The Art and Science of Growing Animal Viruses in the Laboratory: Best Practices in Virus Culture

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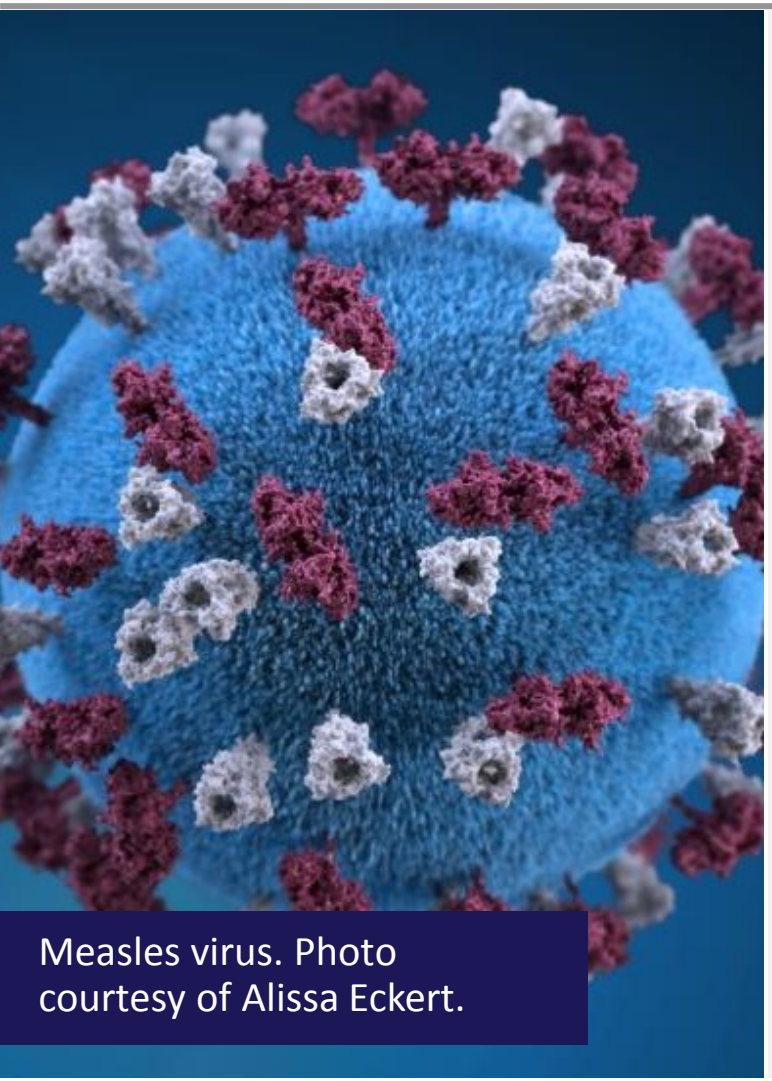
About ATCC

- Founded in 1925, ATCC is a non-profit organization with HQ in Manassas, VA and an R&D and Services center in Gaithersburg, MD
- World's premiere biological materials resource and standards development organization
  - 5,000 cell lines
  - 80,000 microbes
  - Genomic & synthetic nucleic acids
  - Media/Reagents
- ATCC collaborates with and supports the scientific community with industry-standard and innovative biological solutions
  - Growing portfolio of products and services
  - Sales and distribution in 150 countries, 12 International distributors
- Talented team of 450+ employees; over one-third with advanced degrees

An innovative global partner for authentic biomaterials, standards, and services

Outline



Measles virus. Photo courtesy of Alissa Eckert.

- Aseptic technique and best practices for avoiding contamination
- Culturing viruses in tissue culture
  - Choosing a propagation host
  - Cell line authentication
  - Media and reagents
  - Multiplicity of infection
  - Defective interfering particles
  - Influenza growth in tissue culture
- Culturing viruses in eggs
  - Maintenance and source of eggs
  - Allantoic cavity inoculation
  - Hemagglutination assay
- Viral preservation

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Aseptic Technique and Avoiding Contamination

Good aseptic technique is essential for avoiding contamination and cross-contamination

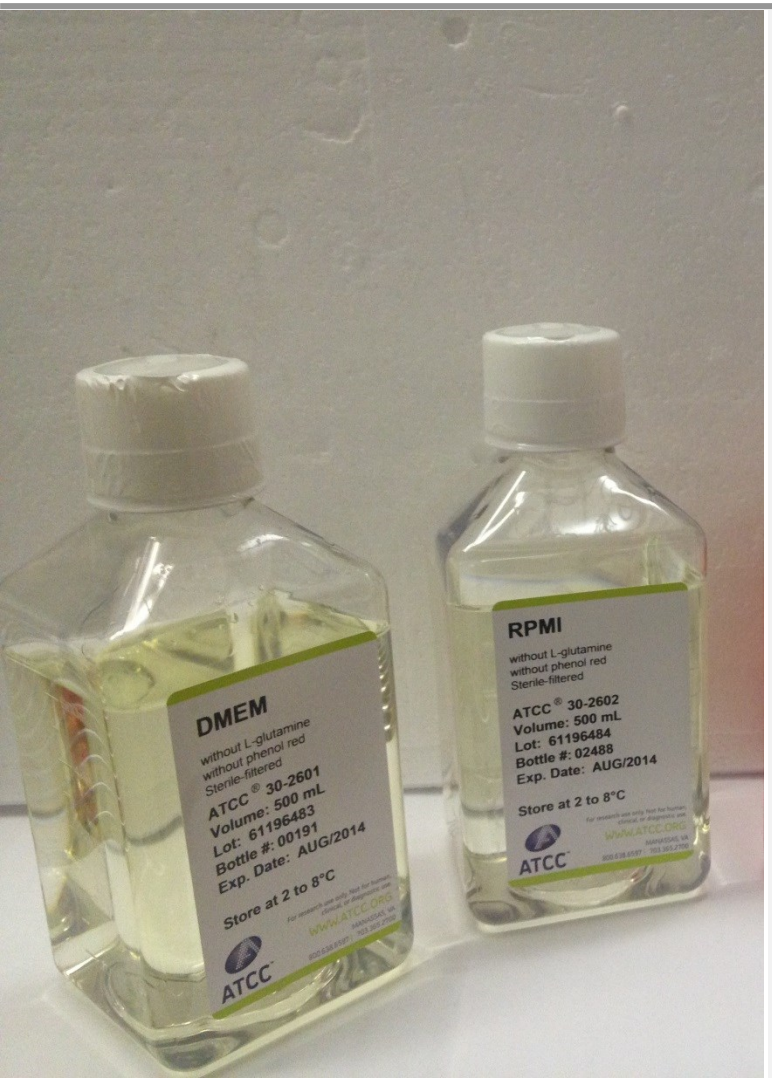
Common sources of contamination:

- Poor laboratory technique
- Working with multiple cultures and cell lines concurrently
- Mislabeling of cultures
- Using one reservoir of growth medium for multiple cultures

Best Practices – Biosafety Cabinets

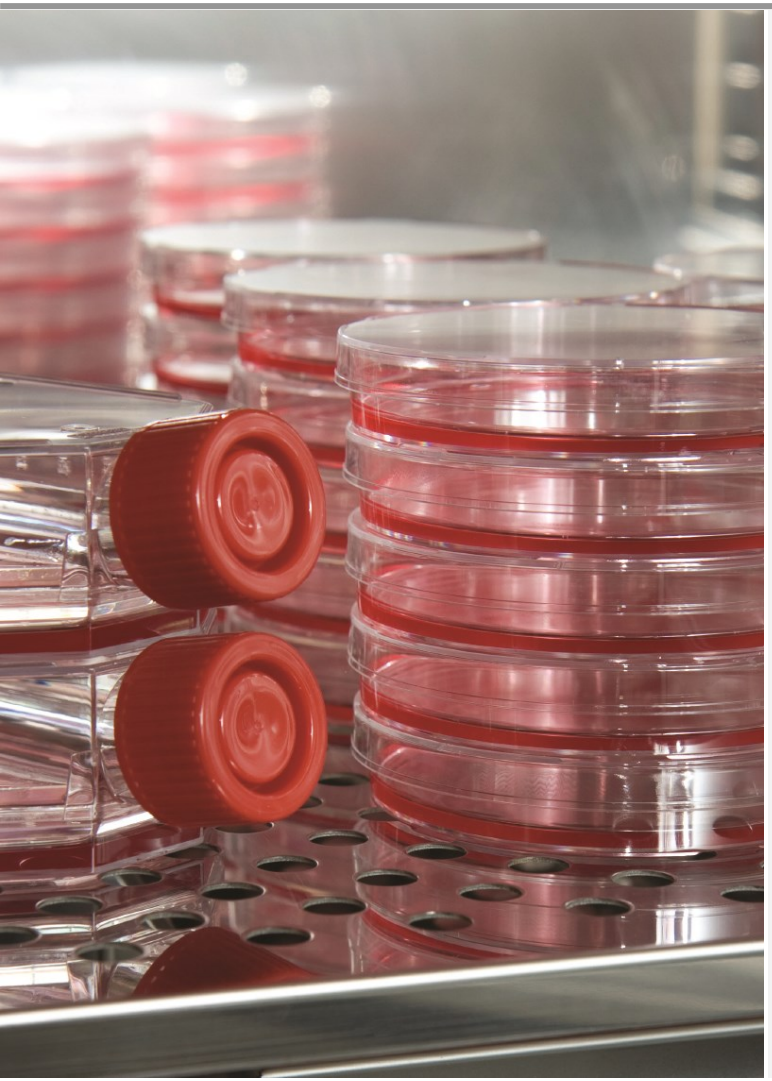
- Learn how the biosafety cabinet (BSC) works to help maintain primary containment
- Check magnehelic gauge and keep a regular record of its function
- Minimize disruption of airflow – keep a small number of articles inside
- Place all your work materials in the BSC before opening containers
- Minimize movement in and around the BSC to reduce air flow disturbance
- Do not reach over items, keep items within reach
- Avoid blocking the airflow, *e.g.*, resting your arms on the front of the BSC
- Perform work ~4 inches back from opening
- Use appropriate disinfectants to wipe BSC surfaces and articles before and after each use

