NC STATE UNIVERSITY

Introduction to Production of Viral Vectors for Gene Therapy

btec
Biomanufacturing Training and Education Center

Presenters

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Golden LEAF Biomanufacturing Training & Education Center

www.btec.ncsu.edu

What is BTEC?

- Education/Training
  - University students
  - Professionals
- Contract Services
- Bioprocess Research

Economic development

Focus on biological products for treatment or prevention of human disease

Presentation outline

- **Gene Therapy Overview**
  - Definitions and basic concepts
  - Products
- **Gene Therapy Vector Processes**
  - Upstream
  - Downstream
- **Analysis of Gene Therapy Vectors**

Gene therapy*

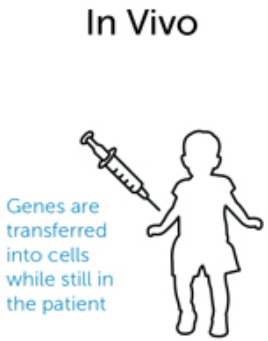
Gene therapy is a technique that modifies a person's genes to treat or cure disease. Gene therapies can work by several mechanisms:

- **Replacing** a disease-causing gene with a healthy copy of the gene
- **Inactivating** a disease-causing gene that is not functioning properly
- **Introducing** a new or modified gene into the body to help treat a disease

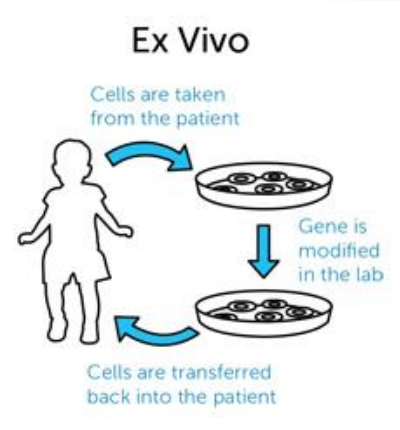
*https://www.fda.gov/BiologicsBloodVaccines/CellularGeneTherapyProducts/ucm573960.htm

In vivo vs. *ex vivo* gene therapy

- **In vivo**: A gene is transferred to cells inside the body.



- **Ex vivo**: Patient cells are harvested and cultivated in the laboratory. A gene is transferred to the cultivated cells. Cells with the new genetic information are then harvested and transplanted back into the patient from whom they were derived.



http://www.danafarberbostonchildrens.org/innovative-approaches/gene-therapy/frequently-asked-questions.aspx

Types of gene therapy products*

- **Plasmid DNA:** Circular DNA molecules can be genetically engineered to carry therapeutic genes into human cells.
- **Viral vectors:** Viruses have a natural ability to deliver genetic material into cells, and therefore some gene therapy products are derived from viruses. Once viruses have been modified to remove their ability to cause infectious disease, these modified viruses can be used as vectors (vehicles) to carry therapeutic genes into human cells.
- **Bacterial vectors:** Bacteria can be modified to prevent them from causing infectious disease and then used as vectors (vehicles) to carry therapeutic genes into human tissues.
- **Human gene editing technology:** The goals of gene editing are to disrupt harmful genes or to repair mutated genes.
- **Patient-derived cellular gene therapy products:** Cells are removed from the patient, genetically modified (often using a viral vector) and then returned to the patient.

*https://www.fda.gov/BiologicsBloodVaccines/CellularGeneTherapyProducts/ucm573960.htm

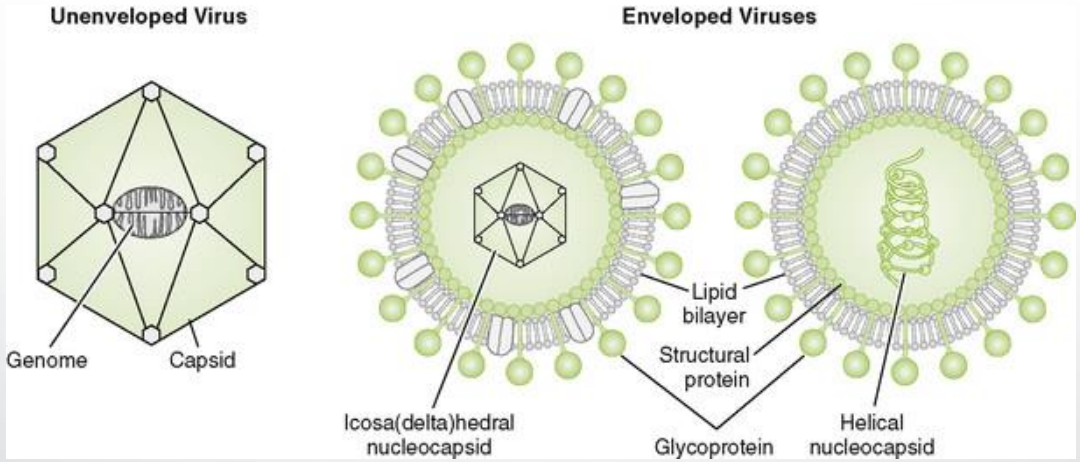
Viruses

Viral particles, also known as **virions**, consist of two or three parts:

(i) a nucleic acid genome (dsDNA, dsRNA, ssDNA, or ssRNA)

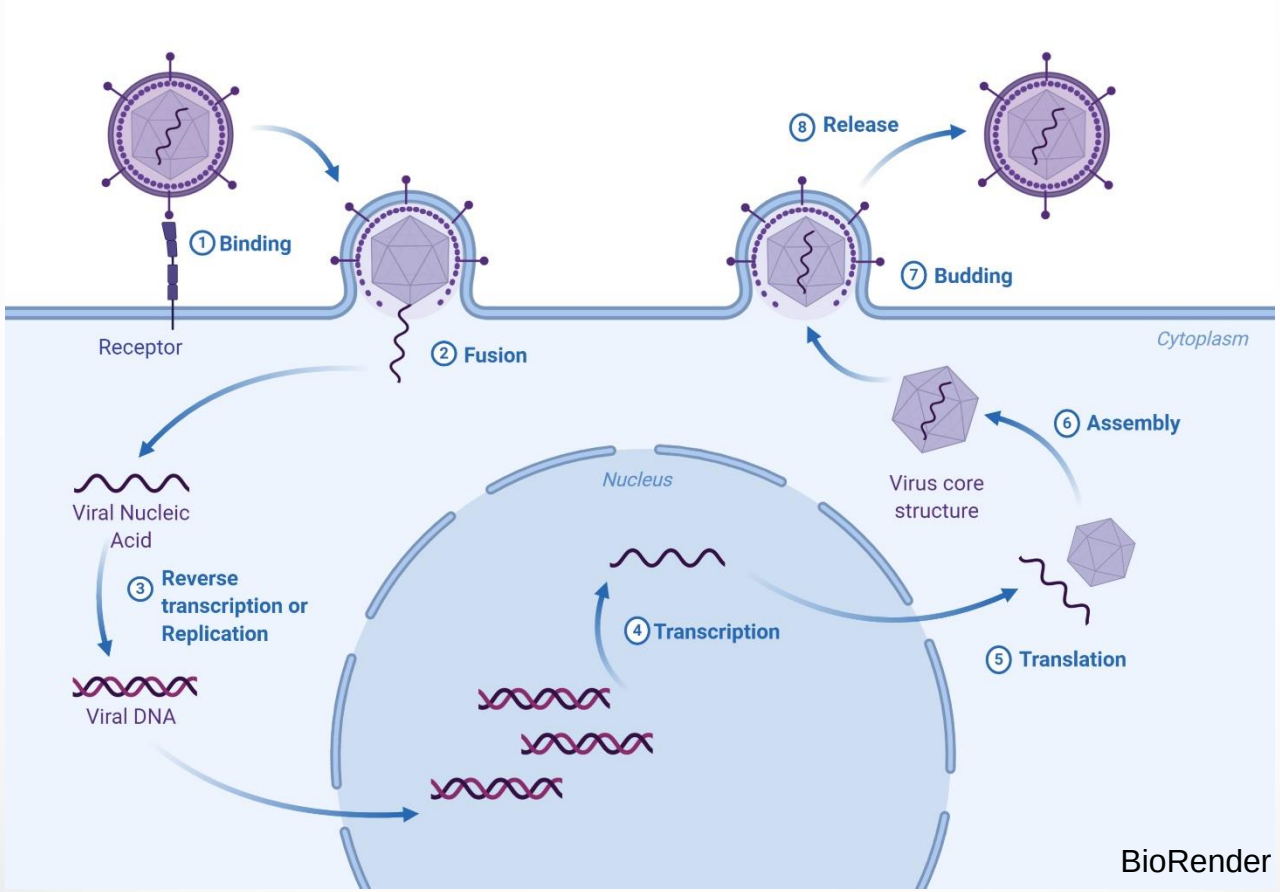
(ii) a protein coat, called the **capsid**, which surrounds and protects the genetic material; and in some cases

(iii) an envelope of lipids that surrounds the protein coat



Why Use Viruses?

Overview of a virus life cycle



The primary function of a virus is to deliver its genes to a host cell causing that host cell to express those genes to make more virus. <u>This makes them a perfect gene delivery vector</u>.

Types of gene therapy viral vectors

Viral vectors

- Adenovirus (AV)
- Adeno-associated virus (AAV)
- Retrovirus (RV)
- Lentivirus

Desirable characteristics:

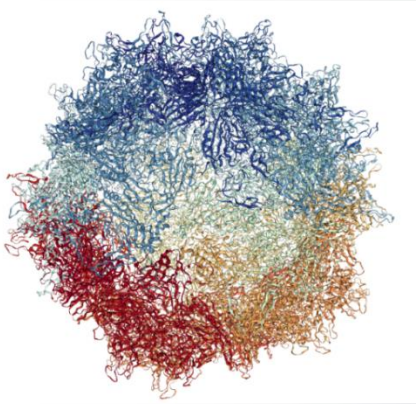
- Ability to target the desired type of cell
- Efficient gene delivery to host cell
- Be maintained successfully for long time periods
- Not elicit an immune response (safe)
- High titer production
- Convenience and reproducibility of production

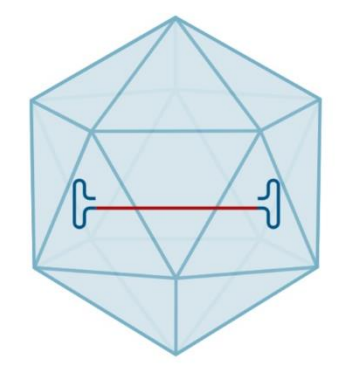
Comparison of most common viral vectors

	Adeno-associated virus (AAV)	Lentivirus (LV)
Genome	ssDNA	ssRNA (+)
Coat	Capsid-only	Envelope and Capsid
Genome size	5kb	9kb
Infection and tropism	Dividing and non-dividing, broad range	Dividing and non-dividing, broad range
Integrating/Non-integrating	Non-integrating	Integrating
Transgene expression	Potentially long-lasting	Long-lasting
Packaging Capacity	4.7 kb	8 kb
Transduction efficiency	Medium	Medium
Immune Response	Very Low	Low

Adeno-associated viral vectors (AAV)

- Smallest gene therapy vector
- Icosahedral assembly of 60 capsid proteins
  - VP1, VP2, VP3 (approx. 1:1:10)
- Size ~ 25 nm; non enveloped
- Single stranded DNA of 4.7 kb
- 11 common serotypes





BTEC produces AAV2 with GFP transgene (AAV2-GFP)

AAV serotypes and the types of cells they infect

Tissue	Optimal Serotype
CNS	AAV1, AAV2, AAV4, AAV5, AAV8, AAV9
Heart	AAV1, AAV8, AAV9
Kidney	AAV2
Liver	AAV7, AAV8, AAV9
Lung	AAV4, AAV5, AAV6, AAV9
Pancreas	AAV8
Photoreceptor Cells	AAV2, AAV5, AAV8
RPE (Retinal Pigment Epithelium)	AAV1, AAV2, AAV4, AAV5, AAV8
Skeletal Muscle	AAV1, AAV6, AAV7, AAV8, AAV9

