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Propagation of Vero Cell-Adapted African Swine Fever Virus (ASFV)

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We use this protocol and it's working

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Abstract

Described and outlined within this protocol is the standardized methodology for the propagation, processing, and titration of Vero cell adapted African Swine Fever Virus (ASFV) to produce viral stocks for experimental use. Utilizing the Vero cell line CCL-81, ASFV Lisbon-60 is propagated until 70% of the monolayer exhibit cytopathic effects (CPE), generally within 5-8 days. To maximum viral harvest yields, infected flasks undergo a freeze-thaw cycle followed by centrifugation to obtain clarified viral supernatant. The virus is then titered using TCID₅₀ assays and stored at -80 °C for future use.

Attachments



TCID50 Example

Sheet...

28KB



titration worksheet....

14KB

Guidelines

****Definitions****

- **African Swine Fever Virus (ASFV):** A large DNA virus affecting swine species which is classified as a select agent.
- **Vero cells:** A continuous anchorage dependent continuous cell line derived from African Green Monkey Kidney Cells often used for viral replication and propagation studies.
- **Cytopathic Effect (CPE):** Visible structural/morphological changes in the host cell resulting from viral infection. Within this protocol this is characterized as rounding cells, syncytia forming, and detachment from the monolayer.
- **Virus Clarification:** The process by which viral particles may be separated from host cellular debris using low-speed centrifugation.
- **TCID₅₀ (50% Tissue Culture Infectious Dose):** A quantitative measurement of viral infectivity based on the dilution at which it is expected that 50% of cells will become infected.
- **Seeding Ratio:** The optimum ratio used to achieve confluency or saturation density within 2 to 3 days. (One container of growing cells can normally be diluted as indicated each time it is passes, i.e. 1:4 means 1 flask may be diluted with appropriate medium to make 4 flasks).
- **Subculture or passage:** The process of transferring or transplantation of cells from one culture vessel to another.
- **Passage frequency:** The normal passage rate to ensure cells maintain optimum growth.
- **Seeding Density:** Cell count required to achieve confluency or saturation density in a desired amount of time.
- **Anchorage Dependent Cell Cultures:** cells which will grow, survive, or maintain their functions only when attached to a surface such as glass or plastic.
- **Suspension Cell Cultures:** Cells or aggregates of cells that multiply while suspended in liquid medium.
- **QH₂O:** Filtered water from Q filtration system (MilliporeSigma™ Milli-Q™ direct water purification system).
- **Quantum Sufficit (Q.S.):** Indicates to add a sufficient amount of water to reach an indicated volume (solids take up space even when dissolved in liquids).

****Personal Qualifications****

- Personnel performing this protocol must be trained and be familiar with BSL-3 practices, aseptic cell culture techniques, virus handling, and the proper handling and requirements of select agents.
- Personnel should be proficient in the use of inverted light microscopy for CPE examination.
- For official diagnostic or regulatory propagation, the personnel should have documented competency in the specific methods utilized for ASFV propagation and titration.

****Contamination Prevention****

- Aseptic Technique is essential and all work must be performed in a Class II Biosafety Cabinet (BSC) to prevent environmental contamination of cultures and cell culture.
- Appropriate Personal Protective Equipment (PPE) not limited to but may include such items as gloves, lab coat, eye protection, appropriate respiratory protection, and front facing gown must be worn while working. These items should be changed immediately if contamination is suspected or when moving from clean areas to contaminated areas.
- Work surfaces and equipment must be disinfected with Virkon S® or other approved disinfections following recommended contact times.
- Use dedicated reagents for clean and infected cultures should be labeled and aliquoted into single use or dedicated containers to prevent cross contamination.
- For work with cell culture it is critical to ensure sterile equipment, supplies, and reagents are used and that aseptic conditions are maintained throughout the process.

****Areas and Equipment****

- It is advised that separate areas/hoods/incubators for cell culture growth/maintenance and virus infection/propagation be used. This ensures cell culture remains virus free.
- Each area should have dedicated calibrated pipettes and equipment.

Calibration:

- All equipment, such as pipettes, centrifuges, incubators, must be calibrated and certified according to manufacturer and institutional SOP instructions and requirements.