Best Practices – Media and Reagents



- Aliquot reagents (media, PBS, trypsin,) and assign each aliquot to one cell line or one virus
- Use a new pipette each time going into stock reagents
- Do not pour reagents such as media (use pipettes) as you will create liquid bridges, increasing the risk of contamination
- Do not move arms/hands/pipettes over open media containers
- Keep containers closed unless they are in immediate use

Best Practices – Virus and Cell Cultures



- Check for contamination of the seed virus prior to growing up a large stock
- Work on only one cell line/virus at a time inside the BSC
- Work with non-infected material first (cells) prior to virus manipulation
- Arrange items “clean to dirty” in an orderly fashion, leaving a clear space to work in
- Keep uninfected cells and virus-inoculated cells in separated incubators
- Clearance time between work to allow of air exchange: Cells (15 min), Viruses (30 min)
- Clean water-baths and incubators regularly
- Quarantine lab: If a material is known to be contaminated with bacteria/fungi, segregate it and work in a different BSC/room

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Best Practices – Personal Protective Equipment (PPE)

- Check PPE requirements for the virus
- Change gloves frequently
- Do not spray disinfectant on gloves, it may compromise the integrity of the glove
- Wear a clean lab coat with long sleeves to prevent the introduction of contaminants from your arms or clothing; tuck sleeves into gloves
- Best Practice: Use disposable lab coats when possible (disposable sleeves is a cheaper option)

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Viral Culturing

Viruses require living cells to support their replication

- Tissue culture system
- Embryonated chicken eggs
- Whole animal systems

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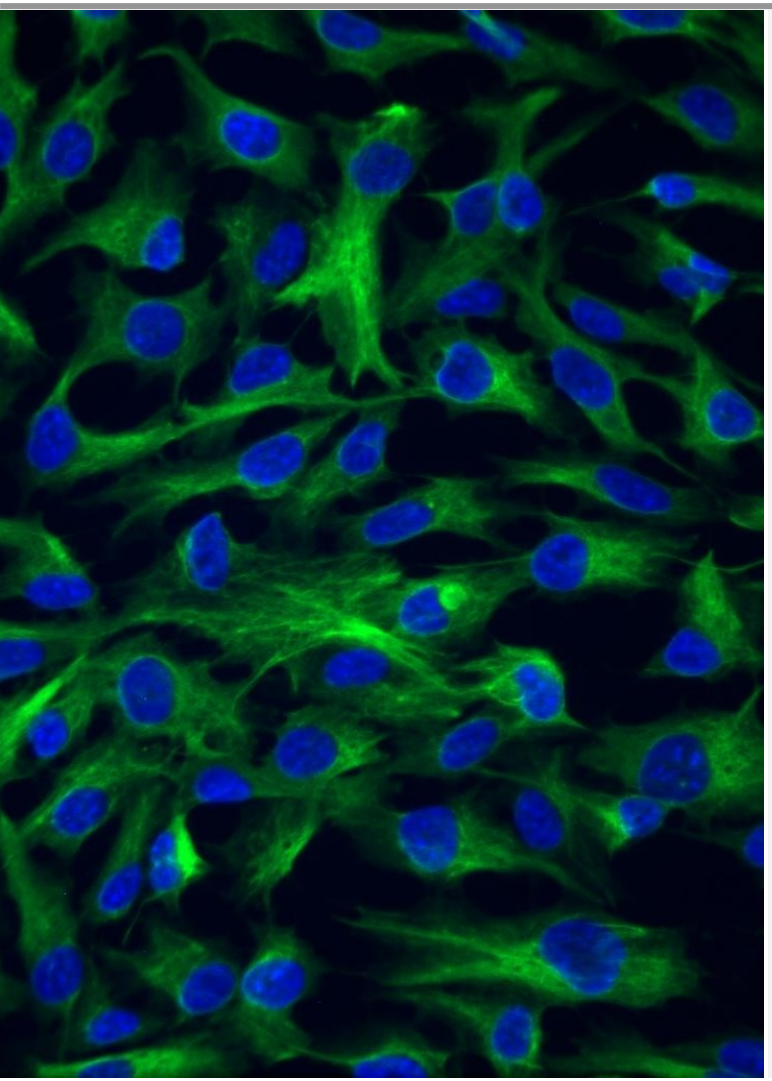
Tissue Culture Systems



- Tissue culture systems comprise cells that originated from a multicellular organism
- Require specialized culture media that contain the essential nutrients and energy sources that are required for cell survival
- Used as the preferred type of growth system for many viruses

Choosing a Propagation Host

If isolating a virus from a clinical specimen:

- Host species and permissibility
- Primary cells vs. Cell lines

If growing a virus from a seed virus:

- Ideally, keep the virus growing in the same host cell to minimize the selection of virus sub-populations
- A selection event (a genetic bottleneck) occurs when a virus is passaged to a new host
- If there is a need to change to another host, ensure that the virus has adapted to the new host by checking for stable titers across several passages

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Know Your Host Cells – Are they Authentic?

Observe cell morphology

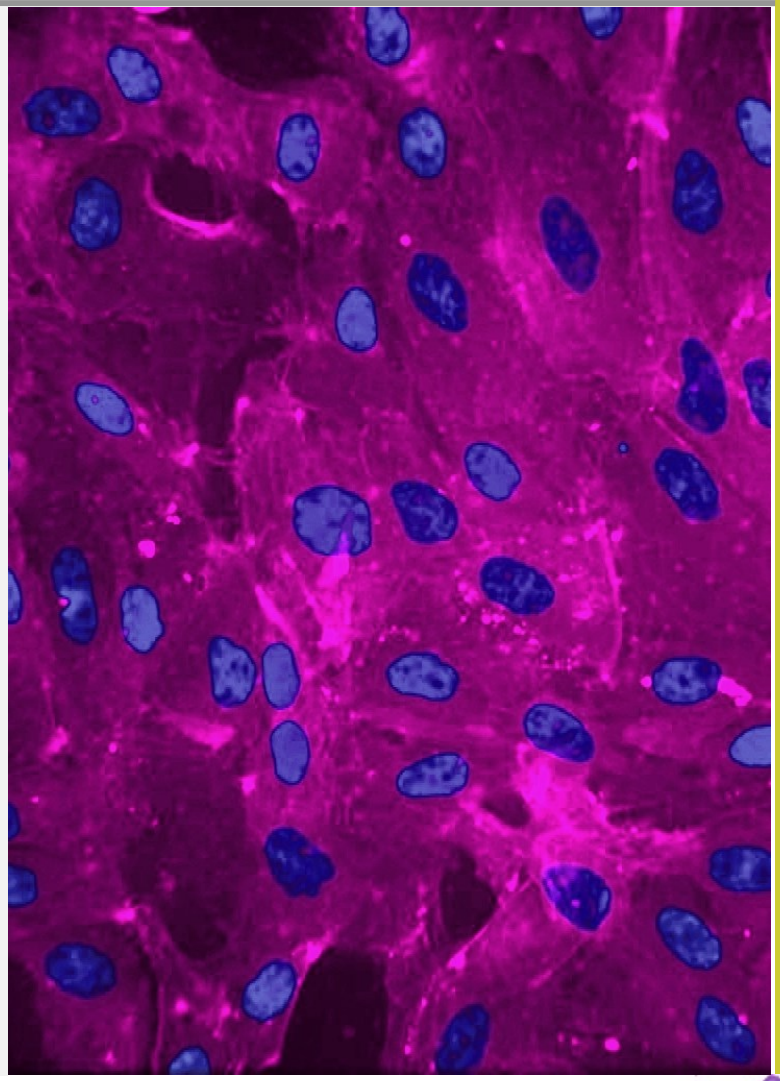
- Shape, membrane structure, and optical properties at various stages of growth

Is there any cross-contamination with other cell lines?