Work taking place to engineer capsids for modifying tropism and evading circulating neutralizing antibodies (e.g., StrideBio)

https://www.addgene.org/viral-vectors/aav/aav-guide/

Gene therapy products approved USFDA, EMA

2017: Kymriah®, Yescarta®, Luxturna® (FDA & EMA)

2015: Imlygic® (FDA & EMA)

2019: Zolgensma® (FDA), Zynteglo® (EMA)

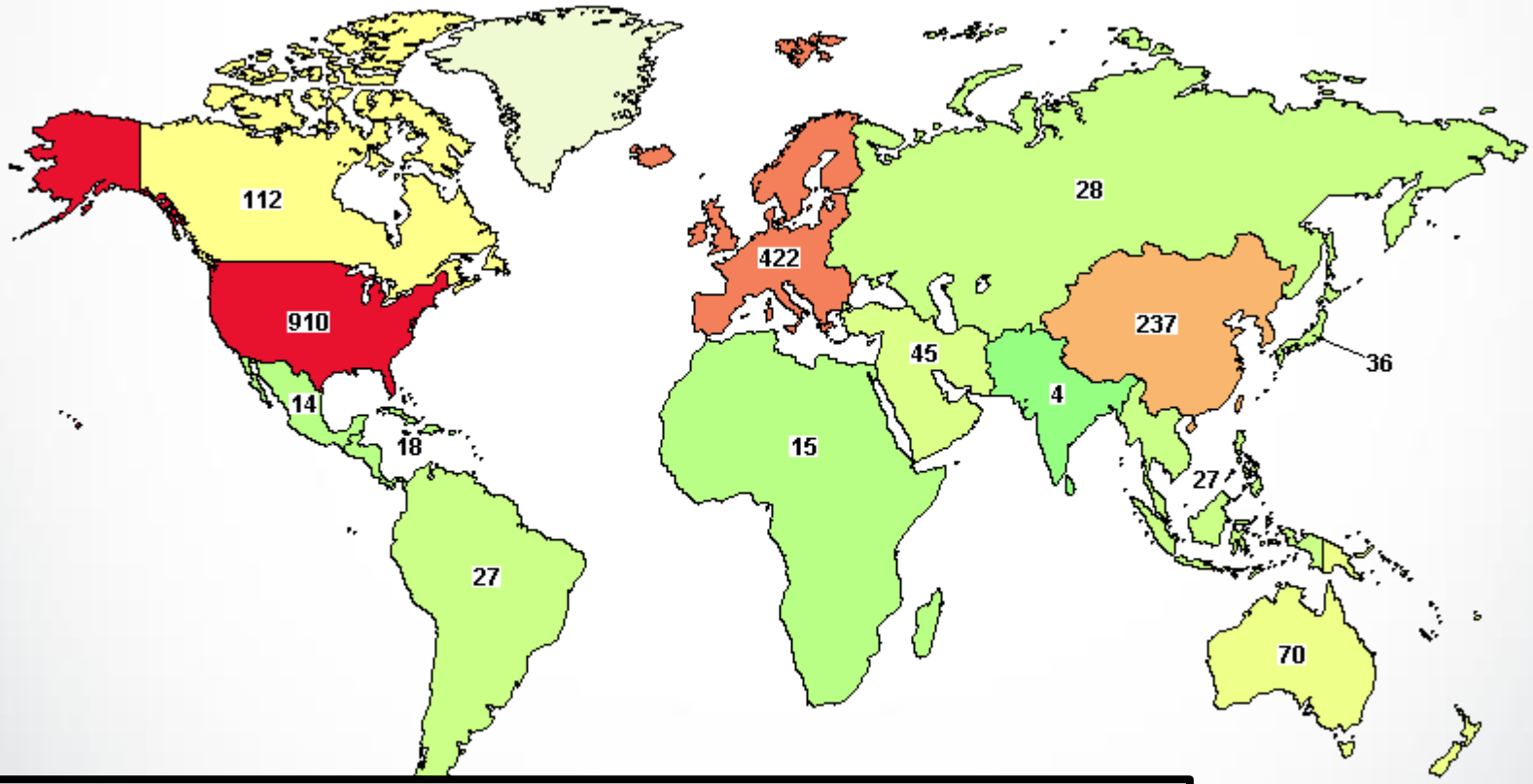
2016: Strimvelis® (EMA)

2012: Glybera® (EMA)

https://www.fiercepharma.com/manufacturing/novartis-still-struggling-kymriah-manufacturing-providing-some-out-spec-doses-to

https://eyewire.news/videos/gene-therapy-luxturna-approved-in-europe-for-inherited-retinal-disease/

Gene therapy clinical trials*



1517 gene therapy clinical trials recruiting, enrolling, and active – growth of gene therapies is inevitable

*from ClinicalTrials.gov

Risks associated with gene therapy

- Unwanted immune system reaction – inflammation; in severe cases, organ failure
- Health problems resulting due to overproduction of enzymes/ proteins by the replaced gene
- Targeting the wrong cells can lead to healthy cells being damaged, causing other illness or diseases, such as cancer (viruses infect more than one type of cell)
- Gene insertion at the wrong place
- Possibility of infection caused by the viral vector and chances of the viral vector being contagious
- Possibility of tumor formation

Gene therapy drug product example - Luxturna®

https://www.fiercepharma.com/regulatory/spark-therapeutics-grabs-fda-ok-for-gene-therapy-luxturna-but-isn-t-disclosing-price

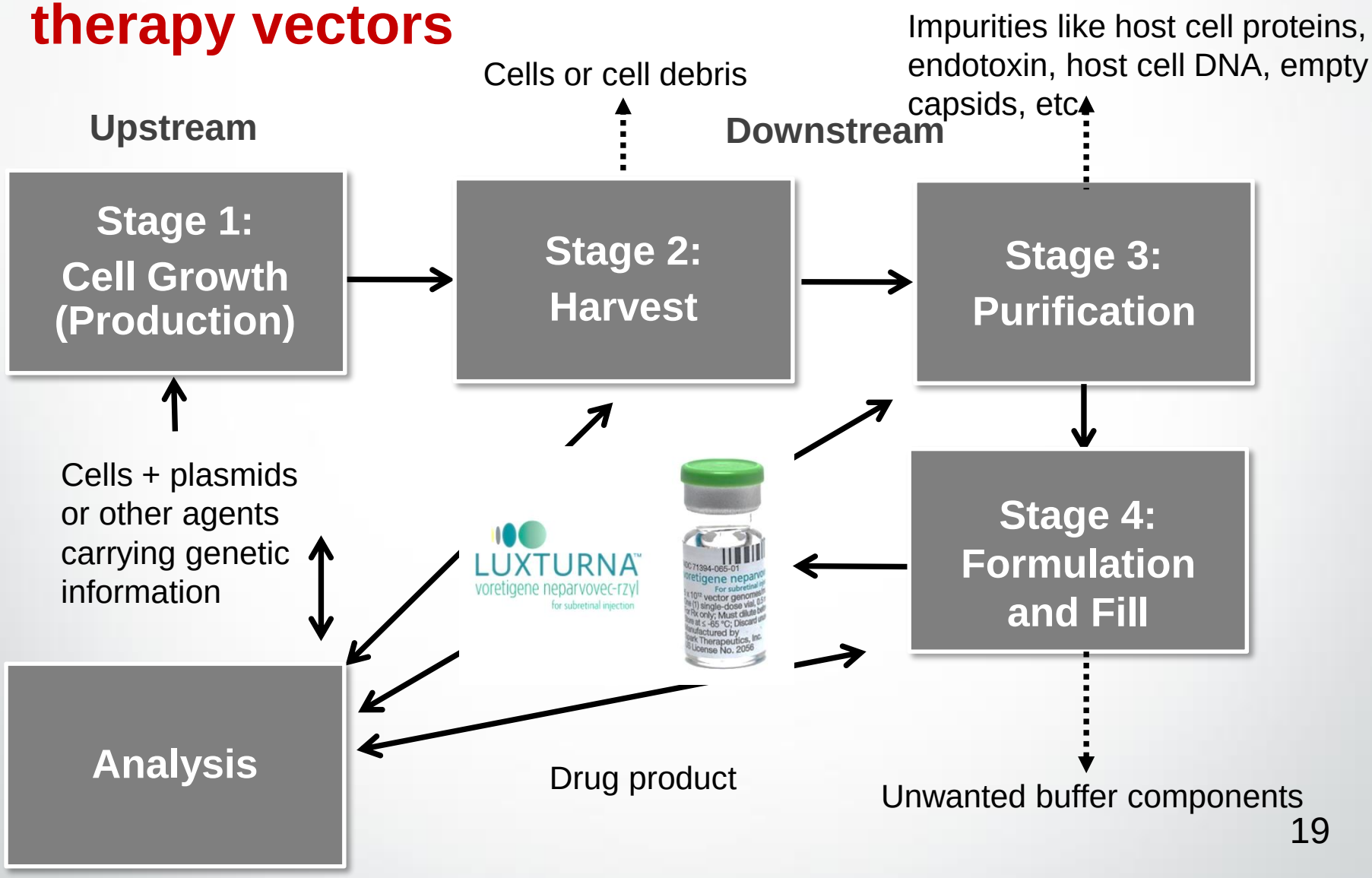
Drug product

- Water for injection (WFI)
- 5 x 10^{12} vector genomes vg/mL
- 180 mM NaCl
- 10 mM sodium phosphate
- 0.001% Poloxamer 188
- pH 7.3
- Impurities: HEK293 DNA, HEK293 proteins, fetal bovine serum
- Total volume=0.5 mL
- Requires 1:10 dilution prior to administration

Diluent

- Sterile water
- 180 mM NaCl
- 10 mM sodium phosphate
- 0.001% Poloxamer 188
- pH 7.3
- Total volume=1.7 mL/vial, 2 vials