Disinfection:

- Work surfaces must be disinfected with Virkon S (or an equivalently validated disinfectant) before and after all procedures. A minimum contact time of 10 minutes is required for Virkon S® for effective ASFV inactivation.

- For cell cultures iodine, 70% ethanol, or phenolic solutions are appropriate for disinfection when prepared according to the manufacturer's recommendations.
- Due to the corrosive nature of some disinfectants a follow-up of 70% ethanol should be performed to protect surfaces and equipment.
- All contaminated instruments, containers, and fluids must be autoclaved before reuse or disposal.

****Reporting and Quality Control****

Select Agent Reporting:

- Any propagation, movement, or destruction of ASFV must be recorded in the appropriate data management systems and reported to the relevant select agent program authorities as required by federal law.

Titration Quality Control:

- A TCID₅₀ assay is considered valid only if the negative control cell culture wells remain healthy and free of CPE throughout the duration of the test. It is important that cells be healthy and fresh to avoid false positive results due to natural cell cycle life and death.

Documentation:

- Record all viral lot numbers, passage levels, and final titers in the institutional tracking system. It is also advisable to record lot numbers, passage levels, and splitting ratios of cell culture as well.

Materials

****Reagents****

- Minimum Essential Media (MEM) with Earles salts and with 0.5% Lactalbumin Enzymatic Hydrolysate (LAH) and sodium bicarbonate (see Cell Culture Media Recipes Appendix, Appendix III).
- Purified and characterized Vero cell-adapted ASFV stock, Lisbon-60 (Obtained from FADDL, TCID₅₀ expected to be 1.00×10^7 /mL)
- Vero cell line (ATCC® CCL-81or CRL-2378.1)
- Fetal Bovine Serum (FBS, gamma irradiated)
- sodium pyruvate (100 mM) (Gibco, REF: 11360-070)
- L-glutamine 200 mM (100X) (Gibco, REF: 25030-081)
- MEM NEAA (100x) (Gibco, REF: 11140-050)
- 0.05-0.1% Amphotericin B, 250 µg/mL (used to protect against yeast and molds in cell production) or Pen-Strep (15.775 g Penicillin, 100.0 g Streptomycin, QH₂O to 1 L)
- Virkon® S disinfectant
- 70% Ethanol
- Trypsin Versene with Saline Solution A (ATV) (see cell culture appendix sheet)
- Trypan Blue Solution (Sigma-Aldrich, Cat: 93595-250 mL)

****Supplies****

- Pipette tips (aerosol-resistant, sterile)
- Serological pipettes (5 mL, 10 mL)
- Centrifuge tubes (1.5–2.0 mL, 15 mL, 50 mL)
- Tissue culture flasks (T-75 cm² preferred, vented or non-vented)
- 96-well flat-bottom plates (tissue culture treated)

- Cryovials (1.5–2.0 mL tubes with O-ring seals for ultra-low temperature storage)
- Laboratory gloves, powder-free
- Disinfectant wipes (for BSC and equipment cleaning)
- Cell Scrapers (depending on the type of cells used or the age of the cells, either passage or age in flask) the cells may stick more efficiently and not be easily removed by use of trypsin solution. Depending on what the cells are being used for it may be appropriate to use a cell scraper to detach them from the flask)
- Parafilm
- Countess Cell counting Slides or Hemocytometer and glass cover slip (advisable to have a counting device of some kind if counting by hand) (Countess, Catalog Number: C10228)

****Equipment****

- Tube racks
- Cryogenic protective gloves
- Protective face shield
- Serological pipette gun
- Class II Biological Safety Cabinet (BSC)
- Humidified Incubator(s) ($37\text{ }^{\circ}\text{C} \pm 5\text{ }^{\circ}\text{C}$, $5\% \text{ CO}_2 \pm 1\%$, unless the specific cell line being used requires different temperature and CO_2 requirements, $37\text{ }^{\circ}\text{C}$ and $5\% \text{ CO}_2$ are standard for Vero cell lines)
- Centrifuge (capable of $1500\times g$ at 4°C)
- Ultra-low freezer ($-80^{\circ}\text{C} \pm 10^{\circ}\text{C}$)
- Refrigerator ($4^{\circ}\text{C} \pm 3^{\circ}\text{C}$)
- Inverted light microscope
- Single and multi-channel pipettes (calibrated; $1\text{ }\mu\text{L}$ – 1 mL range)

- Automated Cell Counter (Countess 3, Invitrogen, or equivalent Invitrogen) (can use a light microscope if counting by hand)

Note(s):

1. Equivalent reagents and equipment may be used with appropriate validation.
2. All work must comply with institutional SOPs and select agent regulations.