- Intraspecies identification by STR analysis
- Interspecies identification by CO1 assay

Passage number of the host cell

- The phenotype of a highly passaged cell line is quite different from their progenitor



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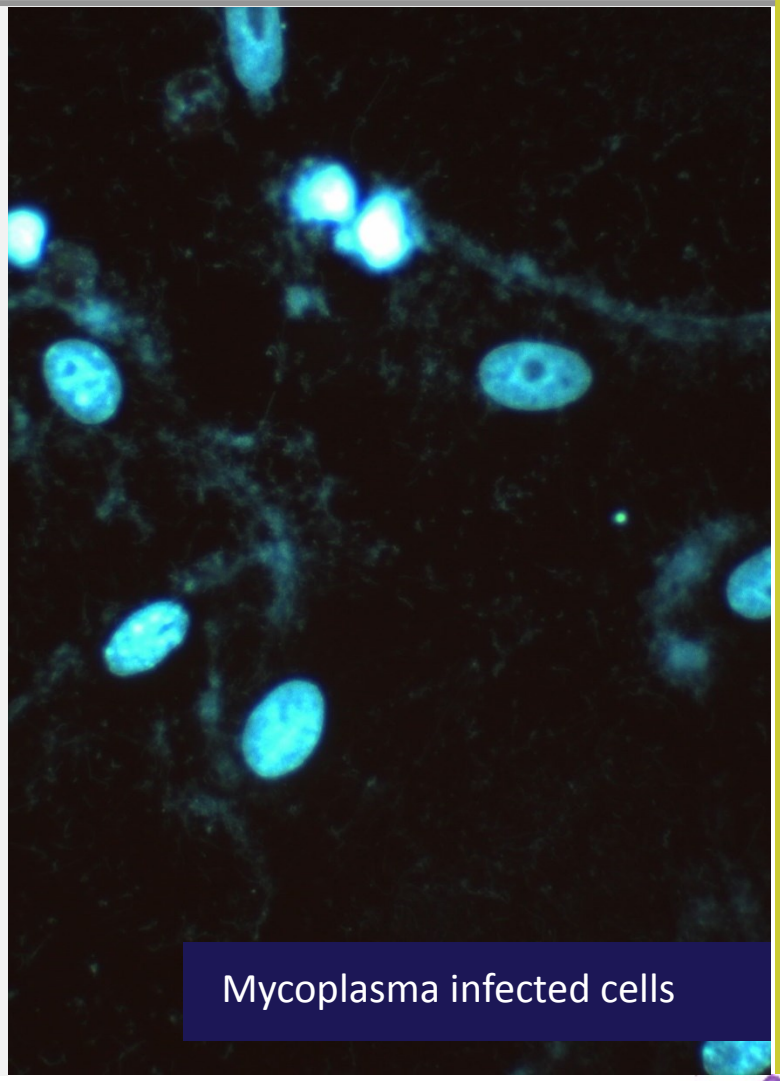
Know Your Host Cells – Are they Authentic?

Is it free of Mycoplasma?

- PCR-based detection
- Direct agar culture
- Indirect Hoechst's stain

Influence of antibiotics/antifungal agents in media

- Grow cells in the absence of antibiotics to unmask contaminants



Mycoplasma infected cells

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Complete Growth Media

- Complete growth medium consists of a basal cell culture medium supplemented with ingredients such as sera, growth factors, trace elements, and hormones
- Numerous formulations:
  - Simple, basic media containing the minimum requirements for growing many cell lines
  - Complex, serum-free media for growing fastidious cell lines
- The type of medium used must be determined empirically for each cell line

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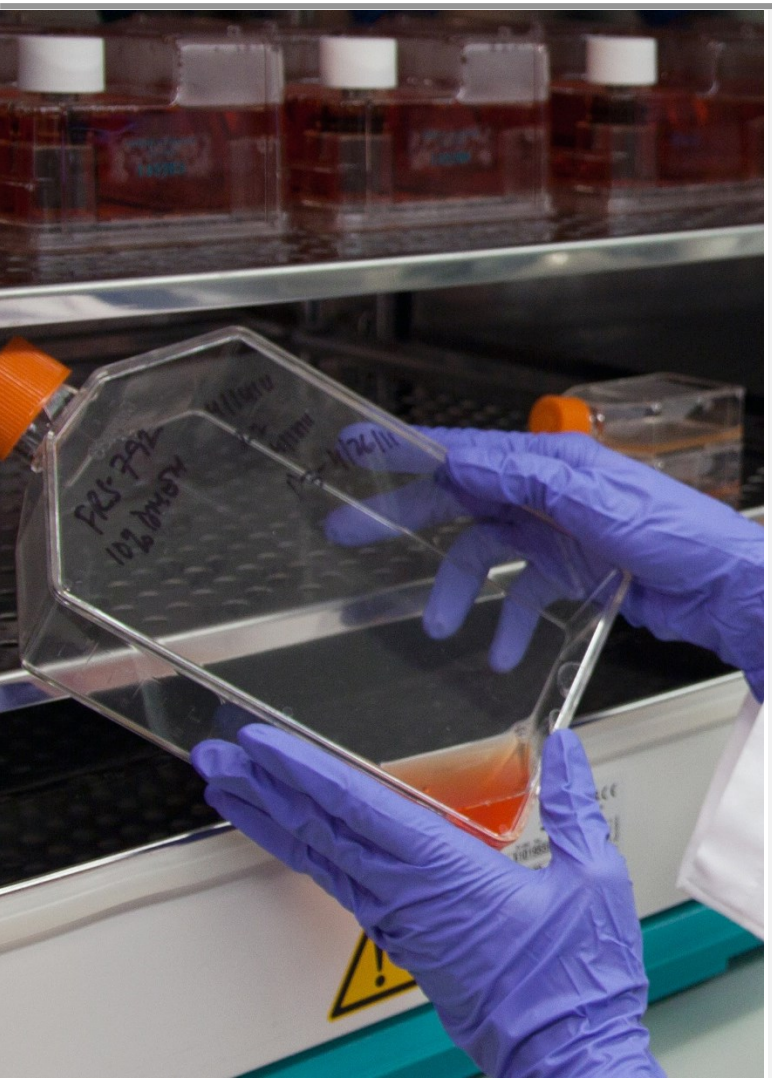
Growth Media Versus Maintenance Media



Growth Media

- Has a higher concentration of FBS, typically 10%
- Used when cells are grown

Maintenance Media (Viral Growth Media)

- Has a lower concentration of FBS, typically 2%
- Used when viruses are propagated

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Common Media

Eagle's Minimum Essential Media (EMEM; ATCC® 30-2003™)

- Contains Earle's balanced salt solution, non-essential amino acids, glycose, and sodium pyruvate
- Because EMEM is a simple medium, it is often fortified with additional supplements or higher levels of serum
- Formulated with a reduced sodium bicarbonate concentration for use with 5% CO_2

Dulbecco's Modified Eagle's Medium (DMEM; ATCC® 30-2002™)

- Contains approximately 4x the amount of vitamins and amino acids and 2-4x as much glucose as the original formula
- Formulated with a reduced sodium bicarbonate concentration for use with 5% CO_2

Antimicrobials

- Antibiotics and/or antimycotics are added to cell culture media as a prophylactic to prevent contamination or as a cure once contamination is discovered
- Antibiotics can mask contamination by mycoplasma and resistant bacteria, and can interfere with the metabolism of sensitive cells
- Antimycotics can be toxic to many cell lines and should be avoided
- Best Practice: Do not use prophylactic antibiotics or antifungals for virus culture

Fetal Bovine Serum (FBS)

Fetal serum is a rich source of growth factors and is appropriate for cell cloning and for the growth of fastidious cells

- Hormonal factors for stimulating cell growth and proliferation as well as promoting differentiated functions
- Transport proteins carrying hormones (*e.g.* transcortin), minerals, trace elements, and lipids
- Attachment factors
- Stabilizing and detoxifying factors needed to maintain pH or to inhibit proteases either directly (*e.g.,* α-antitrypsin inhibitor in serum is an important inhibitor of the protease trypsin) or indirectly by acting as an unspecific sink for proteases and other (toxic) molecules

Primarily because of its rich content of growth factors and its low γ-globulin (antibody) content, FBS has been adopted as the standard supplement of cell culture media

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Fetal Bovine Serum (FBS)

Filtered serum

- Commonly used for culturing cells and viruses
- Filtered through a 0.1 µm pore to remove adventitious agents
- Filters do not remove Mycoplasma or viruses

Gamma-irradiated FBS

- Preferred serum
- Irradiation inactivates viruses and other adventitious agents
- Can be expensive

Heat-inactivated serum

- Should only be used if it is required for a particular cell line
- Heat will reduce or destroy growth factors present in the serum