Generalized process for production of gene therapy vectors



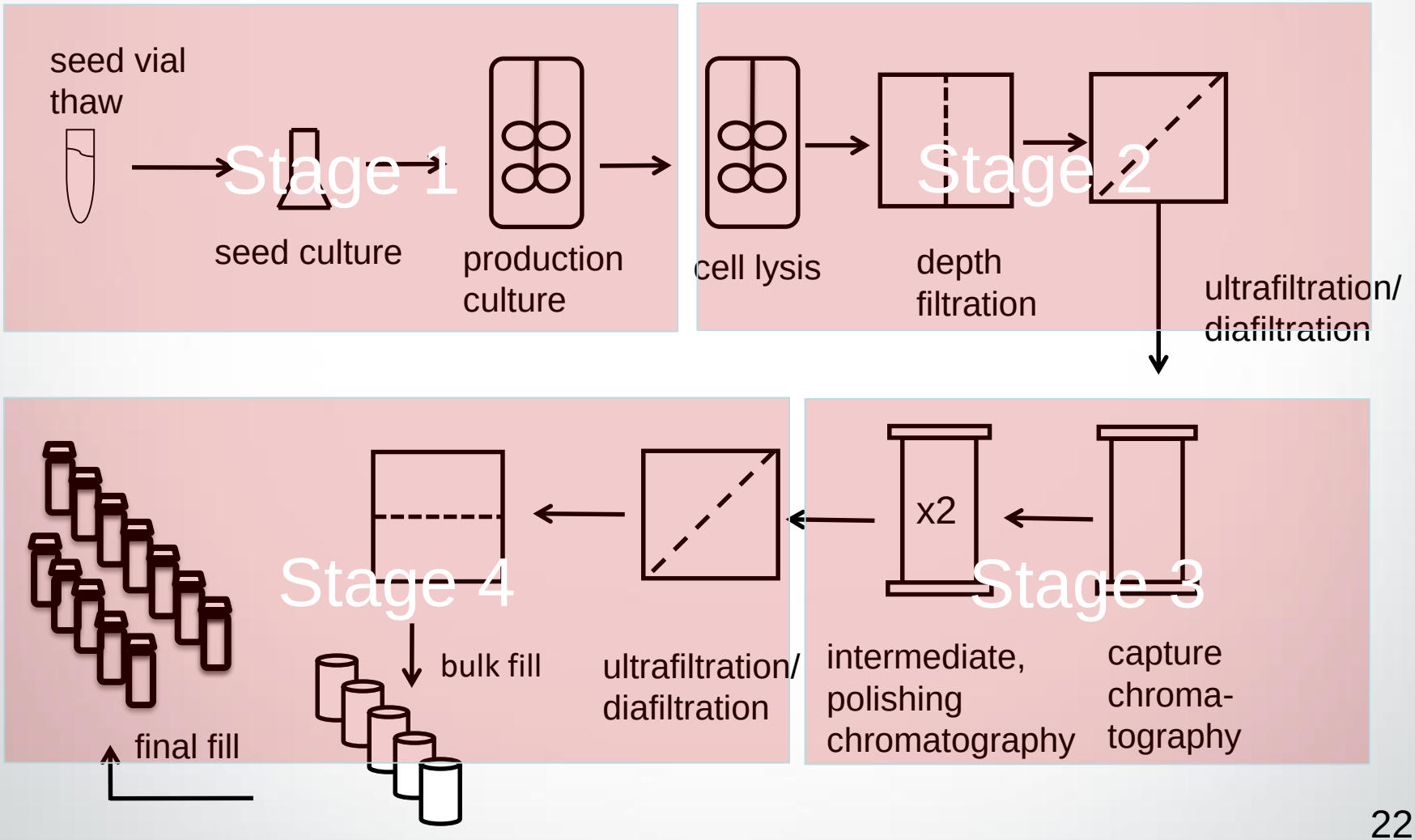
Challenges

- Low Titer
- Wait time to get into a CDMO
  - Plasmid preps
  - Production
- Low % full capsids
  - 1-5% HEK293
  - 60-90% Baculovirus/Sf9
- Separation of full from empty capsids
- Quantifying % full capsids
- CQAs undefined

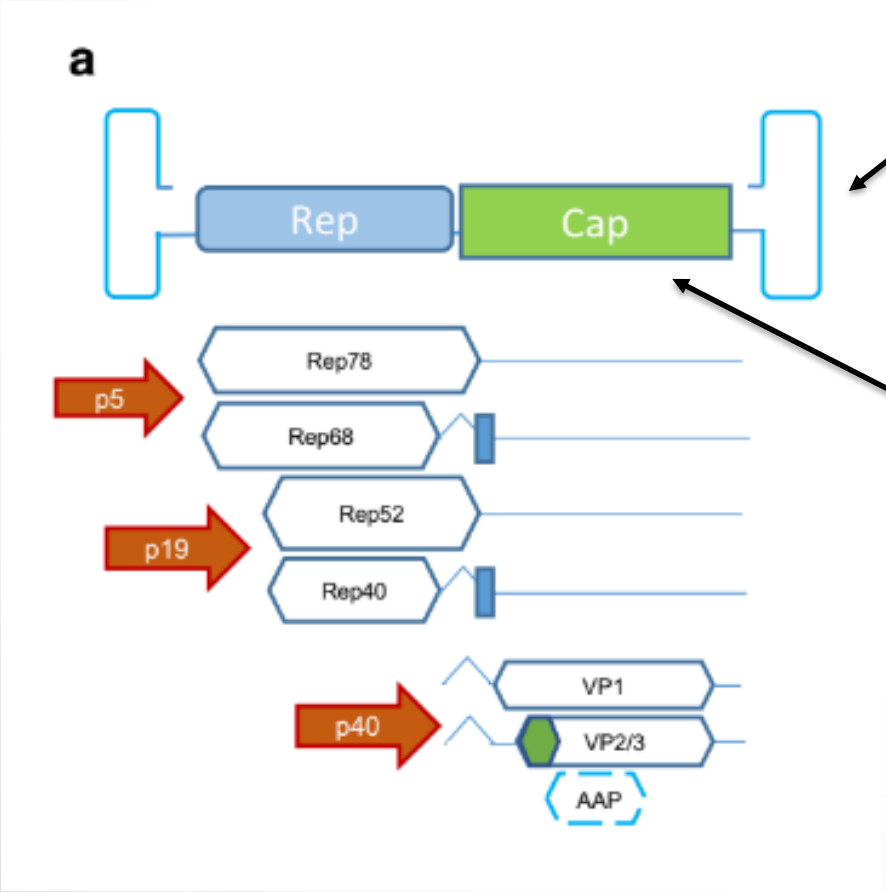
Presentation outline

- **Gene Therapy Overview**
  - Definitions and basic concepts
  - Products
  - Recent history
- **Gene Therapy Vector Processes**
  - Upstream
  - Downstream
- **Analysis of Gene Therapy Vectors**

AAV production process

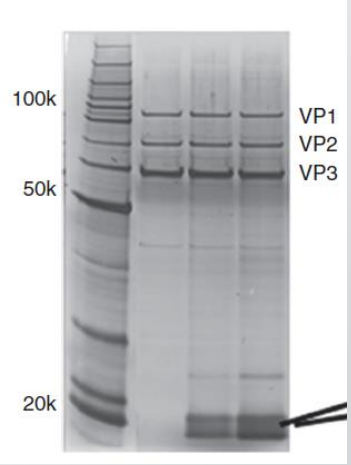


Wildtype adeno-associated virus (AAV)



Inverted terminal repeat (ITR)

Serotype of capsid dictates serotype of virus



Applied Microbiology and Biotechnology
https://doi.org/10.1007/s00253-017-8670-1

Molecular Therapy (2016) 24(2) 287–297

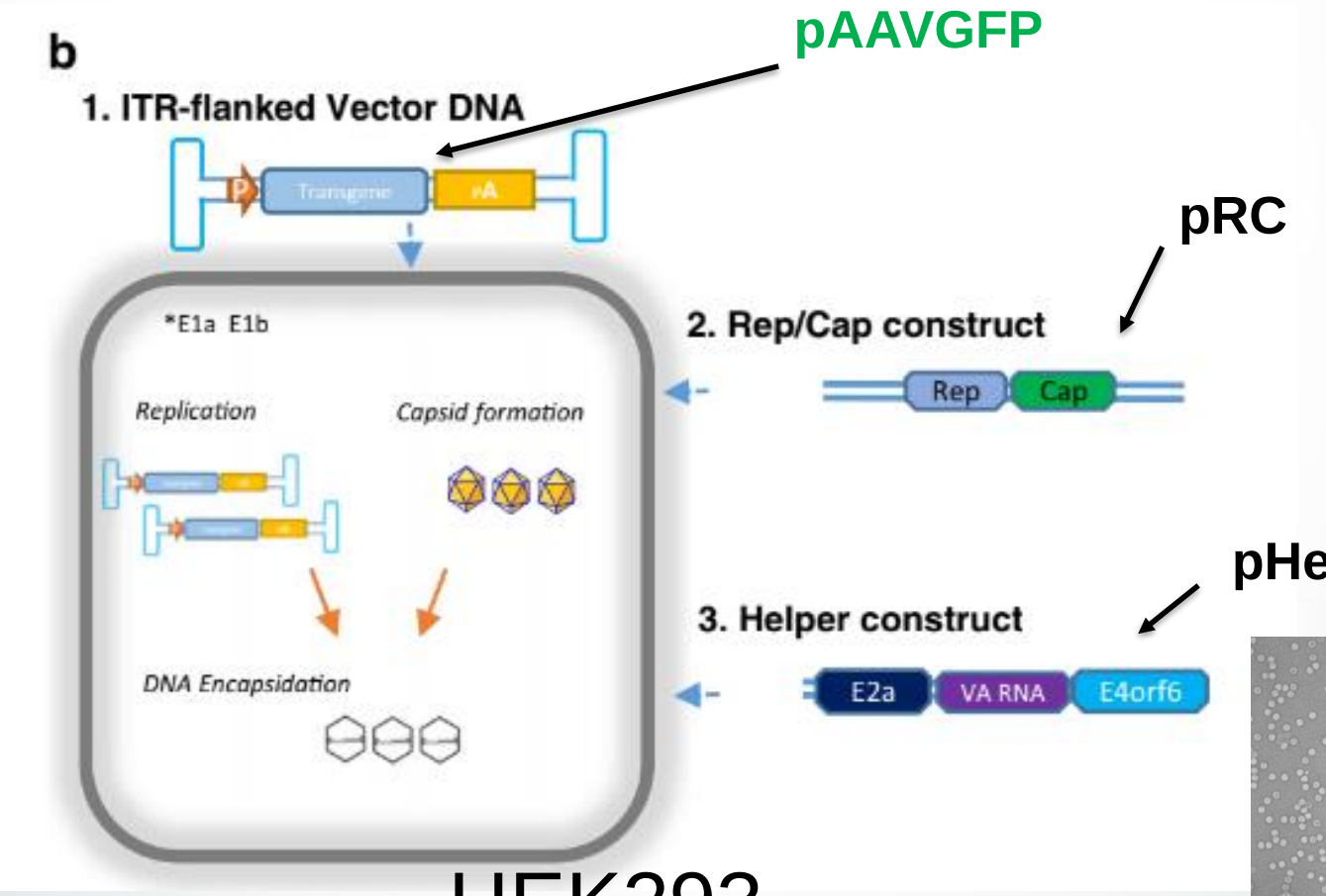
Function of ITR

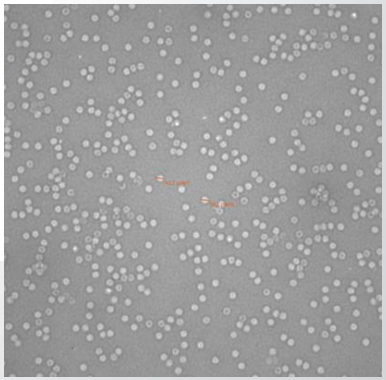
- 145 base pairs
- Form hairpin structure
- Required for:
  - efficient multiplication of the AAV genome
  - efficient encapsidation of the AAV DNA
  - self-priming that allows primase-independent synthesis of the second DNA strand

How do you produce AAV?