Safety warnings

- !** African Swine Fever Virus (ASFV) is a select agent pathogen. Appropriate PPE, BSL facilities, and disinfectant should be utilized accordingly. In addition, all ASFV work should be completed within an appropriate BSC. Virkon® S disinfectant is suitable for use with ASFV and a 10 minute contact time should be used. For cell cultures iodine, 70% ethanol, or phenolic solutions are appropriate for disinfection when prepared according to the manufacturer's recommendations. Proper regulatory oversight, precautions, and record keeping should be followed while working with ASFV. While not harmful or infectious to humans this virus is extremely detrimental to porcine species and prevention of exposure to suide species should be regarded.

Before start

Prior to beginning the procedure annotation of all pertinent information should be performed for accurate select agent standards and inventory records. Laboratory personnel should be properly trained and understand the requirement and associated risks with this procedure. Ensure sufficient training in cell culture and viral propagation has been performed. Appropriate biosafety level should be considered when working with cell cultures and virus.

Cell Preparation

- 1
 - Culture Vero cell line in the appropriate flask size needed for your experiment until the cells are 70 to 90% confluent. While vessel size is up to the discretion of the user a T-75 cm² is recommended.



Note(s):

1. If starting cell culture from frozen cells stored in liquid nitrogen refer to Starting Cells from Frozen Stock Protocol.
2. If subculturing cells from an established monolayer refer to Subculturing Anchorage Dependent section.
3. If your institution has the ability to order confluent subcultured cells, please refer to specific institutional instructions and protocols.
4. Depending on specific Vero cell line and media being used for growth, propagation, or infection appropriate additives should be added to the cell culture media and Subculturing Anchorage Dependent should be referred too.
5. To prevent possible contamination, spillage or leakage, etc. it is preferred to use vented flasks, however, if vented caps are not available non-vented flasks may be utilized. It is important to remember to loosen caps slightly, like one would do with caps of bottles in an autoclave, to enable gas exchange for non-vented flasks.

1.1 Starting Cells from Frozen Stock



- Obtain desired cell stock either from your institutions in house reagent section, institution/labs frozen cell stock holding area, or order from a reputable company (e.g. ATCC® (American Type Culture Collection)).

Note(s):

1. Cells ordered from a company will arrive packaged within dry ice. Ensure proper protective gloves are used when retrieving the tube.
 2. For those obtaining cells from a central cell stock location such as a liquid nitrogen holding tank wearing a protective face shield and gloves is recommended.
 3. Carefully, remove the ampules needed from the appropriate liquid nitrogen freezer and place in a Styrofoam carrier to transport to laboratory room.
 4. While it is debated, cells can be stored in a -80 °C for storage if liquid nitrogen storage is unavailable. This is dependent upon freeze back conditions used, cell type, and concentration of cells frozen. It is advised to wear a glove when retrieving these samples.
- Frozen cells may be thawed in a variety of ways depending on user preference and institutional guidance.

Options:

1. Ampules may be placed in a beaker that had been filled with Place ampules in a beaker 20°- 37°C tap water and allowed to thaw.
2. The laboratorians my use their hands and friction to thaw the cells.
3. 20 - 37 °C di water bath may be used.
4. A 20 - 37 °C metal bead bath may also be used.

Note:

1. To avoid potential contamination, it is recommended that disinfecting agents be placed into any water baths, body heat, or a metal bead bath be used.
- Once the ampule has been thawed carefully transfer to the biosafety cabinet. It is recommended to spray down the ampule with 70% alcohol or wipe down the ampule with sterile gauze soaked in 70% alcohol to disinfect prior to putting the ampule in the biosafety cabinet or immediately after.
 - Ampules may consist of plastic cryovials with O-ring seals, or they may be made of glass. In the case of the later score the glass on the neck of the ampule and snap the top off. Be very careful when doing so to avoid cutting yourself. Additionally, there are available tools on the market which may be purchased which aid in the decapping of the ampule top.
 - Transfer cells to cell culture vessel(s) or appropriately sized centrifuge tube.