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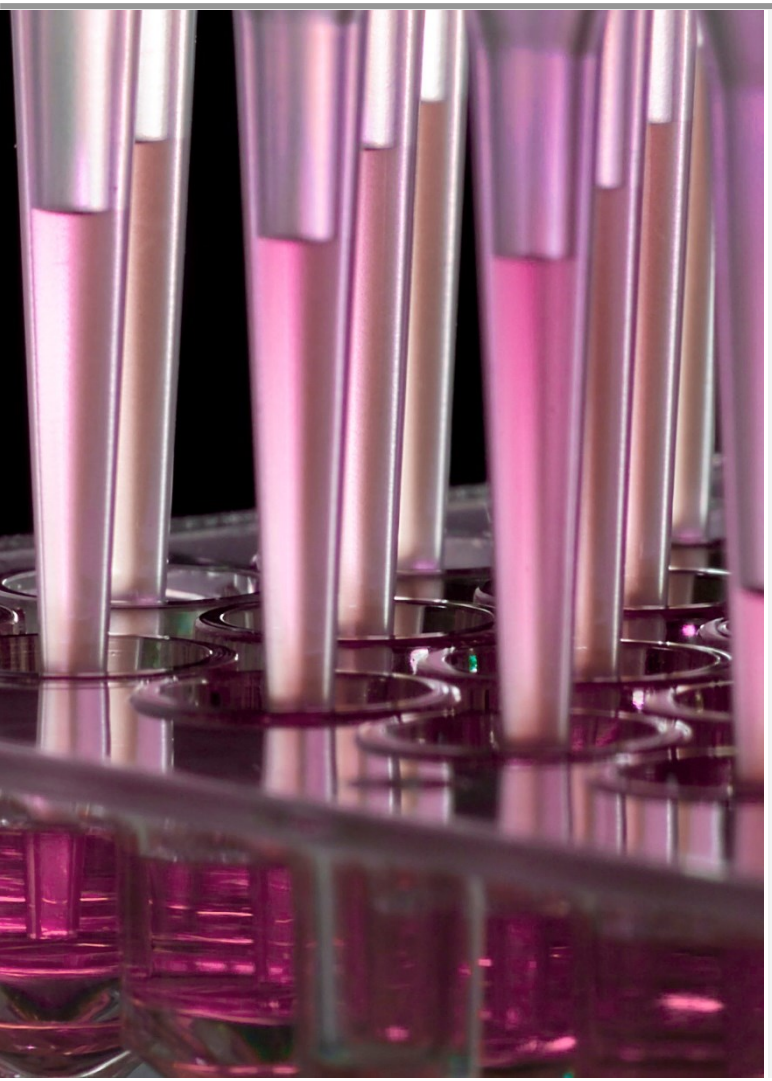
Fetal Bovine Serum (FBS)



Lot-to-lot variation

- The animal source can affect the quality of the serum

Endotoxin levels

- Check endotoxin levels, especially if you are trying to isolate virus from clinical sample

Country of origin

- There may be regulatory implications depending on what country the serum originated from
- *E.g.,* Foot and mouth disease (FMD) is a USDA-regulated livestock pathogen that is highly contagious. Virus cultures grown in serum that originate from FMD-affected countries cannot be imported into the United States.

Multiplicity of Infection (MOI)

MOI refers to the ratio of virus particles to infection targets (*e.g.*, host cells)

- High MOI is used when the experiment requires every cell in the culture to be infected
- Low MOI is used when multiple cycles of infection are required (as in preparation virus stock), typically 0.1 to 0.001 depending on the virus

To calculate MOI, viral titer and the number of cells must be determined

- Viral titer can be determined by plaque assay or any other method of quantifying infectivity
- Cell count can be determined with a cell counter

MOI = Plaque forming units (PFU) or Tissue Culture Infectious Dose ($TCID_{50}$) of virus used for infection / number of cells

$$\text{Example: } \frac{10^4 \text{ PFU/mL} \times 0.25 \text{ mL}}{2.5 \times 10^4 \text{ cells}} = 0.1 \text{ MOI}$$

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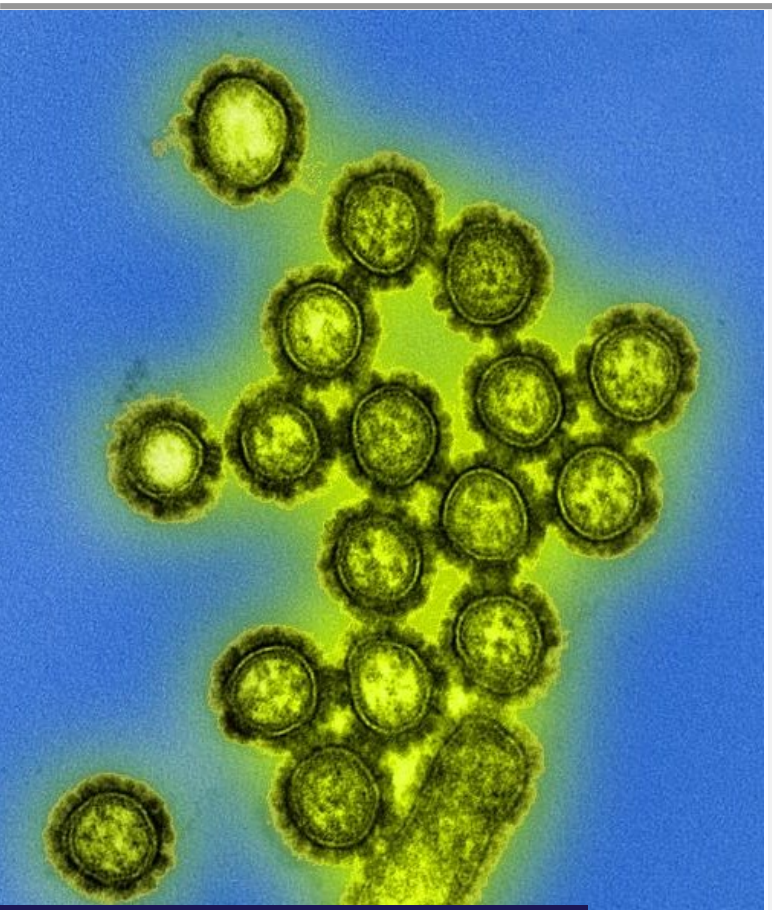
Defective Interfering Particles (DI)



Influenza virus H1N1. Photo courtesy of NIAID.

- Defective interfering (DI) particles are virus-like byproducts that are not infectious due to the presence of mutations in essential viral genes
- DI particles that lack essential genes can co-infect cells with viable virus, which interferes with virus growth
- DI particles are spontaneously produced via error-prone viral replication, and they have a huge impact on virus replication, evolution and pathogenesis
- At higher MOIs, more virus and DI particles are transferred from one passage to the next, enabling the rapid accumulation of DI particles and a greater inhibitory effect on virus growth
- Best practice: Use lower MOI for cultivation of virus stocks

Initiating an Infection in Tissue Culture

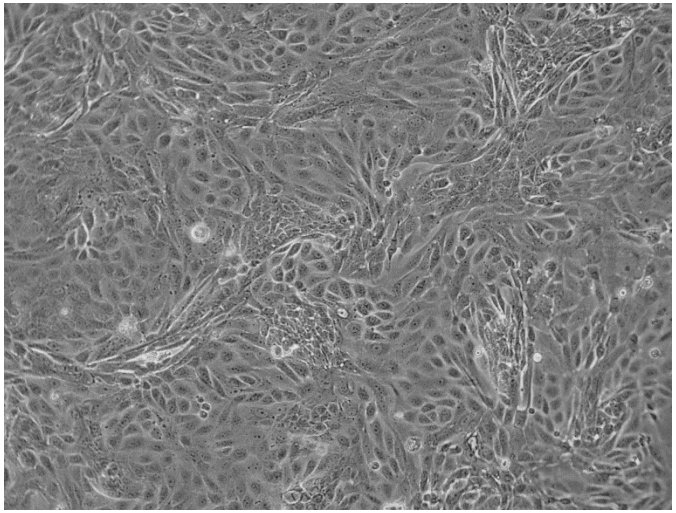
- One to two days prior to viral inoculation, seed the host cells into at least three flasks: one for virus, one for mock infected, and the third for counting cells on the day of infection
- Avoid using cells that were seeded more than 48 hours
- Allow cells to reach the appropriate confluency (within 24 -48 hours)
- Calculate the MOI based on titer of the virus seed
- Quickly thaw the frozen virus in a 37°C water bath and dilute in an appropriate volume of viral growth medium
- Rinse cells to remove traces of FBS prior to adding the virus inoculum

Harvesting a Viral Culture

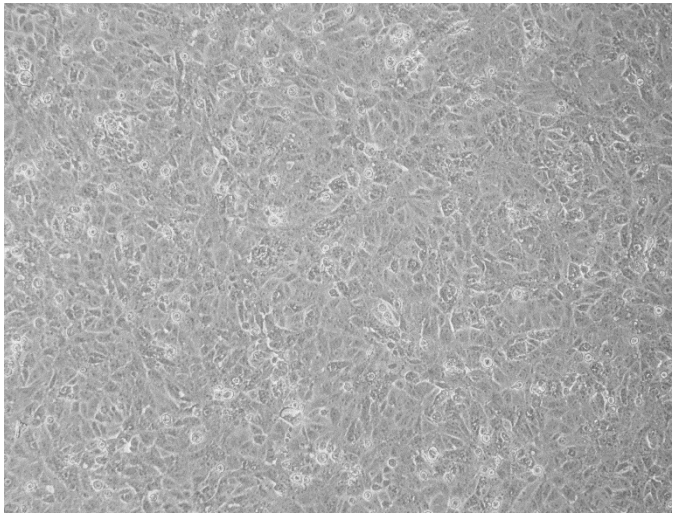
- Incubate the cultures under the appropriate temperature and atmospheric conditions for the recommended incubation period
- Observe infected cells everyday for evidence of cytopathic effects (CPE) or of possible contamination
- Determine the ideal harvest time; for most lytic viruses, harvest when CPE has progressed to ~80%
- Do not let CPE progress to a wipe out of cells as there will be loss of titer
- Freeze-thaw cycles assist in releasing cell associated virus

Influenza Growth in Tissue Culture

- Host cell lines
  - Madin Darby Canine Kidney Cells (MDCK; ATCC® CCL-34™)
  - African Green Monkey Kidney Cells (Vero; ATCC® CCL-81™)
- Cells are maintained in Eagle's Minimum Essential Medium (EMEM; ATCC® 30-2003™) with 10% fetal bovine serum (FBS; ATCC® 30-2020™) at 37°C with 5% CO_2



