Viral Purification: MIC 204

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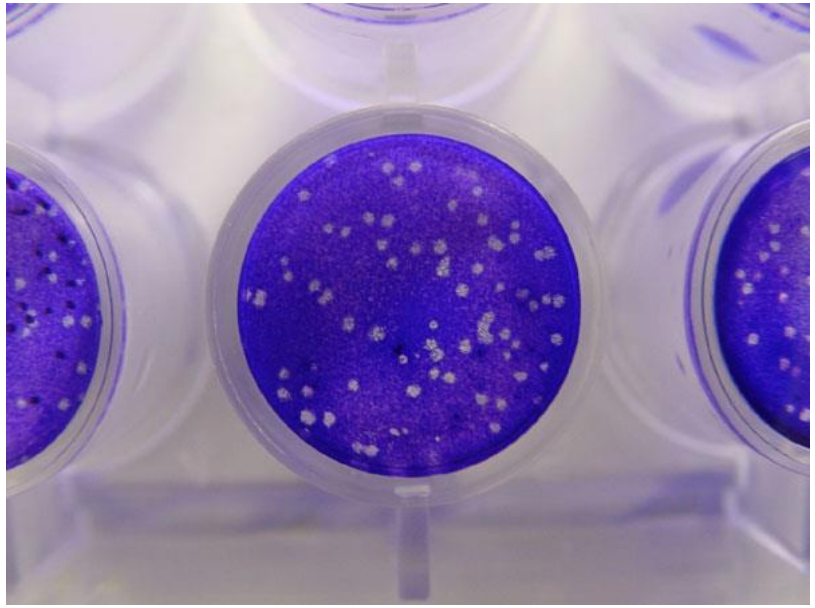
Virus Purification purpose

- Virus purification is the process of separation of virus particles from host cells and other chemicals present in the media
- Virus particles are routinely purified by two methods:
  - biological methods, such as plaque isolation,
  - physical methods, such as centrifugation etc
- Plaque isolation is used for the qualitative purification of (genetically identical) virus particle, while centrifugation is used for the quantitative purification of (biochemical purity) virus particles.
- Purified virus is used for
  - Physico-chemical studies: electron Microscopy
  - Molecular characterization
  - Molecular biology as cloning vectors, phage display libraries
  - Infectious biology studies
  - Vaccine production
  - Gene therapy as viral vectors

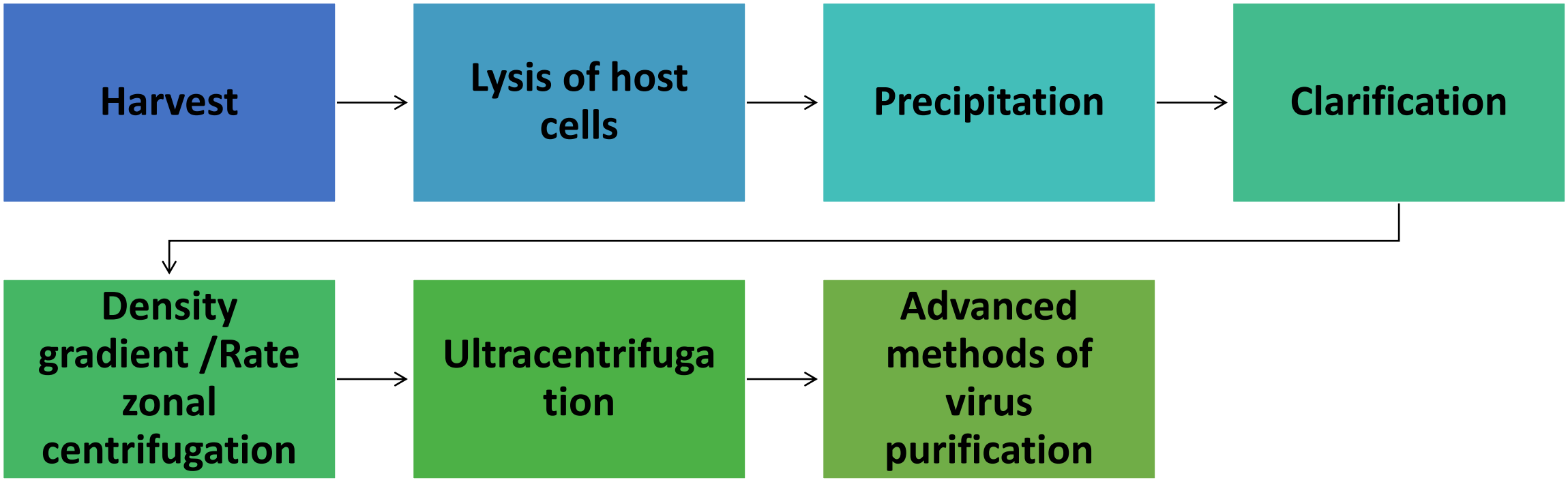
Plaque Isolation

- Virus particles can be purely isolated from a single plaque by plaque assay.
- For instance, a sample can be taken from a single plaque by pipette tip.
- This sample is used as an inoculum to propagate some quantity of virus particles by using cell culture.
- Plaque-purified virus is considered to be genetically identical.
- Bacterial/ animal virus purification can be started from single plaque



https://en.wikipedia.org/wiki/Virus_quantification#/

Steps in Viral Purification



Extraction Buffer

- An extraction medium must enable extraction of maximum amount of virus from the infected host material, keep the virus in stable, infective, un-aggregation condition and minimize host contaminants.
- A buffer of suitable pH and molarity with additives to prevent oxidation and avoid co-precipitation other materials with the virus, yet retain virus infectivity is chosen by trial and error based on the knowledge of the methods used for other viruses.
- Phosphate acetate and borate buffers are commonly used at different pH and molarity. Some virus need a cation like Ca^{2+}or Mg^{2+} to preserve their infectivity, besides the ionic strength of the buffer.

