16	
	Op.1/T.2	8.16	0.18
	Op.2/T.2	7.98	
1:10	*Op.1/T.1*	7.08	0.30
	Op.2/T.1	7.38	
	Op.1/T.1	7.08	0.24
	Op.2/T.2	7.32	
	Op.1/T.2	7.02	0.36
	Op.2/T.1	7.38	
	Op.1/T.2	7.02	0.30
	Op.2/T.2	7.32	
1:100	*Op.1/T.1*	5.89	0
	Op.2/T.1	5.89	
	Op.1/T.1	5.89	0.36
	Op.2/T.2	6.25	
	Op.1/T.2	5.83	0.06
	Op.2/T.1	5.89	
	Op.1/T.2	5.83	0.42
	Op.2/T.2	6.25	
1:1000	*Op.1/T.1*	5.00	0.42
	Op.2/T.1	4.58	
	Op.1/T.1	5.00	0.30
	Op.2/T.2	4.70	
	Op.1/T.2	4.64	0.06
	Op.2/T.1	4.58	
	Op.1/T.2	4.64	0.06
	Op.2/T.2	4.70	
1:10000	*Op.1/T.1*	3.68	0.06
	Op.2/T.1	3.62	
	Op.1/T.1	3.68	0
	Op.2/T.2	3.68	
	Op.1/T.2	3.74	0.12
	Op.2/T.1	3.62	
	Op.1/T.2	3.74	0.06
	Op.2/T.2	3.68	

5.4.3.3 REPEATIBILITY

To test the repeatability parameter, the five samples prepared from the initial pool were analyzed in duplicate:

- Pure (undiluted)
- Diluted 1:10
- Diluted 1:100
- Diluted 1:1000
- Diluted 1:10000

The test was performed (two different titrations for each sample) by two different operators during two independent analytical sessions.

The difference between the titers of each analysis of the same sample, expressed as $Log_{10}TCID_{50}$/ml and measured through two titrations performed by the same operator, should be within the interval of ± 0.5 Log_{10}. This criterion was established based on what is required by ICH Q5A (R1)[1].

The table below shows the obtained results:

		Titration	Measured titer ($Log_{10}TCID_{50}/ml$)	Difference between measured titers ($Log_{10}TCID_{50}/ml$)
OPERATOR 1	***Initial titer***	1	8.1	0.06
		2	8.16	
	1:10	1	7.08	0.06
		2	7.02	
	1:100	1	5.89	0.06
		2	5.83	
	1:1000	1	5.00	0.36
		2	4.64	
	1:10000	1	3.68	0.06
		2	3.74	
OPERATOR 2	***Initial titer***	1	8.16	0.18
		2	7.98	
	1:10	1	7.38	0.06
		2	7.32	
	1:100	1	5.89	0.36
		2	6.25	
	1:1000	1	4.58	0.12
		2	4.70	
	1:10000	1	3.62	0.06
		2	3.68	

5.4.3.4 SPECIFICITY

The specificity of the method was evaluated comparing the CPE with the negative control present on each plate, using an optical microscope.

5.4.3.5 ACCURACY

To test the accuracy parameter, the five samples obtained from the initial pool were analyzed in duplicate:

- Pure (undiluted)
- Diluted 1:10
- Diluted 1:100
- Diluted 1:1000
- Diluted 1:10000

The test was performed (two different titrations for each sample) by two different operators during two independent analytical sessions.

The difference between the expected titer of the sample and the titer obtained using this method (average value $Log_{10}TCID_{50}$/ml) should be within the interval of ± 0.5 $Log_{10}TCID_{50}$. This criterion was established based on what is required by ICH Q5A (R1)[1].

The table below shows the obtained results:

		Expected titer ($Log_{10}TCID_{50}$/ml)	Titration	Measured titer ($Log_{10}TCID_{50}$/ml)	Difference between the measured titer ($Log_{10}TCID_{50}$/ml) and the expected titer ($Log_{10}TCID_{50}$/ml)
Operator 1	***Initial Titer***	Mean of Initial Titer of titration 1 and 2 = 8.13	1	8.10	N.A. To be used as a reference value
			2	8.16	N.A. To be used as a reference value
	1:10	7.13	1	7.08	0.05
			2	7.02	0.11
	1:100)	6.13	1	5.89	0.24
			2	5.83	0.30
	1:1000	5.13	1	5.00	0.13
			2	4.64	0.49
	1:10000	4.13	1	3.68	0.45
			2	3.74	0.39
Operator 2	***Initial Titer***	Mean of Initial Titer of titration 1 and 2 = 8.07	1	8.16	N.A. To be used as a reference value
			2	7.98	N.A. To be used as a reference value
	1:10	7.07	1	7.38	0.31
			2	7.32	0.25
	1:100)	6.07	1	5.89	0.18
			2	6.25	0.18
	1:1000	5.07	1	4.58	0.49
			2	4.70	0.37
	1:10000	4.07	1	3.62	0.45
			2	3.68	0.39

5.4.3.6 Linearity

To test the linearity parameter, the five samples obtained from the initial pool were analyzed in duplicate:

- Pure (undiluted)
- Diluted 1:10
- Diluted 1:100
- Diluted 1:1000
- Diluted 1:10000

The test was performed (two different titrations for each sample) by two different operators during two independent analytical sessions.

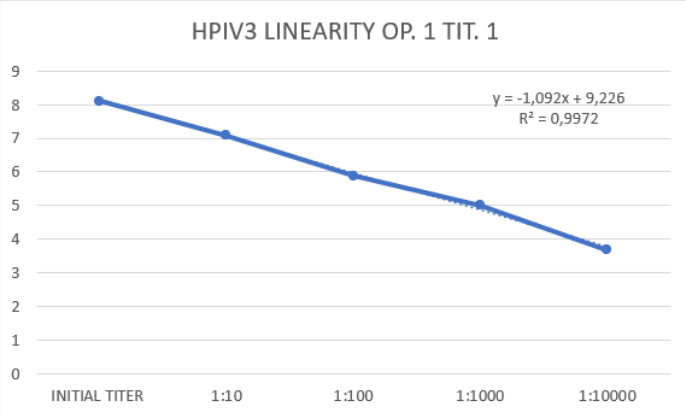
The coefficient of determination (R^2) of the line (described by the linear equation $y=ax+b$) obtained from a graph where the expected titers of the samples are represented on the x axis (INITIAL TITER (A), dilution 1:10 (B), dilution 1:100 (C), dilution 1:1000 (D), dilution 1:10000 (E)) and the titer obtained using this method on the y axis, should be greater than or equal to 0.98.

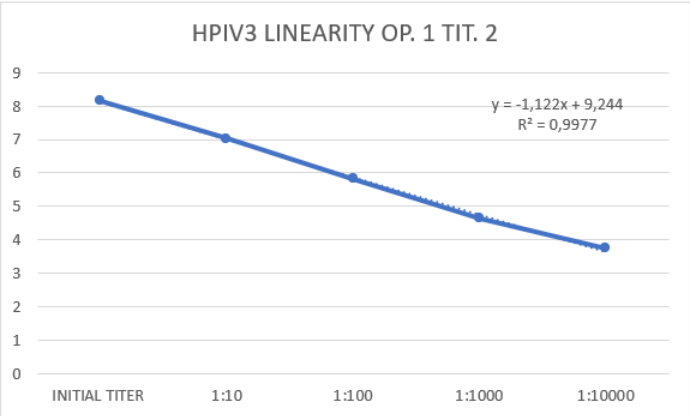
The table below show the obtained results:

LINEARITY		R^2
Operator 1	Titration 1	1.00
	Titration 2	1.00
Operator 2	Titration 1	0.99
	Titration 2	0.98

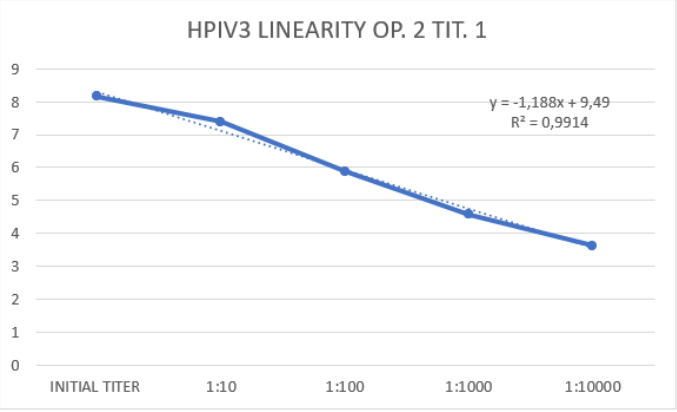
The obtained graphs are shown below:

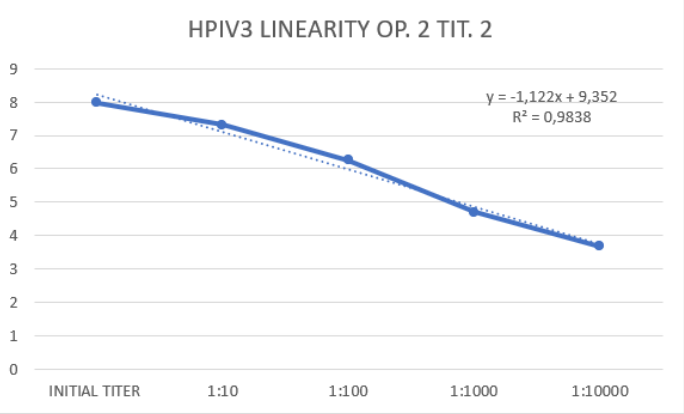
OPERATOR 1:





OPERATOR 2:





5.4.4 titration Method conclusions

The robustness of the method was verified: all parameters were in the respective proposed ranges at the end of all the tests performed, with the support provided by the JMP statistical software.

Cellular concentration for the titration test:

- $0.5*10^5$ cell/ml
- $1.0 *10^5$ cell/ml
- $1.5*10^5$ cell/ml

Different culture medium:

- EMEM 1% FBS
- EMEM 1% FBS with antibiotic and antifungal

Different incubation times:

- 6 days
- 7 days
- 8 days

For Validation the precision, specificity, accuracy and linearity parameters were verified during the method validation process and were compliant with the acceptance criteria.

Following the activities performed during the validation process the method was considered Validated.

5.5 SuHV-1 titration Method validation

5.5.1 Method description

The method is used to determine the titer expressed as $Log_{10}TCID_{50}$/mL of a SuHV-1 viral suspension through the evaluation of the cytopathic effect induced by the virus on the monolayer of VERO cells in adhesion.

5.5.2 Analitical procedure

The method is based on the evaluation of the cytopathic effect that various scalar dilutions of the viral suspension induce on the cell monolayer.

5.5.2.1 preparation of scalar dilution of SuHV-1

A pool of the SuHV-1 viral suspension was used for the preparation of the samples for robustness, specificity, precision, accuracy and linearity. Subsequently, each sample was diluted with 1:3 scalar dilutions prepared as indicated below. Each titration consists of 3 plates of VERO cells (96-well flat bottom plates with low evaporation):

- Plate 1 → infection with dilutions from 3^{-1} to 3^{-11}
- Plate 2 → infection with dilutions from 3^{-12} to 3^{-22}
- Plate 3 → infection with dilutions from 3^{-23} to 3^{-33}

For each titration (Plate 1, 2 and 3) the dilutions are prepared in a deep-well polypropylene plate as described below:

- Fill the first lines of the deep-well polypropylene plates with 600 µl of EMEM 2% FBS medium with antibiotic and antifungal (Penicillin-Streptomycin 1% and Amphotericin B 1 %).

→ rows A and B will be used for the infection of Plate 1 (dilutions from 3^{-1} to 3^{-11} and negative control)

→ rows C and D will be used for the infection of Plate 2 (dilutions from 3^{-12} to 3^{-22} and negative control)
→ rows E and F will be used for the infection of Plate 3 (dilutions from 3^{-23} to 3^{-33} and negative control)

- Add SuHV-1 viral suspension in row A column 1 and in row B column 1 to have a 1:3 diluted suspension.

- Take the defined volume from row A and B column 1 (WELL A1 and WELL B1) and add in row A and B column 2 (WELL A2 and WELL B2), pipette and continue in this way until column 11 (skip column 12 → negative control) → dilutions from 3^{-1} to 3^{-11}

- Take the defined volume from row A and B column 11 (WELL A11 and WELL B11) and add in row C and D column 1 (C1 and D1), continue dilutions in this way until column 11 (skip column 12 → negative control) → dilutions from 3^{-12} to 3^{-22}

- Take the defined volume from row C and D column 11(WELL C11 and WELL D11) and add in row E and F column 1 (WELL E1 and WELL F1), continue dilutions in this way until column 11 (skip column 12 → negative control) → dilutions from 3^{-23} to 3^{-33}

For the preparation of the dilution plate follow the diagram below:

	1	2	3	4	5	6	7	8	9	10	11	12	
A	EMEM 2%FBS + **SuHV-1 (3^{-1})**	EMEM 2%FBS + **WELL A1 (3^{-2})**	EMEM 2%FBS + **WELL A2 (3^{-3})**	EMEM 2%FBS + **WELL A3 (3^{-4})**	EMEM 2%FBS + **WELL A4 (3^{-5})**	EMEM 2%FBS + **WELL A5 (3^{-6})**	EMEM 2%FBS + **WELL A6 (3^{-7})**	EMEM 2%FBS + **WELL A7 (3^{-8})**	EMEM 2%FBS + **WELL A8 (3^{-9})**	EMEM 2%FBS + **WELL A9 (3^{-10})**	EMEM 2%FBS + **WELL A10 (3^{-11})**	EMEM 2%FBS **C-**	**1st PLATE**
B	EMEM 2%FBS + **SuHV-1 (3^{-1})**	EMEM 2%FBS + **WELL B1 (3^{-2})**	EMEM 2%FBS + **WELL B2 (3^{-3})**	EMEM 2%FBS + **WELL B3 (3^{-4})**	EMEM 2%FBS + **WELL B4 (3^{-5})**	EMEM 2%FBS + **WELL B5 (3^{-6})**	EMEM 2%FBS + **WELL B6 (3^{-7})**	EMEM 2%FBS + **WELL B7 (3^{-8})**	EMEM 2%FBS + **WELL B8 (3^{-9})**	EMEM 2%FBS + **WELL B9 (3^{-10})**	EMEM 2%FBS + **WELL B10 (3^{-11})**	EMEM 2%FBS **C-**	
C	EMEM 2%FBS + **WELL A11(3^{-12})**	EMEM 2%FBS + **WELL C1 (3^{-13})**	EMEM 2%FBS + **WELL C2 (3^{-14})**	EMEM 2%FBS + **WELL C3 (3^{-15})**	EMEM 2%FBS + **WELL C4 (3^{-16})**	EMEM 2%FBS + **WELL C5 (3^{-17})**	EMEM 2%FBS + **WELL C6 (3^{-18})**	EMEM 2%FBS + **WELL C7 (3^{-19})**	EMEM 2%FBS + **WELL C8 (3^{-20})**	EMEM 2%FBS + **WELL C9 (3^{-21})**	EMEM 2%FBS + **WELL C10 (3^{-22})**	EMEM 2%FBS **C-**	**2nd PLATE**
D	EMEM 2%FBS + **WELL B11 (3^{-12})**	EMEM 2%FBS + **WELL D1 (3^{-13})**	EMEM 2%FBS + **WELL D2 (3^{-14})**	EMEM 2%FBS + **WELL D3 (3^{-15})**	EMEM 2%FBS + **WELL D4 (3^{-16})**	EMEM 2%FBS + **WELL D5 (3^{-17})**	EMEM 2%FBS + **WELL D6 (3^{-18})**	EMEM 2%FBS + **WELL D7 (3^{-19})**	EMEM 2%FBS + **WELL D8 (3^{-20})**	EMEM 2%FBS + **WELL D9 (3^{-21})**	EMEM 2%FBS + **WELL D10 (3^{-22})**	EMEM 2%FBS **C-**	
E	EMEM 2%FBS + **WELL C11(3^{-23})**	EMEM 2%FBS + **WELL E1 (3^{-24})**	EMEM 2%FBS + **WELL E2 (3^{-25})**	EMEM 2%FBS + **WELL E3 (3^{-26})**	EMEM 2%FBS + **WELL E4 (3^{-27})**	EMEM 2%FBS + **WELL E5 (3^{-28})**	EMEM 2%FBS + **WELL E6 (3^{-29})**	EMEM 2%FBS + **WELL E7 (3^{-30})**	EMEM 2%FBS + **WELL E8 (3^{-31})**	EMEM 2%FBS + **WELL E9 (3^{-32})**	EMEM 2%FBS + **WELL E10 (3^{-33})**	EMEM 2%FBS **C-**	**3rd PLATE**
F	EMEM 2%FBS + **WELL D11(3^{-23})**	EMEM 2%FBS + **WELL F1 (3^{-24})**	EMEM 2%FBS + **WELL F2 (3^{-25})**	EMEM 2%FBS + **WELL F3 (3^{-26})**	EMEM 2%FBS + **WELL F4 (3^{-27})**	EMEM 2%FBS + **WELL F5 (3^{-28})**	EMEM 2%FBS + **WELL F6 (3^{-29})**	EMEM 2%FBS + **WELL F7 (3^{-30})**	EMEM 2%FBS + **WELL F8 (3^{-31})**	EMEM 2%FBS + **WELL F9 (3^{-32})**	EMEM 2%FBS + **WELL F10 (3^{-33})**	EMEM 2%FBS **C-**	
G													
H													

5.5.2.2 Titration procedure

The day before the titration assay, the plates were prepared as described below. Bring VERO cells, grown in EMEM 10 % FBS medium, to a concentration of:

- $1,5*10^5$ cell/ml
- $2,0*10^5$ cell/ml (TARGET)
- $2,5*10^5$ cell/ml

Then take three plates 96-well flat bottom, low evaporation plates (1st, 2nd and 3rd Plate) for each titration to be performed; dispensing cell suspension obtained in each well for each plate as shown below.