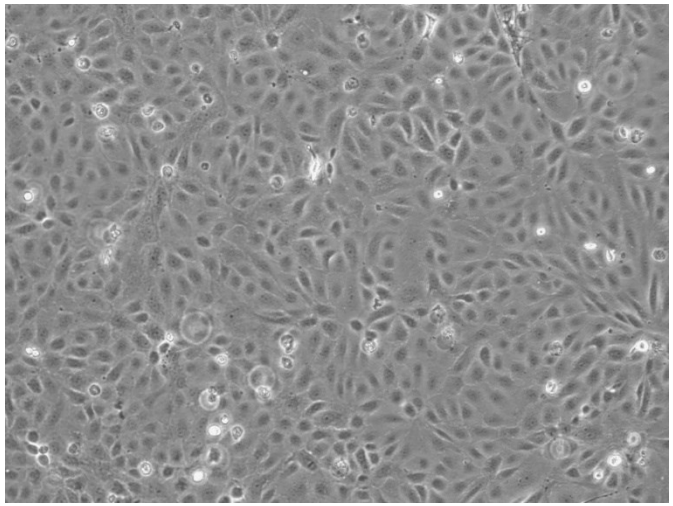
MDCK cells (ATCC® CCL-34™)
100x phase contrast



Vero cells (ATCC® CCL-81™)
100x phase contrast

Influenza Growth in Tissue Culture

Cells are seeded into flasks 1-2 days prior to infection and allowed to reach ~95% confluence



Confluent MDCK cells

100x phase contrast

1 flask to count cells for MOI calculation

1 mock infected control flask

n flasks for infection

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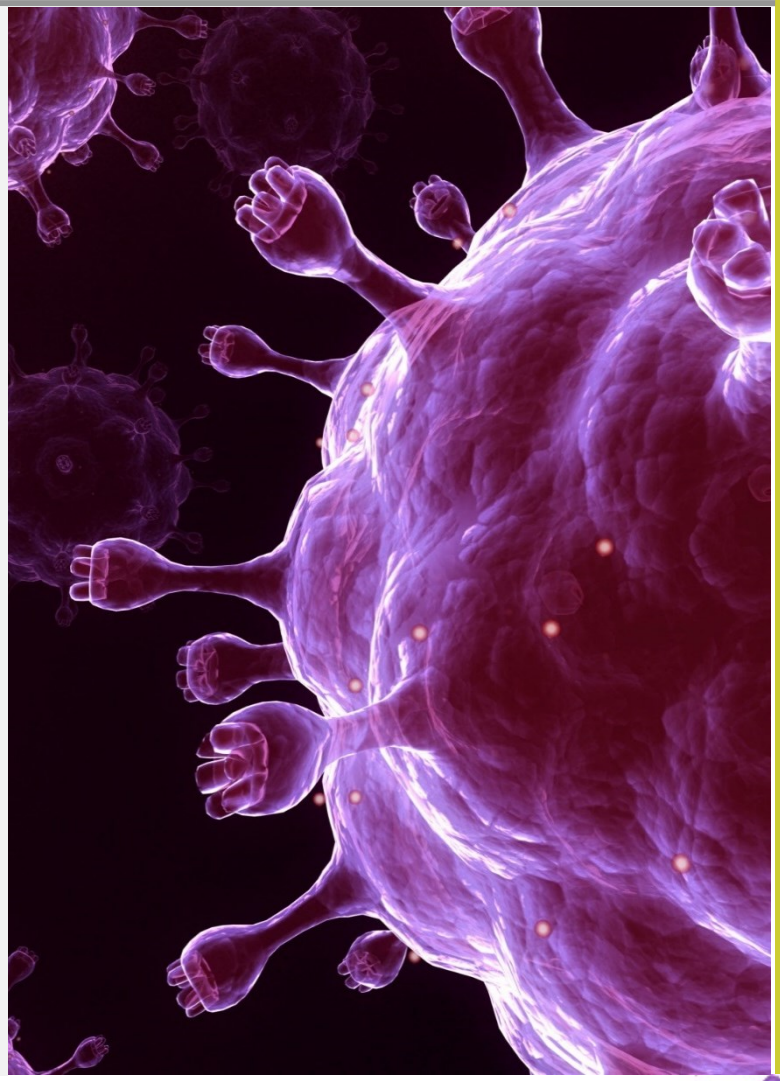
Influenza Growth in Tissue Culture

Prepare virus growth media:

- Virus growth media is EMEM with 10 mM Hepes buffer, 0.125% bovine serum albumin fraction V, and 1-2µg/mL TPCK-treated trypsin
- Trypsin is an endoprotease that cuts specifically on lysine and arginine; this cleaves hemagglutinin into 2 subunits on Influenza virus to allow for viral attachment and entry
- L-1-tosylamide-2-phenylethyl chloromethyl ketone (TPCK) treatment inhibits protease activity of chymotrypsin, a common trypsin contaminant
- Each lot of TPCK-treated trypsin may have a different level of activity and the final concentration of TPCK-treated trypsin is based on 1-2 µg active units/mL



Influenza Growth in Tissue Culture

- Harvest cells from designated MOI flask and count cells
- Calculate volume or dilution of virus inoculum required
- Thaw virus in 37°C water bath until just melted
- Prepare virus dilution in virus growth media
- Rinse flasks designated for the mock infected control and infection with phosphate buffered saline (PBS)
- Add appropriate inoculum volume for flask size (2-5 mL for T-75 cm^2)
- Rock flasks to evenly distribute the inoculum
- Incubate for virus adsorption (33°C for Influenza B, 35°C for Influenza A) in a 5% CO_2 atmosphere
- Feed flasks with virus growth media (8-17 mL for T-75 cm^2) and incubate for the recommended time

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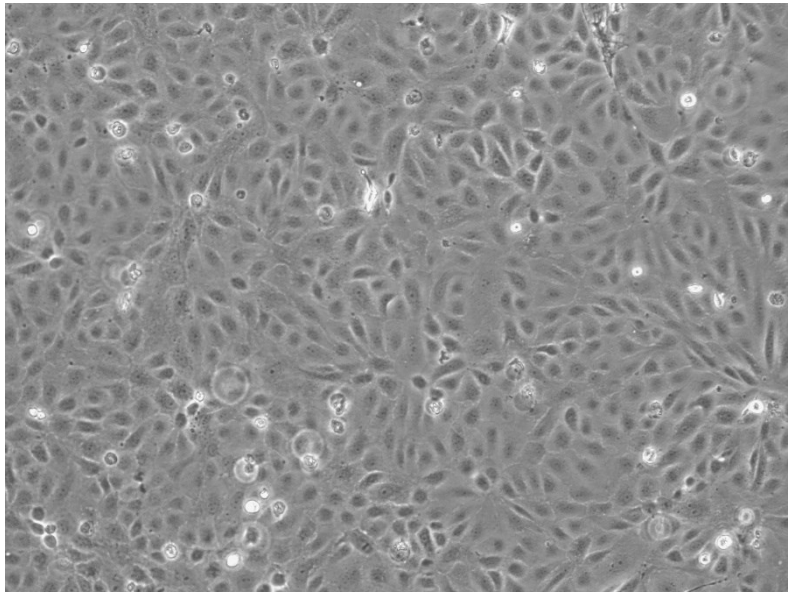
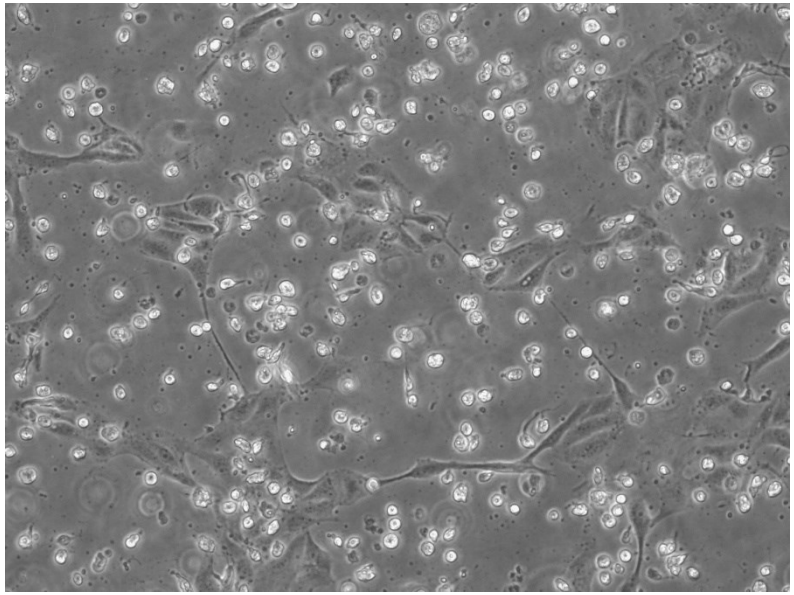
Influenza Growth in Tissue Culture



Mock infected control, MDCK cells

2 days post infection, 100x phase contrast



Influenza A virus A/Hong Kong/8/68 (H3N2) (ATCC® VR-1679™) in MDCK cells

2 days post infection, 100x phase contrast

- Monitor infections daily to look for signs of contamination and for CPE
- Viruses may cause different types of CPE in different host cells, or none at all
- Harvest when CPE is evident in 80% of cells
- Harvest using a cell scraper, then pool and preserve

