Virus Culture Fundamentals: Methods and Strategies For Viral Propagation

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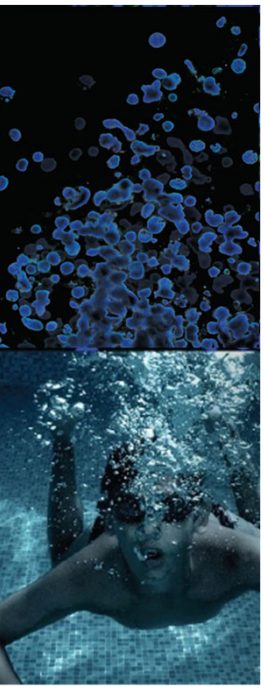
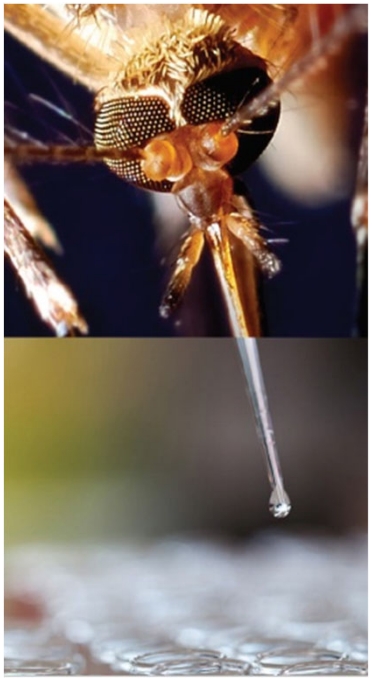
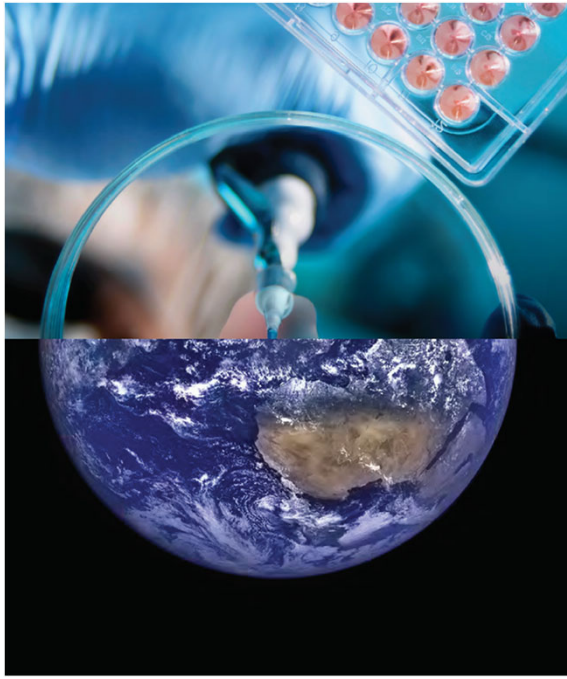
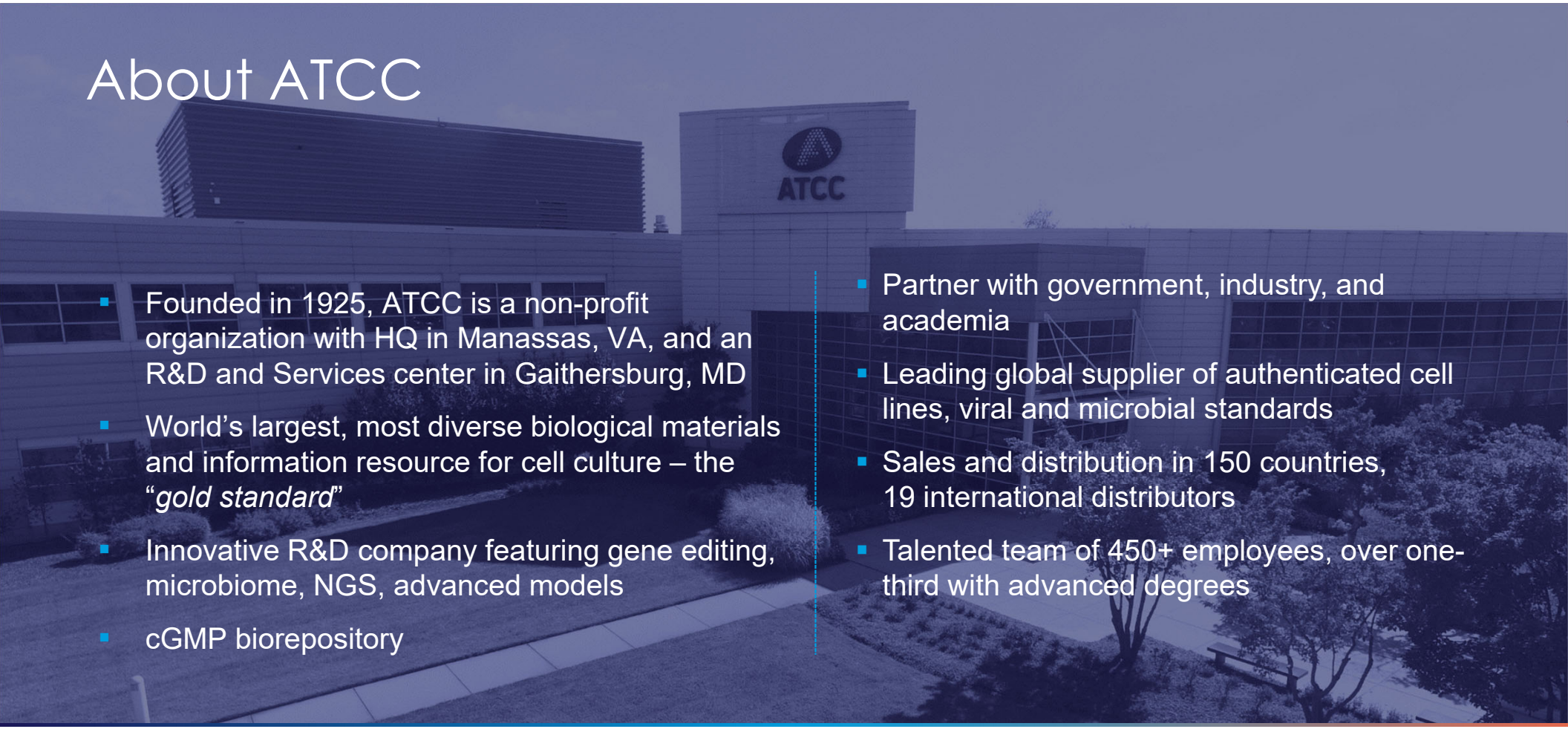






Credible Leads to Incredible™

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 largest, most diverse biological materials and information resource for cell culture – the "*gold standard*"
- Innovative R&D company featuring gene editing, microbiome, NGS, advanced models
- cGMP biorepository
- Partner with government, industry, and academia
- Leading global supplier of authenticated cell lines, viral and microbial standards
- Sales and distribution in 150 countries, 19 international distributors
- Talented team of 450+ employees, over one-third with advanced degrees

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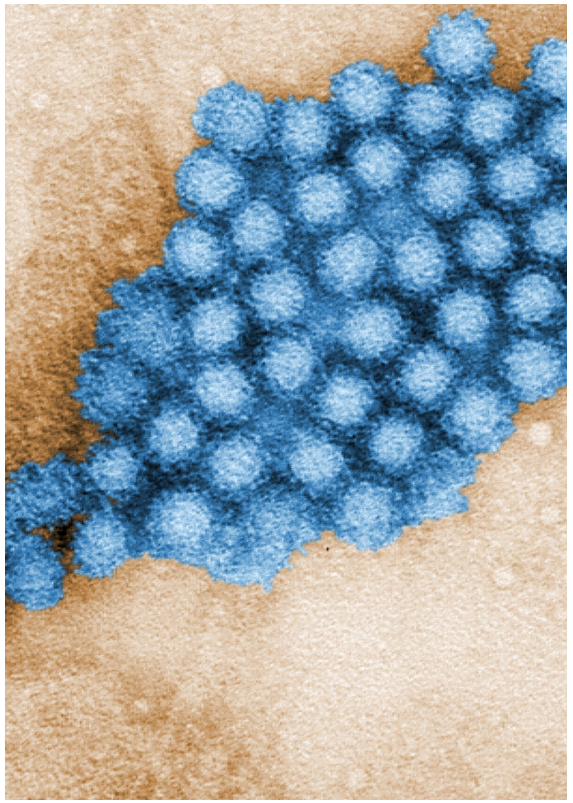
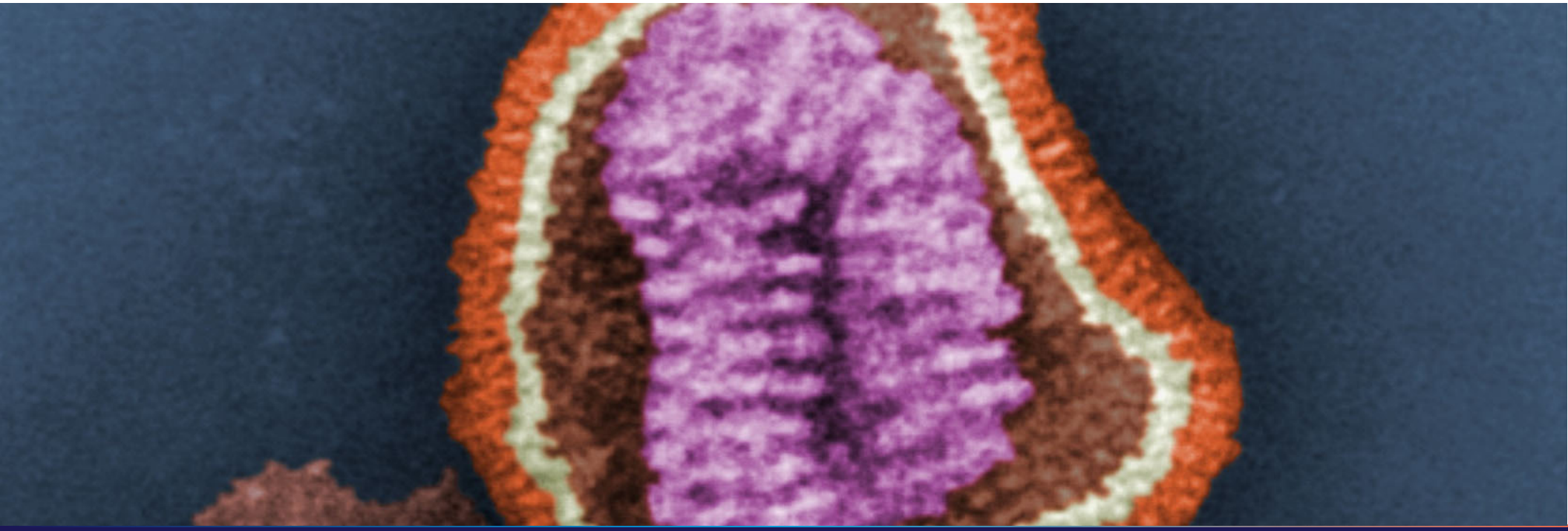
Agenda