	1	2	3	4	5	6	7	8	9	10	11	12
A	VERO + EMEM 10%FBS	VERO + EMEM 10%FBS	VERO + EMEM 10%FBS	VERO + EMEM 10%FBS	VERO + EMEM 10%FBS	VERO + EMEM 10%FBS	VERO + EMEM 10%FBS	VERO + EMEM 10%FBS	VERO + EMEM 10%FBS	VERO + EMEM 10%FBS	VERO + EMEM 10%FBS	VERO + EMEM 10%FBS **C-**
B	VERO + EMEM 10%FBS	VERO + EMEM 10%FBS	VERO + EMEM 10%FBS	VERO + EMEM 10%FBS	VERO + EMEM 10%FBS	VERO + EMEM 10%FBS	VERO + EMEM 10%FBS	VERO + EMEM 10%FBS	VERO + EMEM 10%FBS	VERO + EMEM 10%FBS	VERO + EMEM 10%FBS	VERO + EMEM 10%FBS **C-**
C	VERO + EMEM 10%FBS	VERO + EMEM 10%FBS	VERO + EMEM 10%FBS	VERO + EMEM 10%FBS	VERO + EMEM 10%FBS	VERO + EMEM 10%FBS	VERO + EMEM 10%FBS	VERO + EMEM 10%FBS	VERO + EMEM 10%FBS	VERO + EMEM 10%FBS	VERO + EMEM 10%FBS	VERO + EMEM 10%FBS **C-**
D	VERO + EMEM 10%FBS	VERO + EMEM 10%FBS	VERO + EMEM 10%FBS	VERO + EMEM 10%FBS	VERO + EMEM 10%FBS	VERO + EMEM 10%FBS	VERO + EMEM 10%FBS	VERO + EMEM 10%FBS	VERO + EMEM 10%FBS	VERO + EMEM 10%FBS	VERO + EMEM 10%FBS	VERO + EMEM 10%FBS **C-**
E	VERO + EMEM 10%FBS	VERO + EMEM 10%FBS	VERO + EMEM 10%FBS	VERO + EMEM 10%FBS	VERO + EMEM 10%FBS	VERO + EMEM 10%FBS	VERO + EMEM 10%FBS	VERO + EMEM 10%FBS	VERO + EMEM 10%FBS	VERO + EMEM 10%FBS	VERO + EMEM 10%FBS	VERO + EMEM 10%FBS **C-**
F	VERO + EMEM 10%FBS	VERO + EMEM 10%FBS	VERO + EMEM 10%FBS	VERO + EMEM 10%FBS	VERO + EMEM 10%FBS	VERO + EMEM 10%FBS	VERO + EMEM 10%FBS	VERO + EMEM 10%FBS	VERO + EMEM 10%FBS	VERO + EMEM 10%FBS	VERO + EMEM 10%FBS	VERO + EMEM 10%FBS **C-**
G	VERO + EMEM 10%FBS	VERO + EMEM 10%FBS	VERO + EMEM 10%FBS	VERO + EMEM 10%FBS	VERO + EMEM 10%FBS	VERO + EMEM 10%FBS	VERO + EMEM 10%FBS	VERO + EMEM 10%FBS	VERO + EMEM 10%FBS	VERO + EMEM 10%FBS	VERO + EMEM 10%FBS	VERO + EMEM 10%FBS **C-**
H	VERO + EMEM 10%FBS	VERO + EMEM 10%FBS	VERO + EMEM 10%FBS	VERO + EMEM 10%FBS	VERO + EMEM 10%FBS	VERO + EMEM 10%FBS	VERO + EMEM 10%FBS	VERO + EMEM 10%FBS	VERO + EMEM 10%FBS	VERO + EMEM 10%FBS	VERO + EMEM 10%FBS	VERO + EMEM 10%FBS **C-**

The plates were placed in an incubator at 37.0± 1.0 °C and 5.0± 1.0 % CO_2 for 24 hours.

After this time wash the cell monolayer with PBS to remove any serum or dead cell residues and proceeded with infection of the 1st, 2nd, and 3rd Plates as indicated below.

Infection of the 1st Plate:

With a multichannel pipette, the defined volume was taken from row A of the deep-well polypropylene plates and dispense it into row A of the 1st Plate. Action was repeated 3 more times, respectively into rows B, C and D of the 1st Plate.

Follow the same process with row B of the deep-well polypropylene plates and dispense it into row E, F, G and H of the 1st Plate.

	1	2	3	4	5	6	7	8	9	10	11	12	
A	EMEM 2%FBS + **SuHV-1 (3^{-1})**	EMEM 2%FBS + **WELL A1 (3^{-2})**	EMEM 2%FBS + **WELL A2 (3^{-3})**	EMEM 2%FBS + **WELL A3 (3^{-4})**	EMEM 2%FBS + **WELL A4 (3^{-5})**	EMEM 2%FBS + **WELL A5 (3^{-6})**	EMEM 2%FBS + **WELL A6 (3^{-7})**	EMEM 2%FBS + **WELL A7 (3^{-8})**	EMEM 2%FBS + **WELL A8 (3^{-9})**	EMEM 2%FBS + **WELL A9 (3^{-10})**	EMEM 2%FBS + **WELL A10 (3^{-11})**	EMEM 2%FBS **C-**	**1st PLATE**
B	EMEM 2%FBS + **SuHV-1 (3^{-1})**	EMEM 2%FBS + **WELL B1 (3^{-2})**	EMEM 2%FBS + **WELL B2 (3^{-3})**	EMEM 2%FBS + **WELL B3 (3^{-4})**	EMEM 2%FBS + **WELL B4 (3^{-5})**	EMEM 2%FBS + **WELL B5 (3^{-6})**	EMEM 2%FBS + **WELL B6 (3^{-7})**	EMEM 2%FBS + **WELL B7 (3^{-8})**	EMEM 2%FBS + **WELL B8 (3^{-9})**	EMEM 2%FBS + **WELL B9 (3^{-10})**	EMEM 2%FBS + **WELL B10 (3^{-11})**	EMEM 2%FBS **C-**	
C	EMEM 2%FBS + **WELL A11(3^{-12})**	EMEM 2%FBS + **WELL C1 (3^{-13})**	EMEM 2%FBS + **WELL C2 (3^{-14})**	EMEM 2%FBS + **WELL C3 (3^{-15})**	EMEM 2%FBS + **WELL C4 (3^{-16})**	EMEM 2%FBS + **WELL C5 (3^{-17})**	EMEM 2%FBS + **WELL C6 (3^{-18})**	EMEM 2%FBS + **WELL C7 (3^{-19})**	EMEM 2%FBS + **WELL C8 (3^{-20})**	EMEM 2%FBS + **WELL C9 (3^{-21})**	EMEM 2%FBS + **WELL C10 (3^{-22})**	EMEM 2%FBS **C-**	**2nd PLATE**
D	EMEM 2%FBS + **WELL B11 (3^{-12})**	EMEM 2%FBS + **WELL D1 (3^{-13})**	EMEM 2%FBS + **WELL D2 (3^{-14})**	EMEM 2%FBS + **WELL D3 (3^{-15})**	EMEM 2%FBS + **WELL D4 (3^{-16})**	EMEM 2%FBS + **WELL D5 (3^{-17})**	EMEM 2%FBS + **WELL D6 (3^{-18})**	EMEM 2%FBS + **WELL D7 (3^{-19})**	EMEM 2%FBS + **WELL D8 (3^{-20})**	EMEM 2%FBS + **WELL D9 (3^{-21})**	EMEM 2%FBS + **WELL D10 (3^{-22})**	EMEM 2%FBS **C-**	
E	EMEM 2%FBS + **WELL C11(3^{-23})**	EMEM 2%FBS + **WELL E1 (3^{-24})**	EMEM 2%FBS + **WELL E2 (3^{-25})**	EMEM 2%FBS + **WELL E3 (3^{-26})**	EMEM 2%FBS + **WELL E4 (3^{-27})**	EMEM 2%FBS + **WELL E5 (3^{-28})**	EMEM 2%FBS + **WELL E6 (3^{-29})**	EMEM 2%FBS + **WELL E7 (3^{-30})**	EMEM 2%FBS + **WELL E8 (3^{-31})**	EMEM 2%FBS + **WELL E9 (3^{-32})**	EMEM 2%FBS + **WELL E10 (3^{-33})**	EMEM 2%FBS **C-**	**3rd PLATE**
F	EMEM 2%FBS + **WELL D11(3^{-23})**	EMEM 2%FBS + **WELL F1 (3^{-24})**	EMEM 2%FBS + **WELL F2 (3^{-25})**	EMEM 2%FBS + **WELL F3 (3^{-26})**	EMEM 2%FBS + **WELL F4 (3^{-27})**	EMEM 2%FBS + **WELL F5 (3^{-28})**	EMEM 2%FBS + **WELL F6 (3^{-29})**	EMEM 2%FBS + **WELL F7 (3^{-30})**	EMEM 2%FBS + **WELL F8 (3^{-31})**	EMEM 2%FBS + **WELL F9 (3^{-32})**	EMEM 2%FBS + **WELL F10 (3^{-33})**	EMEM 2%FBS **C-**	
G													
H													

1st PLATE: from 3^{-1} to 3^{-11}

	1	2	3	4	5	6	7	8	9	10	11	12
A	VERO EMEM 2%FBS + SUHV-1 (3^{-1})	VERO EMEM 2%FBS + SUHV-1 (3^{-2})	VERO EMEM 2%FBS + SUHV-1 (3^{-3})	VERO EMEM 2%FBS + SUHV-1 (3^{-4})	VERO EMEM 2%FBS + SUHV-1 (3^{-5})	VERO EMEM 2%FBS + SUHV-1 (3^{-6})	VERO EMEM 2%FBS + SUHV-1 (3^{-7})	VERO EMEM 2%FBS + SUHV-1 (3^{-8})	VERO EMEM 2%FBS + SUHV-1 (3^{-9})	VERO EMEM 2%FBS + SUHV-1 (3^{-10})	VERO EMEM 2%FBS + SUHV-1 (3^{-11})	VERO EMEM 2%FBS
B	VERO EMEM 2%FBS + SUHV-1 (3^{-1})	VERO EMEM 2%FBS + SUHV-1 (3^{-2})	VERO EMEM 2%FBS + SUHV-1 (3^{-3})	VERO EMEM 2%FBS + SUHV-1 (3^{-4})	VERO EMEM 2%FBS + SUHV-1 (3^{-5})	VERO EMEM 2%FBS + SUHV-1 (3^{-6})	VERO EMEM 2%FBS + SUHV-1 (3^{-7})	VERO EMEM 2%FBS + SUHV-1 (3^{-8})	VERO EMEM 2%FBS + SUHV-1 (3^{-9})	VERO EMEM 2%FBS + SUHV-1 (3^{-10})	VERO EMEM 2%FBS + SUHV-1 (3^{-11})	VERO EMEM 2%FBS
C	VERO EMEM 2%FBS + SUHV-1 (3^{-1})	VERO EMEM 2%FBS + SUHV-1 (3^{-2})	VERO EMEM 2%FBS + SUHV-1 (3^{-3})	VERO EMEM 2%FBS + SUHV-1 (3^{-4})	VERO EMEM 2%FBS + SUHV-1 (3^{-5})	VERO EMEM 2%FBS + SUHV-1 (3^{-6})	VERO EMEM 2%FBS + SUHV-1 (3^{-7})	VERO EMEM 2%FBS + SUHV-1 (3^{-8})	VERO EMEM 2%FBS + SUHV-1 (3^{-9})	VERO EMEM 2%FBS + SUHV-1 (3^{-10})	VERO EMEM 2%FBS + SUHV-1 (3^{-11})	VERO EMEM 2%FBS
D	VERO EMEM 2%FBS + SUHV-1 (3^{-1})	VERO EMEM 2%FBS + SUHV-1 (3^{-2})	VERO EMEM 2%FBS + SUHV-1 (3^{-3})	VERO EMEM 2%FBS + SUHV-1 (3^{-4})	VERO EMEM 2%FBS + SUHV-1 (3^{-5})	VERO EMEM 2%FBS + SUHV-1 (3^{-6})	VERO EMEM 2%FBS + SUHV-1 (3^{-7})	VERO EMEM 2%FBS + SUHV-1 (3^{-8})	VERO EMEM 2%FBS + SUHV-1 (3^{-9})	VERO EMEM 2%FBS + SUHV-1 (3^{-10})	VERO EMEM 2%FBS + SUHV-1 (3^{-11})	VERO EMEM 2%FBS
E	VERO EMEM 2%FBS + SUHV-1 (3^{-1})	VERO EMEM 2%FBS + SUHV-1 (3^{-2})	VERO EMEM 2%FBS + SUHV-1 (3^{-3})	VERO EMEM 2%FBS + SUHV-1 (3^{-4})	VERO EMEM 2%FBS + SUHV-1 (3^{-5})	VERO EMEM 2%FBS + SUHV-1 (3^{-6})	VERO EMEM 2%FBS + SUHV-1 (3^{-7})	VERO EMEM 2%FBS + SUHV-1 (3^{-8})	VERO EMEM 2%FBS + SUHV-1 (3^{-9})	VERO EMEM 2%FBS + SUHV-1 (3^{-10})	VERO EMEM 2%FBS + SUHV-1 (3^{-11})	VERO EMEM 2%FBS
F	VERO EMEM 2%FBS + SUHV-1 (3^{-1})	VERO EMEM 2%FBS + SUHV-1 (3^{-2})	VERO EMEM 2%FBS + SUHV-1 (3^{-3})	VERO EMEM 2%FBS + SUHV-1 (3^{-4})	VERO EMEM 2%FBS + SUHV-1 (3^{-5})	VERO EMEM 2%FBS + SUHV-1 (3^{-6})	VERO EMEM 2%FBS + SUHV-1 (3^{-7})	VERO EMEM 2%FBS + SUHV-1 (3^{-8})	VERO EMEM 2%FBS + SUHV-1 (3^{-9})	VERO EMEM 2%FBS + SUHV-1 (3^{-10})	VERO EMEM 2%FBS + SUHV-1 (3^{-11})	VERO EMEM 2%FBS
G	VERO EMEM 2%FBS + SUHV-1 (3^{-1})	VERO EMEM 2%FBS + SUHV-1 (3^{-2})	VERO EMEM 2%FBS + SUHV-1 (3^{-3})	VERO EMEM 2%FBS + SUHV-1 (3^{-4})	VERO EMEM 2%FBS + SUHV-1 (3^{-5})	VERO EMEM 2%FBS + SUHV-1 (3^{-6})	VERO EMEM 2%FBS + SUHV-1 (3^{-7})	VERO EMEM 2%FBS + SUHV-1 (3^{-8})	VERO EMEM 2%FBS + SUHV-1 (3^{-9})	VERO EMEM 2%FBS + SUHV-1 (3^{-10})	VERO EMEM 2%FBS + SUHV-1 (3^{-11})	VERO EMEM 2%FBS
H	VERO EMEM 2%FBS + SUHV-1 (3^{-1})	VERO EMEM 2%FBS + SUHV-1 (3^{-2})	VERO EMEM 2%FBS + SUHV-1 (3^{-3})	VERO EMEM 2%FBS + SUHV-1 (3^{-4})	VERO EMEM 2%FBS + SUHV-1 (3^{-5})	VERO EMEM 2%FBS + SUHV-1 (3^{-6})	VERO EMEM 2%FBS + SUHV-1 (3^{-7})	VERO EMEM 2%FBS + SUHV-1 (3^{-8})	VERO EMEM 2%FBS + SUHV-1 (3^{-9})	VERO EMEM 2%FBS + SUHV-1 (3^{-10})	VERO EMEM 2%FBS + SUHV-1 (3^{-11})	VERO EMEM 2%FBS

Infection of the 2nd Plate:

With a multichannel pipette, the defined volume was taken from row C of the deep-well polypropylene plates and dispense it into row A of the 1st Plate. Action was repeated 3 more times, respectively into rows B, C and D of the 2nd Plate.

Follow the same process with row D of the deep-well polypropylene plates and dispense it into row E, F, G and H of the 2nd Plate.

	1	2	3	4	5	6	7	8	9	10	11	12	
A	EMEM 2%FBS + **SuHV-1 (3^{-1})**	EMEM 2%FBS + **WELL A1 (3^{-2})**	EMEM 2%FBS + **WELL A2 (3^{-3})**	EMEM 2%FBS + **WELL A3 (3^{-4})**	EMEM 2%FBS + **WELL A4 (3^{-5})**	EMEM 2%FBS + **WELL A5 (3^{-6})**	EMEM 2%FBS + **WELL A6 (3^{-7})**	EMEM 2%FBS + **WELL A7 (3^{-8})**	EMEM 2%FBS + **WELL A8 (3^{-9})**	EMEM 2%FBS + **WELL A9 (3^{-10})**	EMEM 2%FBS + **WELL A10 (3^{-11})**	EMEM 2%FBS **C-**	**1st PLATE**
B	EMEM 2%FBS + **SuHV-1 (3^{-1})**	EMEM 2%FBS + **WELL B1 (3^{-2})**	EMEM 2%FBS + **WELL B2 (3^{-3})**	EMEM 2%FBS + **WELL B3 (3^{-4})**	EMEM 2%FBS + **WELL B4 (3^{-5})**	EMEM 2%FBS + **WELL B5 (3^{-6})**	EMEM 2%FBS + **WELL B6 (3^{-7})**	EMEM 2%FBS + **WELL B7 (3^{-8})**	EMEM 2%FBS + **WELL B8 (3^{-9})**	EMEM 2%FBS + **WELL B9 (3^{-10})**	EMEM 2%FBS + **WELL B10 (3^{-11})**	EMEM 2%FBS **C-**	
C	EMEM 2%FBS + **WELL A11(3^{-12})**	EMEM 2%FBS + **WELL C1 (3^{-13})**	EMEM 2%FBS + **WELL C2 (3^{-14})**	EMEM 2%FBS + **WELL C3 (3^{-15})**	EMEM 2%FBS + **WELL C4 (3^{-16})**	EMEM 2%FBS + **WELL C5 (3^{-17})**	EMEM 2%FBS + **WELL C6 (3^{-18})**	EMEM 2%FBS + **WELL C7 (3^{-19})**	EMEM 2%FBS + **WELL C8 (3^{-20})**	EMEM 2%FBS + **WELL C9 (3^{-21})**	EMEM 2%FBS + **WELL C10 (3^{-22})**	EMEM 2%FBS **C-**	**2nd PLATE**
D	EMEM 2%FBS + **WELL B11 (3^{-12})**	EMEM 2%FBS + **WELL D1 (3^{-13})**	EMEM 2%FBS + **WELL D2 (3^{-14})**	EMEM 2%FBS + **WELL D3 (3^{-15})**	EMEM 2%FBS + **WELL D4 (3^{-16})**	EMEM 2%FBS + **WELL D5 (3^{-17})**	EMEM 2%FBS + **WELL D6 (3^{-18})**	EMEM 2%FBS + **WELL D7 (3^{-19})**	EMEM 2%FBS + **WELL D8 (3^{-20})**	EMEM 2%FBS + **WELL D9 (3^{-21})**	EMEM 2%FBS + **WELL D10 (3^{-22})**	EMEM 2%FBS **C-**	
E	EMEM 2%FBS + **WELL C11(3^{-23})**	EMEM 2%FBS + **WELL E1 (3^{-24})**	EMEM 2%FBS + **WELL E2 (3^{-25})**	EMEM 2%FBS + **WELL E3 (3^{-26})**	EMEM 2%FBS + **WELL E4 (3^{-27})**	EMEM 2%FBS + **WELL E5 (3^{-28})**	EMEM 2%FBS + **WELL E6 (3^{-29})**	EMEM 2%FBS + **WELL E7 (3^{-30})**	EMEM 2%FBS + **WELL E8 (3^{-31})**	EMEM 2%FBS + **WELL E9 (3^{-32})**	EMEM 2%FBS + **WELL E10 (3^{-33})**	EMEM 2%FBS **C-**	**3rd PLATE**
F	EMEM 2%FBS + **WELL D11(3^{-23})**	EMEM 2%FBS + **WELL F1 (3^{-24})**	EMEM 2%FBS + **WELL F2 (3^{-25})**	EMEM 2%FBS + **WELL F3 (3^{-26})**	EMEM 2%FBS + **WELL F4 (3^{-27})**	EMEM 2%FBS + **WELL F5 (3^{-28})**	EMEM 2%FBS + **WELL F6 (3^{-29})**	EMEM 2%FBS + **WELL F7 (3^{-30})**	EMEM 2%FBS + **WELL F8 (3^{-31})**	EMEM 2%FBS + **WELL F9 (3^{-32})**	EMEM 2%FBS + **WELL F10 (3^{-33})**	EMEM 2%FBS **C-**	
G													
H													