Clarification

- Removal of host cell constituents
- Mortar pestle, food blenders, meat mincer and the electrically operated glass mortar and pestle are useful to homogenize the virus infected material in a suitable extraction buffer.
- All extractions of virus are done in cold conditions at 4°C or using salt-ice bath around mortar to prevent oxidation reactions.
- Cell components (Ribosomes, RuBP carboxylase protein from chloroplast, fragments of lower molecular weight compounds should be removed in the extraction process, leaving the infective virus in the solution.
- Animal virus in cell culture/ tissue samples: Enzymes such as collagenase/ trypsin EDTA
- Plant viruses: extracting media supplemented with reducing agents (beta mercaptoethanol, Na sulphite) and chelating agents (EDTA)
- Bacteria: treat with chloroform
- Followed by centrifugation 1000 to 10000 rpm- host constituents settle as pellet while virus remains in the supernatant

Concentration

- Precipitation
- Filtration
- Centrifugation
- Chromatography

Precipitation

- **A salt such as ammonium sulphate is added to a protein solution to saturation levels until the virus precipitates. The amount of salt added to achieve this needs to be noted carefully. This method purifies the protein. It can be used with both enveloped and non-enveloped viruses.**
- **Poly ethylene glycol been most frequently used. PEG has shown an enhanced virus yield up to 64% in the case of bovine rotavirus compared to 7% from the ultracentrifugation process.**
- **Some other examples include 8% PEG 8000 has been used as a preliminary step to improve the transduction efficiency and optimize the sequential CsCl gradient ultrafiltration]. PEG and salts can purify virus by altering its solubility causing precipitation or salting out effect .**
- **An alternate concept has been to precipitate the impurities from the virus while leaving the virus in solution. A 750 kDa polyethyleneimine (PEI) of 0.0045 w/v% solution was able to precipitate 85% of the DNA after centrifugation .**
- **Polysorbate 80 or sodium chloride has also been useful during precipitation protocol by breaking up the aggregates between DNA and viruses which are held together by hydrophobic and electrostatic charge interactions. Dissolution of aggregates is followed up with chromatography to achieve a final DNA concentration of 5 pg/dose**
- **Other factors can also affect how well this method works such as the temperature, pH, protein concentrations, and precipitation agents.**

Filtration

- Gel Filtration:
- Sephadex, agar and agarose remove smaller host components by adsorption.