- Virus fundamentals
  - Presented by Adria Allen
- Authentication and quality control
  - Presented by Megan Yockey
- Troubleshooting strategies
  - Presented by Alex Piccirillo



Norovirus courtesy of Dr. Charles D. Humphrey, CDC



The Fundamentals

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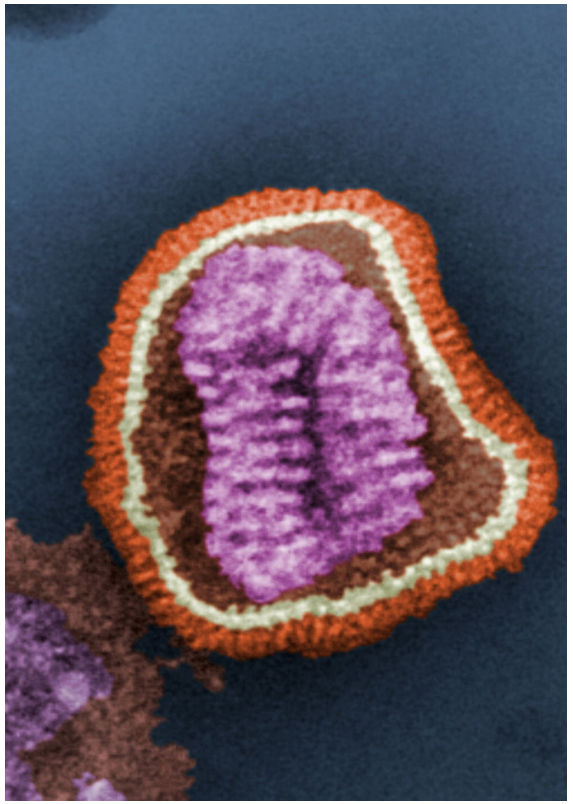
Overview

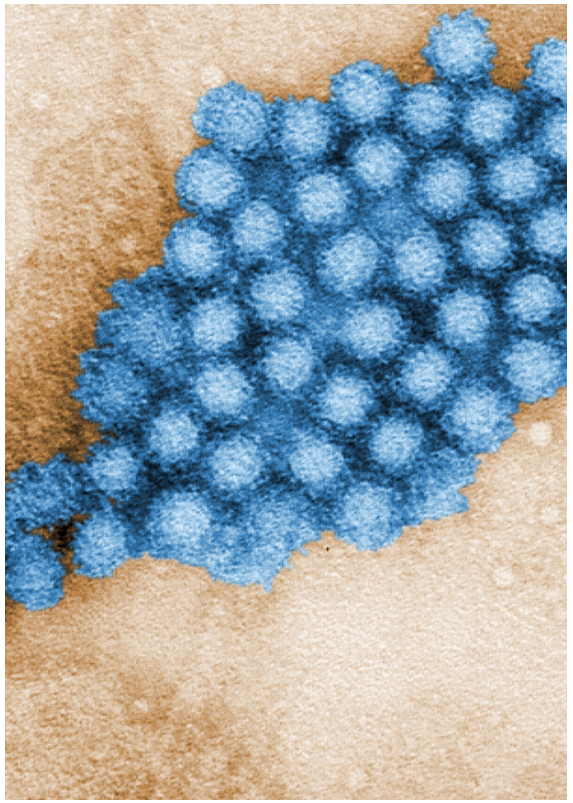
- Virus Basics
  - Structure
  - Life Cycle
- Virus Propagation
- Common Hosts
- Propagation Strategies



Influenza virus courtesy of Dr. Erskine L Palmer and ML Martini, CDC

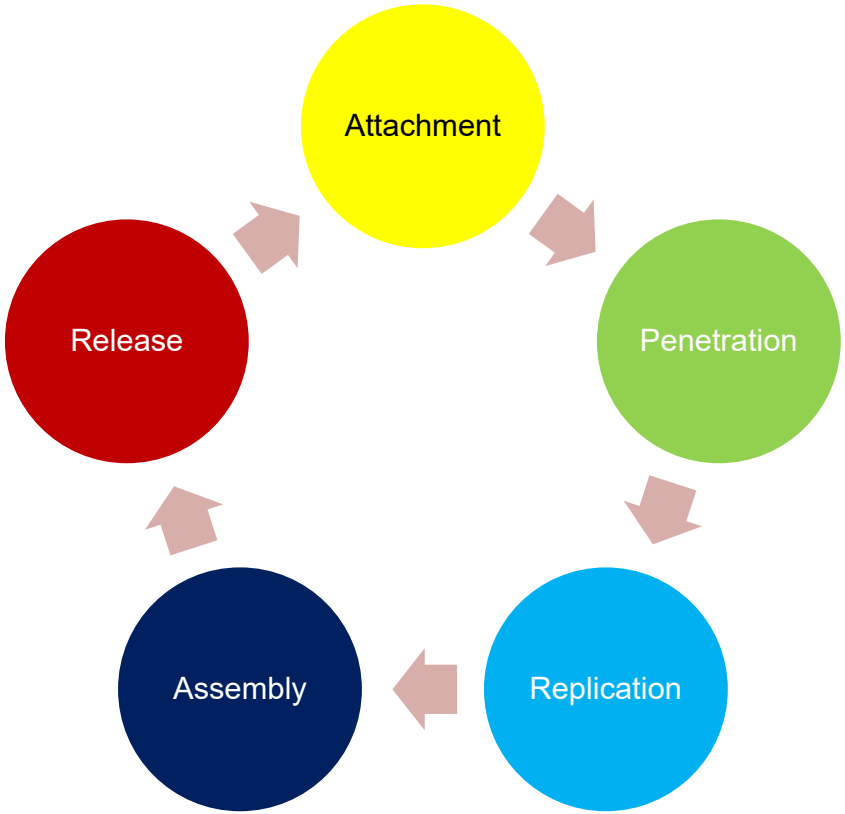
Viruses: The Basics

- Genome
  - DNA or RNA
  - Double stranded or single stranded
  - Segmented or non-segmented
- Structure
  - Enveloped vs non enveloped



Norovirus courtesy of Dr. Charles D. Humphrey, CDC

Virus Life Cycle



- Attachment
  - lock and key
  - Susceptible host
- Penetration
  - Virus gets into cell
- Replication/Assembly
  - Permissive host
  - Virus commandeers cell functions to make more virus
- Release*

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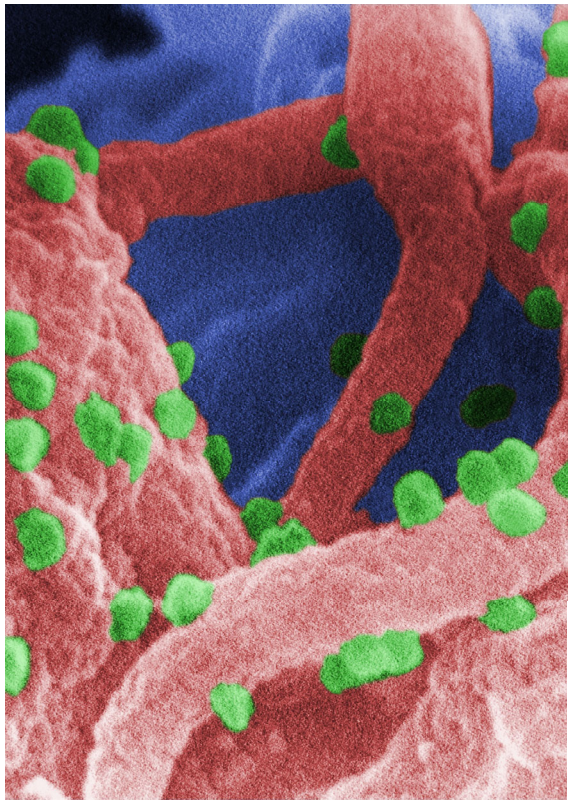
Virus Life Cycle: Release