2^{nd} PLATE: from 3^{-12} to 3^{-22}

	1	2	3	4	5	6	7	8	9	10	11	12
A	VERO EMEM 2%FBS + SUHV-1 (3^{-12})	VERO EMEM 2%FBS + SUHV-1 (3^{-13})	VERO EMEM 2%FBS + SUHV-1 (3^{-14})	VERO EMEM 2%FBS + SUHV-1 (3^{-15})	VERO EMEM 2%FBS + SUHV-1 (3^{-16})	VERO EMEM 2%FBS + SUHV-1 (3^{-17})	VERO EMEM 2%FBS + SUHV-1 (3^{-18})	VERO EMEM 2%FBS + SUHV-1 (3^{-19})	VERO EMEM 2%FBS + SUHV-1 (3^{-20})	VERO EMEM 2%FBS + SUHV-1 (3^{-21})	VERO EMEM 2%FBS + SUHV-1 (3^{-22})	VERO EMEM 2%FBS
B	VERO EMEM 2%FBS + SUHV-1 (3^{-12})	VERO EMEM 2%FBS + SUHV-1 (3^{-13})	VERO EMEM 2%FBS + SUHV-1 (3^{-14})	VERO EMEM 2%FBS + SUHV-1 (3^{-15})	VERO EMEM 2%FBS + SUHV-1 (3^{-16})	VERO EMEM 2%FBS + SUHV-1 (3^{-17})	VERO EMEM 2%FBS + SUHV-1 (3^{-18})	VERO EMEM 2%FBS + SUHV-1 (3^{-19})	VERO EMEM 2%FBS + SUHV-1 (3^{-20})	VERO EMEM 2%FBS + SUHV-1 (3^{-21})	VERO EMEM 2%FBS + SUHV-1 (3^{-22})	VERO EMEM 2%FBS
C	VERO EMEM 2%FBS + SUHV-1 (3^{-12})	VERO EMEM 2%FBS + SUHV-1 (3^{-13})	VERO EMEM 2%FBS + SUHV-1 (3^{-14})	VERO EMEM 2%FBS + SUHV-1 (3^{-15})	VERO EMEM 2%FBS + SUHV-1 (3^{-16})	VERO EMEM 2%FBS + SUHV-1 (3^{-17})	VERO EMEM 2%FBS + SUHV-1 (3^{-18})	VERO EMEM 2%FBS + SUHV-1 (3^{-19})	VERO EMEM 2%FBS + SUHV-1 (3^{-20})	VERO EMEM 2%FBS + SUHV-1 (3^{-21})	VERO EMEM 2%FBS + SUHV-1 (3^{-22})	VERO EMEM 2%FBS
D	VERO EMEM 2%FBS + SUHV-1 (3^{-12})	VERO EMEM 2%FBS + SUHV-1 (3^{-13})	VERO EMEM 2%FBS + SUHV-1 (3^{-14})	VERO EMEM 2%FBS + SUHV-1 (3^{-15})	VERO EMEM 2%FBS + SUHV-1 (3^{-16})	VERO EMEM 2%FBS + SUHV-1 (3^{-17})	VERO EMEM 2%FBS + SUHV-1 (3^{-18})	VERO EMEM 2%FBS + SUHV-1 (3^{-19})	VERO EMEM 2%FBS + SUHV-1 (3^{-20})	VERO EMEM 2%FBS + SUHV-1 (3^{-21})	VERO EMEM 2%FBS + SUHV-1 (3^{-22})	VERO EMEM 2%FBS
E	VERO EMEM 2%FBS + SUHV-1 (3^{-12})	VERO EMEM 2%FBS + SUHV-1 (3^{-13})	VERO EMEM 2%FBS + SUHV-1 (3^{-14})	VERO EMEM 2%FBS + SUHV-1 (3^{-15})	VERO EMEM 2%FBS + SUHV-1 (3^{-16})	VERO EMEM 2%FBS + SUHV-1 (3^{-17})	VERO EMEM 2%FBS + SUHV-1 (3^{-18})	VERO EMEM 2%FBS + SUHV-1 (3^{-19})	VERO EMEM 2%FBS + SUHV-1 (3^{-20})	VERO EMEM 2%FBS + SUHV-1 (3^{-21})	VERO EMEM 2%FBS + SUHV-1 (3^{-22})	VERO EMEM 2%FBS
F	VERO EMEM 2%FBS + SUHV-1 (3^{-12})	VERO EMEM 2%FBS + SUHV-1 (3^{-13})	VERO EMEM 2%FBS + SUHV-1 (3^{-14})	VERO EMEM 2%FBS + SUHV-1 (3^{-15})	VERO EMEM 2%FBS + SUHV-1 (3^{-16})	VERO EMEM 2%FBS + SUHV-1 (3^{-17})	VERO EMEM 2%FBS + SUHV-1 (3^{-18})	VERO EMEM 2%FBS + SUHV-1 (3^{-19})	VERO EMEM 2%FBS + SUHV-1 (3^{-20})	VERO EMEM 2%FBS + SUHV-1 (3^{-21})	VERO EMEM 2%FBS + SUHV-1 (3^{-22})	VERO EMEM 2%FBS
G	VERO EMEM 2%FBS + SUHV-1 (3^{-12})	VERO EMEM 2%FBS + SUHV-1 (3^{-13})	VERO EMEM 2%FBS + SUHV-1 (3^{-14})	VERO EMEM 2%FBS + SUHV-1 (3^{-15})	VERO EMEM 2%FBS + SUHV-1 (3^{-16})	VERO EMEM 2%FBS + SUHV-1 (3^{-17})	VERO EMEM 2%FBS + SUHV-1 (3^{-18})	VERO EMEM 2%FBS + SUHV-1 (3^{-19})	VERO EMEM 2%FBS + SUHV-1 (3^{-20})	VERO EMEM 2%FBS + SUHV-1 (3^{-21})	VERO EMEM 2%FBS + SUHV-1 (3^{-22})	VERO EMEM 2%FBS
H	VERO EMEM 2%FBS + SUHV-1 (3^{-12})	VERO EMEM 2%FBS + SUHV-1 (3^{-13})	VERO EMEM 2%FBS + SUHV-1 (3^{-14})	VERO EMEM 2%FBS + SUHV-1 (3^{-15})	VERO EMEM 2%FBS + SUHV-1 (3^{-16})	VERO EMEM 2%FBS + SUHV-1 (3^{-17})	VERO EMEM 2%FBS + SUHV-1 (3^{-18})	VERO EMEM 2%FBS + SUHV-1 (3^{-19})	VERO EMEM 2%FBS + SUHV-1 (3^{-20})	VERO EMEM 2%FBS + SUHV-1 (3^{-21})	VERO EMEM 2%FBS + SUHV-1 (3^{-22})	VERO EMEM 2%FBS

Infection of the 3rd Plate:

With a multichannel pipette, the defined volume was taken from row E of the deep-well polypropylene plates and dispense it into row A of the 1st Plate. Action was repeated 3 more times, respectively into rows B, C and D of the 3rd Plate.

Follow the same process with row F of the deep-well polypropylene plates and dispense it into row E, F, G and H of the 3rd Plate.

	1	2	3	4	5	6	7	8	9	10	11	12	
A	EMEM 2%FBS + **SuHV-1 (3^{-1})**	EMEM 2%FBS + **WELL A1 (3^{-2})**	EMEM 2%FBS + **WELL A2 (3^{-3})**	EMEM 2%FBS + **WELL A3 (3^{-4})**	EMEM 2%FBS + **WELL A4 (3^{-5})**	EMEM 2%FBS + **WELL A5 (3^{-6})**	EMEM 2%FBS + **WELL A6 (3^{-7})**	EMEM 2%FBS + **WELL A7 (3^{-8})**	EMEM 2%FBS + **WELL A8 (3^{-9})**	EMEM 2%FBS + **WELL A9 (3^{-10})**	EMEM 2%FBS + **WELL A10 (3^{-11})**	EMEM 2%FBS **C-**	**1st PLATE**
B	EMEM 2%FBS + **SuHV-1 (3^{-1})**	EMEM 2%FBS + **WELL B1 (3^{-2})**	EMEM 2%FBS + **WELL B2 (3^{-3})**	EMEM 2%FBS + **WELL B3 (3^{-4})**	EMEM 2%FBS + **WELL B4 (3^{-5})**	EMEM 2%FBS + **WELL B5 (3^{-6})**	EMEM 2%FBS + **WELL B6 (3^{-7})**	EMEM 2%FBS + **WELL B7 (3^{-8})**	EMEM 2%FBS + **WELL B8 (3^{-9})**	EMEM 2%FBS + **WELL B9 (3^{-10})**	EMEM 2%FBS + **WELL B10 (3^{-11})**	EMEM 2%FBS **C-**	
C	EMEM 2%FBS + **WELL A11(3^{-12})**	EMEM 2%FBS + **WELL C1 (3^{-13})**	EMEM 2%FBS + **WELL C2 (3^{-14})**	EMEM 2%FBS + **WELL C3 (3^{-15})**	EMEM 2%FBS + **WELL C4 (3^{-16})**	EMEM 2%FBS + **WELL C5 (3^{-17})**	EMEM 2%FBS + **WELL C6 (3^{-18})**	EMEM 2%FBS + **WELL C7 (3^{-19})**	EMEM 2%FBS + **WELL C8 (3^{-20})**	EMEM 2%FBS + **WELL C9 (3^{-21})**	EMEM 2%FBS + **WELL C10 (3^{-22})**	EMEM 2%FBS **C-**	**2nd PLATE**
D	EMEM 2%FBS + **WELL B11 (3^{-12})**	EMEM 2%FBS + **WELL D1 (3^{-13})**	EMEM 2%FBS + **WELL D2 (3^{-14})**	EMEM 2%FBS + **WELL D3 (3^{-15})**	EMEM 2%FBS + **WELL D4 (3^{-16})**	EMEM 2%FBS + **WELL D5 (3^{-17})**	EMEM 2%FBS + **WELL D6 (3^{-18})**	EMEM 2%FBS + **WELL D7 (3^{-19})**	EMEM 2%FBS + **WELL D8 (3^{-20})**	EMEM 2%FBS + **WELL D9 (3^{-21})**	EMEM 2%FBS + **WELL D10 (3^{-22})**	EMEM 2%FBS **C-**	
E	EMEM 2%FBS + **WELL C11(3^{-23})**	EMEM 2%FBS + **WELL E1 (3^{-24})**	EMEM 2%FBS + **WELL E2 (3^{-25})**	EMEM 2%FBS + **WELL E3 (3^{-26})**	EMEM 2%FBS + **WELL E4 (3^{-27})**	EMEM 2%FBS + **WELL E5 (3^{-28})**	EMEM 2%FBS + **WELL E6 (3^{-29})**	EMEM 2%FBS + **WELL E7 (3^{-30})**	EMEM 2%FBS + **WELL E8 (3^{-31})**	EMEM 2%FBS + **WELL E9 (3^{-32})**	EMEM 2%FBS + **WELL E10 (3^{-33})**	EMEM 2%FBS **C-**	**3rd PLATE**
F	EMEM 2%FBS + **WELL D11(3^{-23})**	EMEM 2%FBS + **WELL F1 (3^{-24})**	EMEM 2%FBS + **WELL F2 (3^{-25})**	EMEM 2%FBS + **WELL F3 (3^{-26})**	EMEM 2%FBS + **WELL F4 (3^{-27})**	EMEM 2%FBS + **WELL F5 (3^{-28})**	EMEM 2%FBS + **WELL F6 (3^{-29})**	EMEM 2%FBS + **WELL F7 (3^{-30})**	EMEM 2%FBS + **WELL F8 (3^{-31})**	EMEM 2%FBS + **WELL F9 (3^{-32})**	EMEM 2%FBS + **WELL F10 (3^{-33})**	EMEM 2%FBS **C-**	
G													
H													

3rd PLATE: from 3^{-23} to 3^{-33}

	1	2	3	4	5	6	7	8	9	10	11	12
A	VERO EMEM 2%FBS + SUHV-1 (3^{-23})	VERO EMEM 2%FBS + SUHV-1 (3^{-24})	VERO EMEM 2%FBS + SUHV-1 (3^{-25})	VERO EMEM 2%FBS + SUHV-1 (3^{-26})	VERO EMEM 2%FBS + SUHV-1 (3^{-27})	VERO EMEM 2%FBS + SUHV-1 (3^{-28})	VERO EMEM 2%FBS + SUHV-1 (3^{-29})	VERO EMEM 2%FBS + SUHV-1 (3^{-30})	VERO EMEM 2%FBS + SUHV-1 (3^{-31})	VERO EMEM 2%FBS + SUHV-1 (3^{-32})	VERO EMEM 2%FBS + SUHV-1 (3^{-33})	VERO EMEM 2%FBS
B	VERO EMEM 2%FBS + SUHV-1 (3^{-23})	VERO EMEM 2%FBS + SUHV-1 (3^{-24})	VERO EMEM 2%FBS + SUHV-1 (3^{-25})	VERO EMEM 2%FBS + SUHV-1 (3^{-26})	VERO EMEM 2%FBS + SUHV-1 (3^{-27})	VERO EMEM 2%FBS + SUHV-1 (3^{-28})	VERO EMEM 2%FBS + SUHV-1 (3^{-29})	VERO EMEM 2%FBS + SUHV-1 (3^{-30})	VERO EMEM 2%FBS + SUHV-1 (3^{-31})	VERO EMEM 2%FBS + SUHV-1 (3^{-32})	VERO EMEM 2%FBS + SUHV-1 (3^{-33})	VERO EMEM 2%FBS
C	VERO EMEM 2%FBS + SUHV-1 (3^{-23})	VERO EMEM 2%FBS + SUHV-1 (3^{-24})	VERO EMEM 2%FBS + SUHV-1 (3^{-25})	VERO EMEM 2%FBS + SUHV-1 (3^{-26})	VERO EMEM 2%FBS + SUHV-1 (3^{-27})	VERO EMEM 2%FBS + SUHV-1 (3^{-28})	VERO EMEM 2%FBS + SUHV-1 (3^{-29})	VERO EMEM 2%FBS + SUHV-1 (3^{-30})	VERO EMEM 2%FBS + SUHV-1 (3^{-31})	VERO EMEM 2%FBS + SUHV-1 (3^{-32})	VERO EMEM 2%FBS + SUHV-1 (3^{-33})	VERO EMEM 2%FBS
D	VERO EMEM 2%FBS + SUHV-1 (3^{-23})	VERO EMEM 2%FBS + SUHV-1 (3^{-24})	VERO EMEM 2%FBS + SUHV-1 (3^{-25})	VERO EMEM 2%FBS + SUHV-1 (3^{-26})	VERO EMEM 2%FBS + SUHV-1 (3^{-27})	VERO EMEM 2%FBS + SUHV-1 (3^{-28})	VERO EMEM 2%FBS + SUHV-1 (3^{-29})	VERO EMEM 2%FBS + SUHV-1 (3^{-30})	VERO EMEM 2%FBS + SUHV-1 (3^{-31})	VERO EMEM 2%FBS + SUHV-1 (3^{-32})	VERO EMEM 2%FBS + SUHV-1 (3^{-33})	VERO EMEM 2%FBS
E	VERO EMEM 2%FBS + SUHV-1 (3^{-23})	VERO EMEM 2%FBS + SUHV-1 (3^{-24})	VERO EMEM 2%FBS + SUHV-1 (3^{-25})	VERO EMEM 2%FBS + SUHV-1 (3^{-26})	VERO EMEM 2%FBS + SUHV-1 (3^{-27})	VERO EMEM 2%FBS + SUHV-1 (3^{-28})	VERO EMEM 2%FBS + SUHV-1 (3^{-29})	VERO EMEM 2%FBS + SUHV-1 (3^{-30})	VERO EMEM 2%FBS + SUHV-1 (3^{-31})	VERO EMEM 2%FBS + SUHV-1 (3^{-32})	VERO EMEM 2%FBS + SUHV-1 (3^{-33})	VERO EMEM 2%FBS
F	VERO EMEM 2%FBS + SUHV-1 (3^{-23})	VERO EMEM 2%FBS + SUHV-1 (3^{-24})	VERO EMEM 2%FBS + SUHV-1 (3^{-25})	VERO EMEM 2%FBS + SUHV-1 (3^{-26})	VERO EMEM 2%FBS + SUHV-1 (3^{-27})	VERO EMEM 2%FBS + SUHV-1 (3^{-28})	VERO EMEM 2%FBS + SUHV-1 (3^{-29})	VERO EMEM 2%FBS + SUHV-1 (3^{-30})	VERO EMEM 2%FBS + SUHV-1 (3^{-31})	VERO EMEM 2%FBS + SUHV-1 (3^{-32})	VERO EMEM 2%FBS + SUHV-1 (3^{-33})	VERO EMEM 2%FBS
G	VERO EMEM 2%FBS + SUHV-1 (3^{-23})	VERO EMEM 2%FBS + SUHV-1 (3^{-24})	VERO EMEM 2%FBS + SUHV-1 (3^{-25})	VERO EMEM 2%FBS + SUHV-1 (3^{-26})	VERO EMEM 2%FBS + SUHV-1 (3^{-27})	VERO EMEM 2%FBS + SUHV-1 (3^{-28})	VERO EMEM 2%FBS + SUHV-1 (3^{-29})	VERO EMEM 2%FBS + SUHV-1 (3^{-30})	VERO EMEM 2%FBS + SUHV-1 (3^{-31})	VERO EMEM 2%FBS + SUHV-1 (3^{-32})	VERO EMEM 2%FBS + SUHV-1 (3^{-33})	VERO EMEM 2%FBS
H	VERO EMEM 2%FBS + SUHV-1 (3^{-23})	VERO EMEM 2%FBS + SUHV-1 (3^{-24})	VERO EMEM 2%FBS + SUHV-1 (3^{-25})	VERO EMEM 2%FBS + SUHV-1 (3^{-26})	VERO EMEM 2%FBS + SUHV-1 (3^{-27})	VERO EMEM 2%FBS + SUHV-1 (3^{-28})	VERO EMEM 2%FBS + SUHV-1 (3^{-29})	VERO EMEM 2%FBS + SUHV-1 (3^{-30})	VERO EMEM 2%FBS + SUHV-1 (3^{-31})	VERO EMEM 2%FBS + SUHV-1 (3^{-32})	VERO EMEM 2%FBS + SUHV-1 (3^{-33})	VERO EMEM 2%FBS

At the end of the infection, the three Plates were placed in an incubator at 37.0 °C and 5.0% CO_2 for 2-4 days. After 2-4 days, the titration was stopped with Methanol and stained with Crystal Violet 1%; then the plates were wash with water and let them dry. The plates were evaluated with an optical microscope to detect the presence/absence of a CPE on the cell monolayer.

