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THE SCIENCE OF WHAT'S POSSIBLE.™

Advanced Therapeutic Medicinal Products:

Adeno-associated Virus (AAV)

The Promise of Viral Vector Gene Therapy using AAV

7000

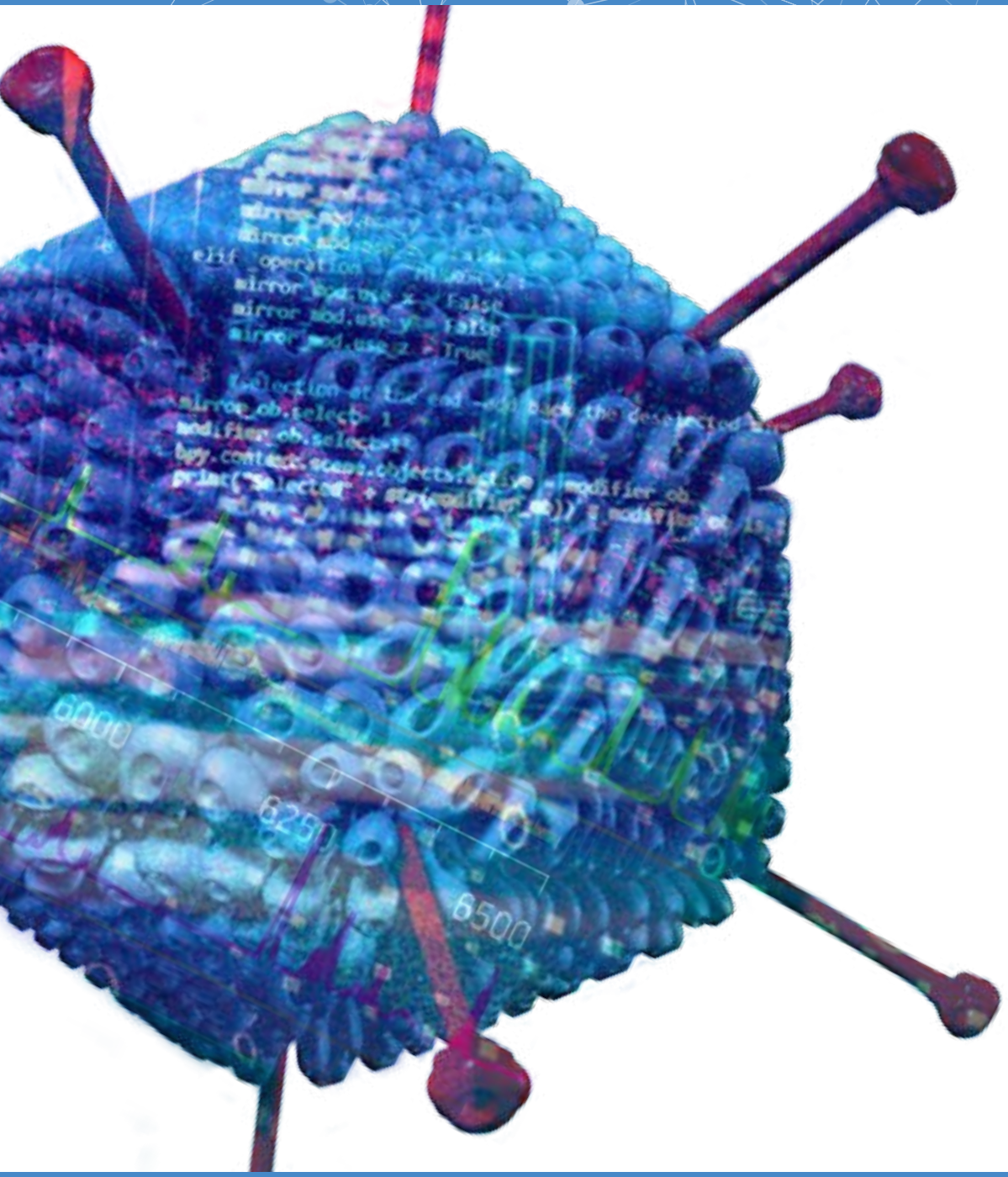
Monogenic Diseases

20 of 22

Children with spinal muscular atrophy (SMA) given the ability to thrive with Zolgensma® (AAV) at 91% efficacy[1].