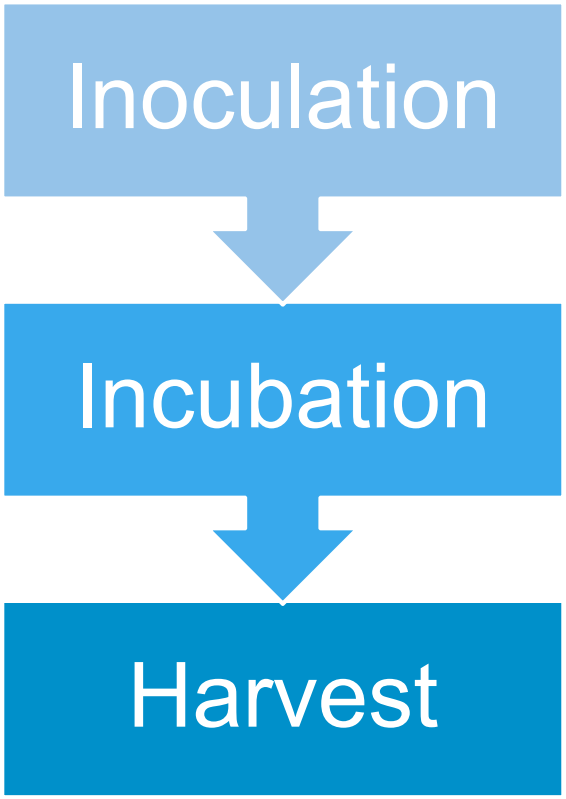
- Cell Lysis
  - Destruction of host cell
  - Viral particles released at same time
  - Usually non-enveloped viruses
- Viral Shedding
  - Exocytosis
  - Budding
- Cell-Associated
  - Virus doesn't completely release from host
  - Host cell aids in infecting new host



HIV (green) budding from lymphocyte courtesy of Public Health Image Library

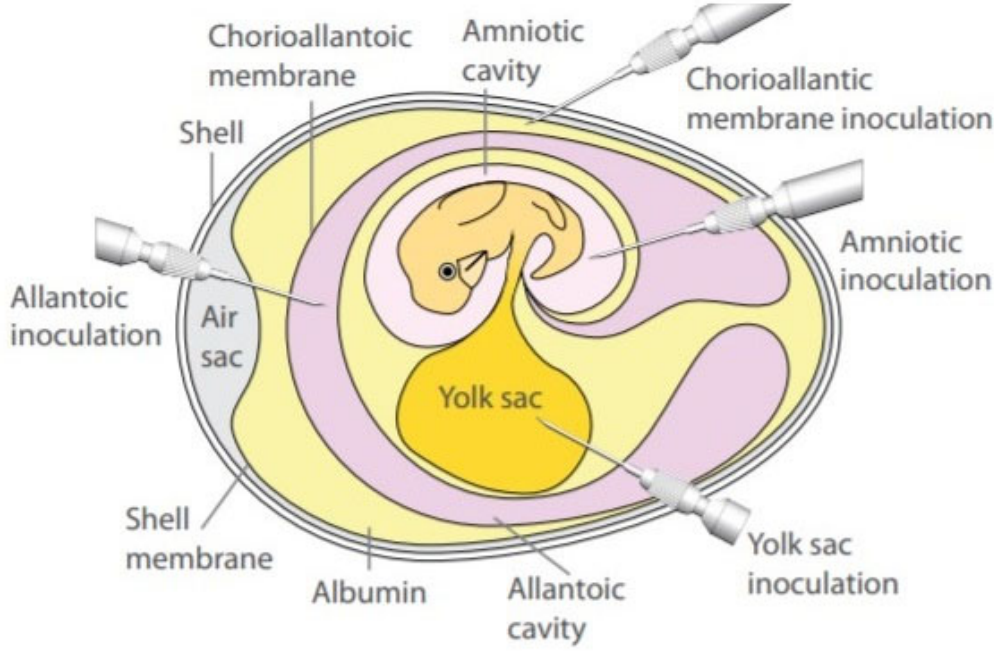
Virus Propagation

- Viral Passage
  - The completion of one cycle of:
    - Inoculation
    - Incubation
    - Harvest
- Passage History
  - A quantitative chronological history of the hosts used to propagate a specific viral product
  - Indicates if different hosts were used
  - Denotes how many times a viral strain was passage



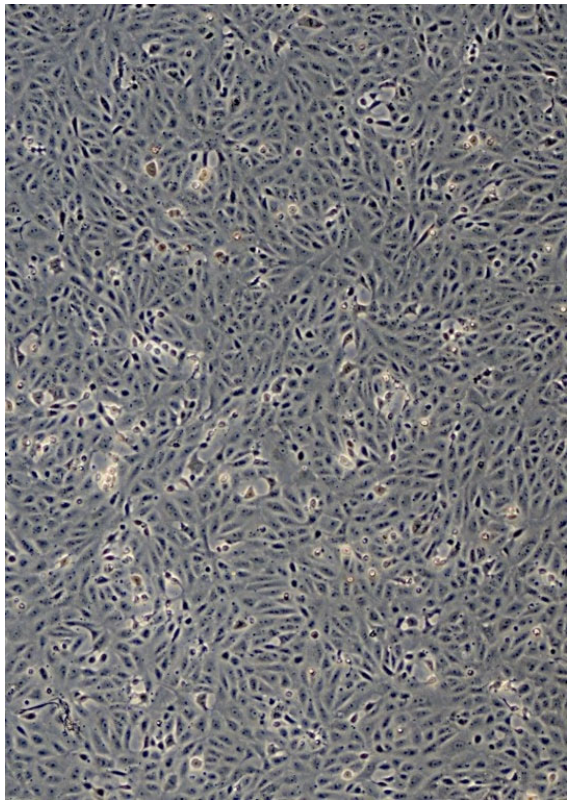
Virus Hosts: Embryonated Eggs

- Embryonated eggs
  - Benefits
    - Sterile environment
    - Multiple tissues
    - Multiple enzymes
    - Able to scale up production easily
    - Simple inoculation
  - Limitations
    - Not suitable for all viruses
    - Possible allergens
- Cell Culture



Virus Hosts: Cell Culture

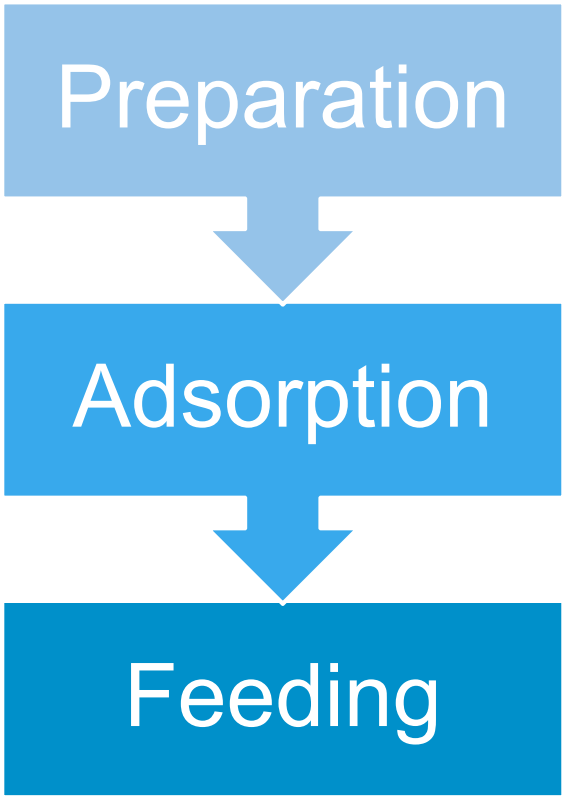
- Cell culture: Growth of tissue or cells in an artificial medium separate from the parent organism
- Cell line: A cell culture developed from a single cell and therefore consisting of cells with a uniform genetic makeup.
- Types of cell lines
  - Adherent
  - Suspension
- Benefits
  - Reproducibility: Grown in a highly controlled environment
  - Ability to scale up production
- Limitations
  - Highly controlled environment
    - Must add everything needed for the virus life cycle



Host cells, Vero (ATCC® CCL-81™)

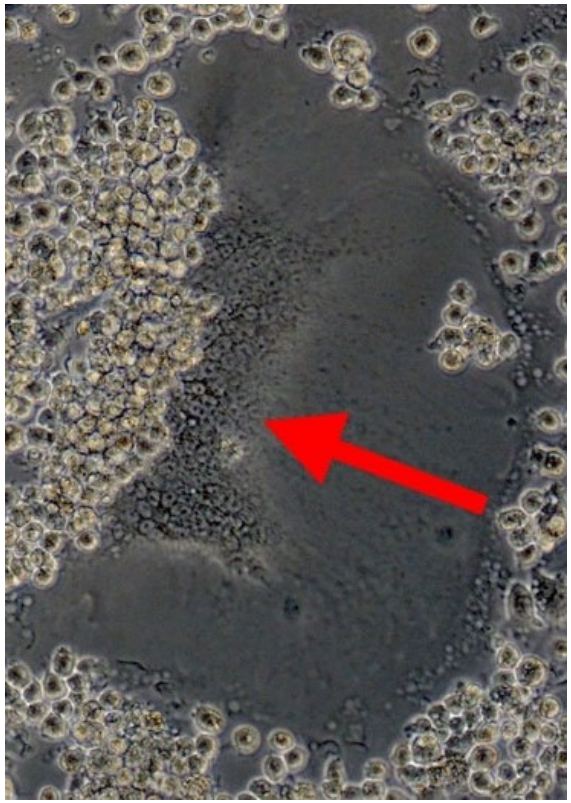
Inoculating Cell Culture

- Preparation
  - Remove cell growth media from cells
  - Inoculum
    - Quantity of virus brought to specific volume
- Adsorption
  - Add inoculum to cell culture
  - Low volume to increase host virus interaction
  - 1 – 3 hours
- Feeding
  - Add viral growth media
  - Keep cells alive long enough to replicate virus