- Numerous ways
- Today we will discuss:
  - Transient transfection into HEK293 cells
  - Recombinant baculovirus infection into insect cells

HEK293 AAV vector production





Applied Microbiology and Biotechnology
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BTEC model system-HEK293

- HEK293 cells
- Plasmids:
  - pAAV-GFP
  - pRC
  - pHelper
- Scale up cells
- Transiently transfect using PEI
- Harvest cells 72 hrs post-transfection
  - Lysis in the bioreactor

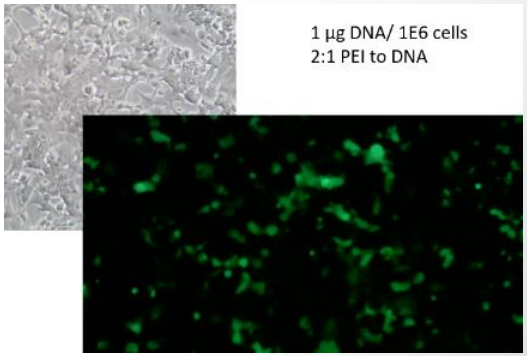
Molecular Therapy (2016) 24(2):287–297

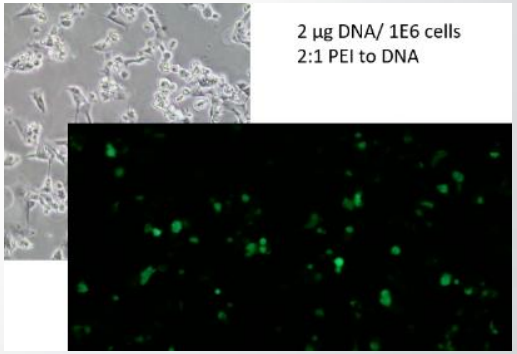
Transfection reagents/methods

- Calcium phosphate
- Polyethyleneimine (PEI)
  - Less toxic
  - inexpensive
  - No method to quantify PEI in final product
- Liposomes
  - Expensive
- Electroporation
  - Hard to scale

Optimization of transfection conditions

- Cell density
- Transfection reagent
- Ratio of DNA to transfection reagent
- Amount of DNA
- Time of expression
- Temperature
- Post-transfection additives
- Ratio of transfection cocktail
- Transfection media

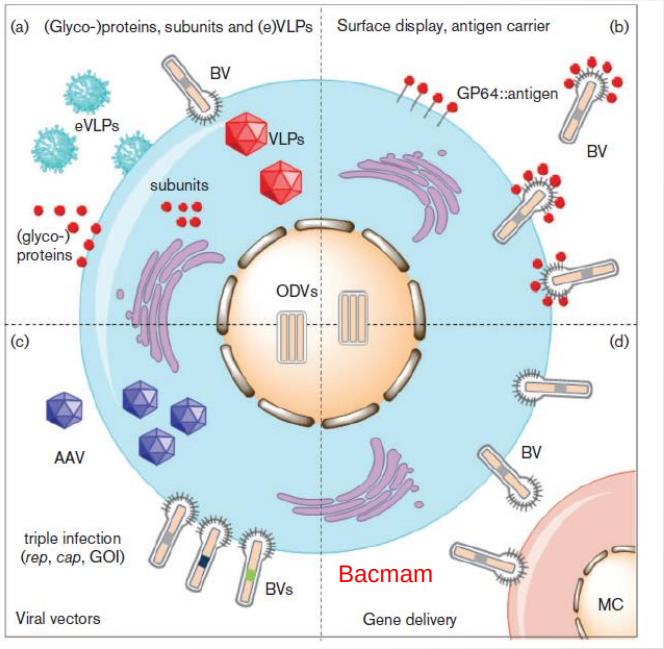




Transfection into HEK293 cells
Courtesy of Brenda Ijeomah

Baculovirus AAV vector production

- Generate 2 or 3 viruses and infect insect cells
  - Cap
  - Rep
  - ITR-gene of interest
- Historically, AVV vectors produced with triple infection were not as infective
  - Incorporation of V1/V2/V3 is stochastic
- Stable Sf-9 cell line expressing cap and rep
  - OneBac 1.0
  - Inducible upon viral infection

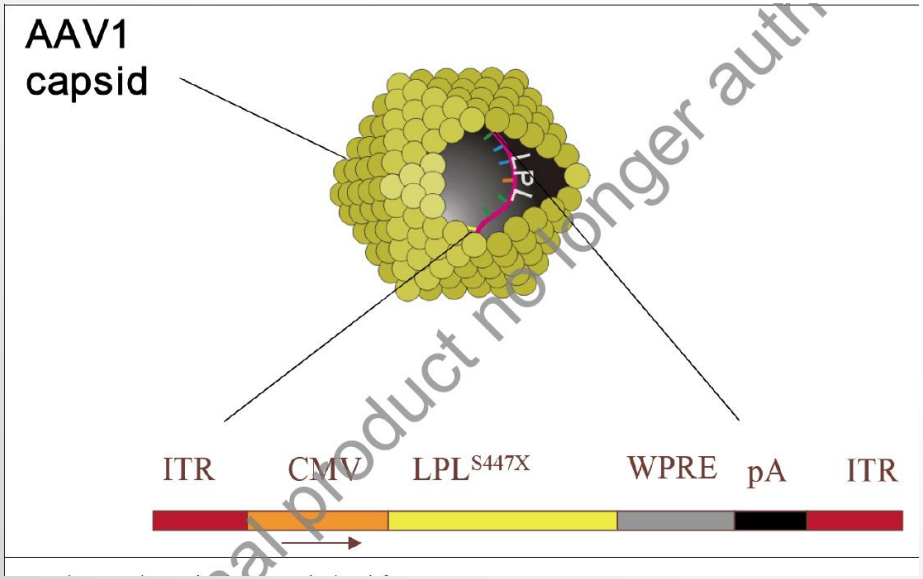


www.pnas.orgcgidoi10.1073pnas.0810614106

Journal of General Virology (2015), 96, 6–23

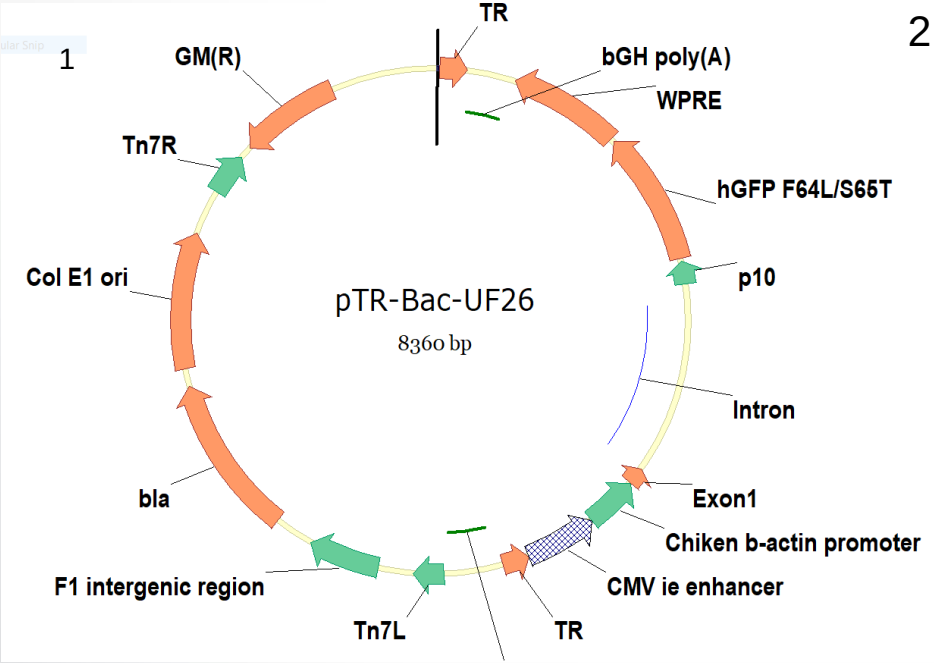
Glybera®

- Produced in Baculovirus
- Compensates for lipoprotein lipase deficiency, a rare inherited disorder which can cause severe pancreatitis
- EU market in 2015
- Cost: one million



AAV2 ITR

Baculovirus model system for production of AAV2-GFP

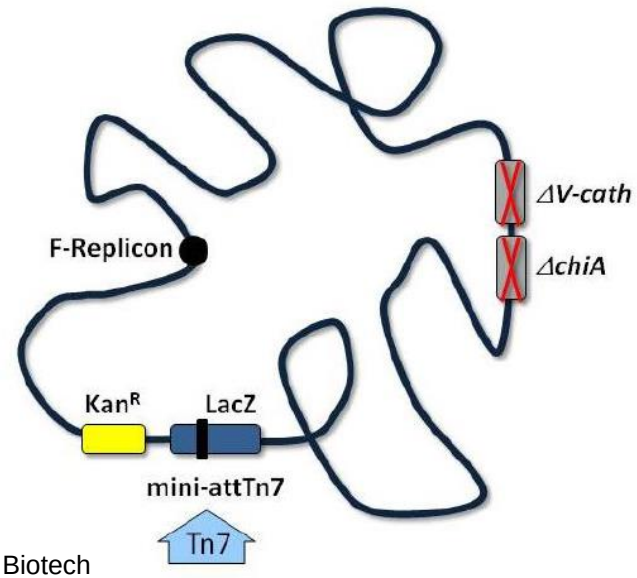


Shuttle plasmid

1 Sergei Zolotukhin, University of Florida Gainsville

2 MultiBac™ baculovirus genome

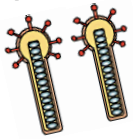
- △cathepsin-type cysteine protease
- △chitinase



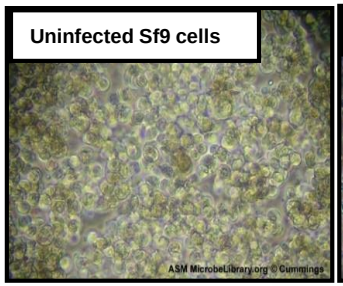
2 Geneva Biotech

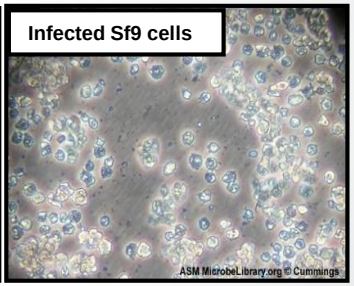
AAV2 production – Sf9/baculovirus

- Baculovirus expression system components
  - Baculovirus
    - *AAV2 ITR-flanked GFP
  - Insect cells
    - *F3: Recombinant Sf-9 cell line containing AAV2 capsid gene and AAV2 replication gene
    - Infection induces the production of cap and rep

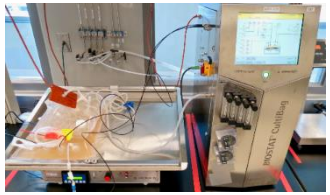


Baculovirus stock

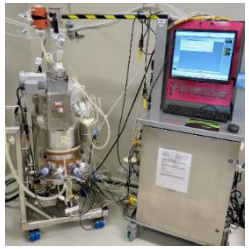




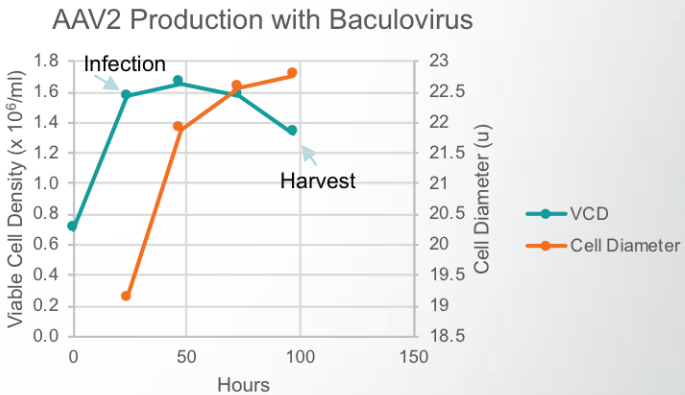
Shake flask



10-L rocker bag

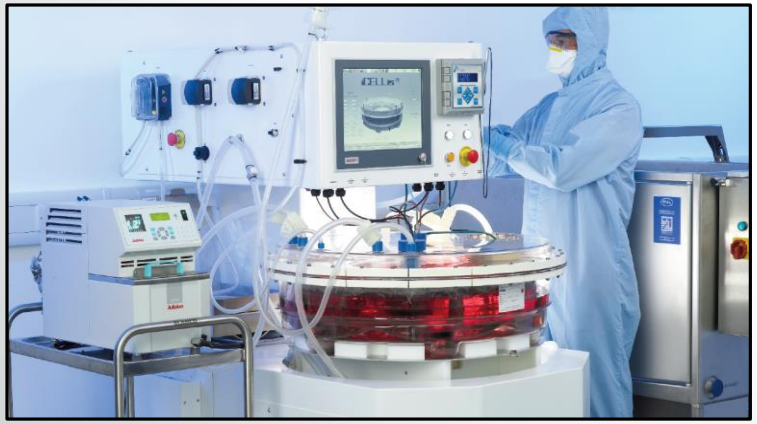


50-L stirred bioreactor



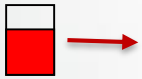
*Obtained under MTA with University of Florida
PNAS (2009) 106(13) 5059-5064

