| ORBIVIRUS ISOLATION IN CELL CULTURE | | |
|---|---|---|
| *Date: 22/06/2021* | Document code: GL-LCV-12 | *Rev. 02* |

## 1. SCOPE

To describe the isolation of Orbivirus (African horse sickness virus, Bluetongue virus and Epizootic haemorrhagic disease virus) in cell culture from clinical samples of animals.

This procedure is applicable to samples obtained accordingly to the PROCEDURE 1 (SAMPLE PREPARATION) from:

-Whole blood collected on anticoagulant (EDTA)

-Tissue samples, specially spleen, lung and lymph nodes

It is also applicable to samples obtained accordingly PROCEDURE 1 (SAMPLE PREPARATION) from tissue samples from embryonated chicken eggs inoculated with the samples described above.

## 2. MATERIALS AND EQUIPMENT

Material and reagents

Cell culture surface (24well plates or 12,5cm$^2$ flask)

Plasticware (sterile): 1,5 ml conical Eppendorf and tubes of 10 and 50 ml

Disposable pipette sterile tips with filter (100, 200 and 1000 µl range)

EMEM: Eagle's Minimum Essential Medium with Earle's balanced salt solution

Schneider's Drosophila Medium, Modified

L-Glutamine

Antibiotic-antimicotic

Non-essential amino acids

Containers for dispensing reagents

Container biosanitary waste

Disinfectant

Inactivated bovine foetal serum (IBFS).

Positive control: stock Virus, previously growth and full titrated



Ctra. M-106 Km 1,4
28110 - ALGETE (MADRID). SPAIN
Phone: +34 *91* 347 8312 / 9270 Fax: +34 91 347 3778
bzn-eurl@mapa.es

| ORBIVIRUS ISOLATION IN CELL CULTURE | | |
|---|---|---|
| *Date: 22/06/2021* | Document code: GL-LCV-12 | *Rev. 02* |

Vero, BHK or KC cells in suspension (prepared removing the attached cells from a flasks using trypsin and resuspending the cells in an appropriate amount of diluent)

Equipment

Laminar Flow Cabin Type II

Stove incubator 37 +/- 2ºC and 5% $CO_2$ (mammal cell lines)

Stove incubator 28 +/- 2ºC (insect cell line)

Inverse Phase Contrast Microscope

Vortex (stirrer)

Refrigerator (2-8º C)

Single-channel micropipettes in ranges 100 - 1000µl

Chronometer

Cell scraper (sterile)

**3. PROCEDURE**

Preparation of the assay (6-24 hours before):

- Calculate the number of plates/flasks needed to test the samples and organize the distribution of samples in the plates/flasks. Consider that to analyze samples in replicate is recommended.
- Prepare the attached cells distributing Vero, BHK or KC cells in suspension to the plates/flasks in an appropriate concentration to have around 80% confluent monolayer of cells.
- Prepare the samples to test according to the PROCEDURE 1 (SAMPLE PREPARATION). Store in refrigeration until inoculation.

Preparation of reagents

- Diluent: will be used to prepare the final dilution of inoculums and the mock inoculum
  - EMEM (Eagle's Minimum Essential Medium) + 1% Antibiotic/Antimycotic (100x) + 1% L-Glutamine + 1% Non - essential Aminoacids (if mammals cells are used)



Ctra. M-106 Km 1,4
28110 - ALGETE (MADRID). SPAIN
Phone: +34 *91* 347 8312 / 9270 Fax: +34 91 347 3778
bzn-eurl@mapa.es

| ORBIVIRUS ISOLATION IN CELL CULTURE | | |
|---|---|---|
| *Date: 22/06/2021* | Document code: GL-LCV-12 | *Rev. 02* |

- Schneider's Drosophila Medium, Modified + 1% Antibiotic/Antimycotic (100x) + 1% L-Glutamine + 1% Non - essential Aminoacids (if insect KC cells are used)

Cell culture medium

Diluent supplemented with:

10% v/v of IBFS if Vero cells are used

10% v/v of IBFS and 5% of Triptose if BHK-21 cells are used

15% v/v of IBFS if KC cells are used

Performance of the assay

- Label plates/flasks containing attached cells accordingly, with permanent marker.
- Prepare inoculum using diluent by adding the appropriate volume (adsorption phase Anexx 1) of sample to achieve:
  - -1/5 dilution if washed blood is used
  - -1/100 if whole blood is used
  - -1/10 dilution if homogenized tissue is used
- Remove the media carefully from the cell culture wells/flask and add inoculums (including mock inoculum) to the wells/flasks.
- Positive control must be prepared and handled always after samples and mock inoculum in each phase. Prepare positive control using diluent by adding the appropriate volume of stock virus to achieve i.e. 10 $TCID_{50}$. Add positive control to the proper flask or wells in a separate plate.
- Incubate at the proper temperature depending of cell line during approximately 1 hour to facilitate the viral adsorption.
- After adsorption phase, remove the inoculum on the monolayer and using a pipette add the appropriate volume of cell culture medium to each flask (proliferation phase volumes: Annex 1).