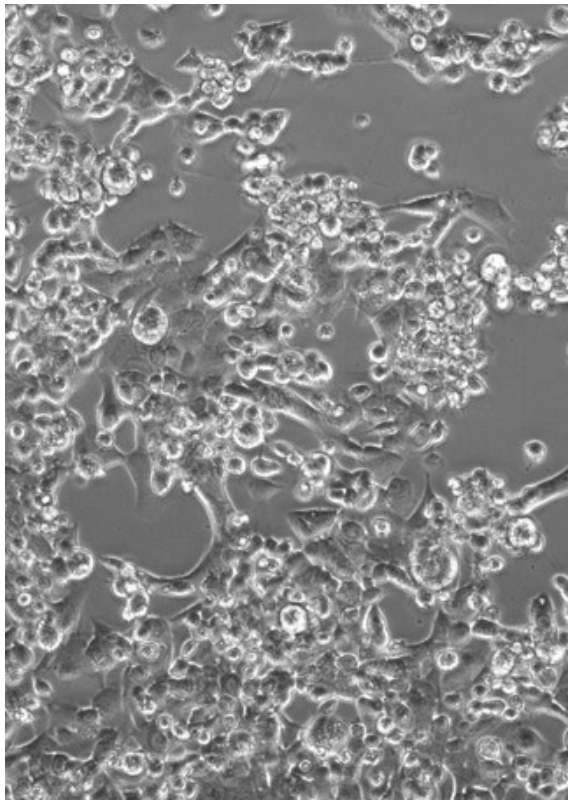


When to Harvest?

- Incubation length
  - Time based
    - No visible CPE
  - Cytopathic effect based
- Common CPE
  - Cell Rounding
  - Cell clumping
  - Syncytia
  - Cell enlargement



Murine hepatitis virus, syncytia formation (ATCC® VR-261™)



Human adenovirus 41, cell rounding and clumping (ATCC® VR-930™)

Harvest

- Scrape
  - Viruses that aren't cell associated
- Dissociation
  - Gently detached adherent cells from vessel wall
  - Cells still alive

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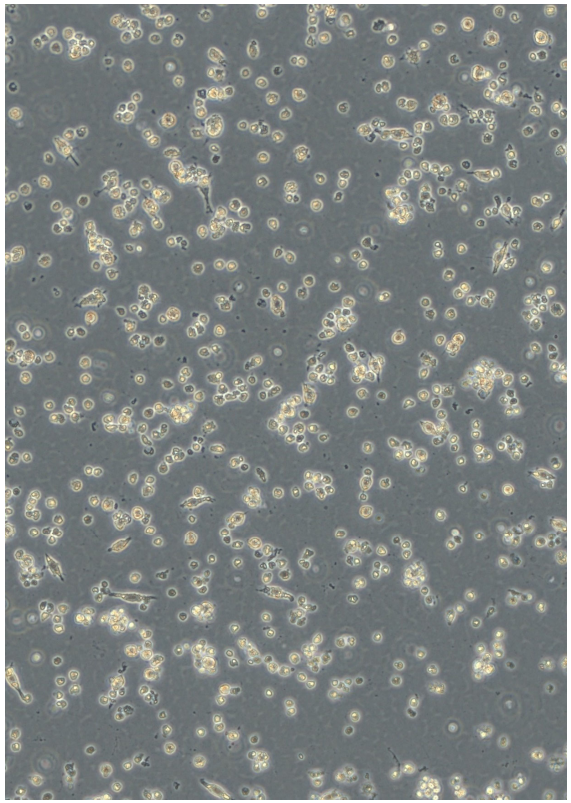
Infection Strategy 1

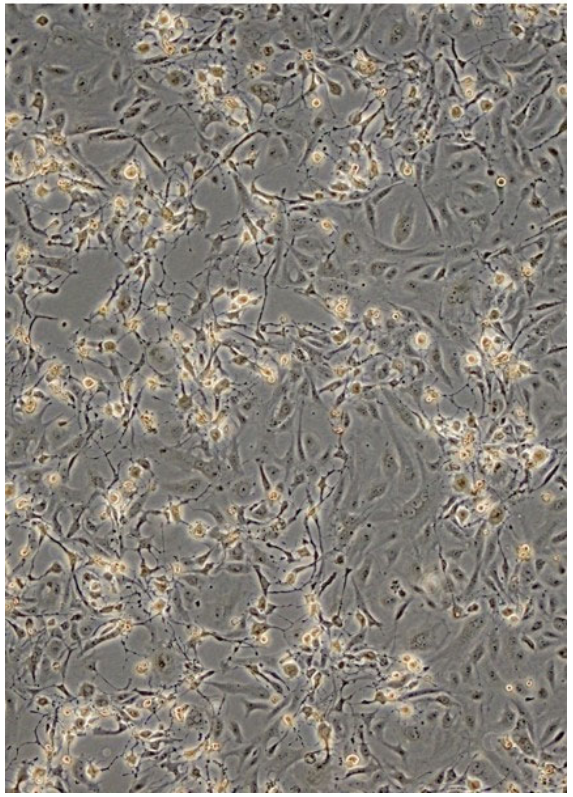
- Multiple rounds of replication within one passage
- Do not want host cells infected at same time
  - Inoculate with less virus
- Build up of viral particles in viral growth media
- Virus Characteristics
  - Non enveloped viruses
  - Viruses that survive outside host for long periods of time



Murine norovirus (ATCC® VR-1937™)

Infection Strategy 2

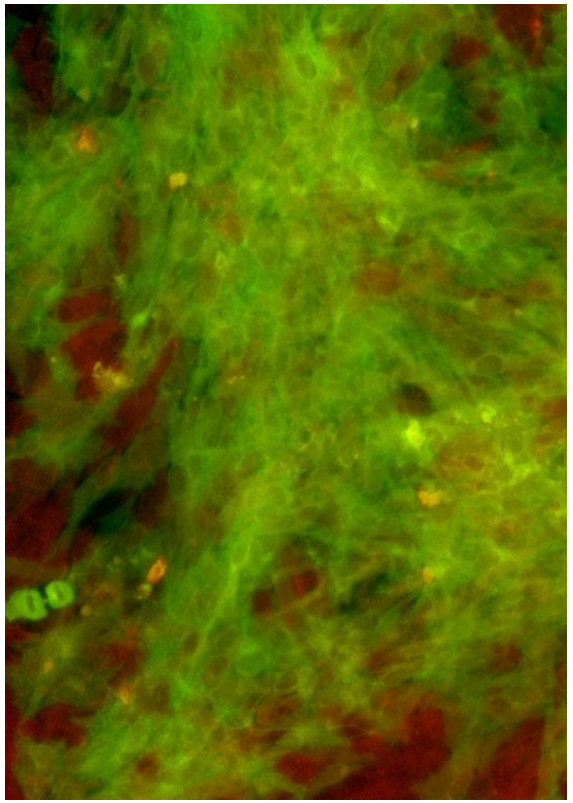
- Few rounds of replication per viral passage
- Host cells should be at the same stage of infection at the same time
- Infect with more viral particles than host cells
- Virus characteristics
  - Virus loses viability quickly after release from cell
  - Cell associated
  - *e.g.*, Varicella zoster (VZV; ATCC® VR-1433™)



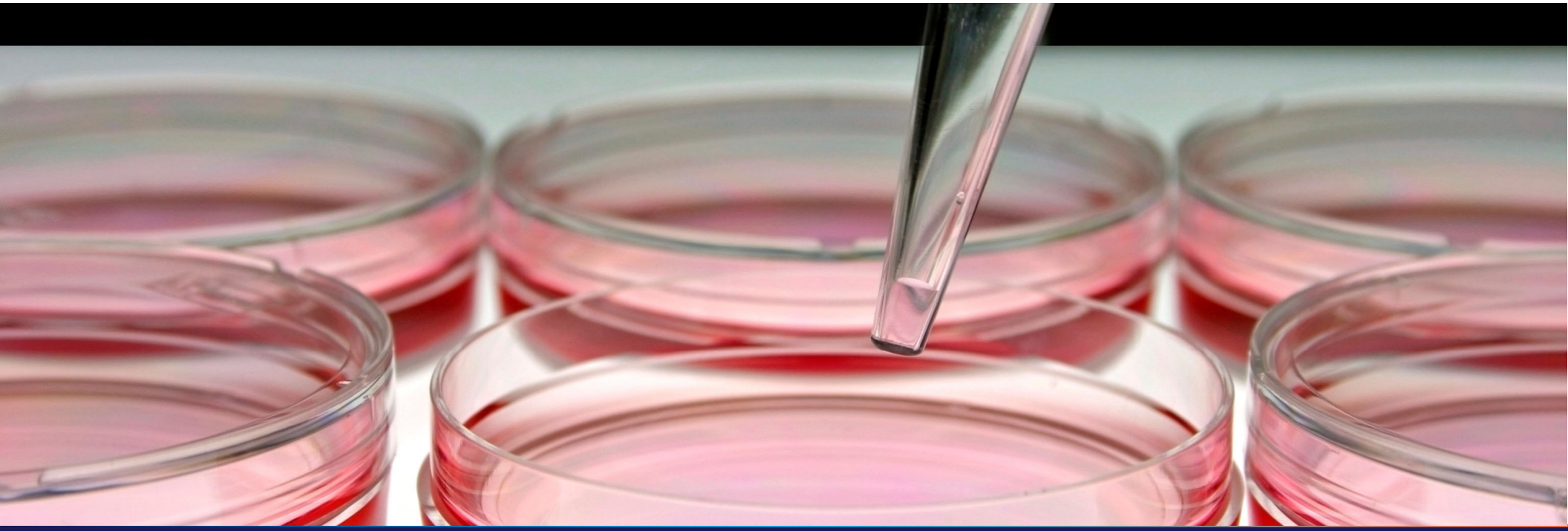
VZV infection of BSC-1 cells (ATCC® CCL-26™)