Note(s):

1. Cells that grow easily after freezing may be transferred directly into appropriate media in a cell culture vessel.
 2. Cells that take longer to start from the freezer should be centrifuged to remove dimethyl sulfoxide (DMSO) before seeding.
- Cells regardless of if they are easy to grow from freezing or not may still be centrifuged to remove old growth media and DMSO (best practice is to centrifuge and remove DMSO).
 - Transfer thawed cell suspension into centrifuge tube and fill with appropriate warmed growth media or irradiated fetal bovine serum.
 - Centrifuge for 5 minutes at 250-500 xg.
 - Discard supernatant and resuspend pellet with appropriate media and transfer to appropriate cell culture vessels.

- Label all cell culture vessels with appropriate information which may include but is not limited to lot number, cell type, passage number, date, and initials of person performing cell culture.

Note:

1. Cells begin to display non typical morphological and growth characteristics the older they are (higher passage number). Use of higher passaged cells is up to the specific user and institution and highly dependent on work being performed. It, however, is advisable that seed stock(s) obtained from a company only be passaged out to 30-35 passages. Thus, creating high number of working stocks from the initial 5 first passages is advised.

1.2 Subculturing Anchorage Dependent Vero Cell Line



Cell Culture Table of Recommende... 28.5KB

Subculturing Anchorage Depend... 35.1KB

Preparation of Reagents

- Prepare necessary supplemental reagent solution and designated growth media for vero cells by adding appropriate ingredients.
- To 860 mL MEM with Earles and with 0.5% LAH add 10 mL sodium pyruvate (1 mM), 10 mL of pen-strep solution (0.157 g/L of penicillin with 1.0 g/L streptomycin solution), 100 mL FBS, 10 mL L-glutamine (2 mM), and 10 mL MEM NEAA (1x).
- Label the bottle of growth media with type, percentage of serum, date, initials of maker, and any additives.
- Thaw ATV (at no warmer than 37 °C) and warm to 24-37 °C. Warm media to 24-37°C.

Subculturing Cells

- Determine the health of the cell culture by observing the culture with a microscope.

Note(s):

1. Look for the typical morphological characteristics of the cell line determined to be normal such as, but not limited to, microscopic appearance, growth rate, and acid production.
2. In general, healthy cells should have proliferated without evidence of morphological deterioration such as granularity around the nucleus or cytoplasmic vacuolation.
3. In addition, healthy anchorage dependent cells should be confluent without evidence of piling or rounding up of cells with detachment from the substrate.

4. However, it must be noted that several factors such as age or seeding ratio may influence the appearance of the cell culture. Most notable is age of cells as with higher passage numbers non-standard morphological characteristics begin to occur.
5. Further, there should be no evidence of bacterial growth or fungal growth within the culture.
6. The presence of one or more of the following characteristics, with the exception of contamination, does not necessarily mean that the culture is non-viable.

- Technicians should use their experience with a particular cell line and or consult with another member in their lab with more familiarity when determining the overall health of any culture as not all cultures act alike.

- If the anchorage dependent cell line is not confluent decide one of the following:

1. whether to split the cells at a lower split ratio
2. delay splitting for 1 to 2 days
3. Feed the cells by replacing the growth media

Note:

1. The decision should be based on experience or in consultation with an appropriate supervisor/coworker.
- If the cells show prolonged deterioration decide whether the cells should be tested for contaminants, discontinued, and/or replaced with fresh cultures.
 - Should the cells be healthy and ready to subculture, decide the split ratio based on:
 1. Previously determined optimal split ratios (see cell culture tables).
 2. Whether the cell line is fragile and slow growing or hardy and fast growing.
 3. When the cells will be subcultured again.
 4. A concentration that will ensure the cells will enter a growth cycle in 24 hr or less.
 5. For anchorage dependent cells when a confluent monolayer is desired (making sure that the cells do not form multiple layers or peel from the flask before the next subculturing)
 - Work in a biosafety cabinet and maintain aseptic conditions throughout the procedure.

Anchorage Dependent Cell Line Protocol

- Remove the media and discard
- Rinse the cell layer by adding 1-2 ATV to the side of the flask opposite the cells and gently move the flask from side to side.