5.5.3 Results

5.5.3.1 Robustness

The aim of robustness was to verify that the performance of the analytical method was not influenced by deliberate changes that can be introduced during the execution of some of the phases of the test, evaluated as potentially critical in the risk assessment phase.

The following tests were performed provided by the Design of Experiment (DoE) and the Risk Assessment, established using the JMP software, to verify the robustness of the analytical method. The order of tests was randomized by the JMP software.

Following the Risk Assessment, the parameters were classified as medium or high-risk were:

- Cell concentration for the titration test
- Different culture medium
- Different incubation times
- Virus suspension pH

Lower and higher values of the set-point:

Parameter	Lower value	Set-point	Higher value
Cell concentration for the titration test	$1.5*10^5$ cell/ml	$2.0*10^5$ cell/ml	$2.5*10^5$ cell/ml
Different incubation times	2	3	4
Virus suspension pH	6.0	7.2	8.4

With regards to the parameter "Different culture medium", the following conditions were used:

- EMEM 2% FBS
- EMEM 2% FBS with antibiotic and antifungal

After executing all the programmed analytical sessions, using the JMP software, starting from the results, the p-values relating to each critical parameter and the parameter interactions were calculated in addition to calculating the "Lack of Fit".

The robustness of the method was verified if all the values are greater than the significance level set at 0.05.

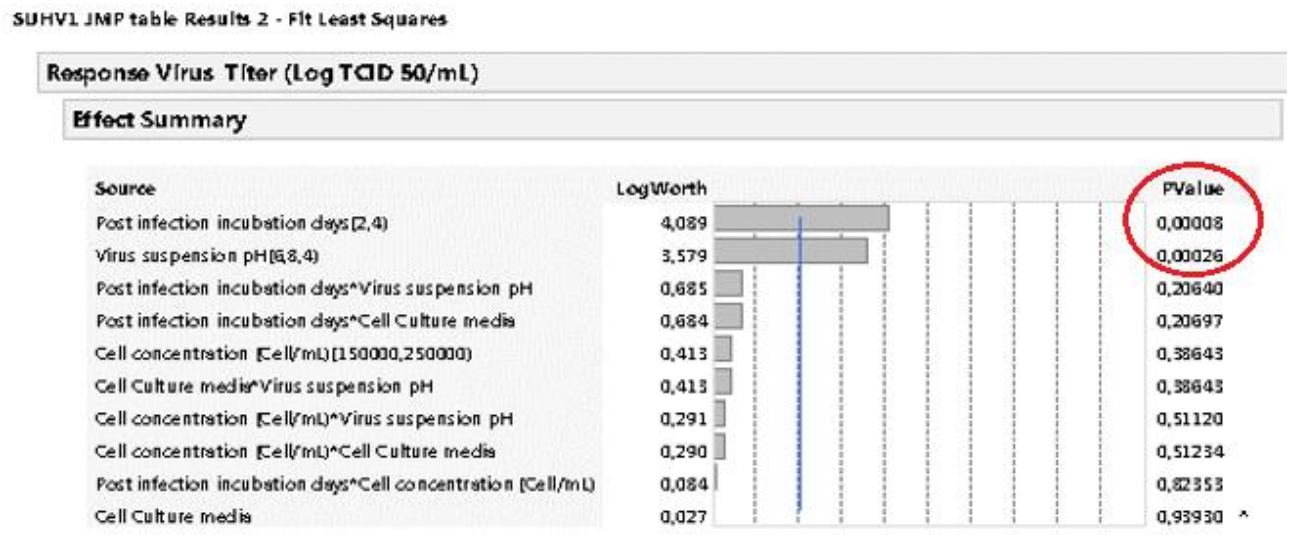
Test number	Cell Culture Medium (1)	Cell Conc. (cell/mL)	Days after infection	Virus suspension pH	Viral Titer ($Log_{10}TCID_{50}$/mL)
1	No AB	150000	2	8,4	8.992
2	AB	250000	4	8,4	9.886
3	AB	150000	2	8,4	8.753
4	No AB	150000	4	8,4	9.767
5	AB	250000	2	6,0	7.799
6	No AB	200000	3	7,2	9.290
7	AB	150000	2	6,0	7.978
8	No AB	250000	4	6,0	8.694
9	No AB	250000	4	8,4	9.350
10	AB	150000	4	6,0	9.290
11	No AB	200000	3	7,2	9.290
12	AB	250000	2	8,4	8.694
13	No AB	150000	2	6,0	7.918
14	AB	250000	4	6,0	9.051
15	AB	200000	3	7,2	9.111
16	No AB	150000	4	6,0	8.992
17	AB	150000	4	8,4	9.529
18	No AB	250000	2	6,0	7.799
19	No AB	250000	2	8,4	8.992

Note:

(1) Cell culture medium: No AB= EMEM 2% FBS

AB= EMEM 2% FBS + antibiotic

The "PValue" value in the "Effect Summary" section (Fig.16) summarizes the significance of each parameter and each interaction. The p-value, "post infection incubation days (2,4)" (0,00008) and "virus suspension pH (6, 8.4)" (0,00026), are less than 0.05 (highlighted in red) and therefore significant.


SUHV1 JMP table Results 2 - Fit Least Squares

Response Virus Titer (Log TCID 50/mL)

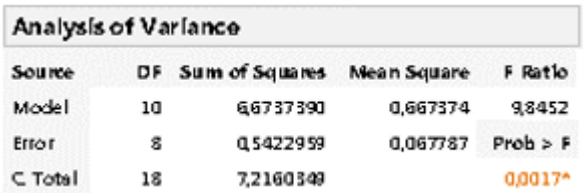
Effect Summary

Source	LogWorth	PValue
Post infection incubation days(2,4)	4,089	0,00008
Virus suspension pH(6,8,4)	3,579	0,00026
Post infection incubation days*Virus suspension pH	0,685	0,20640
Post infection incubation days*Cell Culture media	0,684	0,20697
Cell concentration (Cell/mL)(150000,250000)	0,413	0,38643
Cell Culture media*Virus suspension pH	0,413	0,38643
Cell concentration (Cell/mL)*Virus suspension pH	0,291	0,51120
Cell concentration (Cell/mL)*Cell Culture media	0,290	0,51234
Post infection incubation days*Cell concentration (Cell/mL)	0,084	0,82353
Cell Culture media	0,027	0,93930

Fig.16

For this reason, the global variance was evaluated.

The value "Prob> F" (**0.0017**), Fig.17, in the "Analysis of Variance" section (global p-value of the ANOVA analysis for the total variance of the model) was significant because it was less than 0.05, this means that the study performed so far is not able to demonstrate the robustness of the method.


Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio
Model	10	6,6737390	0,667374	9,8452
Error	8	0,5422959	0,067787	Prob > F
C. Total	18	7,2160349		0,0017*

Fig.17

It was necessary to carry out an increased number of the tests foreseen by the experimental design (6 additional tests), in order to identify precisely the cause of the failure to verify the robustness, introducing the quadratic interactions and inserting between the values of each parameter also their set-point.

Here are the results of all the tests performed including the additional ones (*from test 20 to test 25*):

Test number	Cell Culture Medium (1)	Cell Conc. (cell/mL)	Days after infection	Virus suspension pH	Viral Titer ($Log_{10}TCID_{50}$/mL)
1	No AB	150000	2	8,4	8.992
2	AB	250000	4	8,4	9.886
3	AB	150000	2	8,4	8.753
4	No AB	150000	4	8,4	9.767
5	AB	250000	2	6,0	7.799
6	No AB	200000	3	7,2	9.290
7	AB	150000	2	6,0	7.978
8	No AB	250000	4	6,0	8.694
9	No AB	250000	4	8,4	9.350
10	AB	150000	4	6,0	9.290
11	No AB	200000	3	7,2	9.290
12	AB	250000	2	8,4	8.694
13	No AB	150000	2	6,0	7.918
14	AB	250000	4	6,0	9.051
15	AB	200000	3	7,2	9.111
16	No AB	150000	4	6,0	8.992
17	AB	150000	4	8,4	9.529
18	No AB	250000	2	6,0	7.799
19	No AB	250000	2	8,4	8.992
20	AB	200000	2	6	9.171
21	AB	250000	3	6	9.290
22	AB	200000	4	8,4	9.886
23	AB	150000	3	8,4	9.409
24	AB	150000	4	7,2	9.171
25	No AB	200000	3	7,2	9.409

Note:

(1) Cell culture medium: No AB= EMEM 2% FBS

AB= EMEM 2% FBS + antibiotic

The p-values in the "Effect Summary" section (Fig.18) for "post infection incubation days (2.4)" (0.00002), "cell concentration (Cell / mL)" (0.00813) and "virus suspension pH (6, 8.4) ”(0.00021), are less than 0.05 (highlighted in red) and therefore significant.

Furthermore, the value "Prob> F" (**0.0006**), Fig.18, in the "Analysis of Variance" section is significant because it is less than 0.05. This means that the method is not robust at the proposed work ranges.

Augmented Design SUHV1 JMP Table - Fit Least Squares

Response Virus Titer (Log TCID 50/mL)

Effect Summary

Source	LogWorth	PValue
Post infection incubation days(2,4)	4,624	0,00002
Virus suspension pH(6,8,4)	3,686	0,00021
Cell concentration (Cell/mL)*Cell concentration (Cell/mL)	2,090	0,00813
Virus suspension pH*Virus suspension pH	1,257	0,05527
Post infection incubation days*Post infection incubation days	1,150	0,07072
Cell Culture media*Virus suspension pH	1,144	0,07179
Post infection incubation days*Virus suspension pH	0,562	0,27397
Post infection incubation days*Cell Culture media	0,409	0,38976
Cell concentration (Cell/mL)(150000,250000)	0,371	0,42608
Cell Culture media	0,361	0,43547
Cell concentration (Cell/mL)*Cell Culture media	0,359	0,43780
Cell concentration (Cell/mL)*Virus suspension pH	0,194	0,64026
Post infection incubation days*Cell concentration (Cell/mL)	0,164	0,68596

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio
Model	13	7,8459066	0,603531	8,3285
Error	11	0,7971196	0,072465	Prob > F
C. Total	24	8,6430262		0,0006*

Fig.18

In order to define a working range around the set point of the parameters defined as potentially critical, it is necessary to restrict the ranges of the parameters identified as significant.

The ranges were selected by graphically defining (see Fig.19, vertical green lines) the ranges for which the confidence interval of the response (graphically defined by the blue dashed lines), for each parameter, contained the response obtained at the point (horizontal dashed red line).

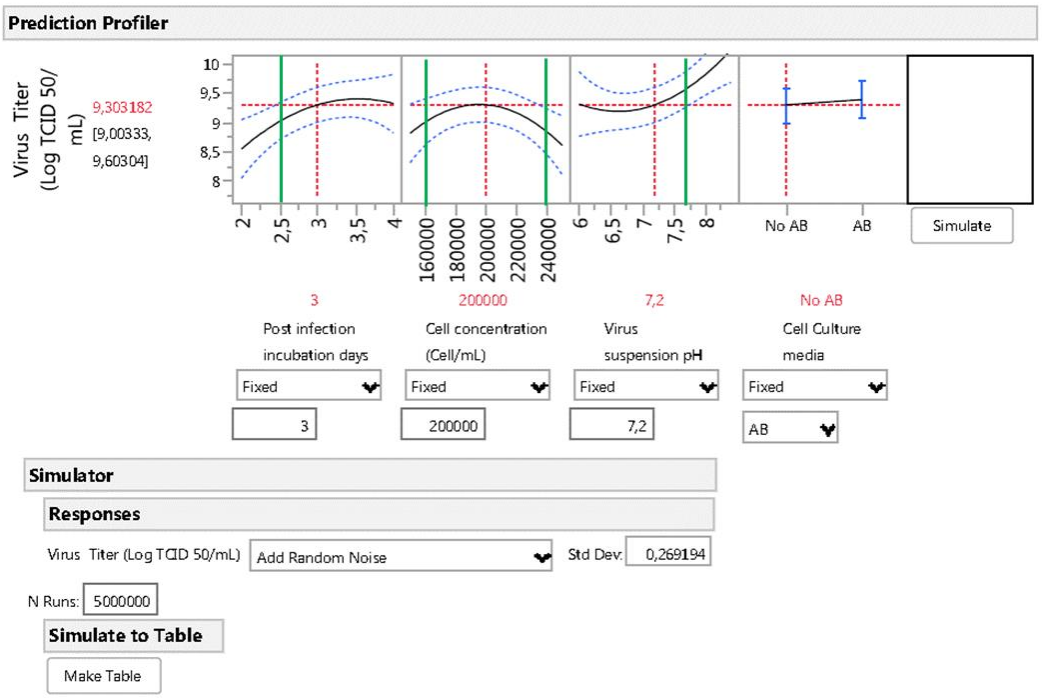


Fig. 19

The defined narrow ranges were:

- 2.5 - 4 days for the "post infection incubation days" parameter (instead of 2 - 4 days)
- 1.6 - 2.4 * 10^5 cell / mL for the "cell concentration (Cell / mL)" parameter (instead of 1.5 - 2.5 * 10^5 cell / mL)
- 6 - 7.7 pH for the "virus suspension pH" parameter (instead of 6 - 8.4 pH)

The "Cell culture media" parameter was not changed.

To confirm the adequacy of the ranges proposed above, including the potential accumulation of variability and the interactions due to the variability of the method parameters, it is possible to use the predictive model of the performance of the method to predict the distribution of results.

To determine the ideal distribution, a simulation with the Monte Carlo method, using the JMP 12.0 software, was first performed (5 million simulations) with all the

parameters set (fixed) at their set point (3 days post infection incubation days, 2 * 10^5 cells / mL, No AB = EMEM 2% FBS and 7.2 pH) as shown in Figure 19.

The distribution obtained (Fig.20) is characterized by a median of the "Virus Titer $Log_{10}TCID_{50}$ / mL" of 9.39 with 95% of the values between 8.87 (2.5 percentile) and 9.92 (97.5 percentile).

Report: Montecarlo simulation fixed

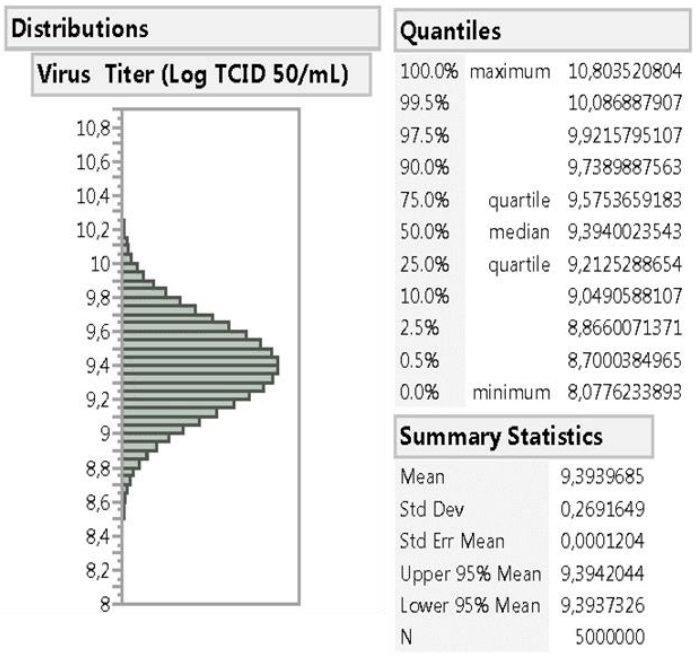
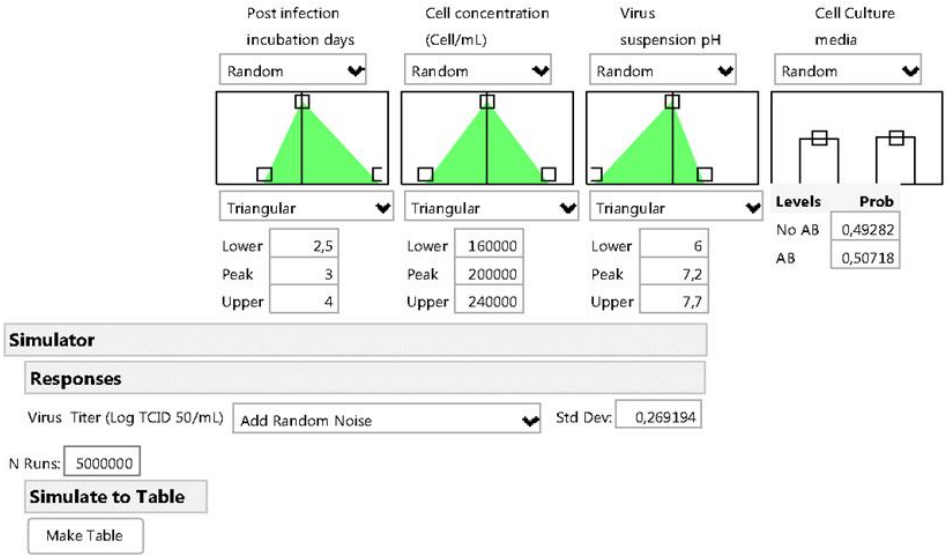


Fig.20

Secondary it was decided to use a triangular distribution of these parameters since most conditions will be close to the standard ones and less frequently close to the outer limits of the ranges (Fig.21).

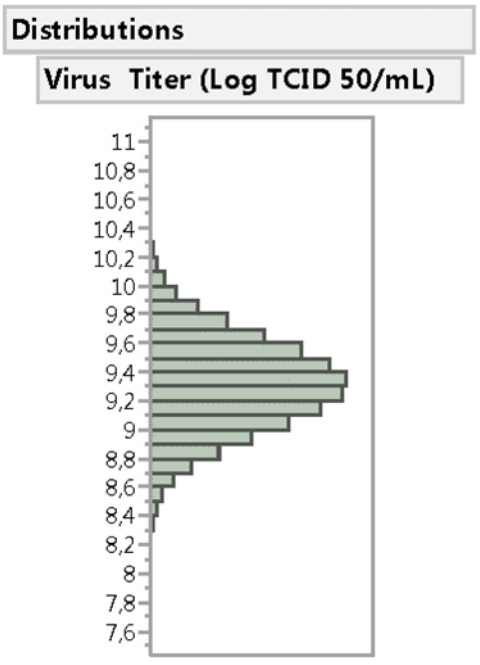
This distribution was preferred to the normal truncated one because it was more adequate in the case of asymmetric ranges.