Infection Strategy 3

- Infect host cells once
  - Infected host cells will divide into two infected daughter cells
  - Uninfected cells will be infected by viral particles budding from infected cells.
- Virus Passage defined by splitting the infected cells
  - Same as cell culture passage
- Virus Characteristics
  - Virus incorporates genome into host cell
  - Persistent infection
  - *e.g.,* retroviruses



Feline Leukemia Virus (ATCC® VR-1889™)



Authentication, Quality Control and Preservation

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Outline

- Viral quantification and viability
  - Plaque assays
  - $TCID_{50}$
    - Titer specifications
    - IFA, RT-PCR/PCR
  - $CEID_{50}$
    - HA Assay
- Authentication methods
  - NGS
  - Sanger sequencing
- QC methods
  - Mycoplasma testing
  - Sterility testing
- Storage conditions for viruses to ensure integrity

Quantification and Viability of Viruses

- Viral quantification involves counting the number of virus particles in a known volume to determine concertation.
  - There are three different methods for viral quantification
    - Inoculating cells or eggs to measure infectivity:
      - Plaque assay, Immunoflurocesent assay (IFA) and Endpoint dilution assay ($TCID_{50}$/$CEID_{50}$)
    - Analyze viral protein or gene expression levels:
      - ELISA, qPCR
    - Counting viral particles:
      - Transmission Electron Microscopy (TEM) and Viral counters

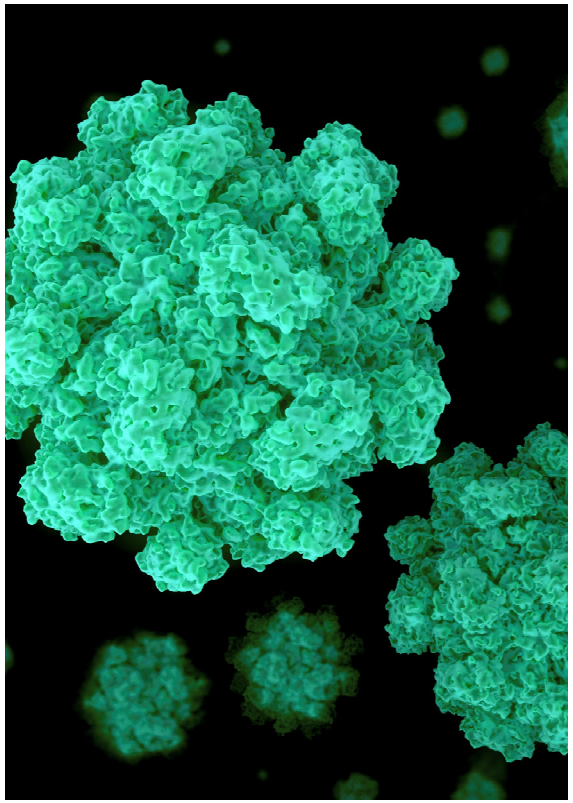

Image of Norovirus

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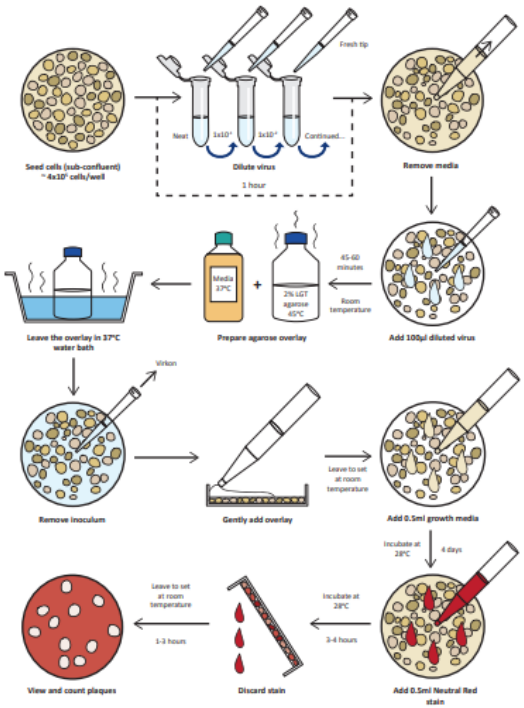
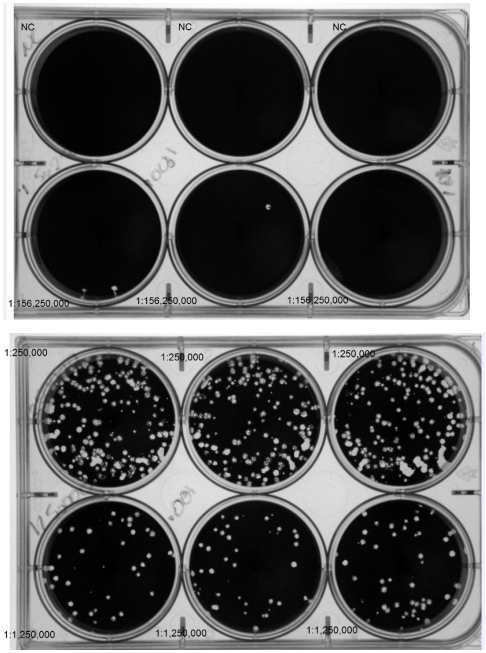
Quantification and Viability of Viruses



baculoQUANT schematic posted in *Biotechnology* 12, February 2019

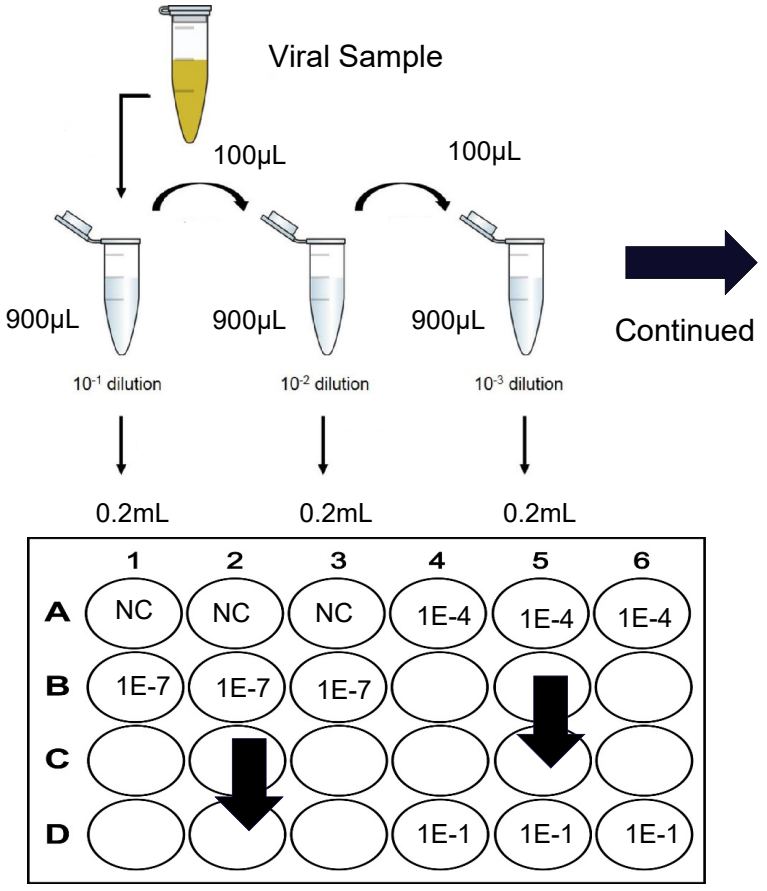
- Plaque Assays
  - Relies on endpoint dilutions series
  - Viral plaques are small areas of cell death.
  - Plaques are counted to determine PFU/mL
  - Plaque formation can take 3-14 days
  - PFU/mL represents number of infected particles within the sample
    - Assumption: each plaque formed is one infective virus particle



Equid herpesvirus 1 (ATCC® VR-2248™) in Vero (ATCC® CCL-81™) cells

Quantification and Viability of Viruses

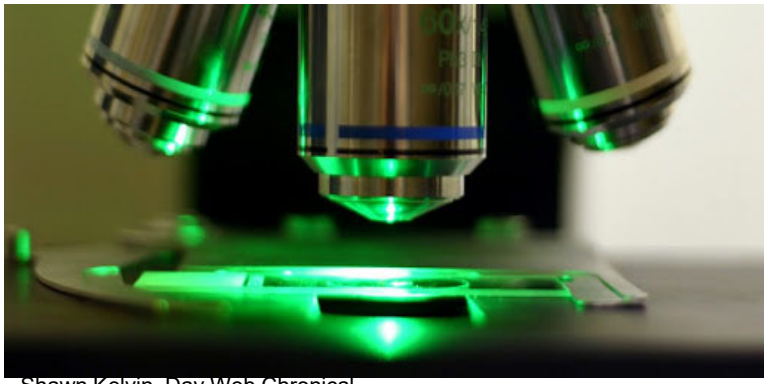
- Tissue Culture Infectious Dose (TCID)
  - Viral titer can be determined in vitro by calculating infectious dose
    - Concentration at which 50% of the cells are infected/well on which cells have been cultured is inoculated
  - Performed using endpoint dilution assay
    - Titer can take 3-14 days
  - Titer generally determined by presence of CPE
    - Accuracy is based on the number of replicates at each dilution
    - Calculated using Reed-Muench method (Burleson et al 1992, Reed and Muench 1938)
  - Minimum titer requirement for ATCC items - 5 x 10^3 $TCID_{50}$/ mL