Single-use bioreactors



Upstream-adherent

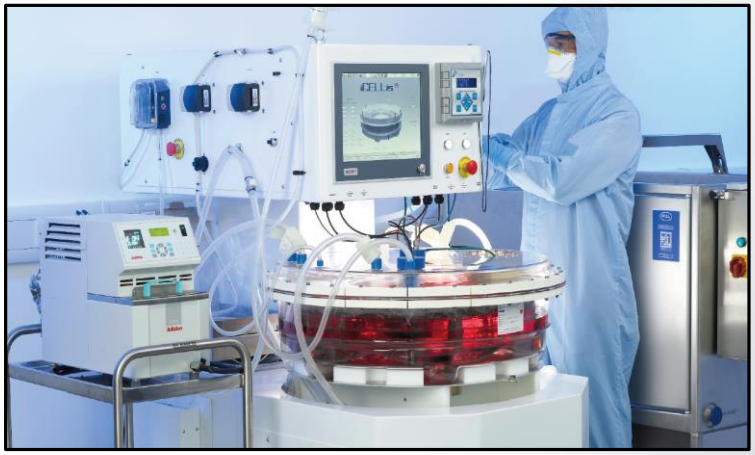
Transfection cocktail



Vial
WCB

T-flask

Cell stack

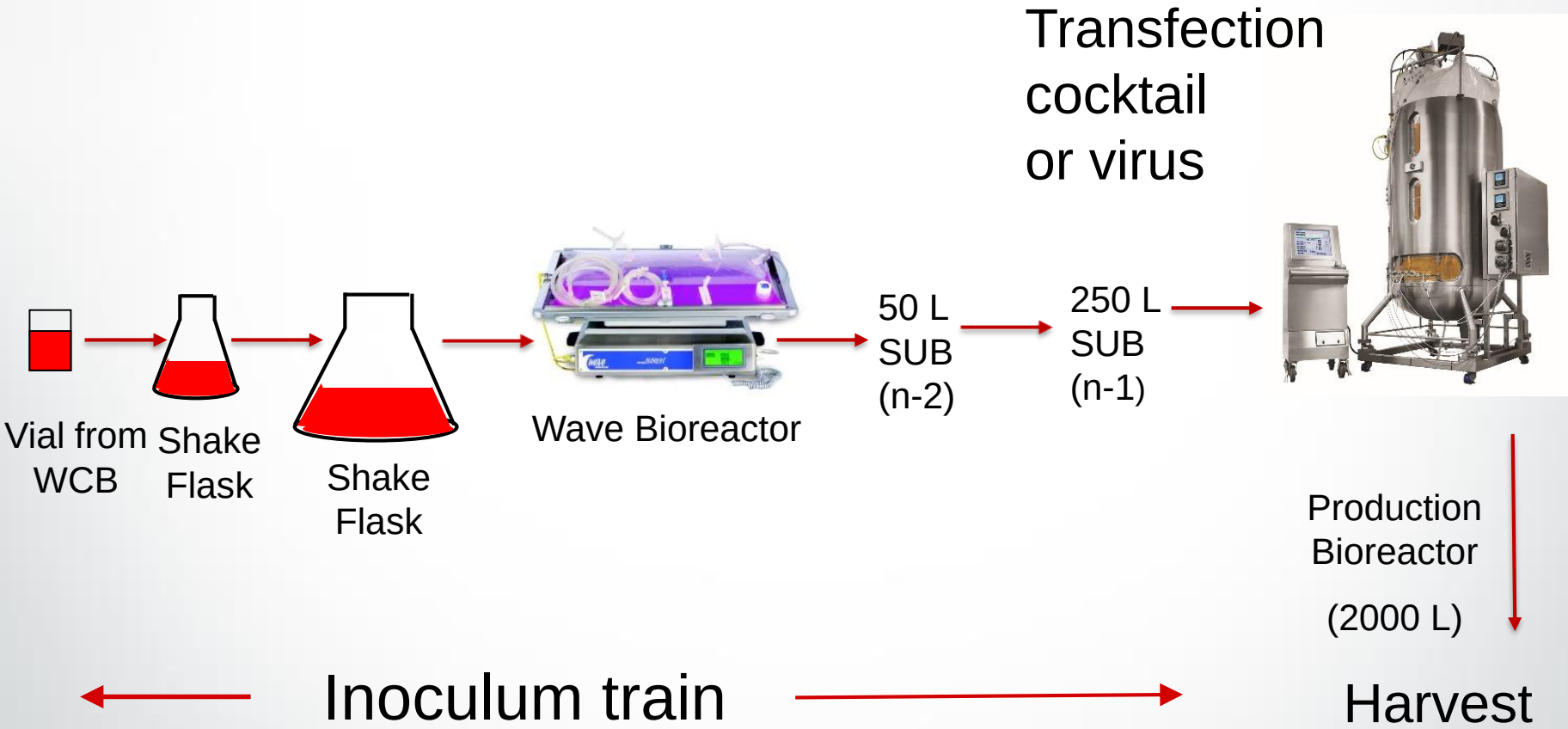


Production bioreactor

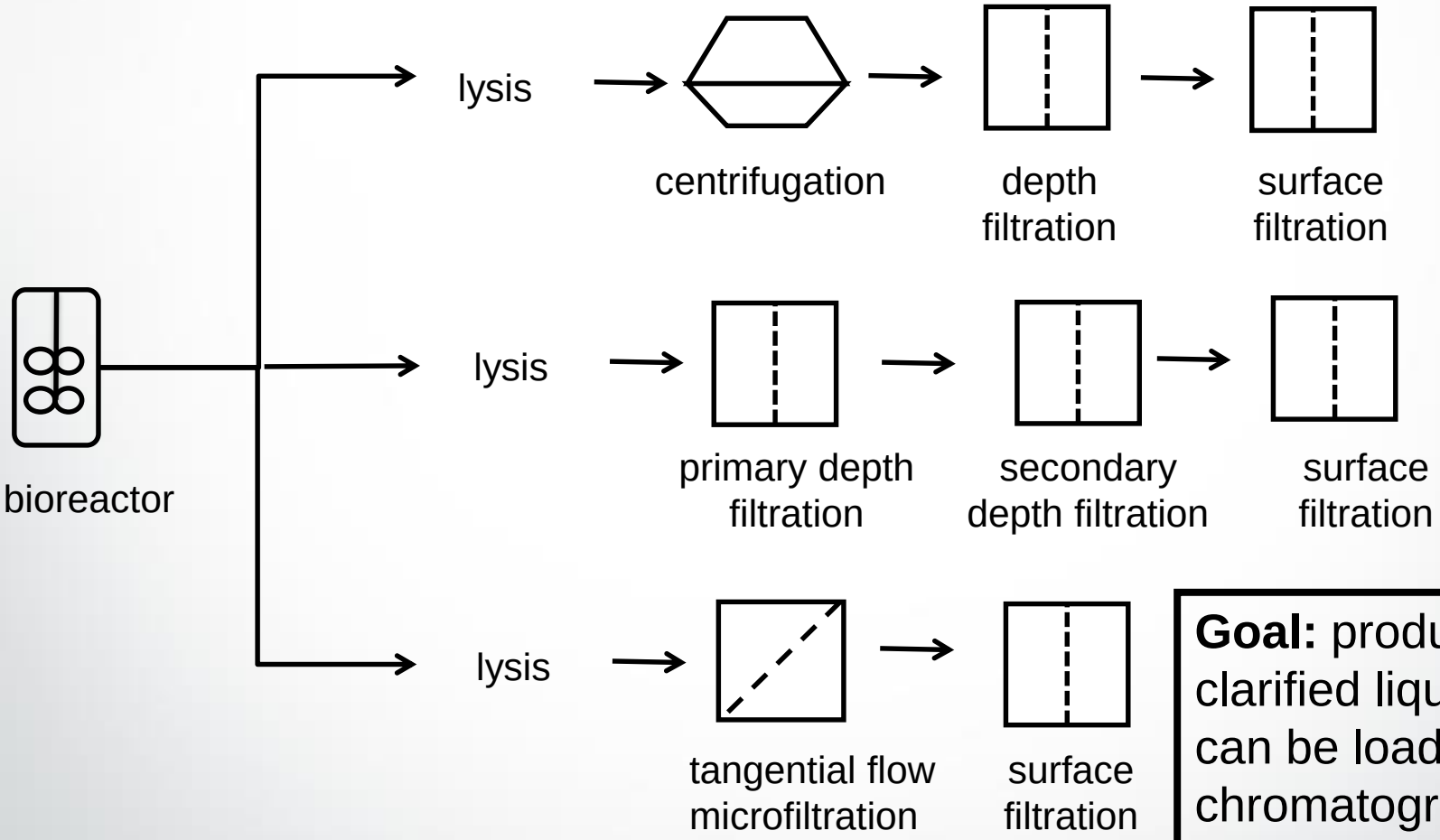
Inoculum train

Harvest

Upstream-suspension



Vector harvest process design options:
intracellular vector, lysis in bioreactor

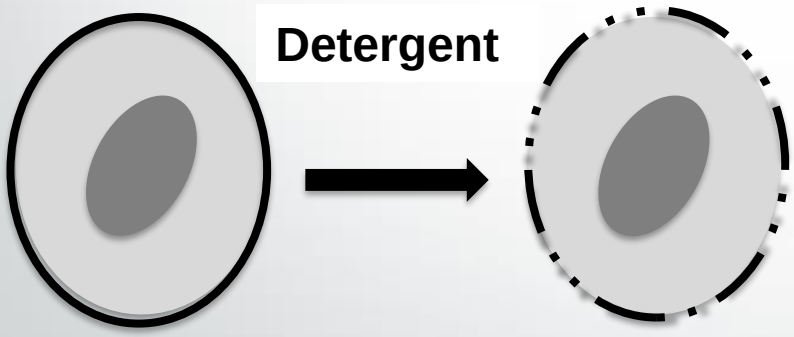


Goal: produce a clarified liquid that can be loaded to a chromatography column

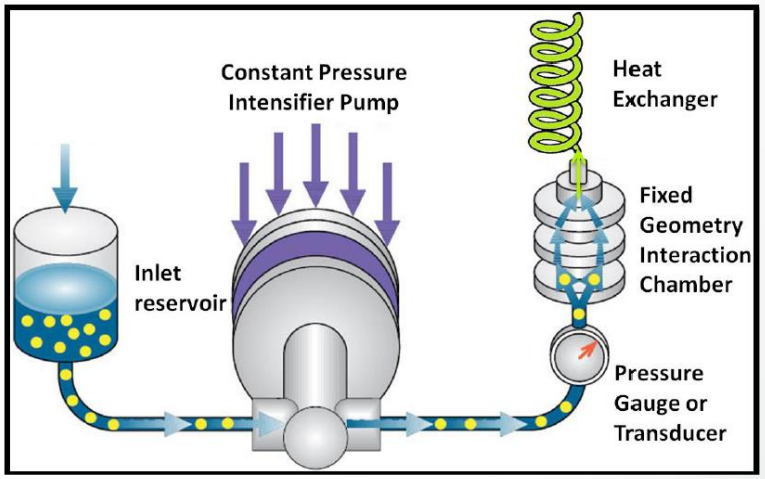
Cell lysis for intracellular product

Detergent-based lysis

incorporation of detergent into the cell membrane, solubilizing lipids and proteins in the membrane, creating pores within the membrane and eventually full cell lysis.