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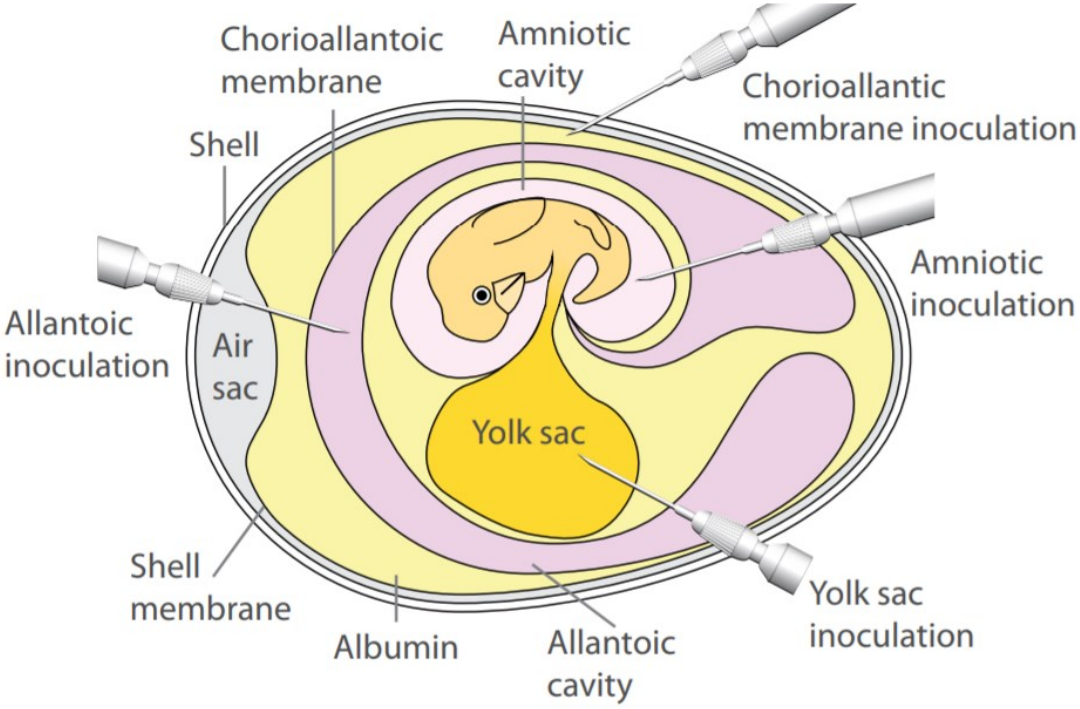
Influenza Growth in Tissue Culture

In cases where the virus does not cause CPE, a fluorescent antibody can be used to detect the virus

- Using a pipette, scrape a small area of the flask surface and transfer infected cells to a slide
- If the fluorescent antibody assay does not show a good proportion of infected cells, continue virus incubation

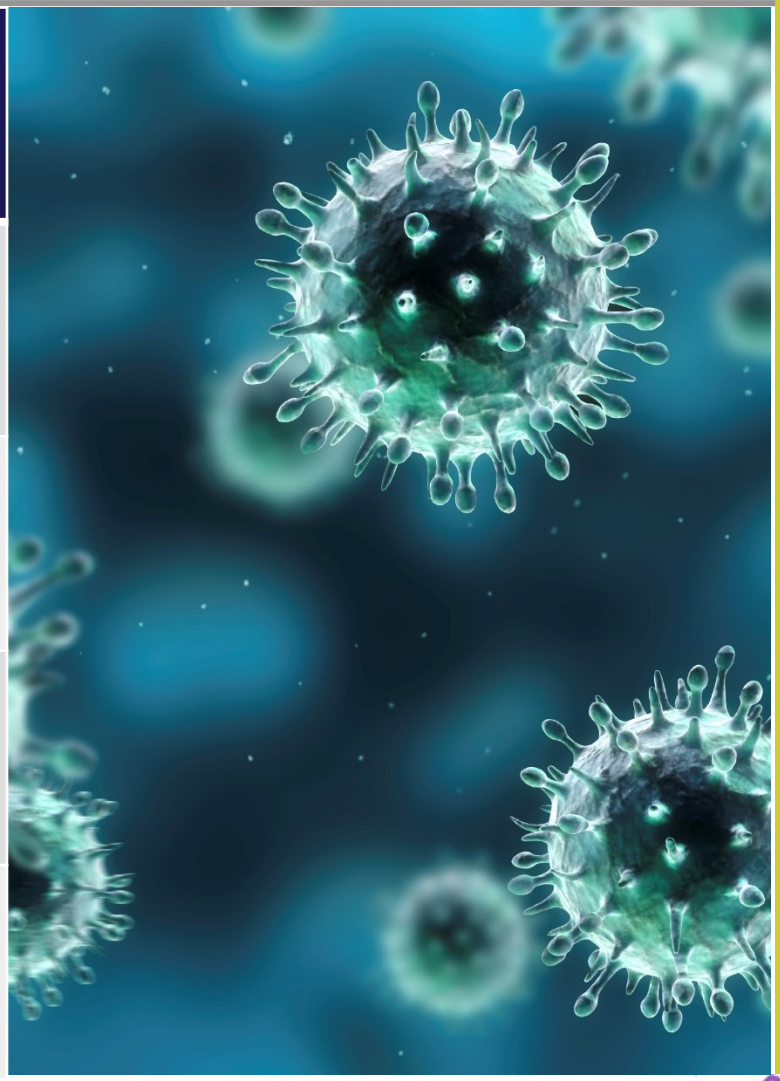
Egg-based Systems

- Convenient and inexpensive host for many viral strains
- There are several membranes and cavities within the egg that can support viral growth; the inoculation site will depend on the growth requirements of the virus
- Viral growth may result in embryo death, embryo cell damage, lesions on the egg membrane, or the agglutination of red blood cells



Viruses Grown in Eggs

Agent	Egg Age (Days)	Inoculation Route	Incubation (Days)	Optimal Temperature
Influenza A virus	10-11	Allantoic	2-3	33-35°C
Influenza B virus	10-11	Allantoic	2-3	33-35°C
Sendai virus	10-11	Allantoic	2-3	35-37°C
Rabies virus	7	Yolk	9-10	36.5°C



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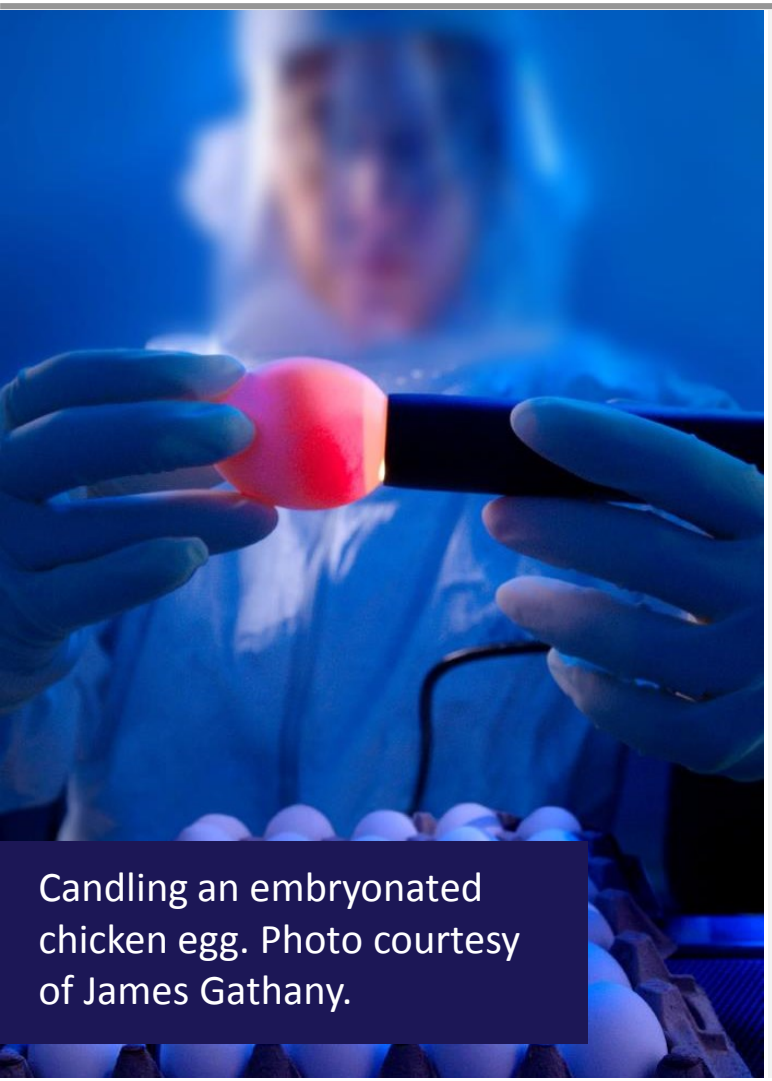
Maintenance and Source of Eggs

- Chicken eggs should be obtained from flocks that are confirmed to be free of specific disease pathogens (**S**pecific **P**athogen **F**ree)
- Eggs should be incubated at 37°C in an atmosphere of 40-70% humidity to ensure proper development of the air sac prior to virus inoculation
- Eggs should be candled a couple of hours after arrival to ensure that embryos are viable and properly developed

Candling Eggs



Candling an embryonated chicken egg. Photo courtesy of James Gathany.