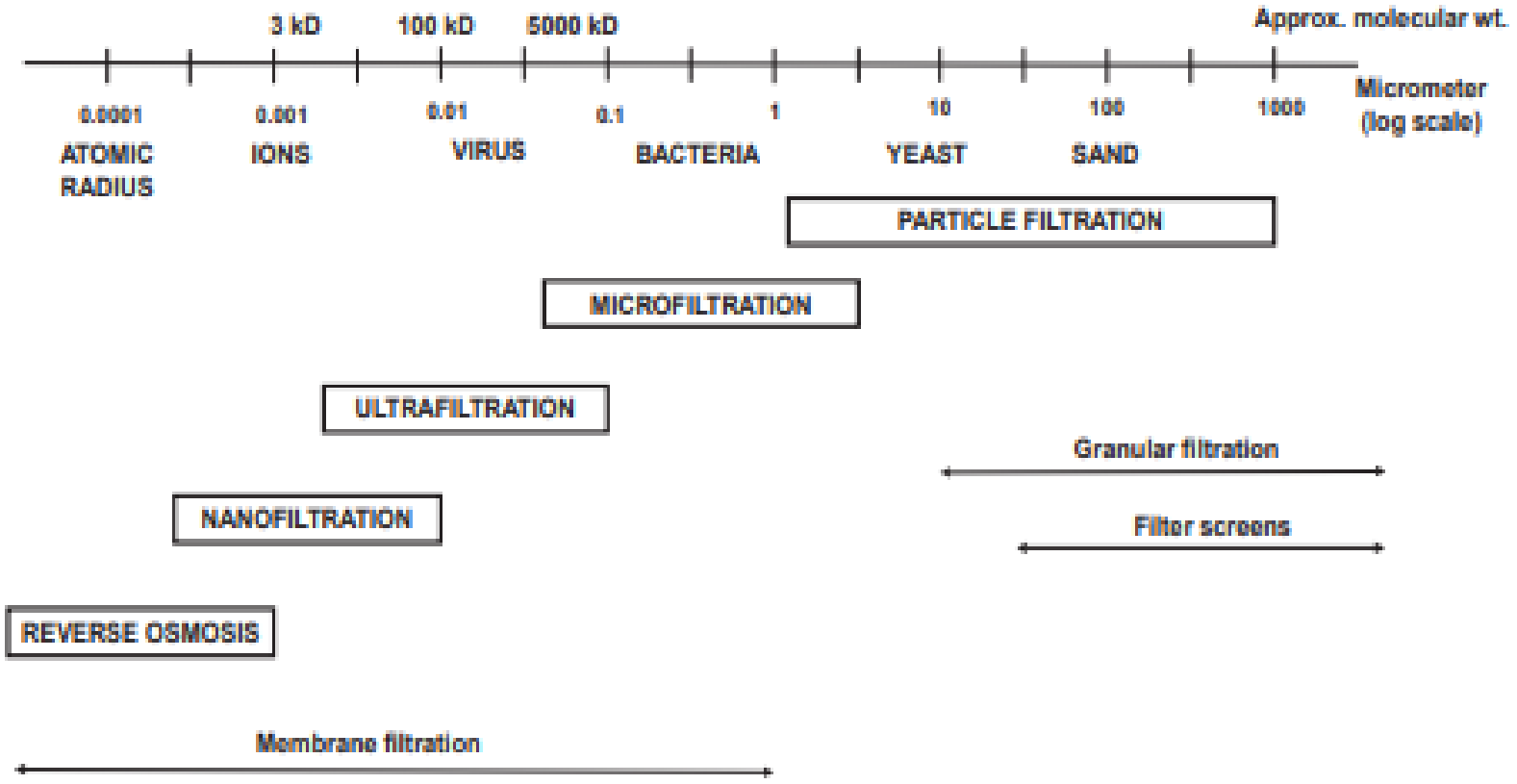
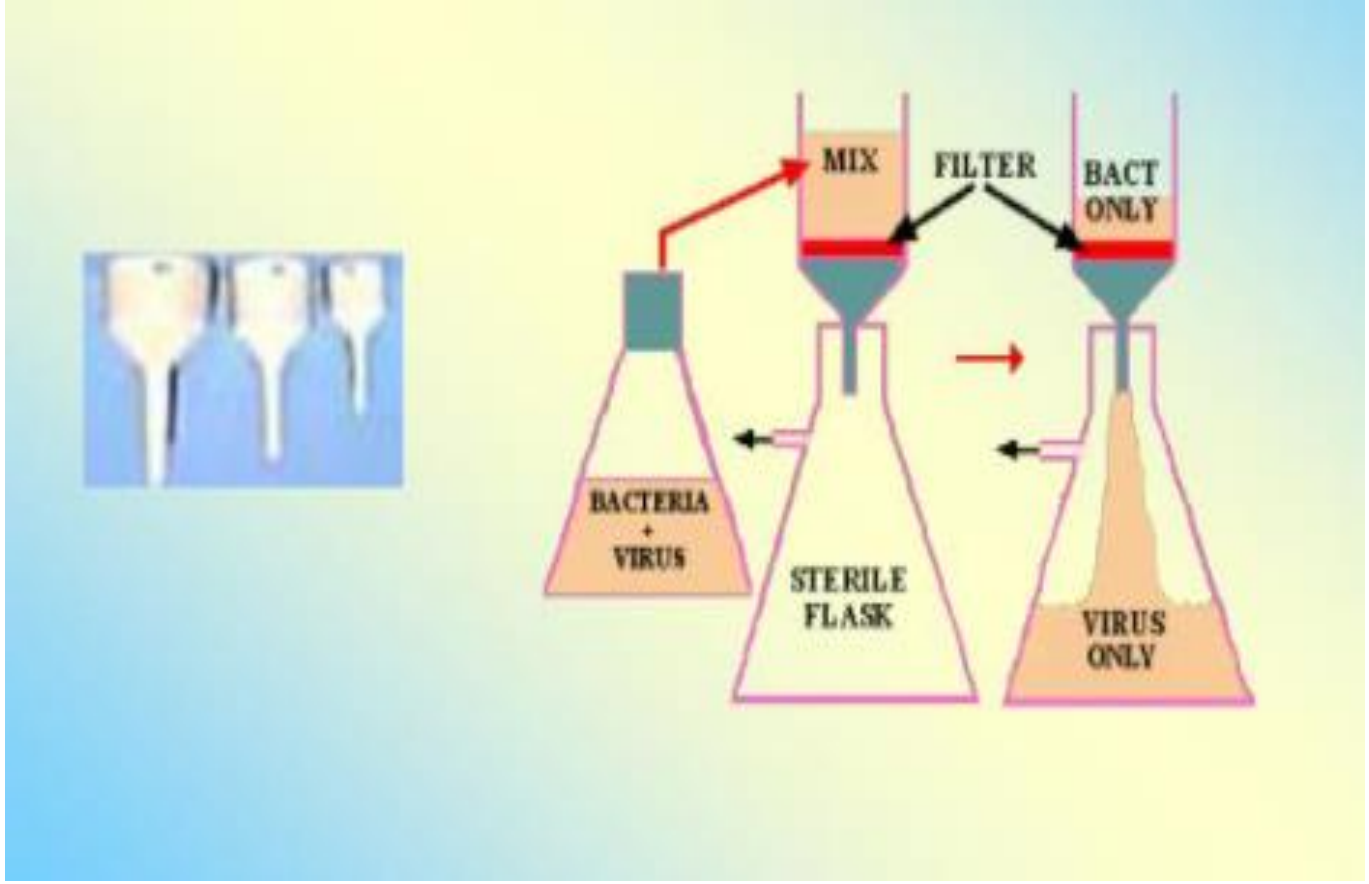


Figure 2.8 Size range of different filtration methods

Filtration- Ultrafiltration

- Ultrafiltration (UF) is a variety of membrane filtration in which hydrostatic pressure forces a liquid against a semi permeable membrane.
- Membrane pores range in size from 0.005 to 0.1 micron.
- Substances with a molecular weight of 100,000 daltons have a size of about 0.05 microns to about 0.08 microns in diameter can be purified.
- UF membranes are used where essentially all colloidal particles (including most pathogenic organisms) must be removed