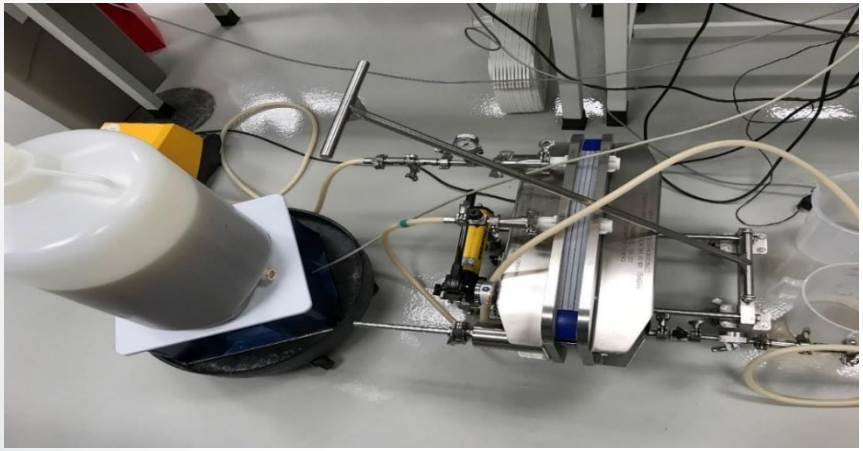


Microfluidizer®



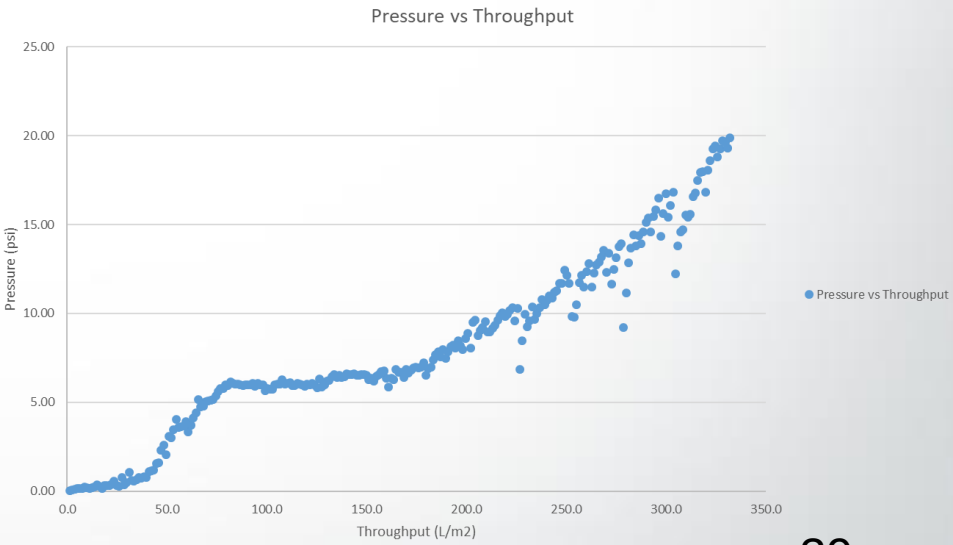
- 50-500 µm microchannels
- Convert pressure to kinetic energy
- Shear and impact forces lead to lysis

Depth filtration of clarified lysate



Depth filtration setup using the Millipore Sigma, Millistak+®HC Pro D0SP

Pressure vs. throughput for the Millipore Sigma, Millistak+®HC Pro D0SP



Product purity: Types of impurities

Process-Related

- Residual host nucleic acid
- Residual plasmid DNA
- Host-cell proteins
- Residual helper virus
- Residual cell culture components
- Residual leachables

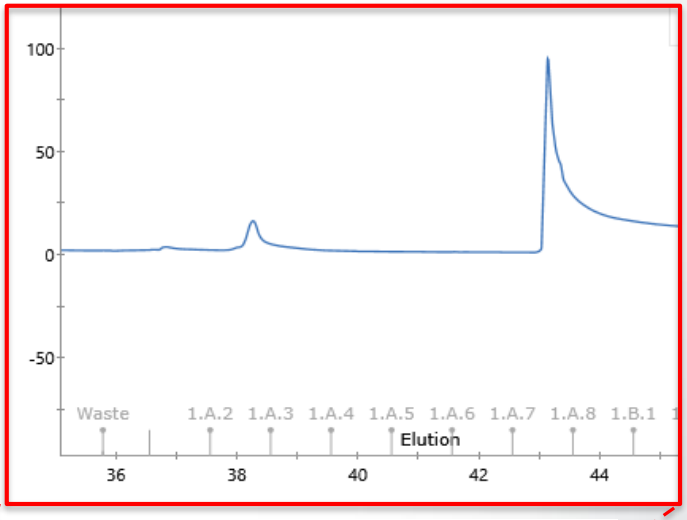
Product-Related

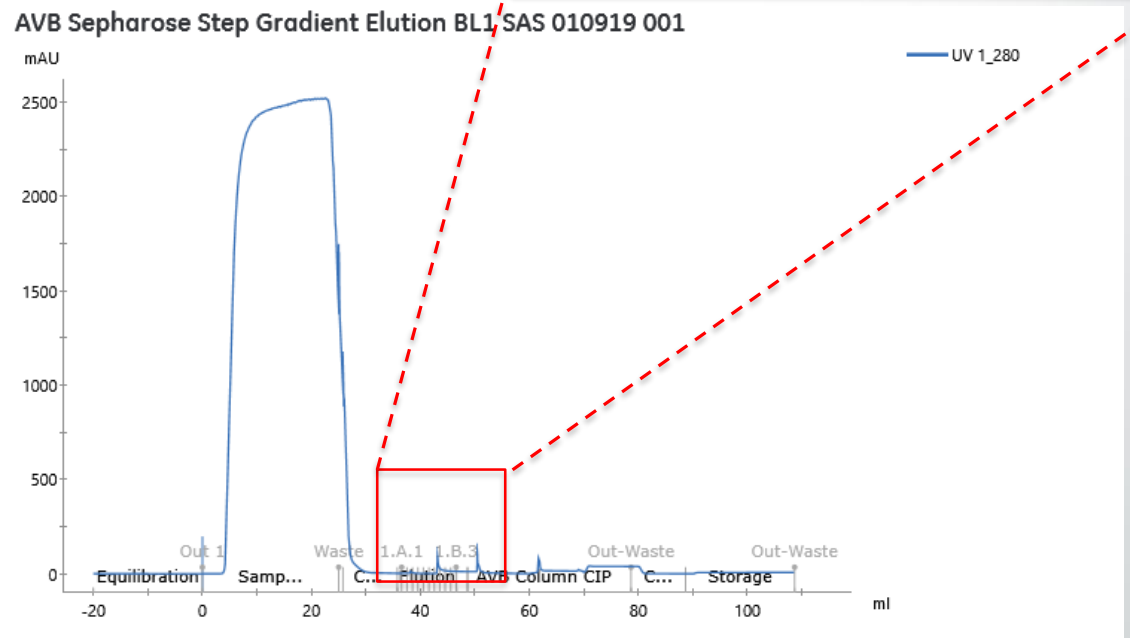
- Aggregates
- Empty capsids
- Noninfectious particles
- Encapsidated non-target DNA (plasmid/host)
- Fragments
- Degradants
- Replication competent virus

AAV Purification using AVB Sepharose resin

AKTA avant

HiTrap AVB columns



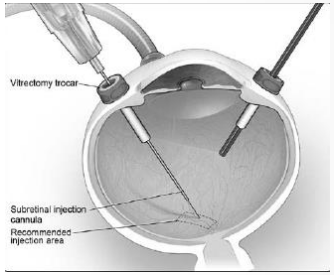


AAV yields

- Baculovirus
  - Voyager Therapeutics: $>10^{12}$ vg/ml
  - 2000 L scale
- HEK 293 Triple Transfection[2]
  - AskBio: 2.4×10^{11} vg/ml
  - 250 L scale

[1]Voyager Therapeutics presentation at Bioprocessing Summit 2019
[2]Grieger NIH presentation on AskBio website

AAV dose is dependent upon route of administration



- Dose delivered locally is “small”
  - Luxturna
    - 1.5 x 10^{11} vector genomes (vg) per eye

- Dose delivered systemically is “large”
  - >1000-fold higher
  - Zolgensma
    - 1.1 x 10^{14} vg per kg of body weight
    - 80 lb. person→ 49 x 10^{14} vg

BioRender and product inserts for drugs

How much AAV do we need to manufacture?

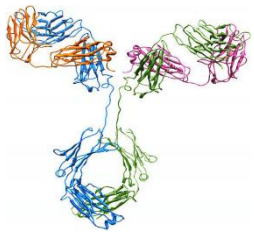
Type of Disease	Number (patients/year)	Route of Administration	Dose (vg/patient)	Demand (vg/year)
Rare	~1000	Local	$\sim 10^{11}$	$\sim 10^{14}$
Rare	~1000	Systemic	$\sim 10^{15}$	$\sim 10^{18}$
Prevalent (Parkinson)	~100,000	Local	$\sim 10^{12}$	$\sim 10^{17}$
Prevalent (Parkinson)	~100,000	Systemic	$\sim 10^{15}$	$\sim 10^{20}$

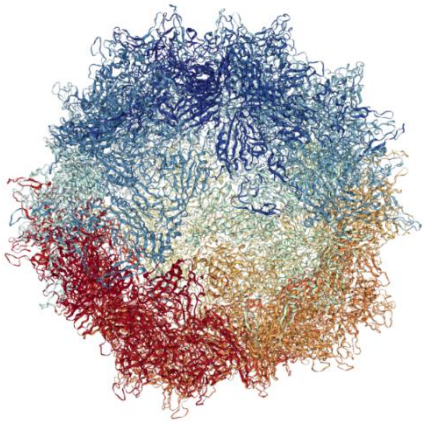
Modified from Voyager Therapeutics presentation at Bioprocessing Summit 2019

Presentation Outline

- **Gene Therapy Overview**
  - Definitions and basic concepts
  - Products
  - Recent history
- **Gene Therapy Vector Processes**
  - Upstream
  - Downstream
- **Analysis of Gene Therapy Vectors**

AAV complexity vs mAb





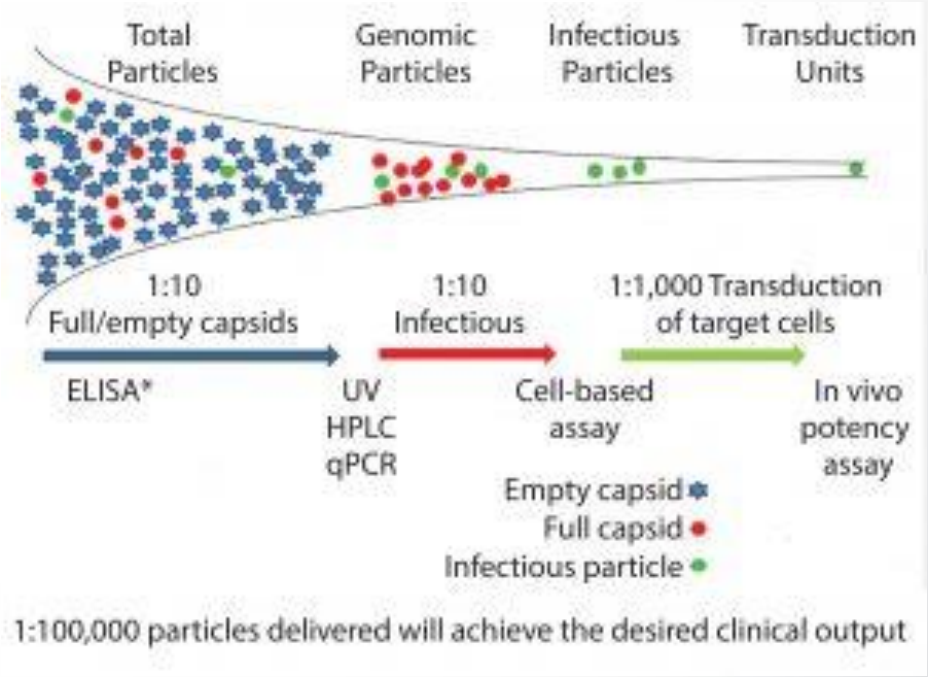
mAb	AAV
2 x Heavy Chain (50 kDa) 2 x Light Chain (25 kDa) Post translational modifications	1 DNA fragment (up to 4.7 kb) 50 x VP3 (62 kDa) 5 x VP2 (72 kDa) 5 x VP1 (87 kDa) Post translational modifications (?)
~ 150 kDa	7.0 MDa (3.9 MDa protein + 3.1 MDa DNA)
CQAs well-defined with available characterization methods	CQAs largely undefined
Well-characterized standard available	No fully-characterized standard