Note(s):

1. 1 mL per 25-cm² flask
2. 1-2 mL per 75-cm² flask
3. 2-4 mL per 150-cm² flask
4. 10-12 mL per 850-cm² roller bottle
5. The quantity will be determined by individual cell line's adherence to the flask and personal preference.

- Pipette off and disc are ATV. Repeat rinse.
- Pipette off most of the ATV from the second rinse and incubate

Note:

1. If the cell line is difficult to displace discard all of the ATV from the second rinse and add appropriate quantity of ATV and leave all of the third application of ATV in the flask.
- Incubate cells at room temperature (20-25 °C) or at 37 °C until cells round up and the monolayer slides off the surface of the flask when it is tilted (Usually 5-15 min).

Note(s):

1. For quicker detachment place in the incubator.
 2. Some cells may require agitation to detach. Gently whack the flask on the palm of your hand or on a solid surface to detach.
- Do not leave the ATV on the cells longer than necessary and do not damage the cells by forcing the cells to detach too soon.
 - Disperse cells by repeating pipetting or syringing over surface monolayer (with a pipette or syringe and cannula).
 - Pipette the suspension up and down several times resting in the bottom corner of the flask until the cells are adequately dispersed.

Note(s):

1. It may be advisable to pipette across the substrate surface if there are clumps or cells still attached and this can help disperse the cells.
2. The degree of pipetting will vary from one cell line to another, and some will disperse easily while others will require more vigorous pipetting. However, too vigorous pipetting can damage the cells. Older cell lines may be harder to break up.

- To ensure that trypsin does not degrade the cellular receptors and to speed up attachment ATV can be inactivated and separated from the culture.
- To inactivate any residual trypsin in the solution, add equal parts to double amount of cell culture growth media to that of ATV used.
- Then centrifuge cellular solution in a centrifuge tube for 5 to 10 min at 200 to 500 xg.
- Decant the supernatant and resuspend the cell pellet in appropriate amount of growth media.

Note:

1. This is dependent on if you are splitting via ratios or if you will be counting cells and splitting based off whole live cell count number.
- If splitting via ratio split:
 1. Resuspend the cellular pellet in 2-5 mL growth media and dilute the cellular suspension to an appropriate seeding concentration (see cell culture tables) by diluting the appropriate volume of cells in the total volume of media required.
 2. Then distributing that suspension among several flasks (This is preferable when using larger quantity of vessels and when concentration of cells should be identical) or desired culture vessels or by adding appropriate volume of suspended cells to media that has been dispensed into desired culture vessels.
 - If splitting via counting live cells:
 1. Take a parafilm strip and remove the backing paper.
 2. Remove 20 μ L of Trypan Blue solution and dispense into a droplet onto the parafilm.
 3. Swirl your tube containing the cell culture solution and using a pipette aspirate 20 μ L of cell solution and dispense into the trypan blue droplet. Pipette up and down.
 4. Take 20 μ L of trypan/blue cell culture solution and dispense into chamber of Countess slide or into the grove of a hemocytometer.
 5. Insert slide into the countess or place hemocytometer onto a light microscope. Follow manufacturer's instructions for cell count instructions.
 6. Depending on desired number of cells needed for desired culture vessels and time when cells are needed calculate required amount of cell solution to mix with cell culture media and then add to cell culture vessels.
 - Label each vessel with the cell line, lot number, passage number, date, and initials of the technician.
 - Incubate vessel(s) until needed use or subculturing. Monitor cell growth occasionally and media acidification.

Virus Propagation

1w 1d

- 2
 - Obtain Vero cell-adapted ASFV stock from an appropriate source

1w 1d

Note:



1. ASFV Vero-adapted Lisbon-60 strain can be supplied by the Foreign Animal Disease Diagnostic Laboratory (FADDL). This is not a commercially available strain and was developed by FADDL. The expected concentration should be at a TCID₅₀ of 1.00×10⁷/mL.
- Allow virus to thaw on ice, if possible. The virus may be thawed at 4 °C ± 3 °C if ice is unavailable.
- Dilute the stock virus in cell culture media containing appropriate additives.