Nanofiltration

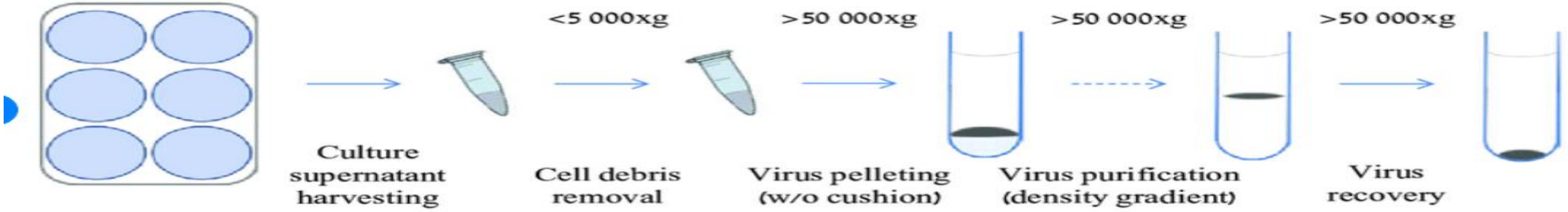
- Nanofiltration is a membrane separation process which involves the diffusion of a solution through a membrane with a very nanopore size. This method can be used for enveloped viruses and non-enveloped viruses. It is particularly good for obtaining small particles.
- It has been more frequently used as a purification for plasma products that contain viruses such as hepatitis B and hepatitis C since the early 90s. It is viewed as a good method of separating out any viruses in the plasma and helping to prevent them entering human plasma stocks.

Tangential flow filtration (TFF)

- Tangential flow filtration (TFF) is the commonly used size based filtration technique for virus purification from cell culture medium. A two stop process was created to purify influenza virus particles.
- A large pore size (0.45 µm) was used to allow the passage of virus while holding cell debris.
- This was followed by a 100 kDa filter that retained the virus and allow host cell proteins to pass through the filter. For smaller viruses, such as the minute virus of mice (MVM) which range in the 20 27 nm size, a smaller filter pore size is required to retain viruses

Centrifugation

- Low speed centrifuge: Supernantant+ lysed cell contain virus particles
- High Speed Centrifugation-1-2 h at 35000-60000 rpm
  - Aqueous phase is discarded and pellet containing virus particles is resuspended in buffer
  - To increase purity virus preparation may be subjected to alternative cycle of low and high speed centrifugation called differential centrifugation
- Ultra centrifugation- 30 min at 100000 rpm
  - A viscous liquid such as glycerol, sucrose, or cellufine sulfate, is used in the process to help control the rate of migration of the different kinds of particles. A slower rate of migration helps to better purify the mixture. The usual aim is for the virus to move from the axis to halfway through the gradient. This is called velocity sedimentation



Leroy, Baptiste & Gillet, Laurent & Vanderplasschen, Alain & Ruddy, Wattiez. (2016). Structural Proteomics of Herpesviruses. Viruses. 8. 50. 10.3390/v8020050.