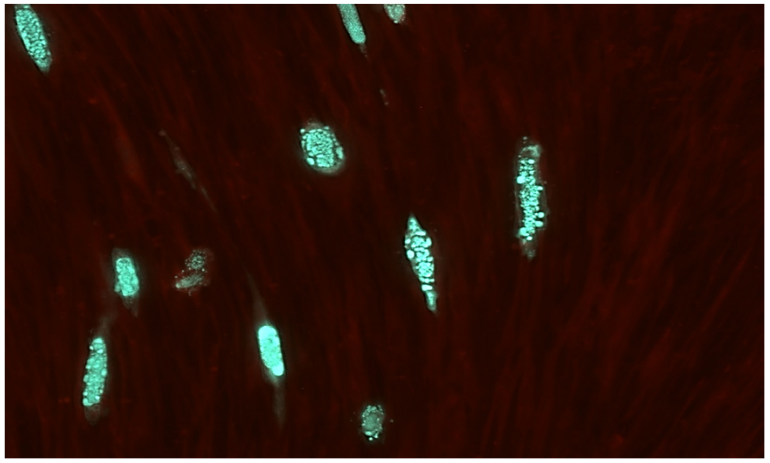
Example of $TCID_{50}$ Assay

Quantification and Viability of Viruses

- Immunoflurocesent assay (IFA)
  - Observed using a microscope equipped to detect fluorescence
  - Direct immunofluorescence assays (DFA) utilize one antibody
  - Indirect immunofluorescence assays (IFA) utilize a primary and secondary antibody
  - Used to interpret $TCID_{50}$ to either confirm presence of CPE, increasing specificity or used for viruses that do not produce CPE



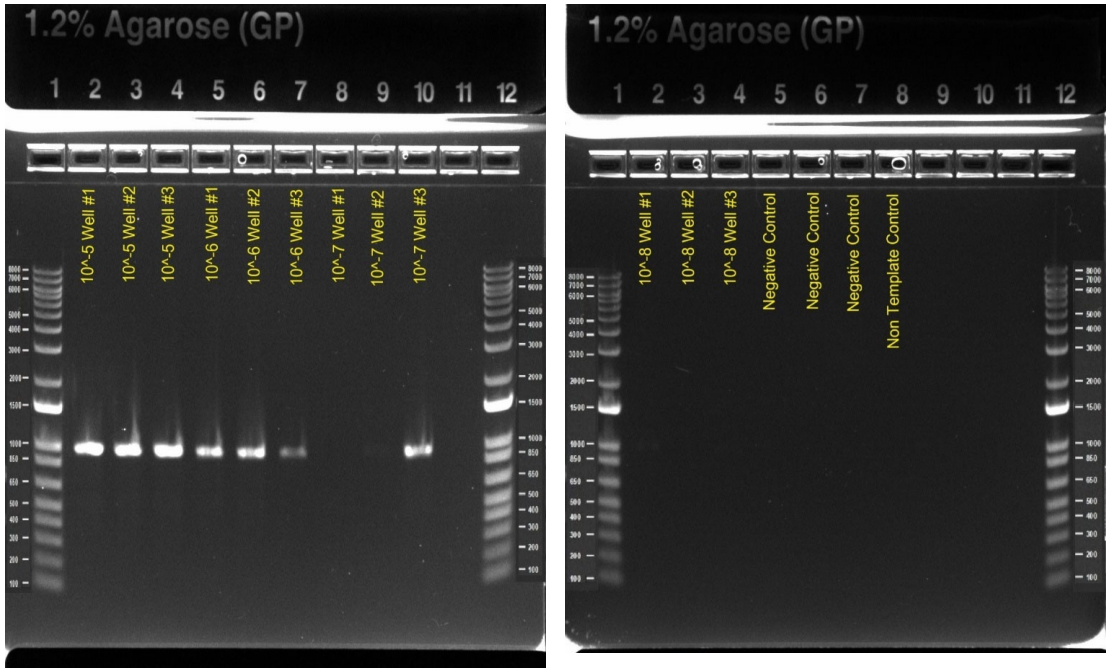
Shawn Kelvin, Day Web Chronical



Human adenovirus 19 (ATCC® VR-1979™) in MRC-5 (ATCC® CCL-171)

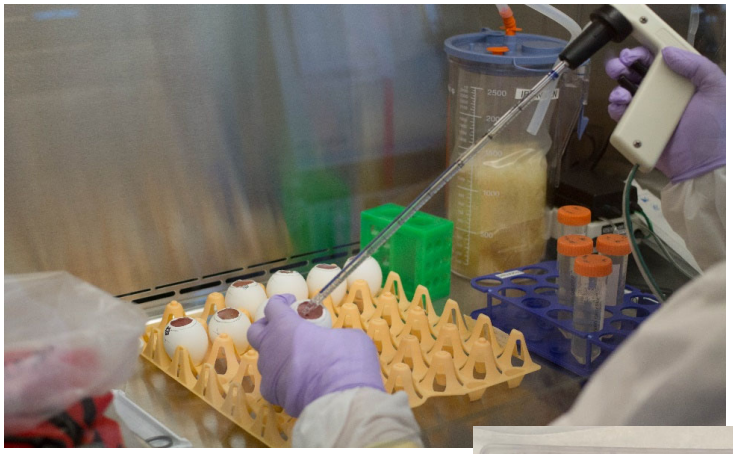
Quantification and Viability of Viruses

- RT-PCR/PCR titer verification
  - Used when viral items do not produce CPE and antibodies unavailable for IFA
  - Design primers specifically for viral product
  - $TCID_{50}$ wells are harvested individually
  - Each well is extracted for RNA or DNA based on viral type.
  - Run RT-PCR for RNA items
  - Run PCR for DNA items
  - $TCID_{50}$ is determined based on the presence or absence of a visible band on E-gel

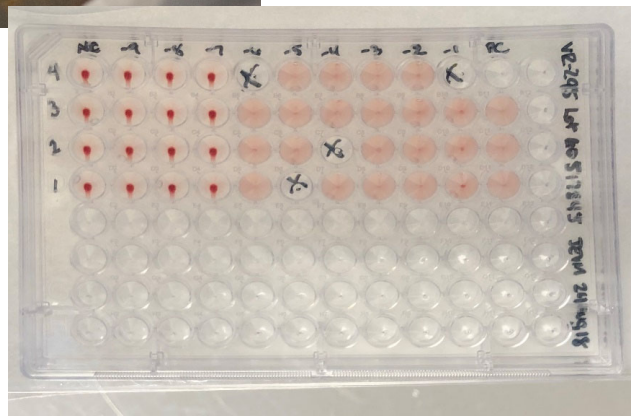


BK polyomavirus (ATCC® VR-837™) in HEL 299 (ATCC® CCL-137™)

Quantification and Viability of Viruses



Harvesting Eggs



Example of $CEID_{50}$ HA Assay- Influenza B virus (ATCC® VR-295™)

- Chicken Embryo Infectious dose ($CEID_{50}$):
- Endpoint serial dilutions are inoculated into embryonated chicken eggs.
  - Embryonated chicken eggs are commonly used to produce Influenza viruses
  - Results are measured using a Hemagglutination assay
    - Button that Lattice indicates positive sample
    - does not drip indicates negative sample
    - Titer calculated using Reed-Muench method (Burleson et al 1992, Reed and Muench 1938)

Authentication of Viruses

- Next Generation Sequencing (NGS) - technology used for DNA and RNA items.
- Whole genome sequencing in a few hours
- ATCC requires a 98% match greater than or equal to 1000 bp to the reference sequence
- ATCC uses NGS as another method to determine if the item is contaminated
  - Alex will go through this as one of our trouble shooting methods

Authentication of Viruses

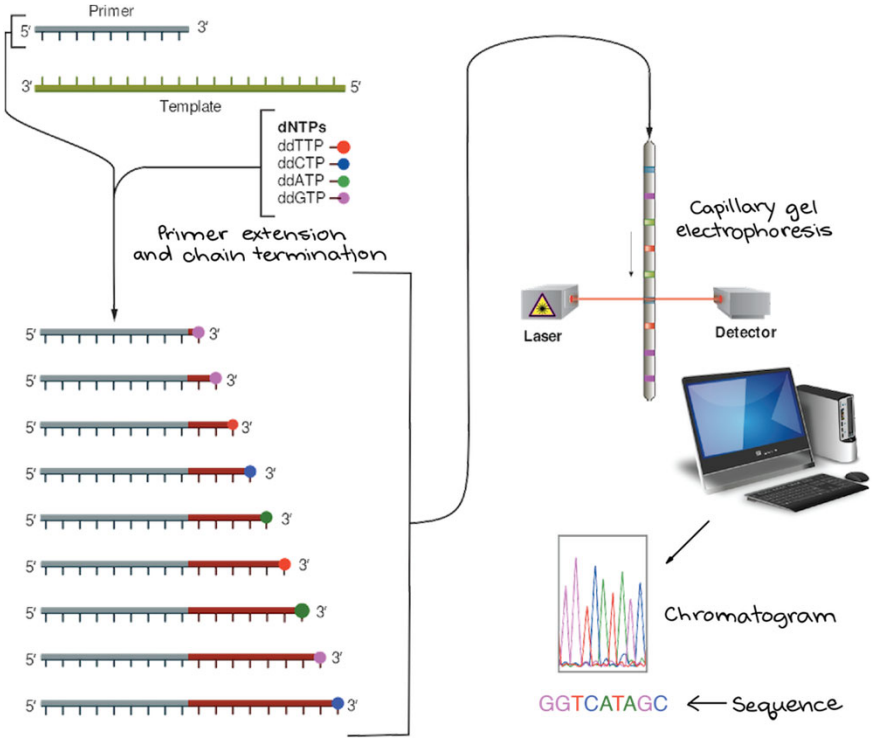


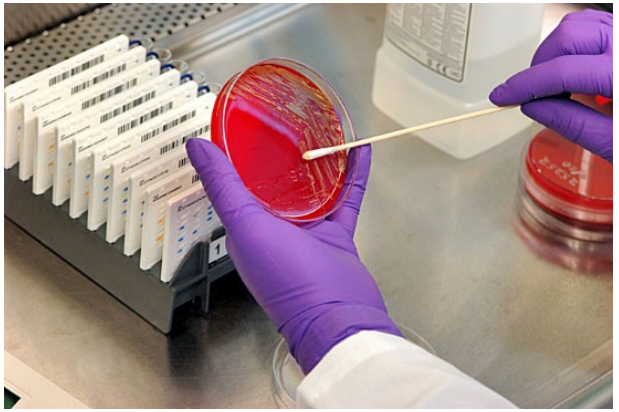
Image modified from "Sanger sequencing," by Estevezj (CC BY-SA 3.0). The modified image is licensed under a (CC BY-SA 3.0) license