- Hold the blunt end of an egg against a light source
- Use a pencil to outline the air sac, which appears transparent
- Use a pencil to mark the embryo eyespot, which appears as the darkest spot
- Influenza A & B viruses should be inoculated into 9-11 day old eggs

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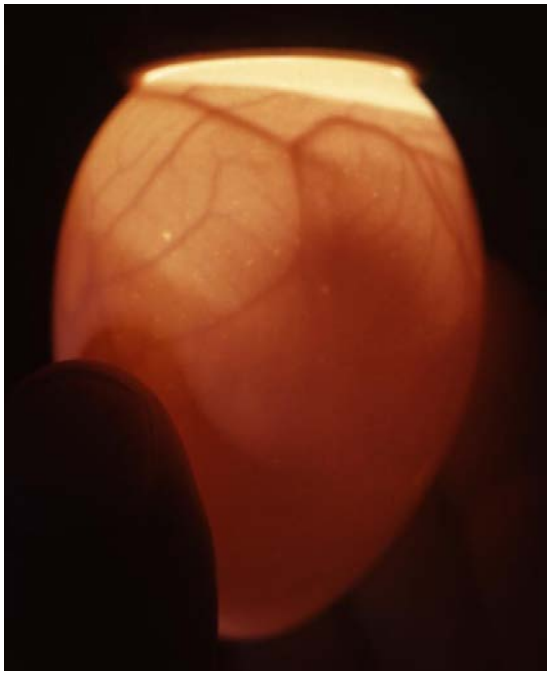
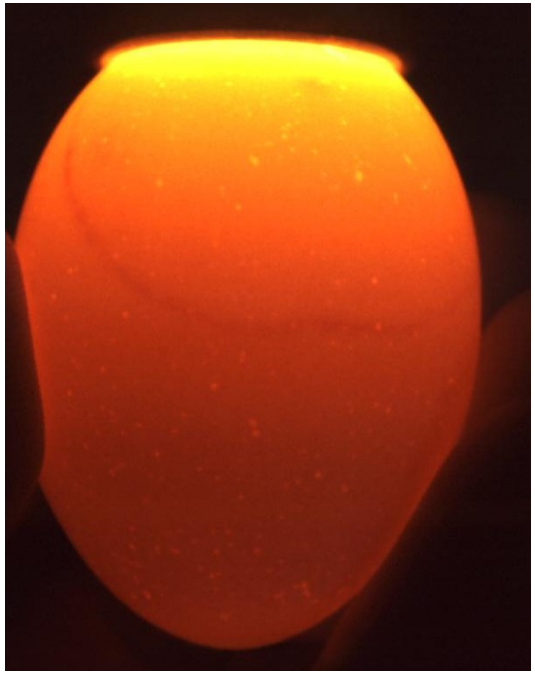
Candling Eggs



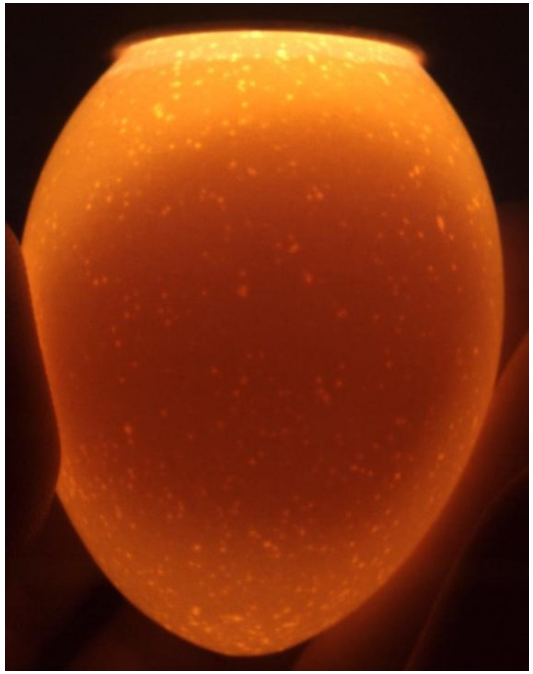
11-day-old fertilized egg

- Air sac
- Blood vessel network
- Embryo movement



Non-viable embryo

- Blood vessels collapsed above yolk sac
- No embryo movement



Unfertilized egg

- No blood vessels visible
- Large yolk sac
- No embryo

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Images courtesy of the CDC and Dr. Joseph McDade

Candling Eggs

- Non-viable embryo
- Collapsed blood vessels

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Allantoic Cavity Inoculation

- Prepare Influenza virus: Quick thaw in a 37°C water bath, dilute in PBS to approximately 1,000 $CEID_{50}$/egg

  (5,000 $CEID_{50}$/mL if 0.2 mL per egg)
- Wipe eggs with ethanol-soaked sterile cotton. Do not spray alcohol!
- Using an 18 gauge needle, pierce a hole into the air sac about halfway from top to pencil outline
- Using a 22 gauge needle and a syringe, inject the viral inoculum through the hole into the allantoic cavity, being careful not to stick the embryo

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Allantoic Cavity Inoculation

- Seal the hole with a drop of nail polish
- Incubate under conditions appropriate for viral replication
- Candle eggs 18-24 hours after inoculation and discard eggs that are non-viable due to needle stick injury

Harvesting Allantoic Fluid

- At the end of incubation period, refrigerate eggs for at least 2 hours
- Wipe the eggs with ethanol-soaked cotton
- Open the egg by tapping on the shell just above the air sac until the shell breaks
- Use sterile scissors or forceps to cut away shell around air sac

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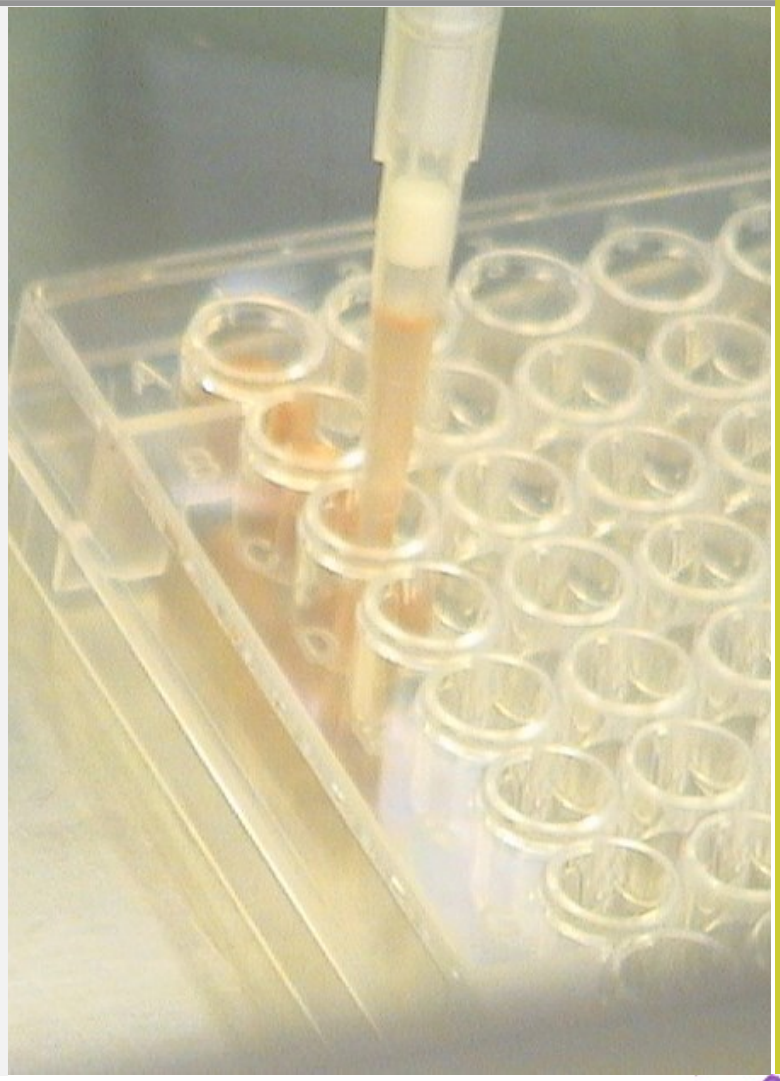
Harvesting Allantoic fluid

- Use a sterile pipette to poke through the allantoic membrane into the allantoic cavity
- Slightly tilt the egg to allow the allantoic fluid to pool
- Harvest the allantoic fluid with a serological pipette
- Check for obvious signs of bacterial/fungal contamination; only harvest clear allantoic fluid
- Egg yolk and blood can cause false negative results by hemagglutination assay (HA)
- Approximately 5-10 mL can be harvested from each egg