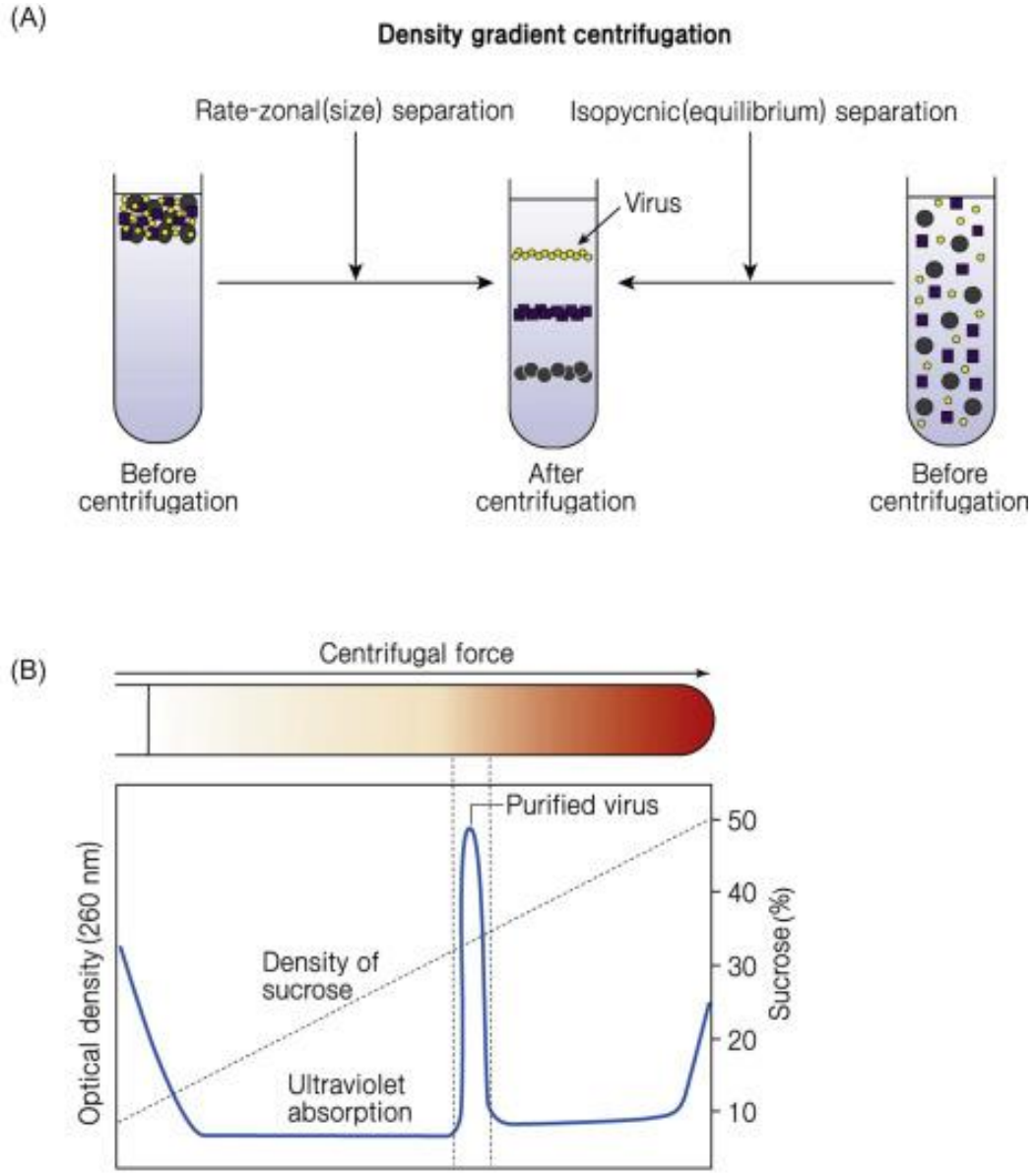
Density Gradient Centrifugation

Density gradient centrifugation is used for isolation of virus particles.
The density gradient of sucrose is prepared from top to bottom of the centrifuge tube. Commonly, sucrose or cesium chloride is used as a density medium.
Then, two methods of separation are used:
(1) rate-zonal separation and
(2) isopycnic (equilibrium) separation (Fig. 4.12).

https://www.sciencedirect.com/topics/biochemistry-genetics-and-molecular-biology/virus-particle

- ***Rate-zonal separation***. The objects in the density medium migrate depending on its size, even though their density is identical. The bigger the size of the particle, the faster it migrates. Specifically, a sample containing viral specimen is loaded onto the top of the preformed density gradient (eg, 10–50% sucrose), and then subjected to centrifugation for a few hours
- ***Isopycnic (equilibrium) separation***. When subjected to centrifugal force, an object having a certain density migrates until the density of the surrounding medium is equal to its own density (ie, equilibrium). This method of separation is termed “isopycnic separation.”
- In the case of sucrose, the solution is highly viscous and hyper osmotic, reducing the overall yield of the virus stock.
- CsCl gradient has known to increase the virus particle to infectious virus ratio up to 1600:1, whereas the requirement is in a much lower range around 20:1 .
- Retrovirus recovery of 37% and a promising 95% purity has been reported using Iodixanol gradient
- What is a practical difference between rate-zonal separation and isopycnic separation? In rate-zonal centrifugation, the density gradient is premade before centrifugation, while in isopycnic separation, the density gradient is made during centrifugation. As a result, the former takes only a few hours (1–4 h), while the latter takes much longer (>16 h). More importantly, the former is used to separate an object by its physical size, while the latter is used to separate an object by its density.

- Isopycnic density centrifugation can also be used , which separates molecules based on their buoyancy density in the viscous fluid. The fluid has a greater density at the bottom than at the top. When the mixture is centrifuged, the particles, including the virus molecules, migrate to the level of the liquid which relates to their density.
- Combination of both velocity sedimentation and isopycnic density centrifugation can be a particularly useful form of purification.



Chromatography

- Useful for large scale virus purification protocols
- Chromatography is useful for purifying both enveloped and non-enveloped viruses. Examples of enveloped viruses are the chickenpox virus and the influenza virus. Non-enveloped viruses do not have the envelope. Examples of these non-enveloped viruses are parvovirus and adeno virus. Non-enveloped viruses are not impacted by heat, drying or acids while enveloped viruses can be affected by these.
- The level of purification varies from one virus to the next. Pore size has an impact on how much of the virus is removed and so does the kind of resin, protein solution, and buffer. It is also more difficult to remove smaller viruses fully with this method.
- Some columns use calcium phosphate, usually at pH7. Elution rates can be changed by altering the pH level and impacting the phosphate concentration.
- An example of a virus that can be treated with this method is the influenza virus. Purity can be improved by 30 to 100 fold. Further improvement can be made by employing an additional chromatography column which can improve the concentration by 10 to 30 fold.

Early Phase Production for Adenovirus Vector-Based Vaccines

Transition to Cost-Effective and Scalable Manufacturing Template

- Suitable for small-scale production
- Lack robustness
- Difficult and expensive to scale up

Single-Use Bioreactor	**DNA Removal**	**Clarification**	**Concentration**	**Membrane Chromatography**	**Diafiltration/ Formulation**	**Final Filtration**
Mobius®	Benzonase®	Millistak+®	Pellicon® 2	Natrix ®	Pellicon® 2	Durapore ®

Next generation Chromatography

Early Phase Production for Adenovirus Vector-Based Vaccines
Transition to Cost-Effective and Scalable Manufacturing Template

NatriFlo® HD-Q Chromatography Membrane

- Functional group: Quaternary amine
- Three-dimentional macroporous hydrogel structure provides
  - A large surface area that contains a high density of functional groups
  - Interconnected pores that provide convective flow channels
  - Excellent accessibility for large molecules (ex. DNA; Virus)

Binding capacities that exceed resin columns with fast flow rates typical of membrane adsorbers

Method

NatriFlo® HD-Q Recon Mini
Flow rate: 2 mL/min (6 sec residence time)
Bind & Elute

Equilibration & wash buffer:
50 mM NaPhosphate pH 6.5, 5% sucrose, 100 mM NaCl, 1 mM MgCl2, 0.1 % Tween20

Elution buffer:
50 mM Tris-HCl pH 8.0, 1M NaCl, 5% sucrose, 1 mM MgCl2, 0.1 % tween 20

Millipore.