- Sanger sequencing
  - Able to analyze short genome fragments, one fragment at a time
  - Highly accurate standard developed 70 years ago
  - Used as a authentication method for ATCC's nucleic acid products
  - Good comparison for NGS as a second method of authentication if needed
  - Limited in ability to detect contamination

Quality Control Methods

- Sterility
  - ATCC items are grown without antibiotics
    - Requires exceptional aseptic technique
    - Bacterial contamination is easy to spot
      - Turbid media
      - Over acidification-media turns yellow/orange
      - Restricted replication or no replication of virus
  - ATCC items require 14 day sterility
    - Bacterial counter system tests for aerobic and anaerobic microorganism contamination
    - Contaminant identified
    - MALDI-TOF used specifically identify contaminates to ensure easy clean up of item if necessary

ATCC Bacteria Counter



ATCC MALDI-TOF

Quality Control Methods

- Mycoplasma contamination
  - Not easy to detect
    - No media turbidity
    - No pH change
    - Cannot be detected microscopically
  - What mycoplasma contamination can cause
    - Inhibits cell growth
    - Affects viral replication
    - Unexplained cell death
- Mycoplasma testing
  - ATCC produces a PCR mycoplasma detection kit
    - Tests for 60 of the most common serotypes

Universal Mycoplasma Detection Kit (ATCC® 30-1012K™)

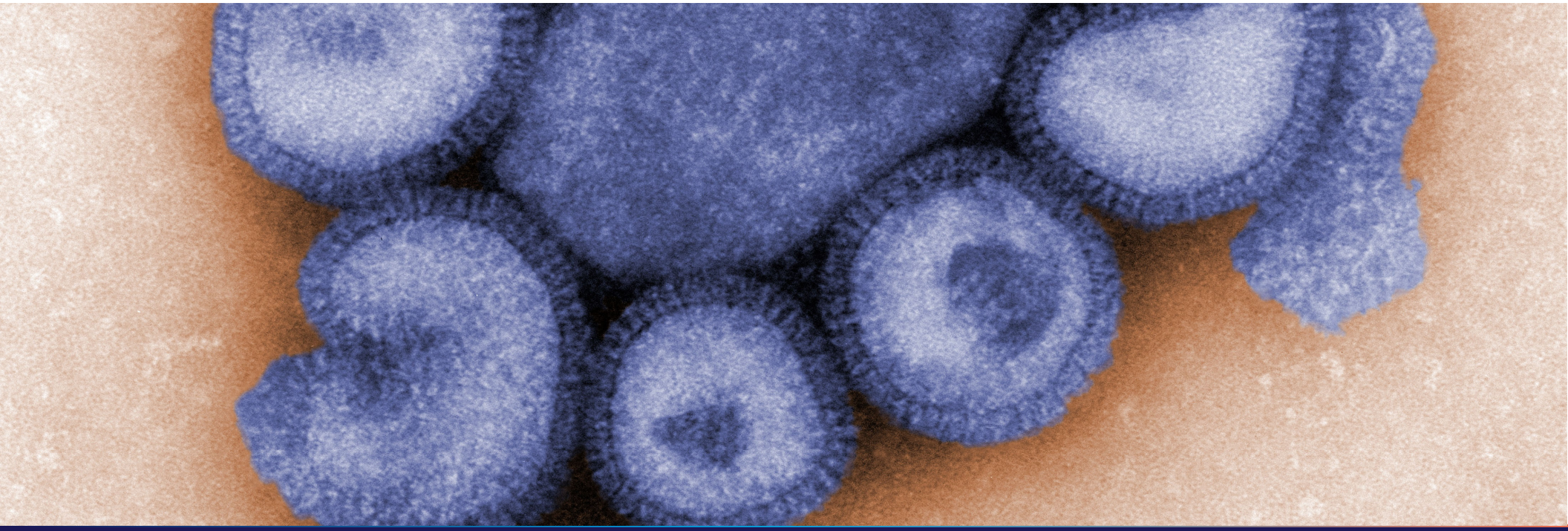
ATCC

Storage

- Cryopreservation-
  - Lytic viruses
    - Flash frozen in LN tank and moved to -80°C for storage
    - Lyophilization - no longer used at ATCC, but extensively used in the past
  - Cell associated viruses
    - Require controlled rate freeze
    - May require cryopreservative such as DMSO
      - Ice formation harmful to cell viability
      - Final product stored in LN tanks ensure integrity of cells

CoolCell® LX Alcohol-Free Cryopreservation Container (ATCC® ACS-6000™)

ATCC Repository, liquid nitrogen tanks



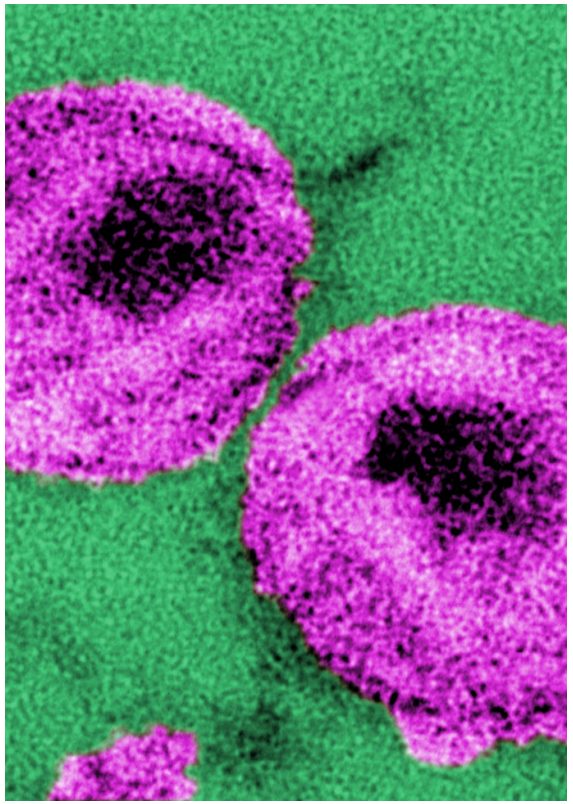
R&D and Troubleshooting

Influenza, courtesy of Dr. FA Murphy, CDC

Why viral replication and propagation fails?

Major Growth Issues

- MOI (multiplicity of infection) is not optimized
- Virus is not adapted to the most appropriate host
- In vitro environment is missing crucial growth factors (*e.g.,* media formulation, enzymes, etc.)
- Contaminants in cell culture and/or virus



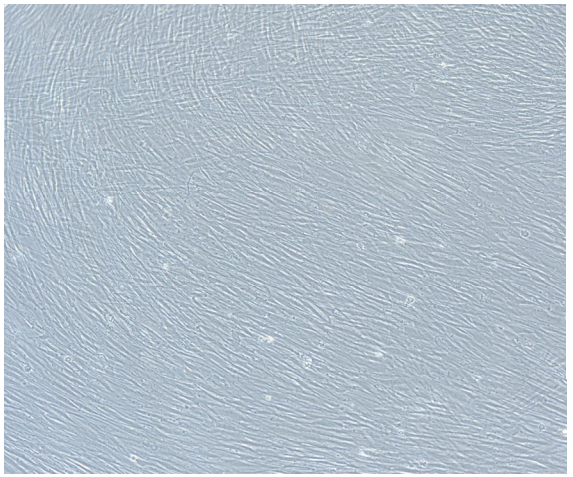
TEM of HIV, courtesy of Drs. Harrison and Feorino, Public Health Image Library

Goal of MSAT Virology

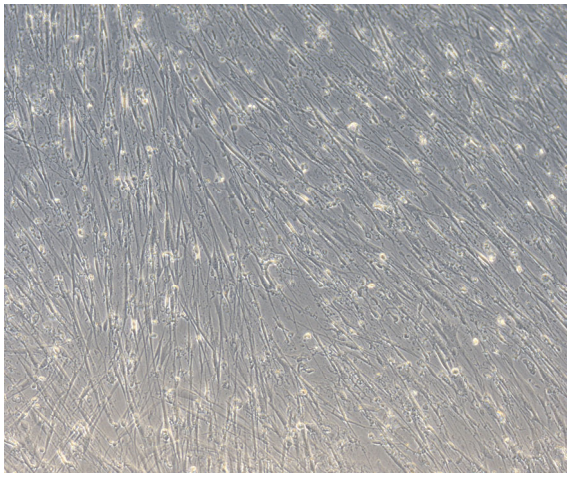
Perform R&D as needed to resolve growth issues and improve the quality of existing viral products