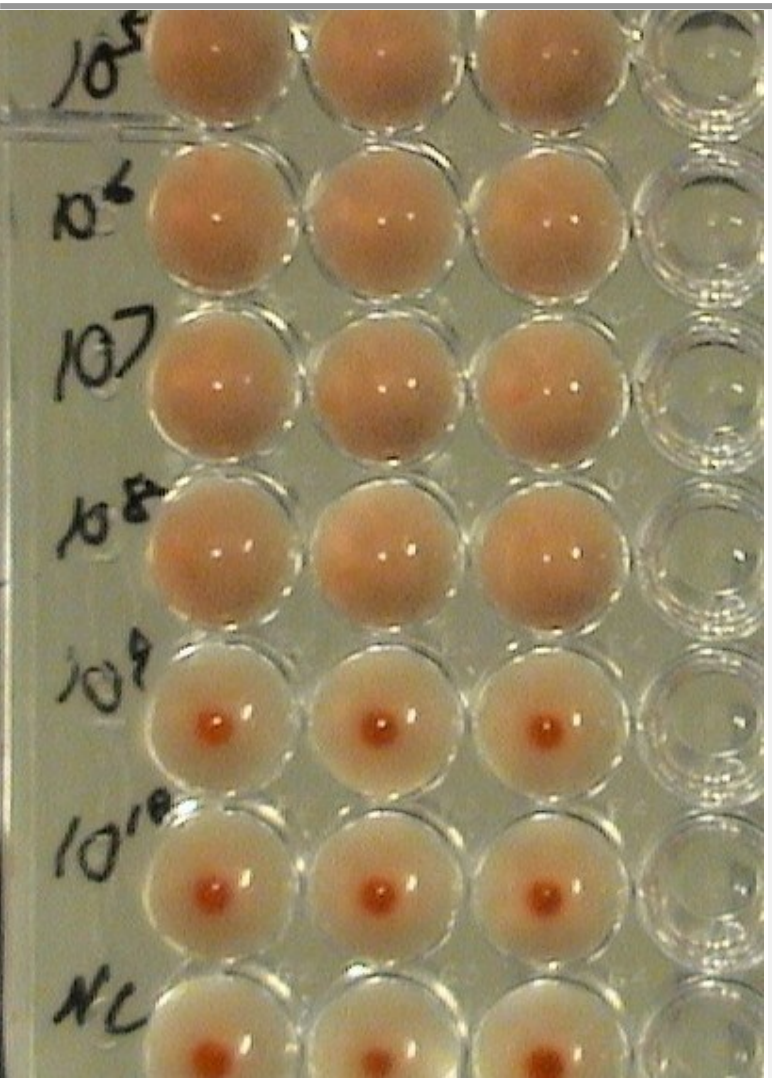
Harvesting Allantoic fluid

Hemagglutination Assay (HA)

- In a 96 well U- or V-bottom plate, add 100 µL NC (uninfected allantoic fluid), virus, or PC
- Add 100 µL of 0.5% Chicken Red Blood Cell Solution to each well
- Allow the plate to sit at room temperature for 30 minutes



Interpretation of Hemagglutination Assay



A positive HA result forms a lattice at the bottom of a well

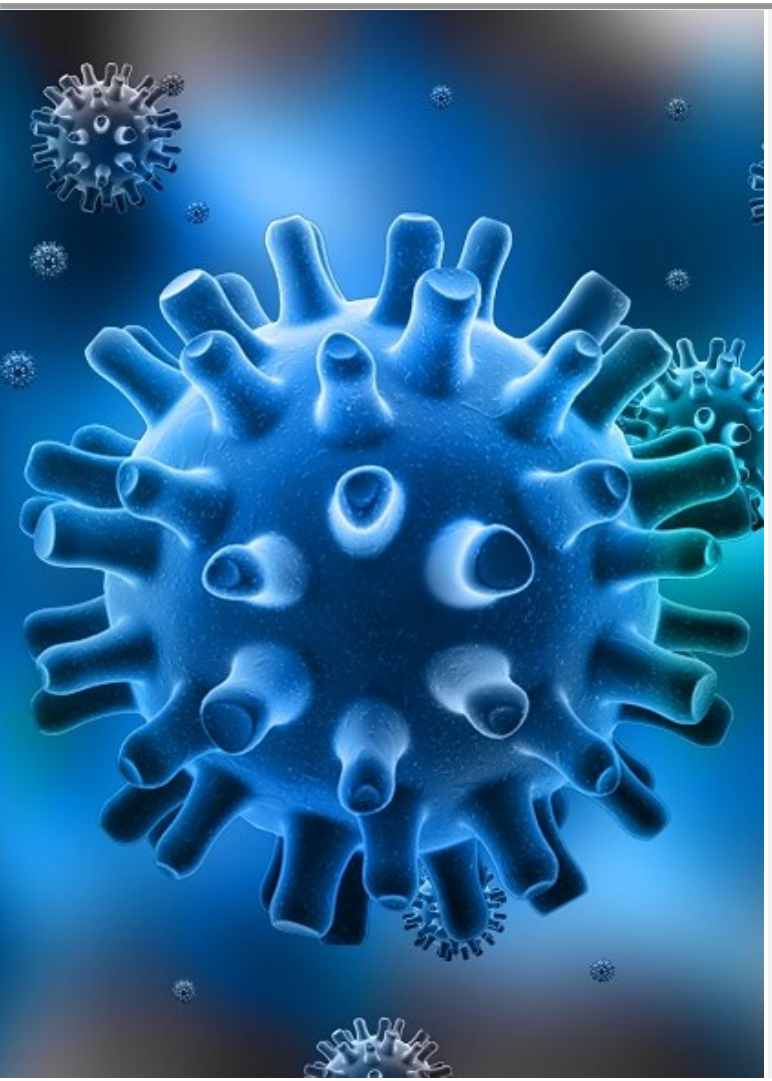
- Red blood cells (RBCs) attach to virus particles, preventing the red blood cells from accumulating at the bottom of the tube
- RBCs stack up in a slightly overlapping arrangement, making the bottom of the tube look fuzzy

A negative HA result is seen as a round button of red blood cells sinking to the bottom of the tube

Viral preservation

- No single procedure is optimal for the preservation of all animal viruses
- For cell-associated viruses, slow freezing is recommended to achieve a cooling rate of -1˚C per minute
- Freeze Medium: Glycerol and DMSO are the most common cryoprotectant agents
- The viability of viruses that are not cell-associated is best maintained by rapid freezing
- Viruses are quickly frozen in a dry ice slurry or liquid nitrogen (N_2) vapor
- Store virus in liquid N_2 vapor or within a mechanical freezer at -80°C
- Ultra-low temperatures (below -120˚C) are required for long-term storage

Summary



- Good laboratory practices and aseptic technique are essential for properly growing and maintaining virus stocks
- When propagating viruses in tissue culture, choosing the ideal propagation host, ensuring host cell authenticity, and using the correct MOI and media are required for viral cultivation
- When propagating viruses in embryonated chicken eggs, use SPF eggs and ensure that each egg contains a viable embryo prior to use
- No single procedure is optimal for viral preservation; this must be determined empirically

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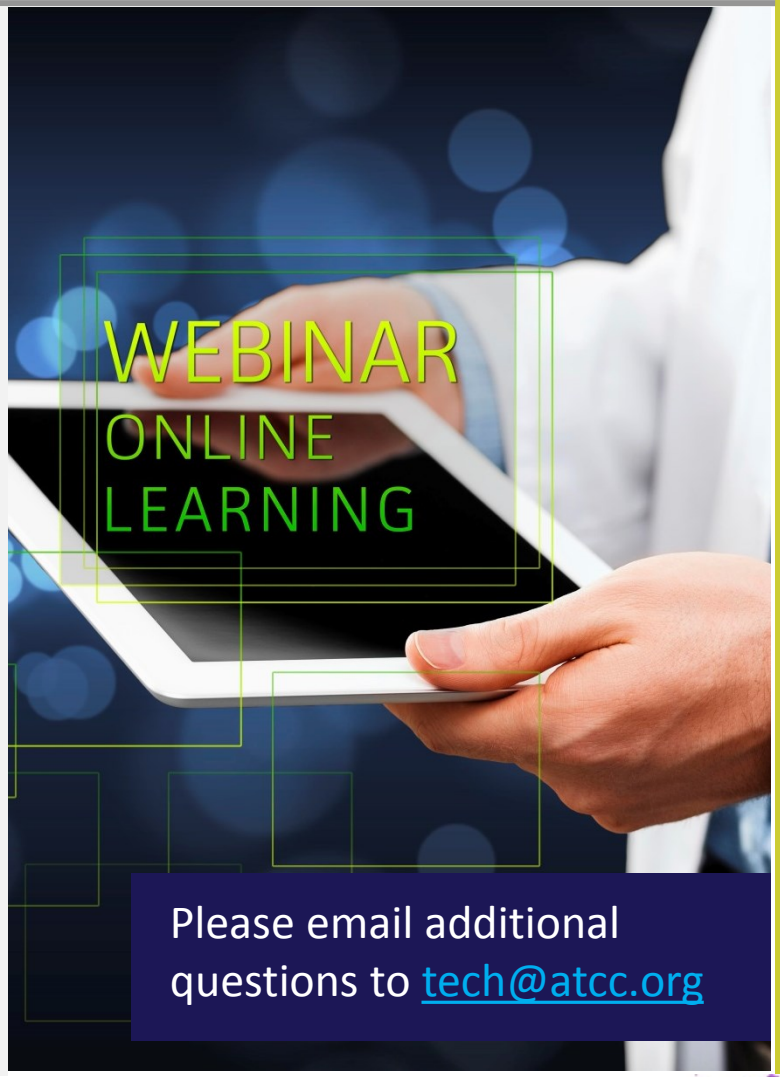

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