4 Years

and counting of sustained efficacy of one dose of Luxturna® (AAV), providing functional vision to those with mutation-associated retinal dystrophy[2].



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Regulatory Situation – Viral Vector Focus 2021

This is a non-exhaustive list and a starting point for further exploration as the regulatory situation continues to evolve. In addition to gene therapy specific regulatory guidance, current good manufacturing practices apply and basic principles of process validation for biological products are commonly referenced.

Agency Name	Specific Documents
FDA Center for Biologics Evaluation and Research (CBER)	FDA Guidance for FDA Reviewers and Sponsors: Content and Review of Chemistry, Manufacturing, and Control (CMC) Information for Human Gene Therapy Investigational New Drug Applications (INDs). FDA Guidance for the Industry: Human Somatic Therapy and Gene therapy (1998).
International Conference on Harmonization (ICH)	Gene Therapy Discussion Groups of the International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use. Additional guiding documents: ICH Q5D Derivation and characterisation of cell substrates used for production of biotechnological/ biological products. ICH Q6B "Stability Testing of Biotechnological/Biological Products. ICH Q5B Analysis of the expression construct in cell lines used for production of r-DNA derived protein products.
US Pharmacopeia (USP)	USP <1047> "Gene Therapy Products" Guidance for gene therapy products that are classified as (1) viral vectors that carry the gene of interest; (2) nucleic acids in a simple form like naked DNA; (3)nucleic acids formulated with agents such as liposomes. USP <1043> "Ancillary Materials for Cell, Gene, and Tissue-Engineered Products" inclusive of raw materials that exerts an effect on the therapeutic material. Ancillary materials are not intended to be present in the final therapeutic product.
Novel and Exceptional Technology and Research Advisory Committee (NExTRAC)	A federal advisory committee that provides recommendations to the NIH Director and a public forum for the discussion of the scientific, safety and ethical issues associated with emerging biotechnologies.

Regulatory Situation – Viral Vector Focus 2021

Since the first gene therapy product was approved in 1993, guidance continues to develop. This non-exhaustive list of European and Chinese regulatory agencies provides examples of documents and definitions for these two regions.

Agency Name	Specific Documents
European Union – Biotechnology (EU) **The European Medicines Agency (EMA)**	In the EU, these advanced therapies are split into four major groups, i.e., gene therapy, somatic cell therapy, tissue-engineered therapies, and combined advanced therapies. Guideline on the quality, non-clinical and clinical aspects of gene therapy medicinal products EMA/CAT/80183/2014. EMA site contains more than ten regulatory guidelines and reflection papers focused on gene therapy products.
Drug Administration of the Ministry of Health (CHN)	"Quality Control Points for Clinical Research on Human Somatic Cell Therapy and Gene Therapy." China food and drug agency: "Technical Guidelines for Human Gene Therapy Research and Formulation Quality Control" 2003.
National Science and Technology Commission (CHN)	Gene Therapy Discussion Groups of the International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use.
Pharmacy Administration of the Ministry of Health (CHN)	"Key Points for Quality Control of Human Somatic and Gene Therapy Clinical Research."
Pharmaceutical Inspection Co-operation Scheme (PIC/S)	GMP guides focusing on the manufacture of Advanced Therapy Medicinal Products (ATMPs).
NIST: Biosystems and Biomaterials Division	Working to develop measurement assurance, stakeholder engagement and contribute to global documentary standards and reference materials for ATMPs.

AAV Gene Therapy Mapping

DISCOVERY

- Understand the disease mechanism
- Design the genome and capsid
- Study transduction and cellular expression

DEVELOPMENT

- Understanding the stability and integrity of a viral vector
- Formulation, capsid design and deep characterization
- Process development for manufacturing

BIOPROCESS

- Produce AAV. Common approach: triple transfection with plasmids for AAV, genome and helper virus
- Adherent cell line HEK293 or alternative Sf9

QA/QC

- Critical quality attributes and release tests: ID, titer, purity, potency, transfection

PRE-CLINICAL

- Cellular disease and animal models
- Toxicity, pharmacokinetics and dosage

INVESTIGATIONAL NEW THERAPY

CLINICAL

- Monitor safety, toxicity, potency and therapy efficacy

NEW DRUG APPROVAL

Discovery

Goal: Determine mechanism of action followed by sequencing, transduction and protein expression.