  NOTE: alternatively, in case of KC cells, after adsorption phase, inoculum could not be removed and appropriate volume (Annex 1) of cell culture medium is added using a pipette. The following day change cell culture medium by removing and adding new.



Ctra. M-106 Km 1,4
28110 - ALGETE (MADRID). SPAIN
Phone: +34 *91* 347 8312 / 9270 Fax: +34 91 347 3778
bzn-eurl@mapa.es

| ORBIVIRUS ISOLATION IN CELL CULTURE | | |
|---|---|---|
| *Date: 22/06/2021* | Document code: GL-LCV-12 | *Rev. 02* |

- Incubate at the proper temperature depending of cell line for 7 days (or 5 days in case of BHK-21). It is recommended to observe at microscopy daily to evaluate the state of cells and, in case of mammals cell lines, the presence of CPE.
- The flasks or wells medium with cells must be harvested if CPE is observed and stored at refrigeration temperature.
- If CPE is not observed after 7 days (or 5 days in case of BHK-21), scrap the attached cells, collect medium and cells and store at refrigeration temperature.

  NOTE: in case of KC cells, it is not necessary to scrap cells. Just collect supernatant and store at refrigeration temperature.

## 4. ANALYSIS AND INTERPRETATION OF RESULTS

The test is accepted if behaviour of cell culture has been the expected:

-Growth of cells in negative control.

-Cell line batch was sensitive enough: virus replication must be evidenced in positive control by CPE in mammals cells or RT-PCR in KC cells

NOTE: to analyse samples in replicate is recommended to increase the chance of isolate the virus, not as a quality control.

Results:

Presence of CPE in mammals cells must be taking into account to consider virus isolation as positive or negative, although it is not definitive and confirmation is always mandatory.

After confirmation by serogroup specific quantitative rRT-PCR:

- Virus isolation is considered as **POSITIVE** when rRT-PCR of harvested medium+cells is positive and the Ct value is significantly lower than Ct value of sample. In case of any doubt, it is recommended to carry out a new passage by inoculating the harvested medium+cells in a new cell monolayer and repeating the complete procedure up to 3 times comparing the Ct value after each passage with the previous one.



Ctra. M-106 Km 1,4
28110 - ALGETE (MADRID). SPAIN
Phone: +34 *91* 347 8312 / 9270 Fax: +34 91 347 3778
bzn-eurl@mapa.es

| ORBIVIRUS ISOLATION IN CELL CULTURE | | |
|---|---|---|
| *Date: 22/06/2021* | Document code: GL-LCV-12 | *Rev. 02* |

- Virus isolation is considered as **NEGATIVE** when rRT-PCR of passage is negative or the Ct value along passages goes significantly increasing (which does not support viral replication).

Virus neutralization test is a classic method of confirmation and typing, which requires virus producing CPE in mammal cells and much more time than RT-PCR. This method is used in EURL as an alternative method (not routinely).

**ANEXX 1 *Volumes***

| | Volume of sample (µl) ADSORTION PHASE | Volume of cell culture medium (ml) PROLIFERATION PHASE |
|---|---|---|
| 24-wells PLATE | 200 (well) | 1(well) |
| Flask 12,5 $cm^2$ | 500 | 5 |



Ctra. M-106 Km 1,4
28110 - ALGETE (MADRID). SPAIN
Phone: +34 *91* 347 8312 / 9270 Fax: +34 91 347 3778
bzn-eurl@mapa.es

| ORBIVIRUS ISOLATION IN CELL CULTURE | | |
|---|---|---|
| *Date: 22/06/2021* | Document code: GL-LCV-12 | *Rev. 02* |

## 5. REFERENCES

CLAVIJO A., HECKERT R.A., DULAC G.C. & AFSHAR A. (2000). Isolation and identification of bluetongue virus. J. Virol. Methods, 87, 13–23.

GARD G.P., WEIR R.P. & WALSH, S.J. (1988). Arboviruses recovered from sentinel cattle using several isolation methods. Vet Microbiol, 18, 119–125.

OIE. Manual for Diagnostic Tests and Vaccines for Terrestrial Animals. English version in force at date
Chapter African horse sickness (Infection with African horse sickness virus)
Chapter: Bluetongue (Infection with Bluetongue virus)
Chapter: Epizootic haemorrhagic disease (Infection with Epizootic haemorrhagic disease virus)

THOMAS F.C. (1984). Comparison of some storage and isolation methods to recover bluetongue virus from bovine blood. Can. J. Comp. Med., 48, 108–110.

Verwoerd D.W., Huismans H., Erasmus B.J. (1979). Orbiviruses. In: Comprehensive Virology, Fraenkel-Conrat H., Wagner R.R., eds. Plenum Press, London, UK, Vol. 14, 285–345.



Ctra. M-106 Km 1,4
28110 - ALGETE (MADRID). SPAIN
Phone: +34 *91* 347 8312 / 9270 Fax: +34 91 347 3778
bzn-eurl@mapa.es