Fig.21



The median of the "Virus Titer $Log_{10}TCID_{50}$ / mL" (9.31), Fig.22, obtained from the random distribution is very similar to that obtained with all the parameters set at their set point, with 95% of the values between 8.68 (2.5 percentile) and 9.93 (97.5 percentile).

Fig.22



Quantiles

100.0%	maximum	10,992340195
99.5%		10,114333406
97.5%		9,9259354155
90.0%		9,7161056285
75.0%	quartile	9,5262828926
50.0%	median	9,3138758751
25.0%	quartile	9,0992498215
10.0%		8,904414554
2.5%		8,6849761793
0.5%		8,4837371449
0.0%	minimum	7,6060125212

Summary Statistics

Mean	9,3116309
Std Dev	0,3165973
Std Err Mean	0,0001416
Upper 95% Mean	9,3119084
Lower 95% Mean	9,3113534
N	5000000

5.5.3.2 INTERMEDIATE PRECISION

To test the intermediate precision parameter, the five samples obtained from the initial pool were analyzed in duplicate as:

- Pure (undiluted)
- Diluted 1:10
- Diluted 1:100
- Diluted 1:1000
- Diluted 1:10000

The test was performed (two different titrations for each sample) by two different operators during two independent analytical sessions.

The difference between the Mean Value of each analysis (expressed as $Log_{10}TCID_{50}$/ml) performed during the entire validation phase (two different operators perform two independent analytical sessions) should be within ± 0.5 Log_{10}. This criterion was established based on what is required by ICH Q5A (R1)[1].

The table below shows the obtained results:

	Titration	*Measured titer ($Log_{10}TCID_{50}$/ml)*	*Difference between measured titers ($Log_{10}TCID_{50}$/ml)*
Initial Titer	*Op.1/T.1*	8.63	0.12
	Op.2/T.1	8.75	
	Op.1/T.1	8.63	0.24
	Op.2/T.2	8.87	
	Op.1/T.2	8.93	0.18
	Op.2/T.1	8.75	
	Op.1/T.2	8.93	0.06
	Op.2/T.2	8.87	
1:10	*Op.1/T.1*	7.50	0.24
	Op.2/T.1	7.26	
	Op.1/T.1	7.50	0.42
	Op.2/T.2	7.08	
	Op.1/T.2	7.38	0.12
	Op.2/T.1	7.26	
	Op.1/T.2	7.38	0.30
	Op.2/T.2	7.08	
1:100	*Op.1/T.1*	6.25	0.06
	Op.2/T.1	6.19	
	Op.1/T.1	6.25	0.18
	Op.2/T.2	6.07	
	Op.1/T.2	5.89	0.30
	Op.2/T.1	6.19	
	Op.1/T.2	5.89	0.18
	Op.2/T.2	6.07	
1:1000	*Op.1/T.1*	5.00	0.12
	Op.2/T.1	5.12	
	Op.1/T.1	5.00	0
	Op.2/T.2	5.00	
	Op.1/T.2	4.88	0.24
	Op.2/T.1	5.12	
	Op.1/T.2	4.88	0.12
	Op.2/T.2	5.00	
1:10000	*Op.1/T.1*	4.16	0.12
	Op.2/T.1	4.04	
	Op.1/T.1	4.16	0.24
	Op.2/T.2	3.92	
	Op.1/T.2	3.80	0.24
	Op.2/T.1	4.04	
	Op.1/T.2	3.80	0.12
	Op.2/T.2	3.92	

5.5.3.3 REPEATABILITY

To test the repeatability parameter, the five samples prepared from the initial pool were analyzed in duplicate:

- Pure (undiluted)
- Diluted 1:10
- Diluted 1:100
- Diluted 1:1000
- Diluted 1:10000

The test was performed (two different titrations for each sample) by two different operators during two independent analytical sessions.

The difference between the titers of each analysis of the same sample, expressed as $Log_{10}TCID_{50}$/ml and measured through two titrations performed by the same operator, should be within the interval of ± 0.5 Log_{10}. This criterion was established based on what is required by ICH Q5A (R1)[1].

The table below shows the obtained results:

		Titration	Measured titer ($Log_{10}TCID_{50}$/ml)	Difference between measured titers ($Log_{10}TCID_{50}$/ml)
OPERATOR 1	***Initial titer***	1	8.63	0.30
		2	8.93	
	1:10	1	7.50	0.12
		2	7.38	
	1:100	1	6.25	0.36
		2	5.89	
	1:1000	1	5.00	0.12
		2	4.88	
	1:10000	1	4.16	0.36
		2	3.80	
OPERATOR 2	***Initial titer***	1	8.75	0.12
		2	8.87	
	1:10	1	7.26	0.18
		2	7.08	
	1:100	1	6.19	0.12
		2	6.07	
	1:1000	1	5.12	0.12
		2	5.00	
	1:10000	1	4.04	0.12
		2	3.92	

5.5.3.4 SPECIFICITY

The specificity of the method was evaluated comparing the CPE with the negative control present on each plate, using an optical microscope.

5.5.3.5 ACCURACY

To test the accuracy parameter, the five samples obtained from the initial pool were analyzed in duplicate:

- Pure (undiluted)
- Diluted 1:10
- Diluted 1:100
- Diluted 1:1000
- Diluted 1:10000

The test was performed (two different titrations for each sample) by two different operators during two independent analytical sessions.

The difference between the expected titer of the sample and the titer obtained using this method (average value $Log_{10}TCID_{50}$/ml) should be within the interval of ± 0.5 $Log_{10}TCID_{50}$. This criterion was established based on what is required by ICH Q5A (R1)[1].

The first time the accuracy didn't match with the acceptance criteria for some samples.

It was necessary to go back to set-up phase to investigate better. After this, the tendency of the SuHV-1 to aggregate was identified as the cause.

For this reason, it was decided to use the purified virus (SuHV-1 VPL) as a sample.

The table below shows the obtained results:

		Expected titer ($Log_{10}TCID_{50}$/ml)	Titration	Measured titer ($Log_{10}TCID_{50}$/ml)	Difference between the measured titer ($Log_{10}TCID_{50}$/ml) and the expected titer ($Log_{10}TCID_{50}$/ml)
Operator 1	***Initial Titer***	Mean of Initial Titer of titration 1 and 2 = 8.37	1	8.34	N.A. To be used as a reference value
			2	8.40	N.A. To be used as a reference value
	1:10	7,37	1	7,56	0,19
			2	7,38	0,01
	1:100	6,37	1	6,49	0,12
			2	6,67	0,30
	1:1000	5,37	1	5,47	0,10
			2	5,29	0,08
	1:10000	4,37	1	4,10	0,27
			2	4,04	0,33
Operator 2	***Initial Titer***	Mean of Initial Titer of titration 1 and 2 = 8.25	1	8,28	N.A. To be used as a reference value
			2	8,22	N.A. To be used as a reference value
	1:10	7,25	1	7.14	0,11
			2	7.08	0,17
	1:100	6,25	1	6,19	0,06
			2	6,25	0
	1:1000	5,25	1	5,00	0,25
			2	5,06	0,19
	1:10000	4,25	1	3,86	0,39
			2	3,98	0,27

5.5.3.6 LINEARITY

To test the linearity parameter, the five samples obtained from the initial pool were analyzed in duplicate:

- Pure (undiluted)
- Diluted 1:10
- Diluted 1:100
- Diluted 1:1000
- Diluted 1:10000

The test was performed (two different titrations for each sample) by two different operators during two independent analytical sessions.

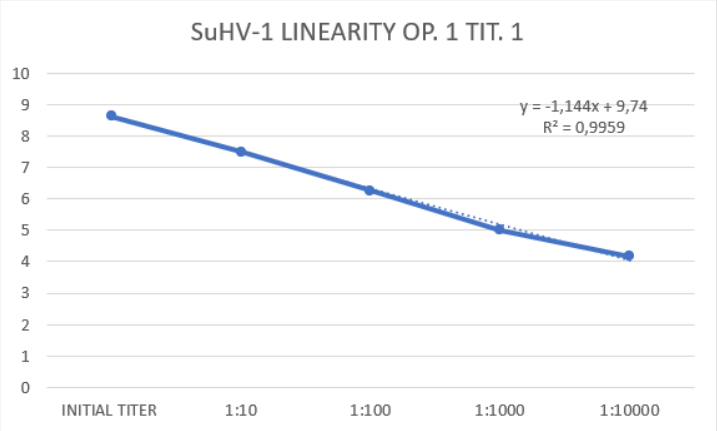
The coefficient of determination (R^2) of the line (described by the linear equation $y=ax+b$) obtained from a graph where the expected titers of the samples are represented on the x axis (INITIAL TITER (A), dilution 1:10 (B), dilution 1:100 (C), dilution 1:1000 (D), dilution 1:10000 (E)) and the titer obtained using this method on the y axis, should be greater than or equal to 0.98.

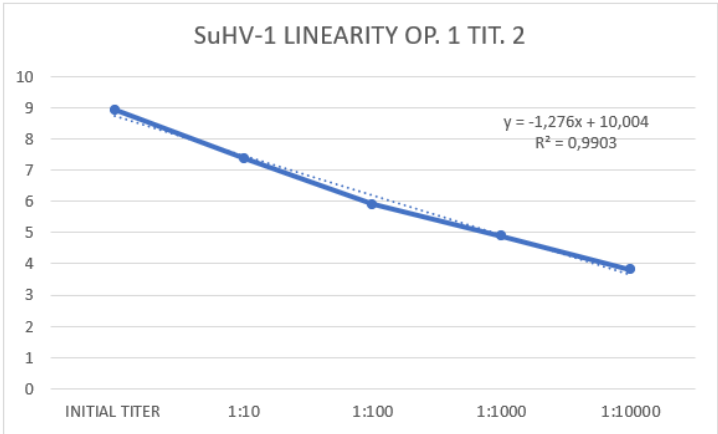
The table below show the obtained results:

LINEARITY		R^2
Operator 1	Titration 1	1.00
	Titration 2	0.99
Operator 2	Titration 1	0.99
	Titration 2	0.99

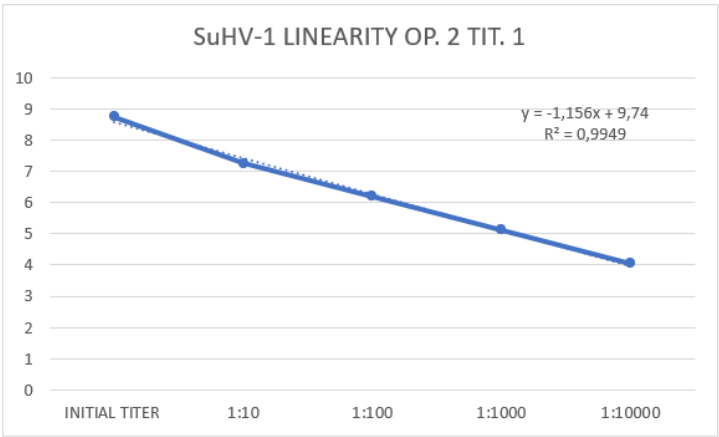
The obtained graphs are shown below:

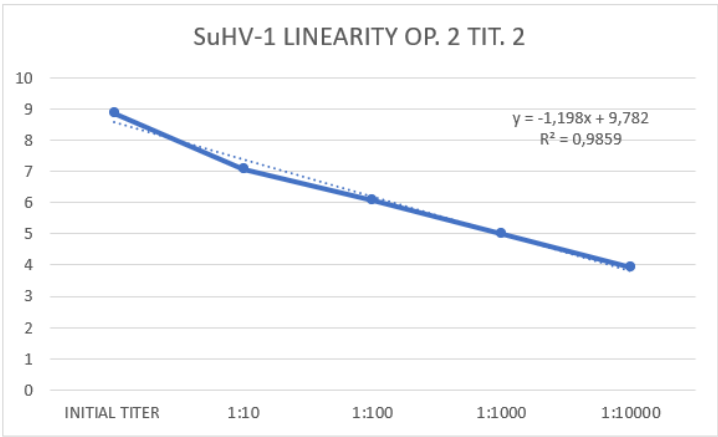
OPERATOR 1:





OPERATOR 2:





5.5.4 titration Method conclusions

The results carried out from Monte Carlo simulations, with the support provided by the JMP statistical software, differed to a maximum of 2% compared to those obtained in the simulation carried out without modifying the method parameters.

This difference is negligible.

Percentile / Parameters	2.5% (Virus Titer $Log_{10}TCID_{50}/mL$)	50% (Virus Titer $Log_{10}TCID_{50}/mL$)	97.5% (Virus Titer $Log_{10}TCID_{50}/mL$)	
Fixed	8.87	9.39	9.92	
random	8.68	9.31	9.93	
	2%	0.9%	0,1%	Difference between fixed and random

Therefore, the robustness of the method was verified within the proposed new ranges.

Cellular concentration for the titration test:

- $1.6*10^5$ cell/ml
- $2.0*10^5$ cell/ml
- $2.4*10^5$ cell/ml

Different culture medium:

- EMEM 2% FBS
- EMEM 2% FBS with antibiotic and antifungal

Different incubation times:

- 2.5 days
- 3 days
- 4 days

For Validation the precision, specificity, accuracy and linearity parameters were verified during the method validation process and were compliant with the acceptance criteria.

Following the activities performed during the validation process the method was considered Validated.

6 PROPAGATION AND PURIFICATION METHOD

The quality of the viral stocks to be used during Viral Clearance Studies depends on the method used to isolate them. Virus quality is crucial for the studies: it can have an impact on the performance of the spikes and on the repeatability, impacting the quality of the obtained results.

An ultrapure viral stock, defined as a **Virus Production Lot** (**VPL**), should have the following characteristics:

- **High titer**: which allows, by making a spike within the regulatory limits, to obtain a high starting titer. To avoid excessive dilution of purification intermediate involved, which could change its properties and compromise the applicability of the validation study.

- **High purity grade**: meaning the reduced presence of aggregates, proteins, and DNA cellular residues.

  The aggregates could compromise the results of Viral Clearance Validation studies. For example, the retention of viruses by nanofiltration could be affected; in addition, it has been shown that virus aggregates are more resistant to chemical inactivation. Before being used in the study, viral spike should therefore be appropriately pre-filtered on filters with a pore dimension depending on the size of the viral particles: pore dimension should be higher than the diameter of the virus to be filtered.

  The proteins and DNA cellular residues could also compromise the results, for same steps such nanofiltration that are extremely sensitive to impurities. Since viral stocks are prepared starting from cell cultures, the purification method used to isolate the viral stocks should be as selective as possible to avoid any impact on the performance of the process step to be assessed during the Viral Clearance Validation Study.

Viruses are infective particles; they are obligatory intracellular parasites, which means that they need a host cell for their replication since they do not have all the biochemical structures needed for replication. Viruses can infect all types of life forms, from animals and plants to microorganisms.

Virus replication may or may not lead to death of the host cell, from which new copies of the virus are formed and released.

Each virus has an elective cellular substrate for its propagation and titration, and these hosts are generally indicated on a certificate provided by their supplier.

To obtain viral stocks of REO3, HPIV3 and SuHV-1, it is necessary to proceed with a precise sequence of steps in the propagation phase and a series of purification steps aimed to reduce the content of impurities derived from the propagation process (e.g. death of host cell).

Based on literature[27] there are different steps useful to produce a VPL with the criteria mentioned before:

- **Pelleting:** this procedure successfully removes most of the amount of e.g. death of host cells in a pellet. A clarified supernatant deriving from the propagation of viruses will be obtained.
- **Ultracentrifugation** is the most critical step of the purification process, since ultracentrifuge speed determines pelleting of virus and, at the same time, could decrease virus infectivity. Consequently, it is necessary set the parameters (speed, temperature, time) based on the characteristic of the viruses (dimensions, presence of envelop or not). The virus pellet obtained could be resuspended in buffer or media.
- **Sonication** could be helpful to reduce virus aggregation. Also, in this case, the optimal conditions of sonication are virus dependent because sonication could disrupt virus membrane that lead to lost in virus infectivity and titer.
- **Filtration** may be used as a final step in the purification process to reduce aggregates and/or remove possible contaminants. The pore size is set based on the virus characteristics.

Taking into consideration these steps and based on Company procedures for other virus purification methods, different purification steps were chosen in common for the REO3, HPIV3 and SuHV-1 virus described in the scheme below (Table 1), as starting point.

Moreover, for each purification conditions applied and tested, different samples were collected.

Samples titer and purity were evaluated by titration assays and determination of the residual protein contents.

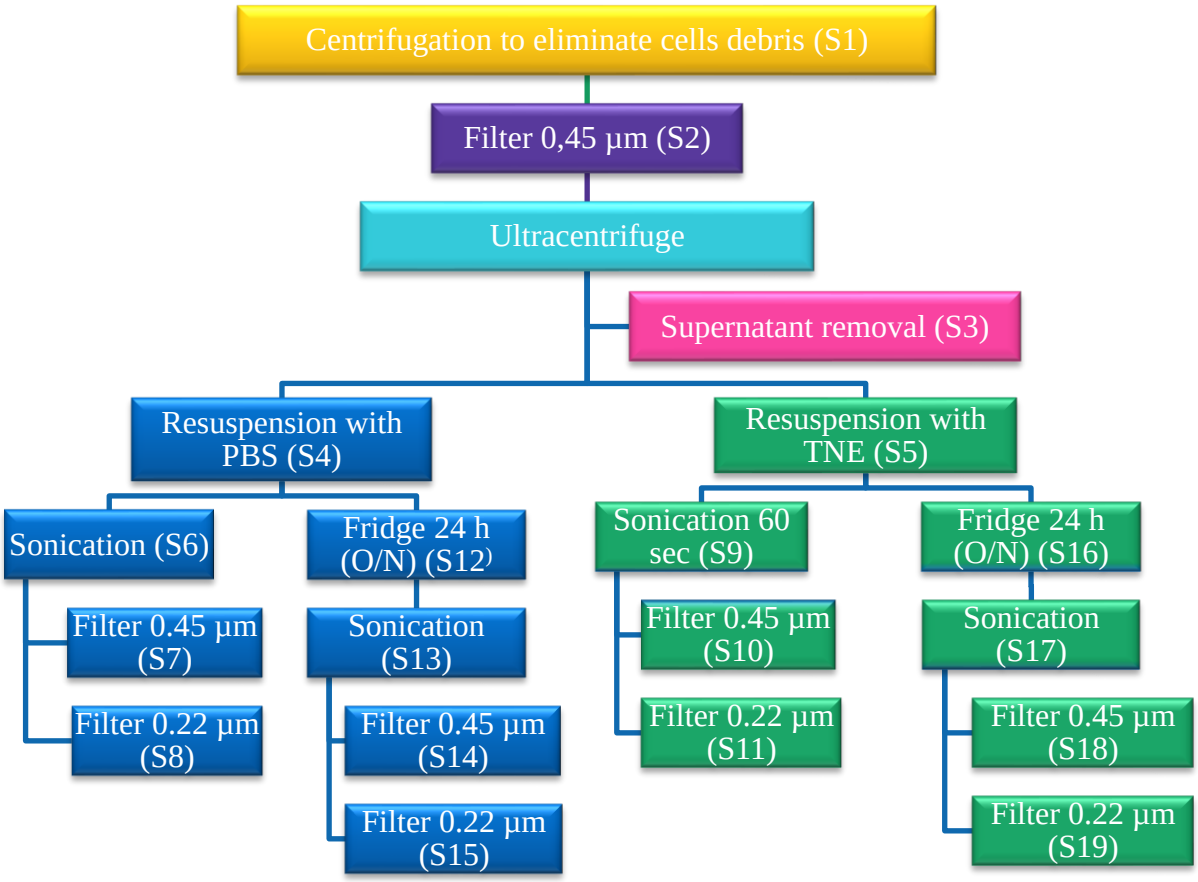


Table 1

Initial purification steps for REO3, HPIV3 and SuHV-1 and the schematic name of the samples.