Next generation Process Intensification

Purification of Engineered NDV for Oncolytic Virotherapy
Improve Productivity and Scalability without Compromising Purity

	Traditional Process	Next Generation Process
Purification Process	 Depth filter → TFF → Ultracentrifugation (UC)	 Depth Filter → AEX (NatriFlo® HD-Q)
Process Duration	9 hours*	0.5 hours
Product Recovery	65-70%	>90%
Scalability	UC difficult and expensive to scale up	Depth filter and AEX easily scalable

*** An additional 2 day dialysis and PEG concentration is required to remove concentrated sucrose from UC**

Traditional Process

- Low virus recovery = need for oversized upstream units & poor overall process economics
- Lengthy process reduced productivity

Next Generation Process

- High virus recovery with great impurity reduction
  - High recovery enables downsize of upstream by 7X
- Simplified, faster process promotes overall productivity and better process economics

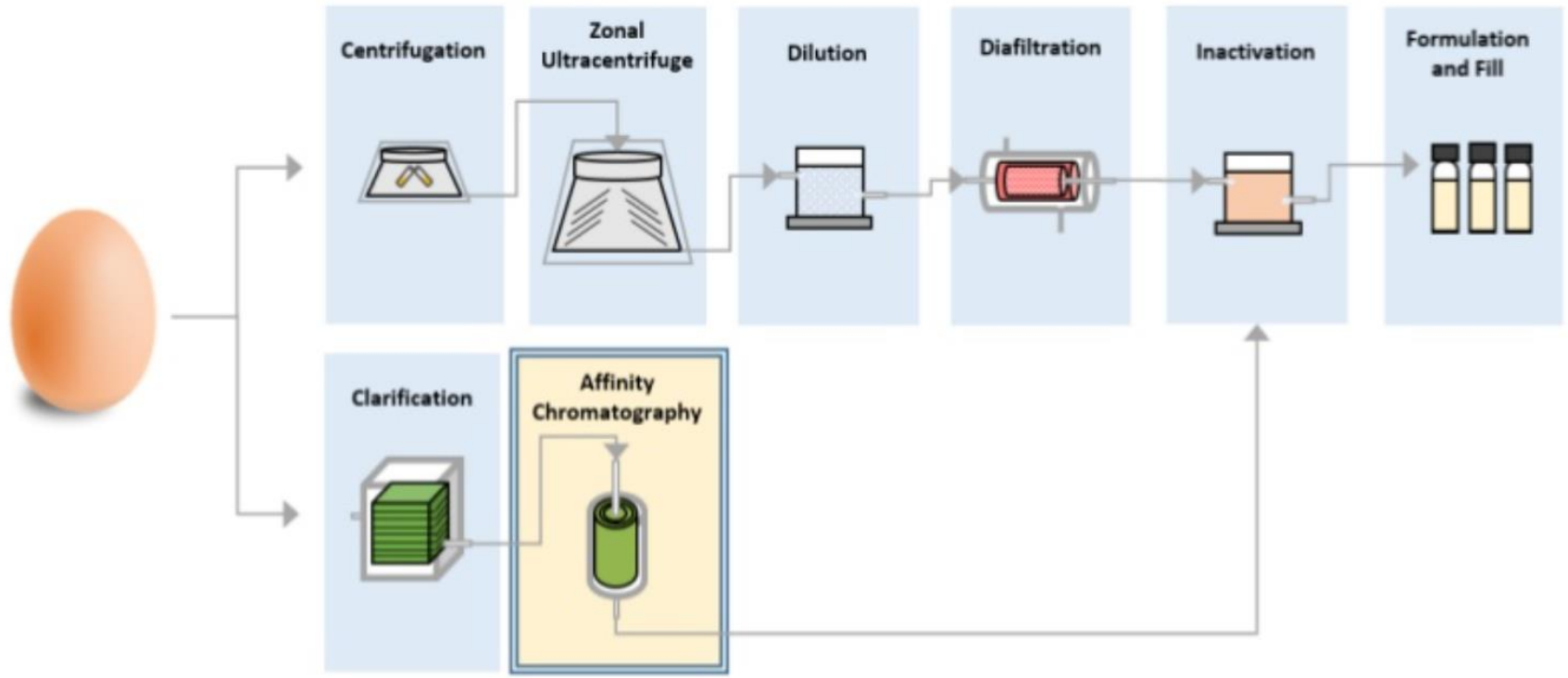
Millipore.