AAV analytical tests performed

Category		Example Assays
Quantity	Total Capsid	ELISA
	Full Capsid (viral genomes)	qPCR, ddPCR
Purity	Host Cell Protein	SDS-PAGE, HPLC
	Full/empty Capsid ratio	ELISA/qPCR, TEM, Spectrophotometry, AUC, CDMS
	Residual DNA (encapsidated and non)	qPCR, pico-green
	Aggregates and fragments	TEM, HPLC
Identity	VP1, VP2, VP3 fingerprint	Western blot, SDS-PAGE
	Viral genome sequence	PCR, DNA Sequencing
Potency	Transduction and Expression	Cell-based assay, and target gene expression/activity
	Infectivity	TCID50
Safety	Endotoxin, Sterility, Adventitious agents, Mycoplasma, etc.	Various methods, as with traditional biotherapeutics

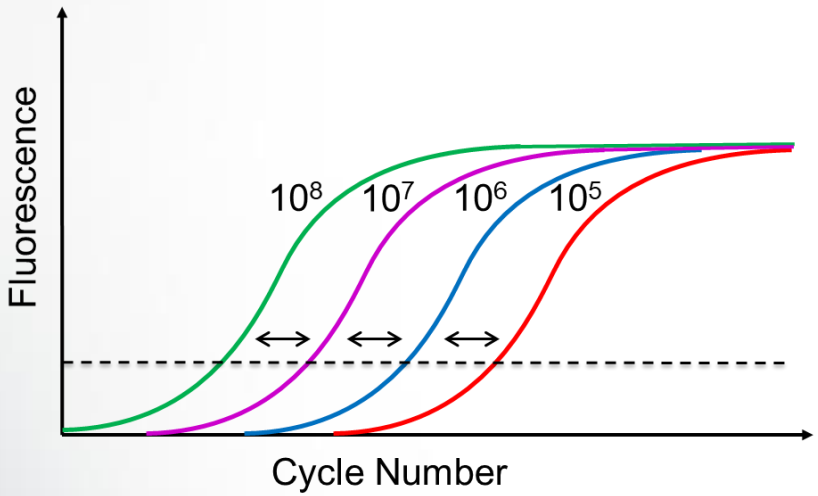
AAV quantification

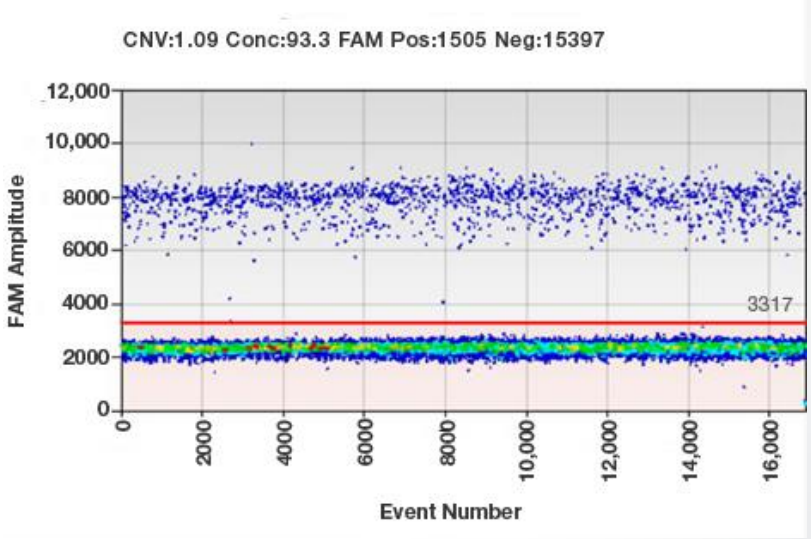
- Viral Genomes
- Capsids
- Infectious Titer
- Transduction Titer



Hitchcock, 2017, BioProcess International

Viral genome titer methods





qPCR	ddPCR
Relative Quantification	Absolute Quantification
Requires a Standard Curve	No Standard Curve
Lower Precision	Higher Precision
Sensitive to Inhibitors	Less Sensitive to Inhibitors

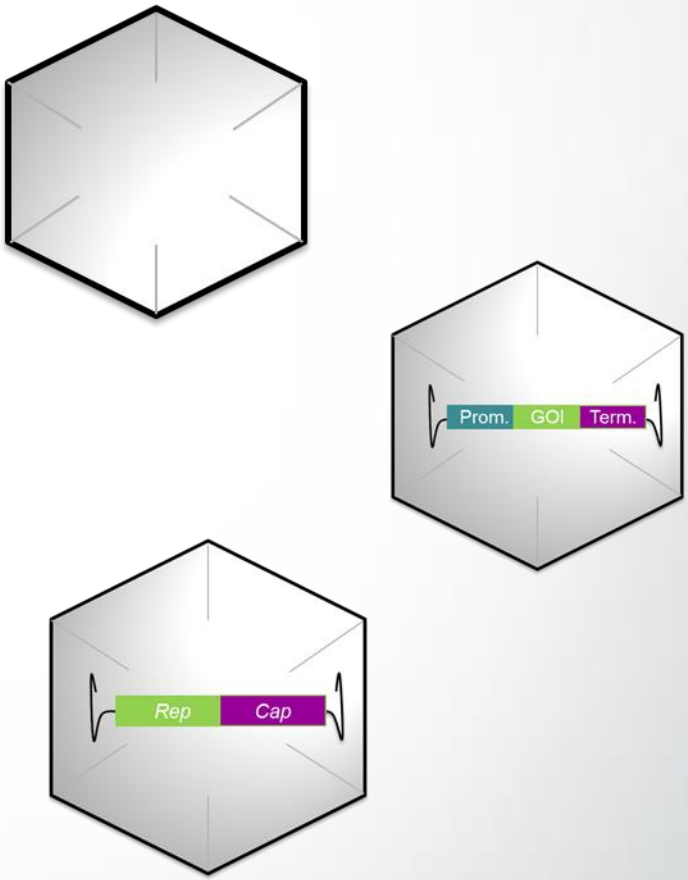
AAV potency assays

- Infectivity assay:
  - $TCID_{50}$ utilizing Adenovirus and a rep/cap expressing cell line
  - Measures AAV infectivity by qPCR
  - Does not demonstrate delivered gene expression or MOA
  - Variability

- Transduction assay:
  - *In vitro* assay transducing target cell type or similar
  - Measures expression or activity of delivered gene through RT-PCR, western blot/ELISA, activity assay, etc.
  - Some AAV serotypes do not transduce well *in vitro*
  - Variability

Product related impurities

- Empty capsids
- Noninfectious particles
- Encapsidated non-target DNA (plasmid/host)
- Replication competent virus
- Aggregates
- Fragments
- Degradants

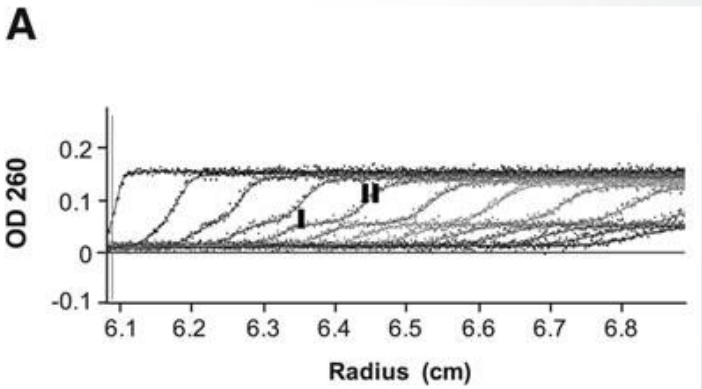


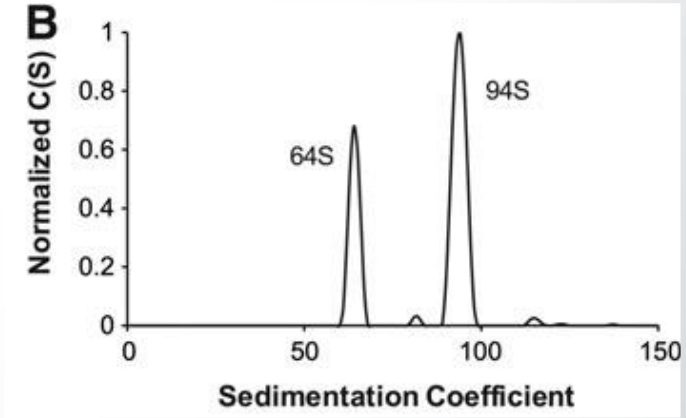
Empty capsids

- AAV capsids which lack the vector genome
- Comprise 50-98% of vector preparations from transient transfection
- Packaging efficiency is effected by factors such as transfection efficiency, size/sequence of vector genome, etc.
- Potential Risks:
  - Immunogenicity
  - Inhibition of vector transduction (competition)
  - Aggregation

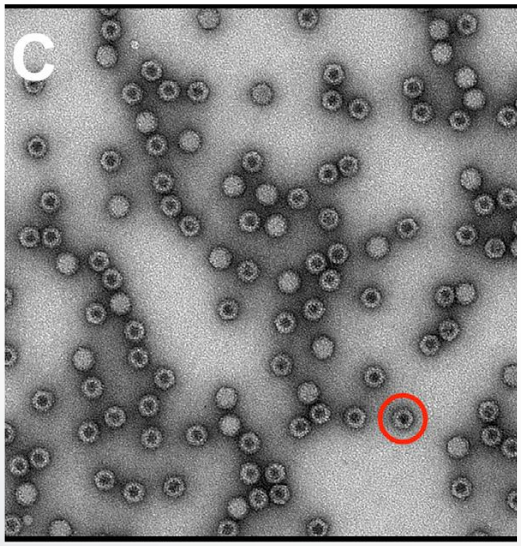
Empty capsid quantification

- Analytical ultracentrifugation
- TEM
- Anion exchange HPLC
- A_{260}/A_{280}
- qPCR / ELISA





Burnham, 2015, Human Gene Therapy Methods



Blessing, 2019, Molecular Therapy

Encapsidated non-target DNA

- AAV containing host, plasmid, or incomplete genomic DNA
- Packaging of host cell or plasmid DNA reported from 1-3% and 1-8%, respectively.
- The plasmid containing the ITR sequence is most common contaminant.
- Extremely difficult to eliminate by purification
- Potential Risks:
  - Immunogenicity
  - Oncogenes
  - Viral genes from host cells
  - Reduction in potency

Product quality attributes AAV

Composition

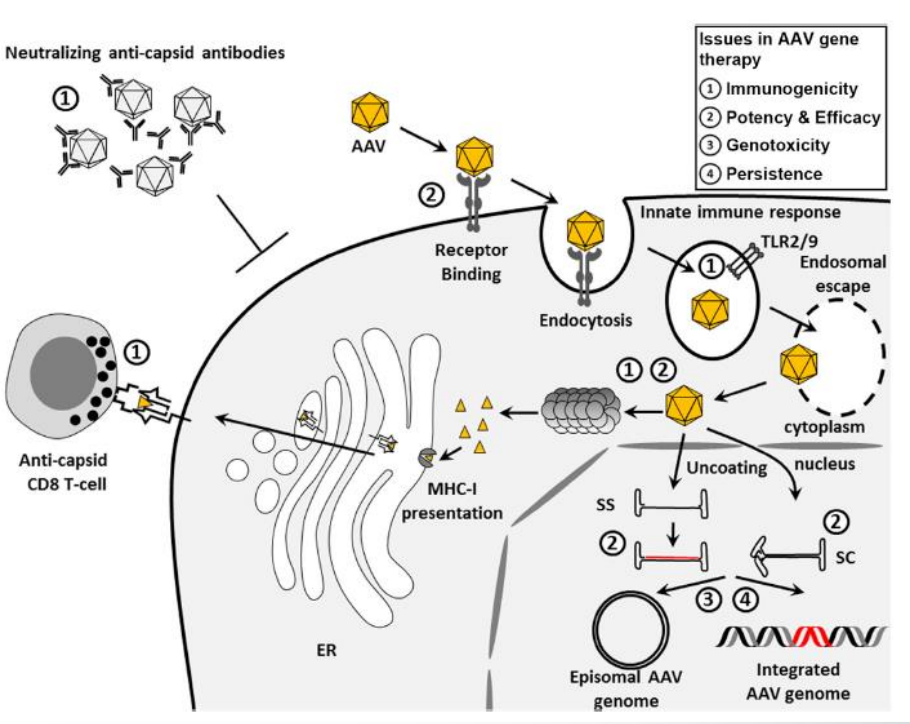
- VP1, 2, 3 ratio
- Full vs. Empty ratio
- DNA sequence
- Titer

Activity

- Infectivity/Transduction
- Expression of target gene
- Transgene function

Others?