As described above samples were collected for each step condition of tested purification (see Table 1).

After the first centrifuge performed for clarification (S1), all supernatants were collected together and it was made a homogenous solution, the pool obtained was filtered on 0.45 µm Polyethersulfone (PES) membrane filter to remove any cellular residue still present from the propagation (S2).

Therefore, the filtered pool was then aliquoted into ultracentrifuge tubes using a syringe. Tubes need to be paired and weight balanced each other because of ultracentrifuge weight sensitivity.

Then the tubes should be placed in the rotor in the ultracentrifuge and left to run.

At the end of each ultracentrifuge run, tubes were opened, supernatant was removed and collected in cryovials (S3).

The pellet obtained was resuspended with PBS or TNE buffer.

Initially a very small volume should be used to resuspend the pellet, to disrupt the aggregates as much as possible. Then, the remaining PBS or TNE volume was slowly added to the suspension to achieve the total volume needed.

The resuspended solution in PBS and TNE was split into:

- viral pool resuspended in PBS (S4);
- viral pool resuspended in PBS O/N;
- viral pool resuspended in TNE (S5);
- viral pool resuspended in TNE O/N.

Where "O/N" stands for "Over Night", indicating that these two aliquots were kept in refrigerator at 4.0 °C for the night. This samples were collected the day after and proceeded with the other purification steps.

The two viral pool resuspended in PBS or TNE were sonicated in an ultrasound water bath. This process generated other 2 different samples: viral pool resuspended in PBS and sonicated (S6) and viral pool resuspended in TNE and sonicated (S9).

Each of them, S6 and S9, was split again and filtered with or a 0.22 µm or a 0.45 µm PES membrane filter to remove any viral aggregates and cellular debris.

The following samples were obtained:

- viral pool resuspended in PBS, sonicated and filtered on 0.45 µm filter (S7);
- viral pool resuspended in PBS, sonicated and filtered on 0.22 µm filter (S8);
- viral pool resuspended in TNE, sonicated and filtered on 0.45 µm filter (S10);
- viral pool resuspended in TNE, sonicated and filtered on 0.22 µm filter (S11).

The obtained samples were dispensed in cryovials and stored at -80°C.

The day after the ultracentrifuge the two O/N samples were taken: the viral pool resuspended in PBS O/N (S12) and viral pool resuspended in TNE O/N (S16).

The two viral pool resuspended in PBS O/N or TNE O/N were sonicated in an ultrasound water bath. This process generated other 2 different samples: viral pool

resuspended in PBS and sonicated (S13) and viral pool resuspended in TNE and sonicated (S17).

Each of them, S12 and S16, was split again and filtered with or a 0.22 µm or a 0.45 µm PES membrane filter to remove any viral aggregates and cellular debris.

The following samples were obtained:

- viral pool resuspended in PBS O/N, sonicated and filtered on 0.45 µm filter (S14);
- viral pool resuspended in PBS O/N, sonicated and filtered on 0.22 µm filter (S15);
- viral pool resuspended in TNE O/N, sonicated and filtered on 0.45 µm filter (S18);
- viral pool resuspended in TNE O/N, sonicated and filtered on 0.22 µm filter (S19).

The obtained samples were dispensed in cryovials and stored at -80°C.

The Methods for the preparation of viral stocks of REO3, HPIV3 and SuHV-1 are described below.

6.1 REO3

6.1.1 Propagation

The original vial of REO3, purchased from ATCC, should be propagated on the host cell LLC-MK2 DERIVATIVE (recommended by Supplier[7]) to prepare the MVB (Master Virus Bank), while an MVB vial of REO3 should be propagated on LLC-MK2 DERIVATIVE cells for the preparation of the WVB (Working Virus Bank), as described below.

Defrost a vial of LLC-MK2 DERIVATIVE cells in a sterile flask and keep them cultured using EMEM 10% FBS culture medium.

The day before the infection, detach the cells from 100% confluent flasks and plate them. Leave to incubate for about 24 hours at 37.0 °C in 5.0 % CO_2.

On the day of the infection, observe the cell monolayer and ensure it has reached 80-90% confluence, then proceed with the infection[7].

Defrost a vial of REO3 virus (original or MVB) at 37.0 °C and bring it to a proper volume using EMEM 0% FBS culture medium.

Aspirate the culture medium from the culture flasks of LLC-MK2 DERIVATIVE cells, wash the monolayer with PBS and inoculate each flask with the proper volume of the prepared viral suspension.

Incubate the infected flasks in an incubator set at 37.0 °C and 5.0 % CO_2 for the inoculation time.

After the inoculation time the onset of incubation, add EMEM 2% FBS culture medium to each flask and incubate again in the incubator set at 37.0 °C and 5.0 % CO_2 until complete cytopathic effect on the cell monolayer.

From ATCC, the effect on host could be CPE, refractile rounding and cell sloughing.

Until complete cytopathic effect on the cell monolayer, collect the supernatant from each flask and centrifuge to eliminate any cellular residue. Collect together all supernatants and mix with a serological pipette to make a homogenous suspension.

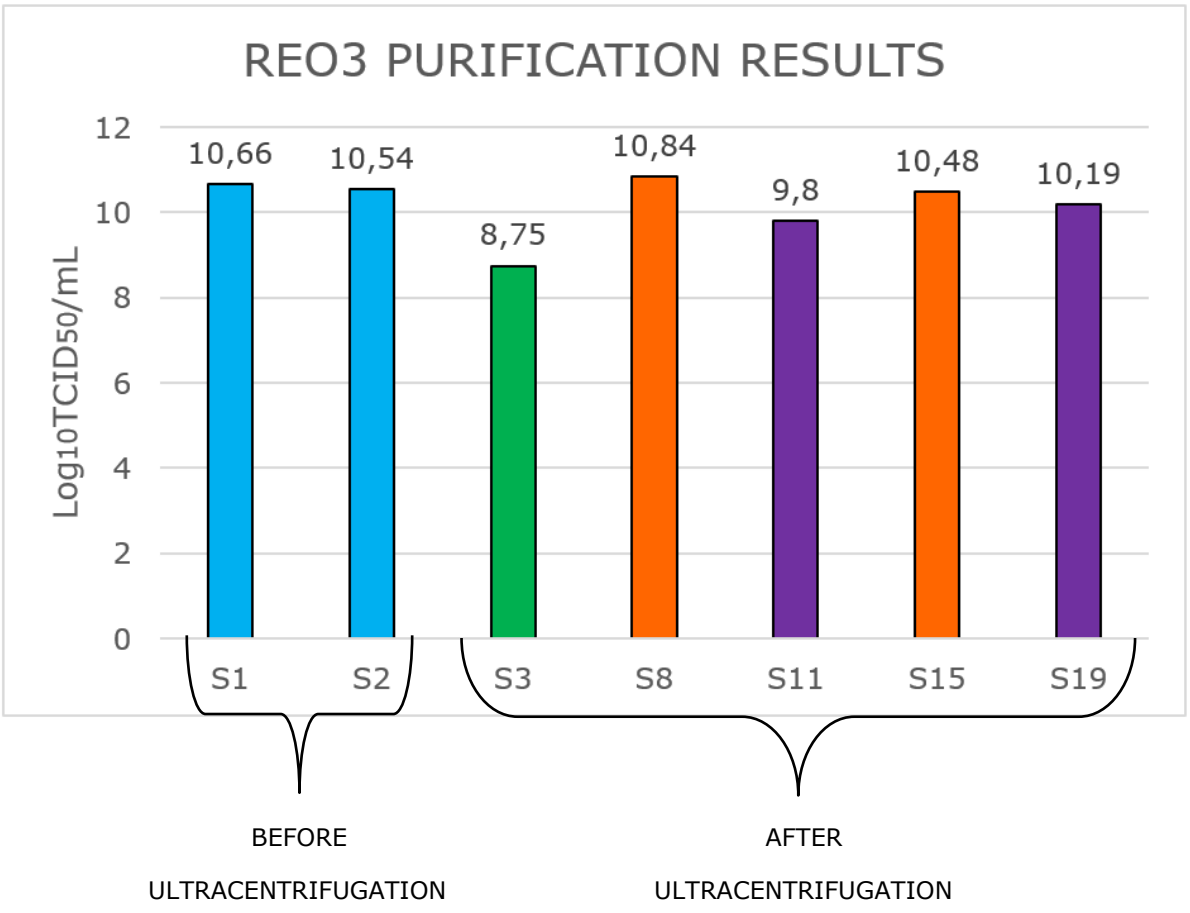
For the preparation of MVB or WVB distribute the solution into cryovials and store them in the freezer at -80 °C.

6.1.2 Purification

Firstly, for REO3 VPL preparation were applied the steps of purification describe in the table 1.

The same method (e.g. speed and time) has been applied, based on Company procedures, for other virus purification that have in common the absence of envelopes such as REO3 virus.

The first ultracentrifugation runs gave the desired results (Graph. 1), for this reason different runs were made to confirm the results obtained before.

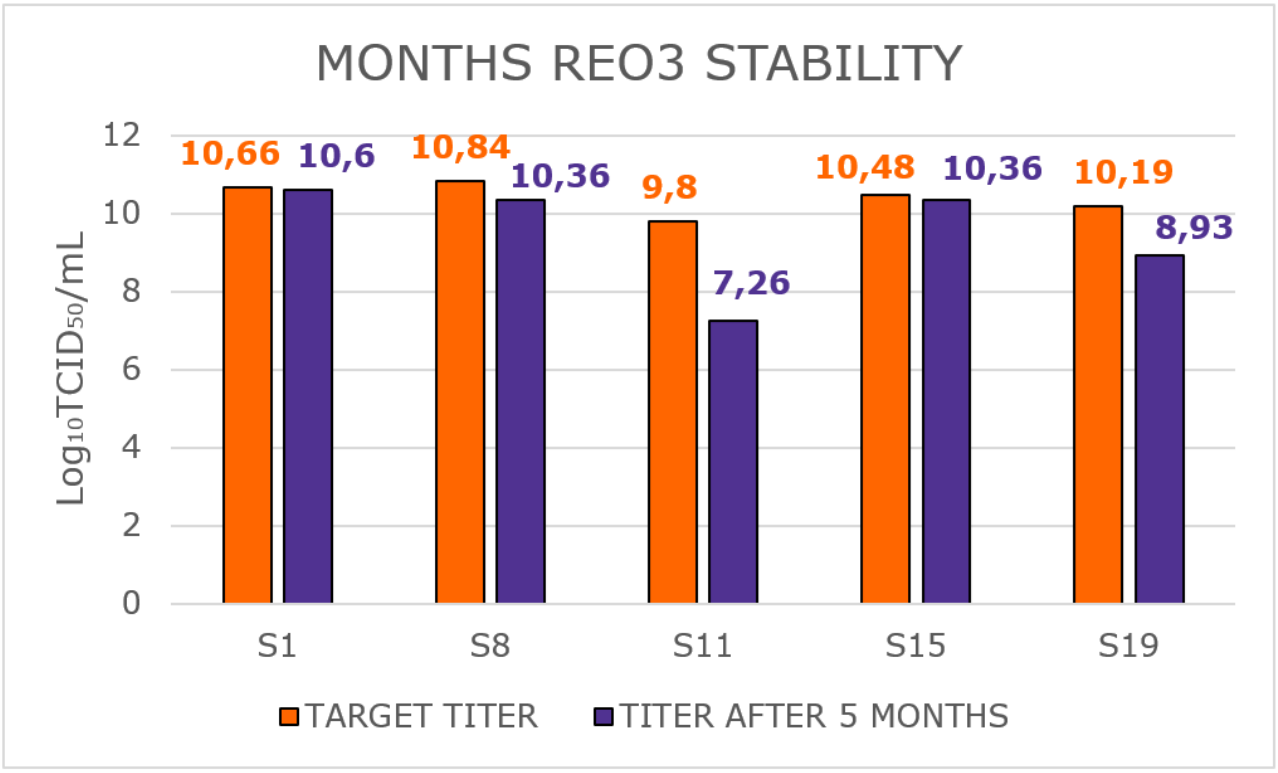


Graph. 1

S1= after first centrifuge of clarification
S2= pool filtered on 0.45 µm filter
S3= supernatant after ultracentrifugation
S8= viral pool resuspended in PBS, sonicated and filtered on 0.22 µm filter
S11= viral pool resuspended in TNE, sonicated and filtered on 0.22 µm filter
S15= viral pool resuspended in PBS O/N, sonicated and filtered on 0.22 µm filter
S19= viral pool resuspended in TNE O/N, sonicated and filtered on 0.22 µm filter

6.1.2.1 stability data

After five months, the most significant samples obtained from the first runs were titrated again to have the stability long term storage data (Graph. 2) of the REO3 virus resuspended in the PBS and TNE buffers at -80 ° C.



Graph. 2

S1= after first centrifuge of clarification

S8= viral pool resuspended in PBS, sonicated and filtered on 0.22 µm filter

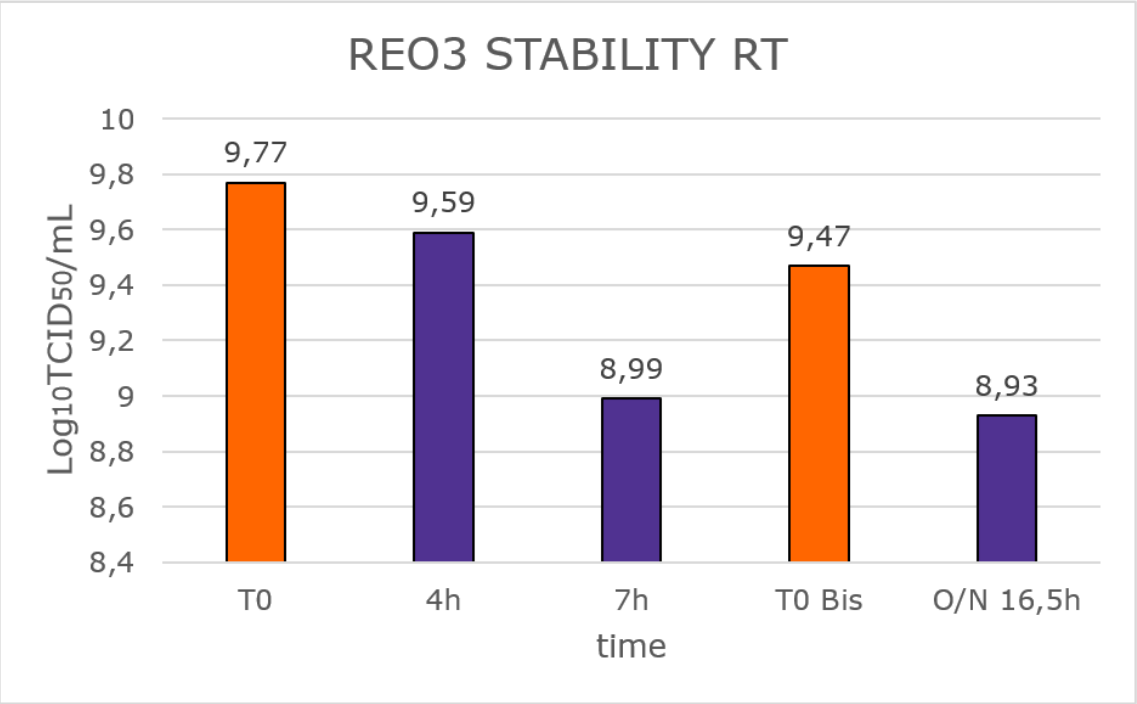
S11= viral pool resuspended in TNE, sonicated and filtered on 0.22 µm filter

S15= viral pool resuspended in PBS O/N, sonicated and filtered on 0.22 µm filter

S19= viral pool resuspended in TNE O/N, sonicated and filtered on 0.22 µm filter

In addition to the stability test listed above, since there are steps in a viral clearance study that can last several hours, it was necessary to carry out stability tests (Graph. 3) at room temperature at different time points to evaluate the virus titers.

The virus should not lose its titer, beyond acceptance criteria of ± 1.0 $Log_{10}TCID_{50}$/mL, from the moment of defrost, at the start of the phase, until the end of the experimental session.



Graph. 3

T0= defrost and filtration of the REO3 virus and titration. After the titration the virus was left at RT (room temperature).

4h= titration of REO3 virus after 4h from defrost

7h= titration of REO3 virus after 7h from defrost

T0 Bis = second defrost and filtration of the REO3 virus and titration. After the titration the virus was leave at RT (room temperature).

16.5h= titration of REO3 virus after 16.5h from defrost.

6.1.3 propagation and purification conclusion

Based on the results carried out from titration and protein content determination the purification steps to produce high titer and purified REO3 stocks (REO3 VPL) were selected and describe below (Table 2).

As resuspension buffer, the choice was the PBS, confirmed by the stability data, it demonstrated a better cryopreservation and higher titer compared to TNE buffer.

For the filtration step both the 0.45 µm and 0.22 µm filter showed a similar titer, therefore the selected option was to adopt 0.22 µm filter, because it assured a purer sample.

The O/N did not show to impact enough pellet resuspension and viral titer. For this reason, to speed up purification process, the overnight period was avoided.

Finally, the BCA results (not included) confirmed the resuspended sample in PBS, sonicated and filtered on 0.22 µm pore membrane, as a suitable sample to be used in Viral Clearance Studies, due to its low residual protein content.