Chromatography

- **Affinity chromatography** is capable of separating **viral** particles from protein and DNA contaminants based on a reversible interaction between the **viral** capsid and a specific biological ligand or receptor coupled to a **chromatographic** matrix
- Useful as ultracentrifugation cannot be used at manufacturing scale
- Useful for vaccine production eg.
  - Immunoaffinity chromatography for Hepatitis A, poliovirus type 1, measle virus, AAV-2
  - Lectin affinity chromatography: Influenza virus (HA), Baculovirus, Herpes Simplex virus

Affinity Membrane Improves Productivity Further

Proof-of-Concept: Purification of Egg-Based Influenza Virus



Process Reference:
Kon, Theone C et al. "Influenza Vaccine Manufacturing: Effect of Inactivation, Splitting and Site of Manufacturing. Comparison of Influenza Vaccine Production Processes." *PLoS One* vol. 11,3 e0150700. 9 Mar. 2016, doi:10.1371/journal.pone.0150700

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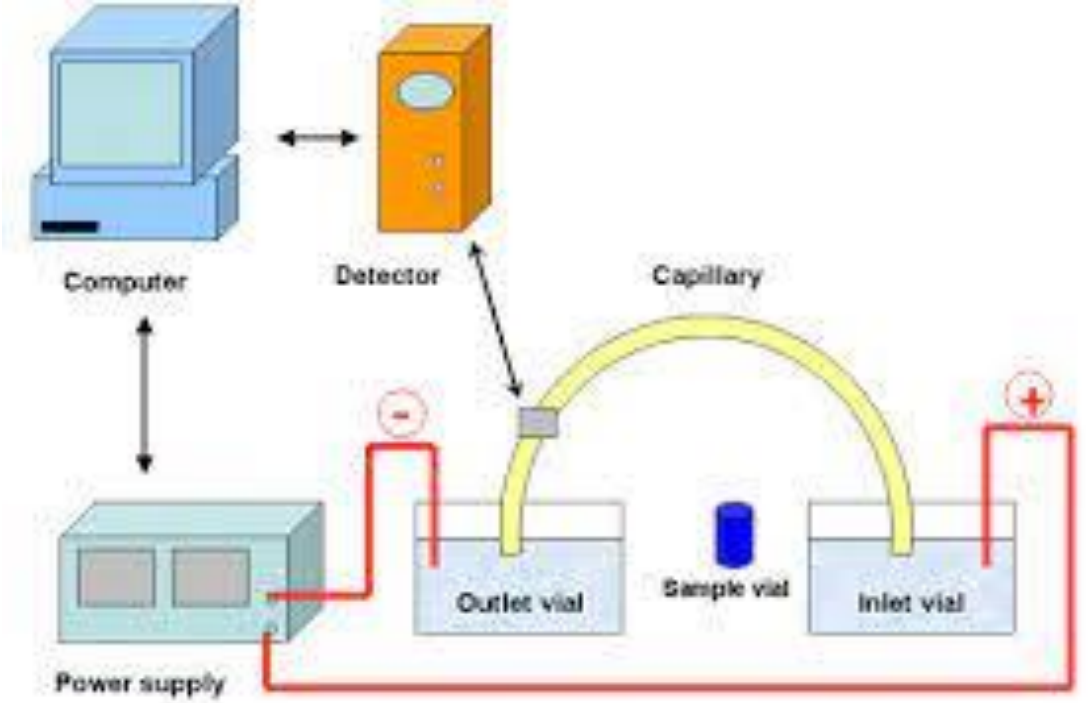
Electrophoresis

- **Capillary zone electrophoresis** with laser-induced fluorescent detection to separate intact virus particles from DNA and RNA impurities.
- Capillary electrophoresis is a method for separating and identifying fragments of fluorescently-labeled DNA by passing them through very thin polymer-filled capillaries. The use of capillaries instead of a conventional slab gel makes it possible to apply a much stronger electric field, resulting in faster separation and higher throughput. The process features single-base resolution and requires small sample quantities.



Viral quantitative capillary electrophoresis for counting and quality control of RNA viruses. Anal Chem. 2012 Nov 6;84(21):9585-91.

Electrophoresis

- Typically used for testing purity of virus or virus proteins
- **Capillary Zone Electrophoresis**
- Using capillary zone electrophoresis and fractionation (Fig 1) viruses are sorted via their charge-size ratio and placed into separate chambers on a sampling tray, making it easy for the user to begin processing.
- **Market Advantages**
- Separates and fractionates rare and closely related bacteriophages with a 3 times better sensitivity than current solutions
- High efficiency and speed can be achieved by combining with an automated fraction collection system to separate mixed phage populations prior to conventional genomic sequencing analysis
- Cost-saving due to reduced labor hours to manually isolate phages

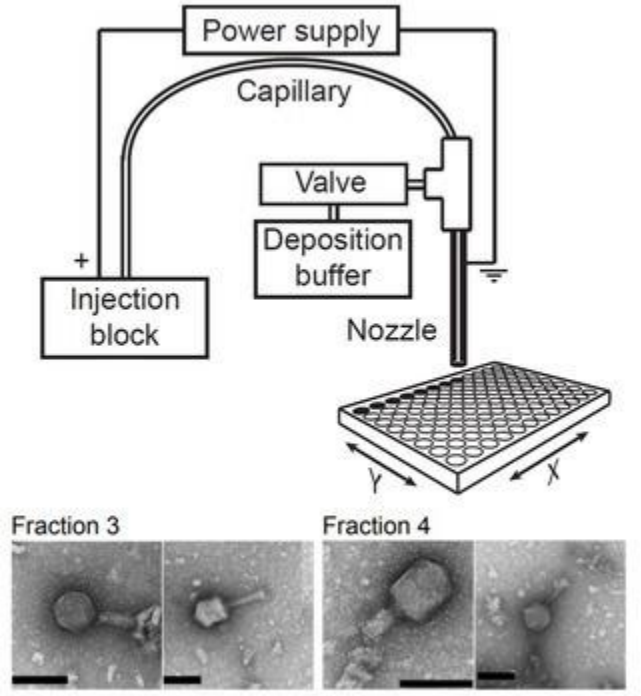


Figure 1: Viruses are divided into specific fractions via CZE

ISOLATION OF VIRUSES USING CAPILLARY ZONE ELECTROPHORESIS

Tech ID: 20-009

https://ideacenter.nd.edu/

Criteria of Purity

- **Infectivity assay-** plaque assay
- **Electron microscopy**
- **Protein assays: serologicall, electrophoresis**
- **Spectrophotometry:**