MOI Optimization

- Optimal MOI will vary depending on:
  - Number of infecting agents,
  - How fast those agents attach to host cells
  - How much time is allowed for attachment
  - Number of host cells
- MOI vs. dilution
- High MOI requires every cell in the culture to be infected; low MOI is used when multiple cycles of infection are needed



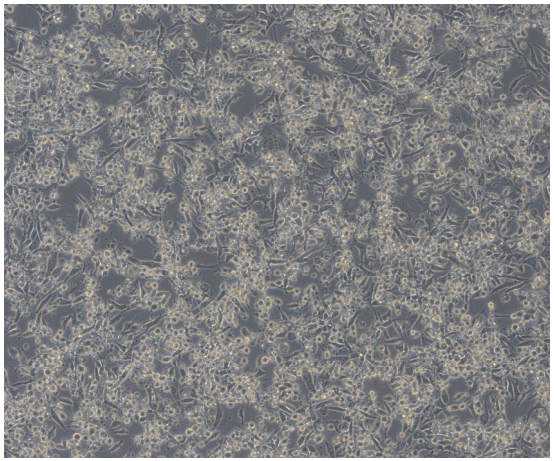
BK polyomavirus
(ATCC® VR-837™)
MOI 0.01
15 days post-infection



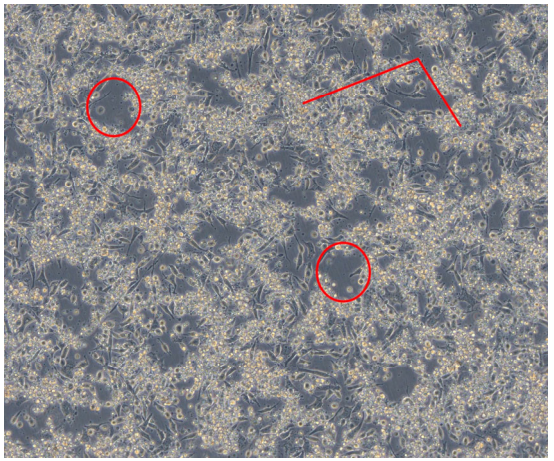
BK polyomavirus
(ATCC® VR-837™)
MOI 1.0
15 days post-infection

Adapting to Host Cells

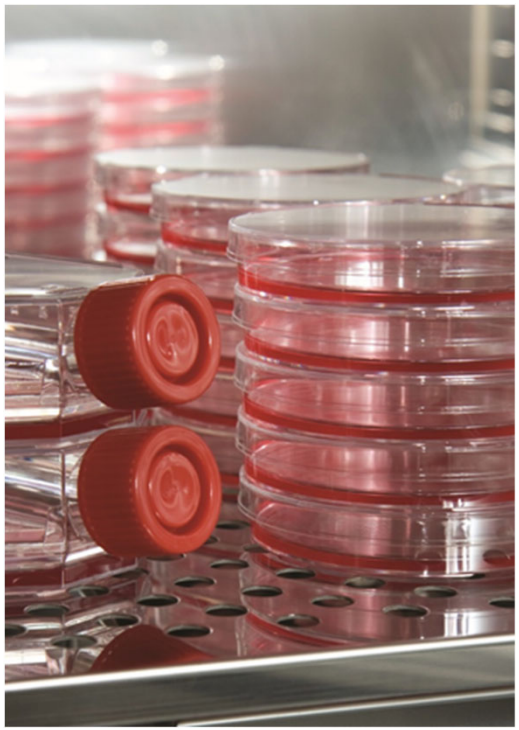
- Many viruses are host specific, meaning they only infect certain types of cells within tissues (tropism); some may have more than one host
  - *e.g.*, poliovirus exhibits tropism for tissues of brain and spinal cord, influenza virus has primary tropism for the respiratory tract
- Adapt depositor materials from: primary cells, eggs, in vivo models, etc.
- Importance of performing multiple passages in host



Human coxsackievirus adapting to RD cells (ATCC® CCL-136™) from Passage 2 to Passage 3



Environmental Growth Factors



- Appropriate medium is important to grow and maintain the host cell line and the virus itself
  - EMEM vs. DMEM
  - Percentage of serum
- Is trypsin needed?
  - Some viruses such as influenza and rotaviruses are dependent on trypsin
  - Acts as proteolytic cleavage for virus proteins to allow for successful attachment to host cells

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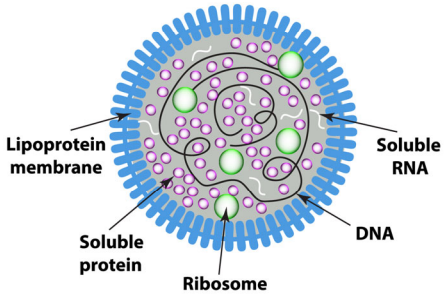
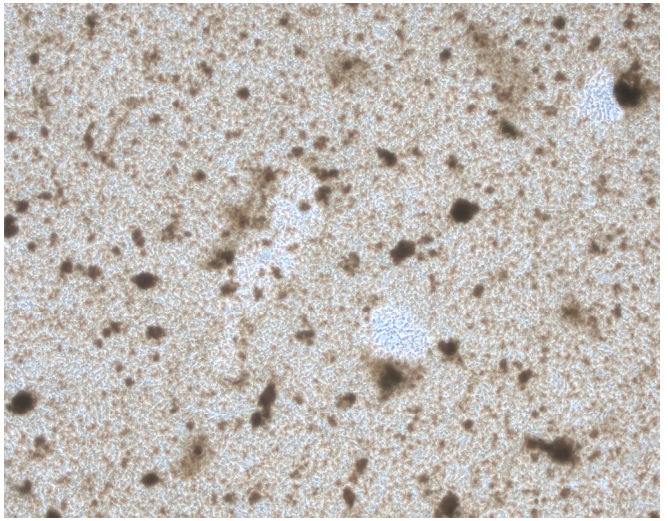
Contaminants

- Viruses can become contaminated with various bacteria, fungi and/or other virus DNA/RNA cultures, especially much older items in the ATCC catalog
- Virus cross-contamination can be detected by NGS and resolved by plaque purification
- MRA (mycoplasma removal agent) vs. diethyl ether
- Other bacteria/fungi can be removed by filtration or use of antibiotics/antimycotics

Mycoplasma





What about new accession items?

- Viral samples are sent to ATCC from researchers and scientists across the globe to be deposited for eventual propagation and authentication

- With more than 2,000 viruses, including type strains from different host species, ATCC ensures that every preparation made is as close as possible to the original culture deposit, while taking any necessary measures (MOI optimization, adapting to more beneficial host, etc.) to provide the best product available for customers

- Have a sample to drop off? Contact the Central Accessioning Unit at CAU@atcc.org or visit https://www.atcc.org/en/Services/Deposit_Services.aspx

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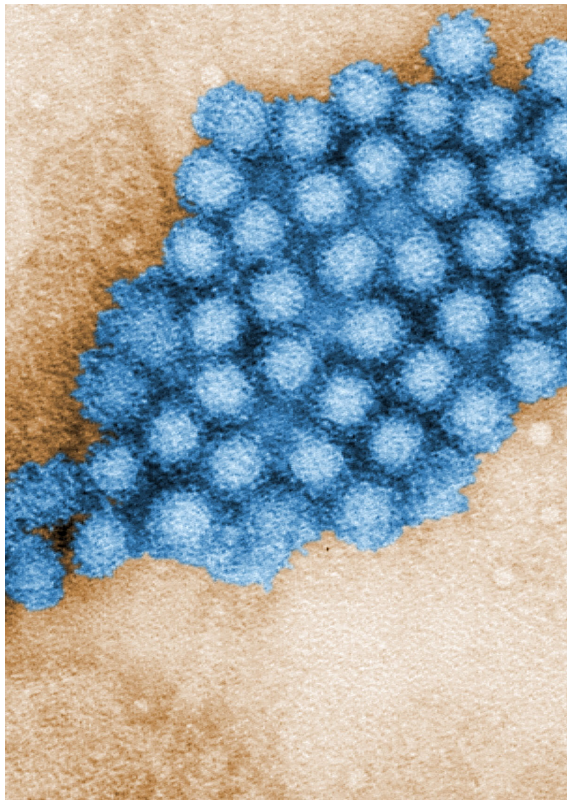
Summary

- Virus fundamentals
  - Propagation strategies depend on how a virus infects and is released from host cells
  - Host cells must be susceptible and permissive for viral propagation to be successful
  - Virus propagation can be broken down into 3 steps: inoculation, incubation, and harvest
- Authentication and quality control
  - ATCC uses a variety of methods to ensure viability and authentication of viral products
  - We also ensure that our products undergo proper quality control procedures
  - Integrity of ATCC viral items is preserved through proper storage techniques
- Troubleshooting strategies
  - Main goal of MSAT Virology is to perform R&D to fix replication and propagation issues
    - Optimizing for MOI, adapting to most appropriate host cell line, adding ideal environmental growth factors, and removing contaminants from virus culture
  - Viral samples deposited to ATCC will be properly replicated and authenticated
  - MSAT Virology uses a variety of best practices to ensure successful propagation



Norovirus courtesy of Dr. Charles D. Humphrey, CDC

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Questions?

From Our Lab To Yours

Safety Tips for Returning to the Lab

To help you get back to work, we have put together a free kit containing best practices to help keep your lab infection-free:

- Poster
- Infographic
- Decals

Order the kit here:

www.atcc.org/backtothelab