Note(s):

1. Volume of cell culture media should be appropriate to flask size being utilized. 10 mL of cell culture media is sufficient for a T-75 cm² flask.
2. Dilution may vary depending on titer of source virus. Virus obtained from FADDL with an expected TCID₅₀ of 1.00×10⁷/mL may be diluted 1:10.
3. A higher dilution may be needed, 1:20, if the titer is different such as TCID₅₀ of 1.00×10^{7.7}/mL
- Remove the media from the flask containing Vero cells.
- Add virus suspension to the flask contain Vero cells.

Note(s):

1. Avoid pipetting directly on the cell monolayer to prevent detachment of cells.
2. Tighten the cap for vented flasks and for non-vented flasks caps should be closed but loosened 1/2 turn.
3. Gently rock the flask side to side to ensure full coverage over cellular monolayer.
- Transfer the inoculated flask to the incubator, 37 °C with 5% CO₂, following appropriate institutional biosafety requirements and select agent requirements.
- Allow the infection to proceed until cytopathic effects (CPE) is observed in approximately 70% of cells.

Note(s):

1. Observe CPE under the microscope according to appropriate biosafety procedures.



2. It is typical for infection to proceed from 5 to 7 days sometimes 8.

- Once 70% of cellular monolayer exhibits CPE remove flasks from incubator and freeze the flask at -80 °C according to appropriate biosafety procedures.

Virus Clarification

1w 1d

- 3
- Allow the flask to thaw according to appropriate biosafety procedures. It is recommended to allow the flask to thaw within the BSC.
 - Once flask has thawed transfer viral/cellular solution to an appropriately sized and select agent approved centrifuge tube.
 - Centrifuge at 1500 xg $\pm$ 200 rpm for 10 min at 4 °C $\pm$ 3 °C according to appropriate biosafety procedures to remove cellular debris. It is recommended to use centrifugal buckets with lids with O-rings to provide an airtight seal in the event of aerosols.
 - Transfer the clarified supernatant to an appropriately labeled storage container(s).



Note(s):

1. Typically, 1 mL aliquots are created from the clarified supernatant and transferred into cryovials containing O-ring seals.
 2. Appropriate data management and select agent record keeping of viral inventory should be performed and followed.
- Virus aliquots should be stored at -80 °C $\pm$ 10 °C according to appropriate biosafety and select agent guidance procedures until use.

3.1 TCID₅₀

1w 1d

- It is recommended that virus culture be titered via TCID₅₀ methodology.
- Culture Vero cells within a 96 well plate until cells are 70 to 90% confluent.
- Once cells are confluent prepare viral serial dilutions. To do this take a sterile tube and mix 450 μ L of cell culture media and inoculate with 50 μ L of the clarified virus supernatant stock, creating your 1:10 first serial dilution.
- Mix gently.
- Remove growth media from all wells within the cell culture plate and add 100 μ L of fresh media to all wells except for wells in column 1.



- To column 1 add 111.1 μL of your 10^{-1} virus dilution into 4 wells, creating your 4 replicates.
- From column 1 transfer 11.1 μL using a multi-channel pipette into column 2 wells containing 100 μL of media, giving a total of 111.1 μL and your 10^{-2} dilution.
- Pipette up and down a few times to mix. Avoid pipetting directly onto the cells and disturbing the monolayer.
- Repeat this transfer pattern across the rest of the plate until you reach your final desired dilution (column 10 for 10^{-10} or column 11 for a 10^{-11} dilution). Mixing each time before moving to the next well.
- Once you have reached the final dilution column discard 11.1 μL from the wells. Every well should now have 100 μL within it.
- Column 12 will serve as your control wells.
- Transfer the plate to the incubator, 37 °C with 5% CO_2 , following appropriate institutional biosafety requirements and select agent requirements.
- Allow the infection to proceed for 5 to 8 days. Record and check the CPE daily.

Note(s): The assay is considered complete when one of the following occurs:

1. The CPE results remain consistent and identical across three consecutive days.
 2. Your uninfected cells begin to show more than 10-20% detachment or rounding indicating age and normal growth life cycle and death. Stop immediately to avoid false positive results.
 3. There is extensive CPE where cells are detaching and rounding in lower dilution wells.
- TCID_{50} values should be calculated using the Spearman-Kärber method.



Protocol references

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ATCC® CCL-81™: Vero Cell Line, African Green Monkey Kidney. American Type Culture Collection.

Vero - CCL-81 | ATCC