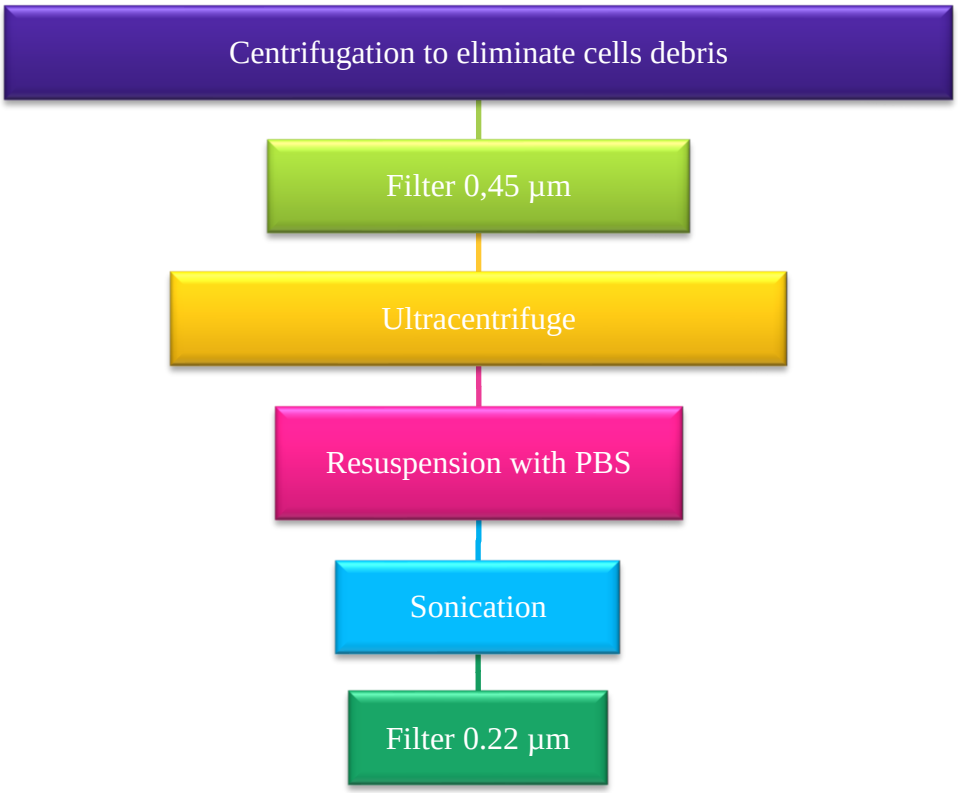


Table 2

Purification steps for REO3.

6.2 SuHV-1

6.2.1 Propagation

Two different approaches were carried out for the propagation of SuHV-1 virus.

Firstly, the original vial of SuHV-1 should be propagated on the host cell MDBK (NBL-1) to prepare the MVB.

Defrost a vial of MDBK (NBL-1) cells in a sterile flask and keep them cultured using EMEM 10% HS culture medium.

The day prior to the infection, detach the cells from 100% confluent flasks and plate them. Leave to incubate for about 24 hours at 37.0 °C in 5.0 % CO_2.

On the day of the infection, observe the cell monolayer and ensure it has reached 80-90% confluence, then proceed with the infection.

Defrost a vial of SuHV-1 virus (original) at 37.0 °C and bring it to a proper volume using EMEM 0% HS culture medium.

Aspirate the culture medium from the culture flasks of MDBK (NBL-1) cells, wash the monolayer with PBS and inoculate each flask with the proper volume of the prepared viral suspension.

Incubate the infected flasks in an incubator set at 37.0 °C and 5.0 % CO_2 for the inoculation time.

After the inoculation time the onset of incubation, add EMEM 2% HS culture medium to each flask and incubate again in the incubator set at 37.0 °C and 5.0 % CO_2 until complete cytopathic effect on the cell monolayer.

The effect on host could be CPE, refractile rounding, clumping and cell sloughing.

Until complete cytopathic effect on the cell monolayer, collect the supernatant from each flask and centrifuge to eliminate any cellular residue. Combine all supernatants and mix with a serological pipette to make a homogenous solution.

For the preparation of MVB distribute the solution into cryovials and store them in the freezer at -80 °C.

Secondly based on the preliminary results of virus purification the propagation for the WVB was changed because it is necessary to have a higher titer than the MVB.

A comparison was made with ST cells (another host cell for SuHV-1 virus).

The ST and MDBK (NBL-1) cells were tested both for propagation with different

amount of virus, then propagation of MVB, on cells split the day before and with the MOI (Multiplicy Of Infection) calculation.

MOI means the ratio between the number of viral particles for infection with respect to the number of cells to be infected.

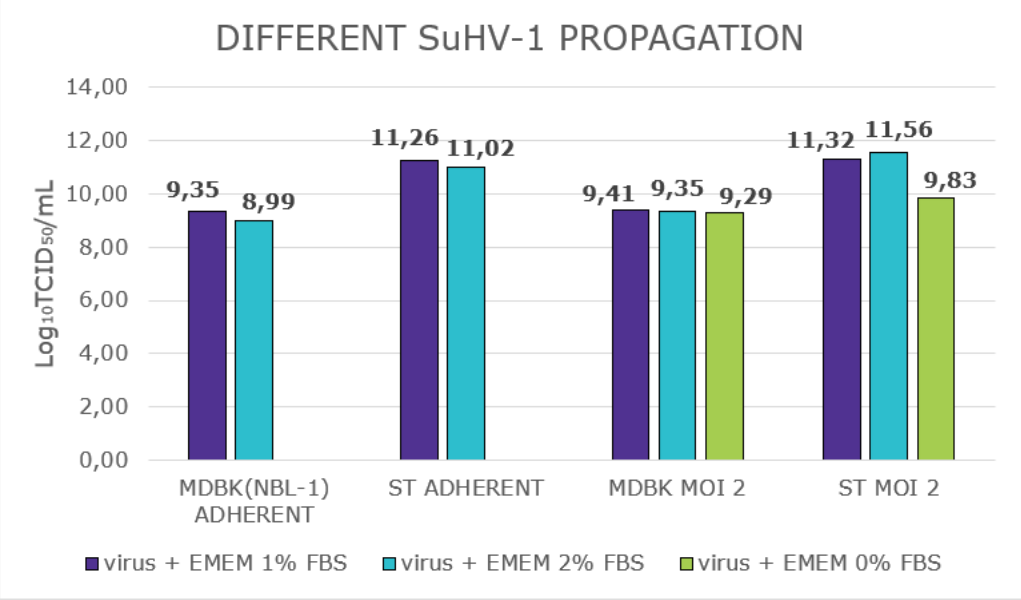
The MOI value defined for the propagation of SuHV-1 was 2 and to calculate the volume necessary to infect the cell suspension obtained is described below:

$$TCID_{50} \text{ Initial} : \text{Initial Volume} = TCID_{50} \text{ Final} : \text{Final Volume (x)}$$

$TCID_{50}$ INITIAL= this is the titer of the MVB vial used
$TCID_{50}$ FINAL = total cells in each flask * 2 (MOI)
INITIAL VOLUME = 1 mL (this is the volume inside the chosen vial)

Moreover, the comparison was made with different concentration of FBS in the media, EMEM 0%, 1% and 2% FBS.

Below, in the Graph.4, the results of the different propagation performed.



Graph.4

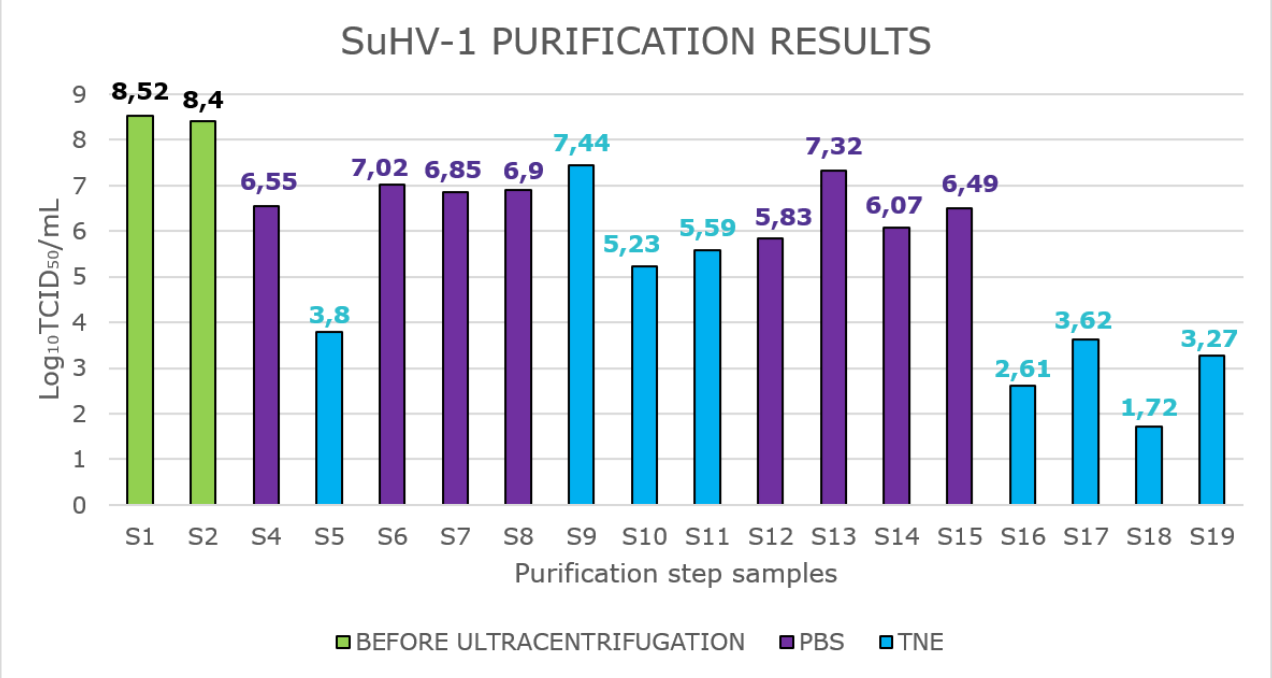
Different SuHV-1 propagations.

For the preparation of VPL the step of purification describes below (par.6.2.2) should be followed.

6.2.2 Purification

Firstly, for SuHV-1 VPL preparation were applied the steps of purification described in the table 1.

The same method (e.g. speed and time) was applied, based on Company procedures, for other virus purification that have in common envelope such as SuHV-1 virus (Graph.5).



Graph. 5

S1= after first centrifuge of clarification

S2= pool filtered on 0.45 µm filter

S4= viral pool resuspended in PBS

S5= viral pool resuspended in TNE

S6= viral pool resuspended in PBS, sonicated

S7= viral pool resuspended in PBS, sonicated and filtered on 0.45 µm filter

S8= viral pool resuspended in PBS, sonicated and filtered on 0.22 µm filter

S9= viral pool resuspended in TNE, sonicated

S10= viral pool resuspended in TNE, sonicated and filtered on 0.45 µm filter

S11= viral pool resuspended in TNE, sonicated and filtered on 0.22 µm filter

S12= viral pool resuspended in PBS O/N

S13= viral pool resuspended in PBS O/N, sonicated

S14= viral pool resuspended in PBS O/N, sonicated and filtered on 0.45 µm filter

S15= viral pool resuspended in PBS O/N, sonicated and filtered on 0.22 µm filter

S16= viral pool resuspended in TNE O/N

S17= viral pool resuspended in TNE O/N, sonicated

S18= viral pool resuspended in TNE O/N, sonicated and filtered on 0.45 µm filter

S19= viral pool resuspended in TNE O/N, sonicated and filtered on 0.22 µm filter

First ultracentrifugation runs didn't provide the desired results (Graph.5), for this reason different runs were made to obtain other results.

Different parameters of ultracentrifugation were tested: speed (rpm), time and buffer in several days to obtain the best method to purify the SuHV-1 virus.

The runs didn't provide the desired results because the results reflected a good purification method, but the final titer was always at the limit of the acceptance criteria for viral clearance studies, e.g. steps nanofiltration.

For these reasons it was necessary to change to starting point, having an higher titer of virus.

Based on the results from the different SuHV-1 propagation tests (par.6.2.1) was chosen the propagation with ST cells with EMEM 2% FBS and MOI of 2, before the purification step.

After this propagation, the purification was performed applying the better parameters of ultracentrifugation tested before.

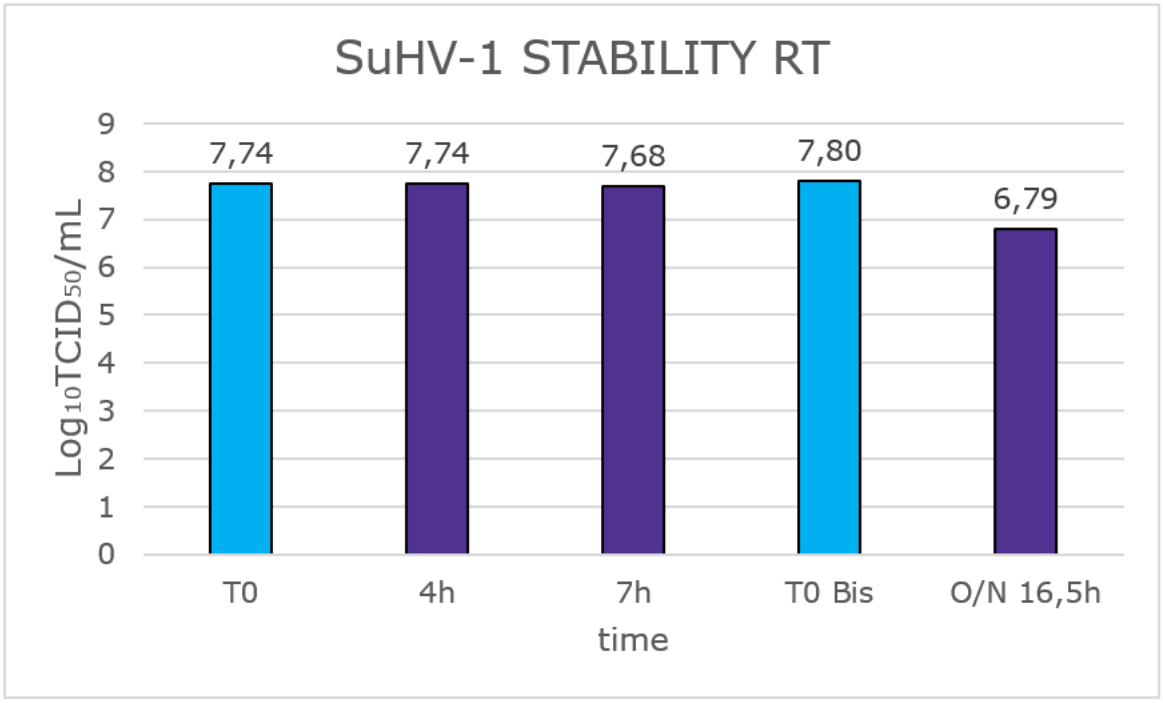
The final titer obtained for SuHV-1 was 9.53 ± 0,5 $Log_{10}TCID_{50}$/mL that was in acceptance criteria for viral clearance studies.

6.2.2.1 stability data

After six and seven months, the most significant samples obtained from the first run were titrated again to have the stability long term storage data (Graph. 6) of the virus resuspended in the PBS and TNE buffers at -80 ° C.

In addition to the stability test listed above, since there are steps in a viral clearance study that can last several hours, it was necessary to carry out stability tests (Graph. 7) at room temperature at different time points to evaluate the virus titer.

The virus should not lose its titer, beyond acceptance criteria ± 1.0 $Log_{10}TCID_{50}$/mL, from the moment of defrost, at the start of the phase, until the end of the experimental session.



Graph. 7

T0= defrost, sonication and filtration of the SuHV-1 virus and titration. After the titration the virus was leave at RT (room temperature).

4h= sonication and titration of SuHV-1 virus after 4h from defrost

7h= sonication and titration of SuHV-1 virus after 7h from defrost

T0 Bis = second defrost, sonication and filtration of the SuHV-1 virus and titration. After the titration the virus was left at RT (room temperature).

16.5h= sonication and titration of SuHV-1 virus after 16.5h from defrost

6.2.3 Propagation and purification conclusion

Based on the results carried out from titration and protein content determination the purification steps to produce high titer and purified SuHV-1 stocks (SuHV-1 VPL) were selected and describe below (Table 3).

Before the purification step was necessary the propagation of SuHV-1 on ST cells with the EMEM 2% FBS media and a MOI of 2.

As resuspension buffer, the choice was the TNE, confirmed by the stability data, it demonstrated a better cryopreservation and high titer compared to PBS buffer.

For the filtration step both the 0.45 µm and 0.22 µm filter showed a similar titer, therefore the selected option was adopted 0.22 µm filter, because it assured a purer sample.

The O/N did not show to impact enough pellet resuspension and viral titer. For this reason, to speed up purification process, the overnight period was avoided.

Finally, the BCA results (not included) confirmed the resuspended sample in TNE, sonicated and filtered on 0.22 µm pore membrane, as a suitable sample to be used in Viral Clearance Studies, due to its low residual protein content.

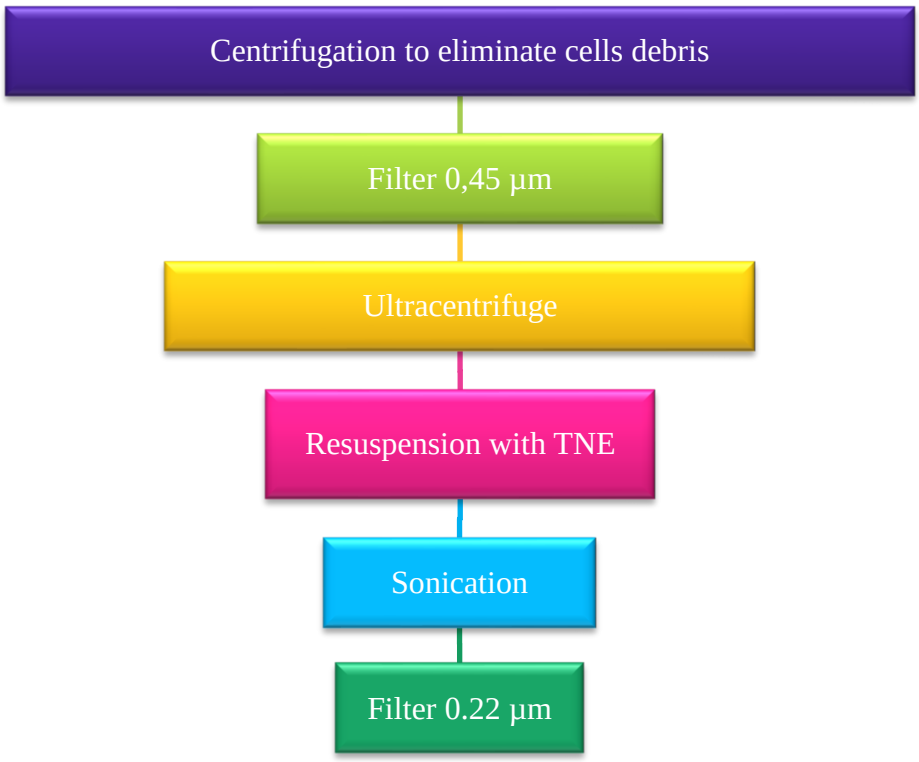


Table 3

Purification steps for SuHV-1.