Measure 260:280 ratio to know approximate nucleoprotein ration

Purified virus	0.29-0.31 OD value at 260 nm
	0.86 to 1.012 at 280nm

SDS PAGE/ELISA/Western blotting for virus/VLP purity

SDS-PAGE & Western blot Analysis:

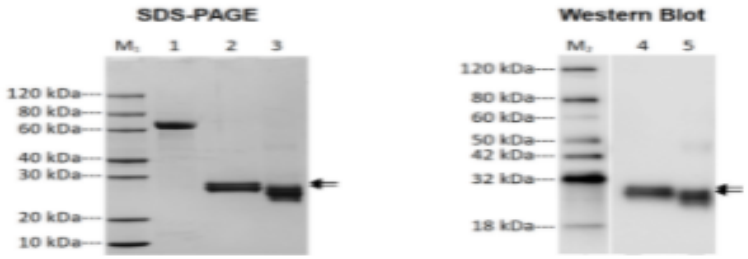


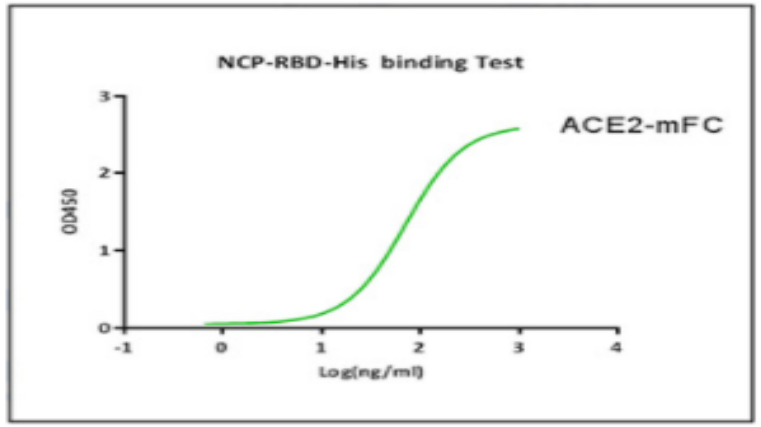
Fig.1 SDS-PAGE and Western blot analysis of RBD Protein (His tag)

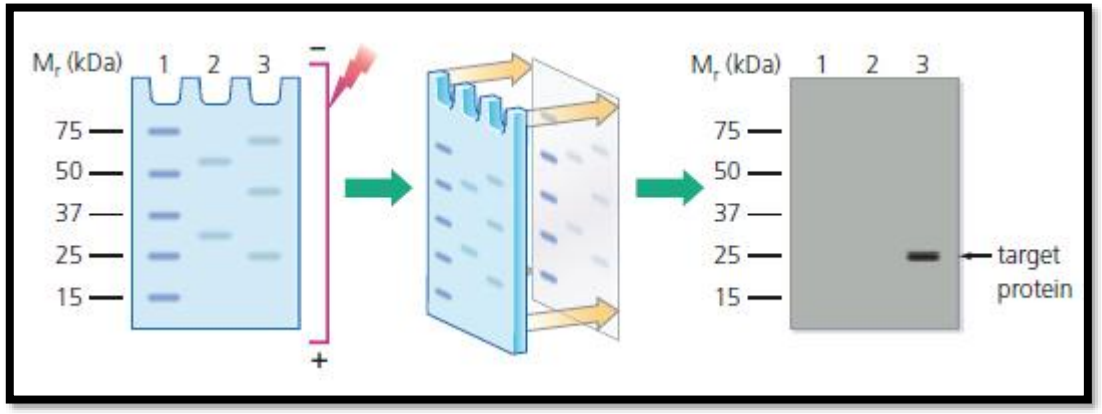
Lane M1: Protein Marker, GenScript, Cat. No. M00516
Lane M2: Protein Marker, GenScript, Cat. No. M00521
Lane 1: BSA (2.00 μg)
Lane 2: RBD Protein (Reducing condition, 2.00 μg)
Lane 3: RBD Protein (Non-reducing condition, 2.00 μg)
Lane 4: RBD Protein (Reducing condition)
Lane 5: RBD Protein (Non-reducing condition)
Primary antibody: Mouse-anti-His mAb (GenScript, Cat.No. A00186)

Purity >90%

https://www.genscript.com/covid-19-protein-expression.html

ELISA Results Binding against ACE2





https://www.bio-rad-antibodies.com/western-blotting-immunoblotting-introduction.html

Virus yield

- The total weight of purified virus is called virus yield. It varies with the virus and plant used.
- It ranges from 0.05 ug/g leaf with Barley yellow Dwarf-virus (BYDV) to 2000 µg TMV/g tobacco.

Maintenance/Storage of virus

- Lyophilization
- Deep Freezer: Store in Na azide, chlorobutanol to prevent microbial growth and stabilize virus
- Extracted or purified virus is stored in small quantities in vials with equal volume of glycerol and 3 or 4 crystals of thymol or sodium azide (to prevent microbial growth) at 4°C or frozen.
- Add liquid nitrogen in equal volume as storage buffer

References

- https://en.wikipedia.org/wiki/Virus_quantification#/
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