- Critical quality attributes for AAV is a current area of research.



Colella, et al. 2018, Mol.Ther. Methods & Clinical Development

Short Course: Hands-on GMP Manufacturing of Vectors for Gene Therapy

NC STATE UNIVERSITY

Training needs? Think BTEC.

BTEC's open-enrollment training program provides job-focused instruction in the country's largest, most advanced biomanufacturing training facility. Guided by **experienced instructors**, participants engage in **classroom and hands-on learning** in **bench- and pilot-scale labs**, which are equipped with **industry-standard equipment**. Check the schedule below and visit our website for more information: **www.btec.ncsu.edu/industry**.

btec Biomanufacturing Training and Education Center

2020 Professional Development Short Courses

COURSE TITLE	DATE	TRACK
Hands-On cGMP Biomanufacturing Operations	Jan 21–24	Biomanufacturing
Hands-On cGMP Biomanufacturing Operations	Mar 9–12	Biomanufacturing
Foundations of Downstream Processing and Formulation	May 19–21	Bioprocess Development
Hands-On cGMP Biomanufacturing Operations	May 19–22	Biomanufacturing
Chromatography Column Packing: Foundations and Applications	Jun 2–4	Biomanufacturing
Fermentation Engineering	Jun 2–4	Bioprocess Engineering
Hands-On cGMP Biomanufacturing of Vectors for Gene Therapy	Jun 8–11	Biomanufacturing
Downstream Biopharmaceuctical Processes: Fundamentals and Design	Jun 16–18	Bioprocess Development
Fundamentals of Mammalian Cell Line Development	Jun 23–25	Bioprocess Development
Hands-On cGMP Biomanufacturing Operations	Jul 7–10	Biomanufacturing
Hands-On Essentials of Automation for Biomanufacturing	Jul 8–9	Bioprocess Engineering
Cell Culture Engineering: A Single-Use Perspective	Jul 21–23	Bioprocess Engineering
Biopharmaceutical Assay Essentials	Jul 21–24	Analytical Technologies
Applied Cleaning Validation Practices: A STERIS Master Class	Jul 28–29	Biomanufacturing
Fermentation Engineering	Jul 28–30	Bioprocess Engineering
Hands-On cGMP Biomanufacturing of Vectors for Gene Therapy	Aug 3–6	Biomanufacturing
Introduction to Design of Experiments (DoE) for Bioprocess Analysis and Optimization	Sep 22–24	Bioprocess Development
Hands-On Single-Use Processing for Biopharmaceuticals	Oct 6–8	Biomanufacturing
Hands-On cGMP Biomanufacturing Operations	Oct 13–16	Biomanufacturing
Applied Principles and Techniques of Depth Flow Filtration (DFF) and Tangential Flow Filtration (TFF) for BioPharm Downstream Purification	Oct 20–23	Bioprocess Development
Microbial Contamination Control in Bioprocessing Operations	Dec 1–3	Biomanufacturing
SELF-PACED ONLINE COURSE		
Fundamentals of Biomanufacturing	Enrollment ongoing	

- 2020 offerings:

  Jun 8-11, **Aug 3-6**

- **To register:**

  https://www.btec.ncsu.edu/industry/short_courses/index.php

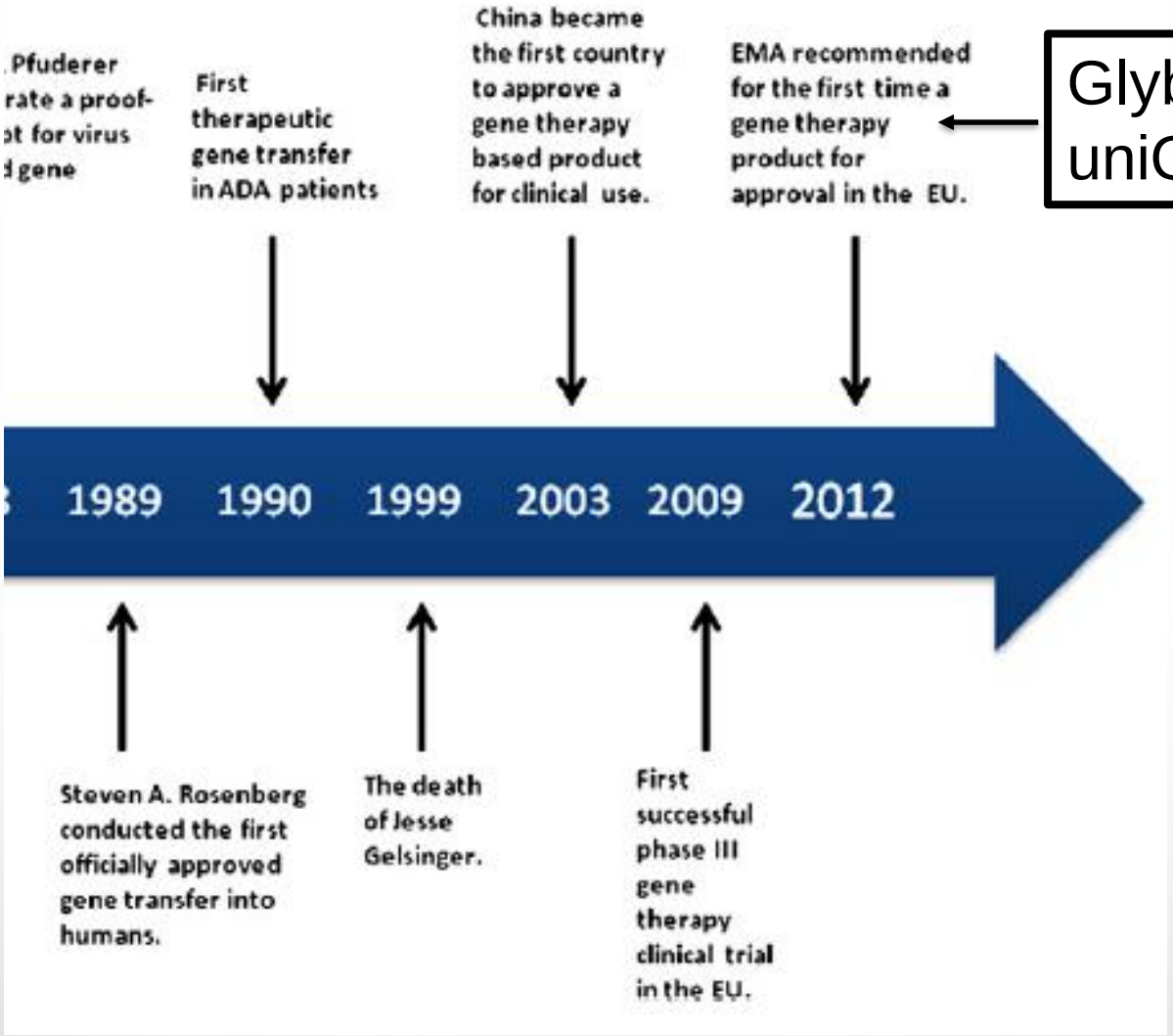
Conclusions

- Gene therapy is a rapidly growing biopharmaceutical product class
- AAV is a popular viral vector for *in vivo* gene therapy produced in HEK-293 and insect cells
- Many challenges exist in production and analysis of AAV vectors including:
  - Cost and material availability
  - Low yield
  - Product-related impurities
  - Undefined CQAs

The Life Force of Tomorrow's Industry

Extra Slides

Recent history of gene therapy



Glybera, from uniQure

Wirth, Thomas, et al., "History of gene therapy," Gene, 525, 2013, 162-169

Manufacturing of Gene Therapy Vectors: Synopsis

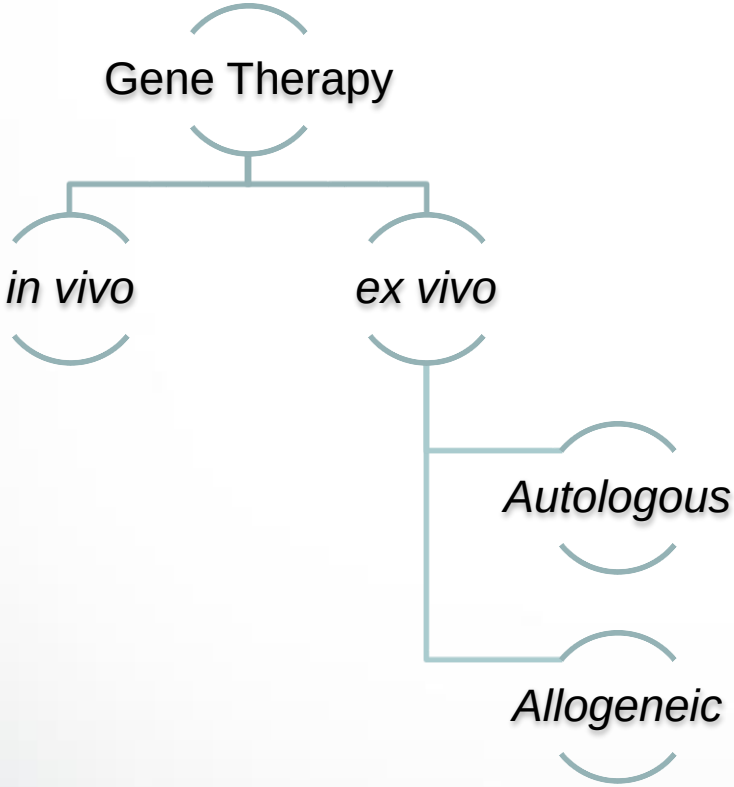
- Multiple different unit operations used – cell culture bioreactors, depth filtration, chromatography, ultrafiltration, etc.
- No template for the process exists because virus vectors are diverse in terms of size, structure, chemical properties, and host systems.
- Significant (?) process development required, using **scalable** unit operations.
- Batch processing typically used.
- Combination of single-use and reusable equipment used.

  According to BioPharm International's 2015 Manufacturing Trends Survey* **71.4%** of respondents use hybrid manufacturing systems, which feature both traditional stainless steel and disposable, single-use products

- Extensive QC (analytical testing) performed.

* R. Peters, *"Technologies and Practices Must Evolve to Meet Demand," *BioPharm International*, Vol **28** (1) 2015

Ex vivo gene therapy: allogeneic vs. autologous



Cells taken from/administered to the same individual

Cells derived from a donor