6.3 HPIV3

6.3.1 propagation

The original vial of HPIV3, purchased from ATCC, should be propagated on the host cell LLC-MK2 DERIVATIVE (recommended by supplier[28]) to prepare the MVB, while an MVB vial of REO3 should be propagated on LLC-MK2 DERIVATIVE cells for the preparation of the WVB, as described below.

Defrost a vial of LLC-MK2 DERIVATIVE cells in a sterile flask and keep them cultured using EMEM 10% FBS culture medium.

The day before the infection, detach the cells from 100% confluent flasks and plate them. Leave to incubate for about 24 hours at 37.0 °C in 5.0 % CO_2.

On the day of the infection, observe the cell monolayer and ensure it has reached 80-90% confluence, then proceed with the infectio[28].

Defrost a vial of HPIV3 virus (original or MVB) at 37.0 °C and bring it to a proper volume using EMEM 0% FBS culture medium.

Aspirate the culture medium from the culture flasks of LLC-MK2 DERIVATIVE cells, wash the monolayer with PBS and inoculate each flask with the proper volume of the prepared viral suspension.

Incubate the infected flasks in an incubator set at 37.0 °C and 5.0 % CO_2 for the inoculation time.

After the inoculation time the onset of incubation, add EMEM 2% FBS culture medium to each flask and incubate again in the incubator set at 37.0 °C and 5.0 % CO_2 until complete cytopathic effect on the cell monolayer.

From ATCC, the effect on host could be CPE, refractile rounding, clumping and cell sloughing.

Until complete cytopathic effect on the cell monolayer, collect the supernatant from each flask and centrifuge to eliminate any cellular residue. Collect together all supernatants and mix with a serological pipette to make a homogenous solution.

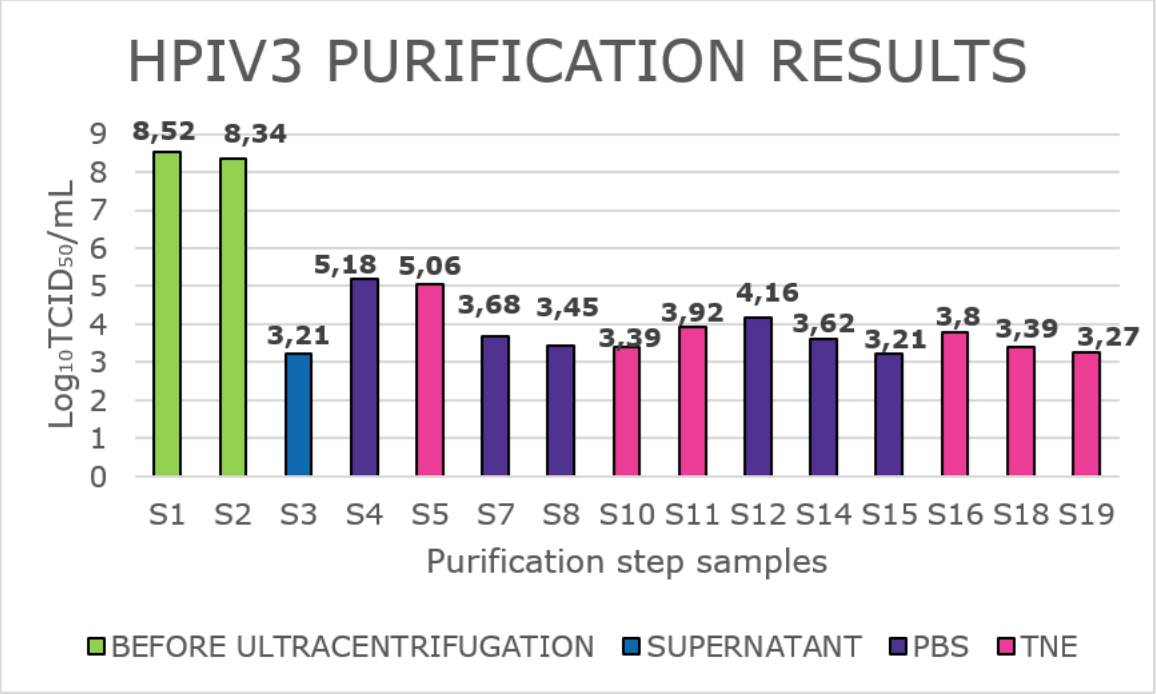
For the preparation of MVB or WVB distribute the solution into cryovials and place them in the freezer at -80 °C.

For the preparation of VPL follow the step of purification describe below (par. 6.3.2).

6.3.2 purification

Firstly, for HPIV3 VPL preparation were applied the steps of purification describe in the table 1.

The same method (e.g. speed and time) was applied, based on Company procedures, for other virus purification that have in common envelope such as HPIV3 virus (Graph.8).



Graph. 8

S1= after first centrifuge of clarification

S2= pool filtered on 0.45 µm filter

S3= supernatant

S4= viral pool resuspended in PBS

S5= viral pool resuspended in TNE

S7= viral pool resuspended in PBS, sonicated and filtered on 0.45 µm filter

S8= viral pool resuspended in PBS, sonicated and filtered on 0.22 µm filter

S10= viral pool resuspended in TNE, sonicated and filtered on 0.45 µm filter

S11= viral pool resuspended in TNE, sonicated and filtered on 0.22 µm filter

S12= viral pool resuspended in PBS O/N

S14= viral pool resuspended in PBS O/N, sonicated and filtered on 0.45 µm filter

S15= viral pool resuspended in PBS O/N, sonicated and filtered on 0.22 µm filter

S16= viral pool resuspended in TNE O/N

S18= viral pool resuspended in TNE O/N, sonicated and filtered on 0.45 µm filter

S19= viral pool resuspended in TNE O/N, sonicated and filtered on 0.22 µm filter

First ultracentrifugation run didn't provide the desired results (Graph.8), for this reason different runs were made to obtained additional results.

Different parameters of ultracentrifugation were tested: speed (rpm) and time in several days to perform the best method to purify the HPIV3 virus.

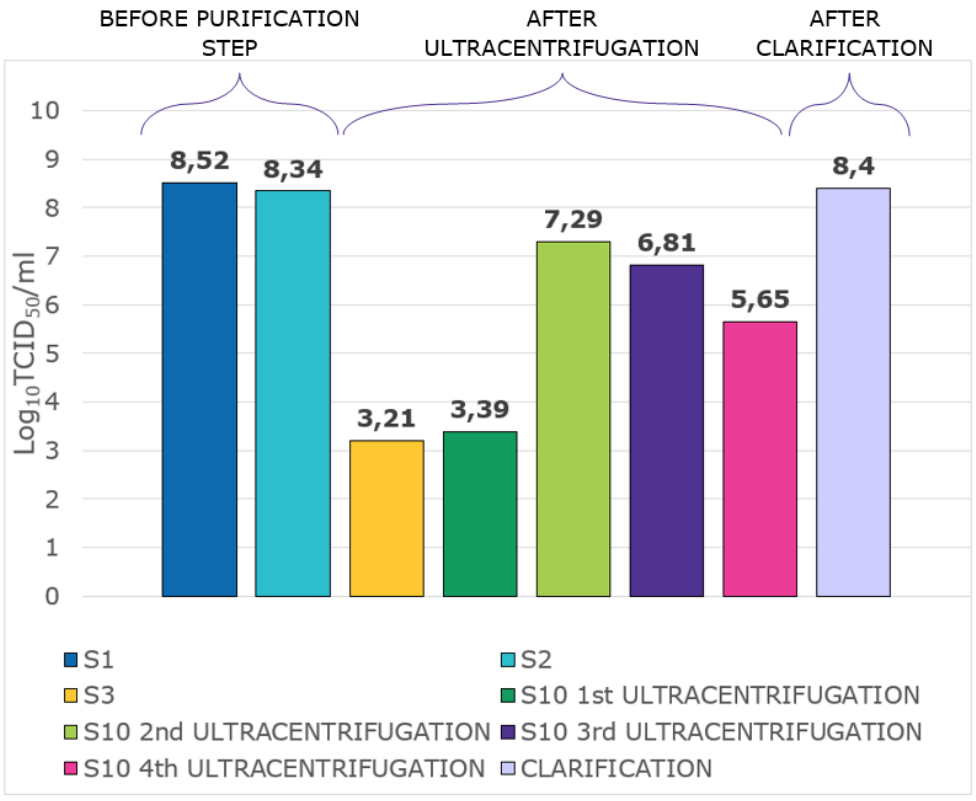
Therefore, the runs didn't provide the desired results.

Also, at low speed the run didn't reach good results of virus titer, as if the ultracentrifugation step led to lost in viral infectivity.

For these reasons it was necessary to change completely the method.

Another method, described below, to purified HPIV3 virus is the clarification step.

Virus clarification was performed instead of ultracentrifugation, and then the better parameters tested before were applied in terms of sonication, TNE buffer and 0.45 µm filter.



Graph.9

Comparison of different purification HPIV3 tests.

S1= after first centrifuge of clarification

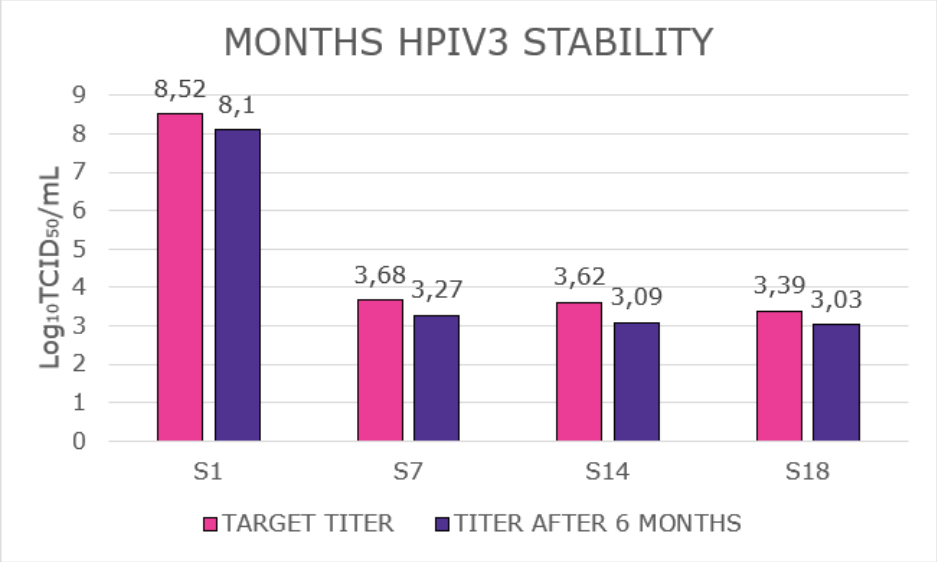
S2= pool filtered on 0.45 µm filter

S3= supernatant

S10= viral pool resuspended in TNE, sonicated and filtered on 0.45 µm filter

6.3.2.1 stability data

After six months, the most significant samples obtained from the first runs were titrated again to have the stability long term storage data (Graph. 10) of the virus resuspended in the PBS and TNE buffers at -80 ° C.



Graph. 10

S1= after first centrifuge of clarification

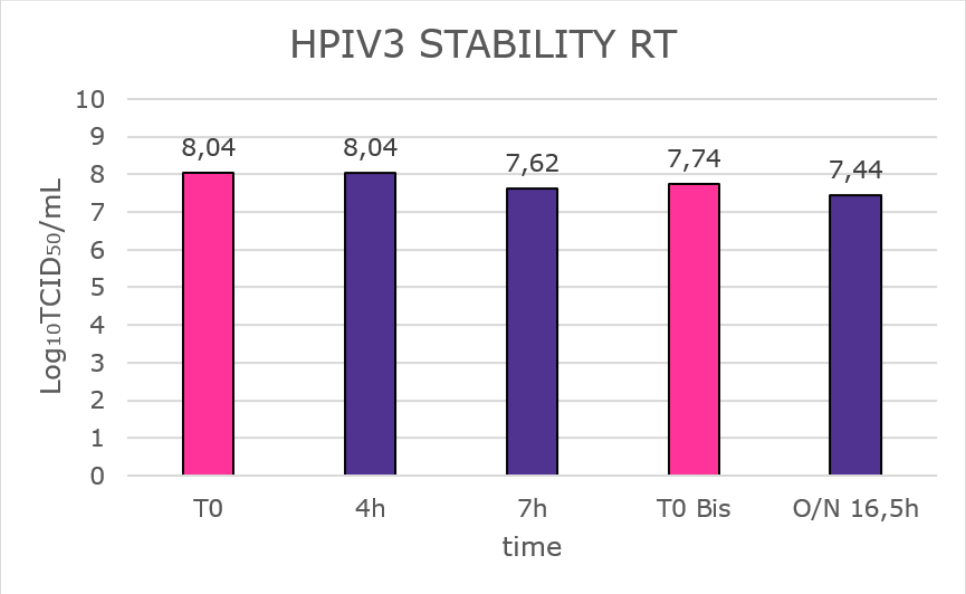
S7= viral pool resuspended in PBS, sonicated and filtered on 0.45 µm filter

S14= viral pool resuspended in PBS O/N, sonicated and filtered on 0.45 µm filter

S18= viral pool resuspended in TNE O/N, sonicated and filtered on 0.45 µm filter

In addition to the stability test listed above, since there are steps in a viral clearance study that can last several hours, it was necessary to carry out stability tests (Graph. 11) at room temperature in different time points to evaluate the virus titer.

The virus should not lose its titer, beyond acceptance criteria ± 1.0 $Log_{10}TCID_{50}$/mL, from the moment of defrost, at the start of the phase, until the end of the study.



Graph.11

T0= defrost and filtration of the HPIV3 virus and titration. After the titration the virus was leave at RT (room temperature).

4h= titration of HPIV3 virus after 4h from defrost

7h= titration of HPIV3 virus after 7h from defrost

T0 Bis = second defrost and filtration of the HPIV3 virus and titration. After the titration the virus was left at RT (room temperature).

16.5h= titration of HPIV3 virus after 16.5h from defrost

6.3.3 propagation and purification conclusion

Based on the results carried out from titration and protein content determination the purification steps to produce high titer and purified HPIV3 stocks (HPIV3 VPL) were selected and describe below (Table 4).

For the purification method was necessary to perform a clarification step.

As resuspension buffer, the choice was the TNE, confirmed by the stability titrations, it demonstrated a better cryopreservation and higher titer compared to PBS buffer.

For the filtration step, it was decided to adopt 0.45 µm filter, based on the size of the virus.

Finally, the BCA results (not included) confirmed the resuspended sample in TNE, sonicated and filtered on 0.45 µm pore membrane, as a suitable sample to be used in Viral Clearance Studies, due to its low residual protein content.

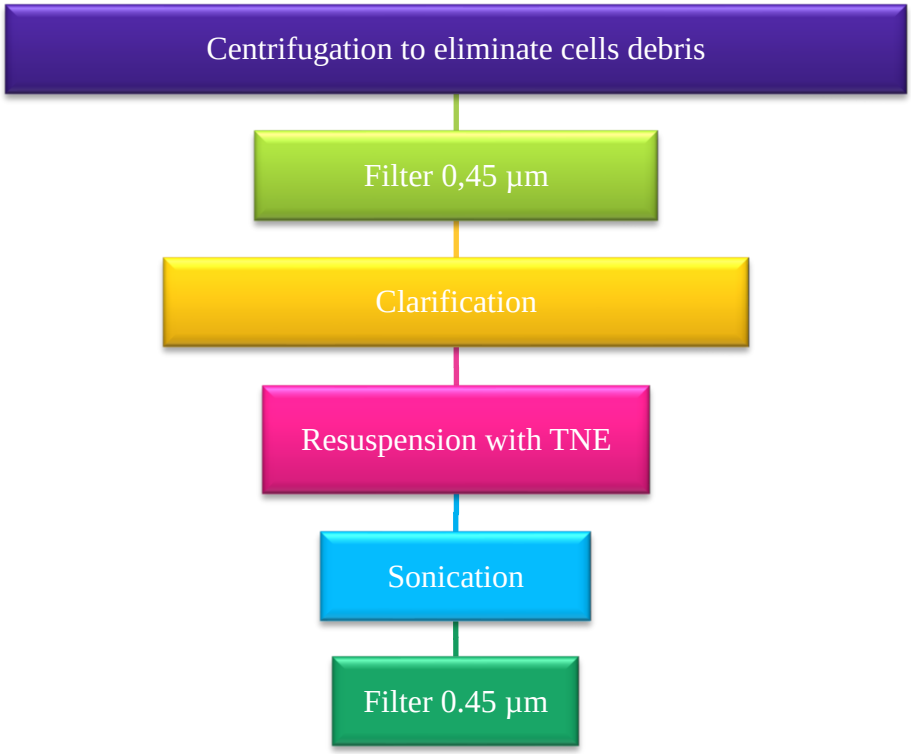


Table 4

Purification steps for HPIV3.

7 CONCLUSIONS

A Viral Clearance Study should evaluate the capability of the overall purification process to remove or inactivate a broad spectrum of virus types, including viruses that are known to contaminate or have the potential to contaminate the raw materials, and those that can be introduced during manufacturing[2].

The output of the Viral Clearance Validation Studies, as described previously, is the LRF, that is determined in a logarithmic scale, by calculating the virus titer in the loading and in the product fraction after the purification step.

To reach this output, validated viral titration methods are needed for the different tested viruses.

The first aim of PhD project was on viral titration methods development and validation for three viruses: REO3, SuHV-1 and HPIV3, according to ICH-Q2 (R1) and GMP.

Moreover, to performed Viral Clearance Validation Studies high titer and high purified virus stocks are needed.

For this reason, the second aim of PhD project was on viral propagation and purification methods development for the three viruses: REO3, SuHV-1 and HPIV3.

Following the activities performed during the PhD project, the tested titration methods for REO3, SuHV-1 and HPIV3 can be considered in a validated state with respect to the considered parameters and the relative acceptance criteria.

In addition, the propagation and purification methods carried out for REO3, SuHV-1 and HPIV3 can be considered efficient for reaching high titer and pure viral stocks.

These methods will be used during Viral Clearance Studies in the Merck RMB company in Ivrea.

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