Attribute	Description	Technology/Mechanism
Sequence	Introduce the correct gene Viral DNA replaced with a transgene, ~4.7 kb limit for AAV	Next Generation Sequencing, dPCR, qPCR Promoter | Intron | Coding Sequence | polyA
Capsid selection	Tissue tropism linked to viral serotype Selective engineering to optimize for better transduction, manufacturability, and establish intellectual property	 AAV1 AAV5 AAV6 | AAV1 AAV6 | AAV1 AAV5 AAV7 AAV9 | AAV2 AAV6 AAV8 | AAV1 AAV6 AAV7 AAV8 AAV9 | AAV7 AAV8 AAV9
Expression	Check point for transduction efficiency Ensure the adequate and correct gene expression via transgene analysis, analysis of expressed protein(s), or functional assays	ELISA, PCR, Microarray, Bioanalytical MS

Capsid Development

Goal: Optimize the stability and integrity of the AAV viral vector used for transgene delivery. Formulations can include: organic carbohydrates to protect the native conformation, amino acids (Leu, Arg), and/or surfactants (Pluronic) to prevent aggregation.

Attribute	Description	Technology	Practical Guidance
Empty/Partial/Full	Full genome required for correct protein expression UV 260/280 Ratio: < 0.7 Empty and > 1.3 Full IEX – robust & ability to validate	**Instrument:** IEX, ACQUITY UPLC H-Class PLUS Bio System. ACQUITY UPLC TUV, ACQUITY UPLC FLR Detector **Column:** IEX Protein-Pak Hi Res-Q **Informatics:** Empower CDS	**Application Note:** Anion-Exchange Chromatography for Determining Empty and Full Capsid Contents in Adeno-Associated Virus **Complimentary technologies:** CDMS, AUC
Impurities	Host cell impurities: Residual host viral DNA or RNA Residual host cell protein Product impurities: subvisible particles Process related impurities, i.e. detergent, anti-foam, leachables (HPLC), transfection reagents	**Instrument:** Arc HPLC, ACQUITY UPLC H-Class PLUS Bio System. ACQUITY UPLC TUV, QTOF (Xevo G2-XS Qtof MS or SYANPT XS), Atmospheric Pressure Gas Chromatography (APGC) **Column:** dependent on system selected **Informatics:** Empower CDS	**Application Note (mAb):** Identification of Host Cell Proteins (HCPs) in Monoclonal Antibodies at Sub-ppm Levels Using the SYNAPT XS Mass Spectrometer **Complimentary Technologies:** ELISA, qPCR/dPCR, infectious titer assay, NGS
Capsid ID, Post-translational modifications, sequence variants	Ensure the correct gene is expression via protein expression or functional assays Check point for transduction efficiency Common PTMs: glycosylation, deamidation & phosphorylation	**Instrument:** BioAccord LC-MS System incorporating ACQUITY UPLC FLR Detector, QTOF (SYANPT XS), **Columns:** Peptide Mapping Column: BEH C18 300Å; Desalting: SEC Columns, BEH SEC 200Å **Chemistry:** RapiGest **Informatics:** waters_connect	Supporting literature, open access article: Zhang, X. et al. "Optimized reversed phase LC/MS methods for intact protein analysis and peptide mapping of adeno-associated virus (AAV) proteins" Human Gene Therapy. 2021

Capsid Development

Aspiration: Optimizing the stability and integrity of the AAV viral vector used for transgene delivery. Formulations can include organic carbohydrates to protect the native conformation and amino acids (Leu, Arg) and/or surfactants (Pluronic) to prevent aggregation.

Attribute	Description	Technology	Practical Guidance
 **Capsid titer**	Full contain the drug substance. Optimize: empty AAVs induce immunogenicity	**Instrument:** SEC, ACQUITY UPLC TUV, ACQUITY UPLC FLR Detector **Column:** SEC Columns, BEH SEC 125 Å Informatics: Empower CDS	**Application Note:** Rapid AAV Concentration Detection Using Fluoresence and Dual UV Detection
 **Size and aggregation**	Tissue tropism linked to viral serotype Design further to optimize for better transduction or establish IP	**Instrument:** UPLC-SEC, ACQUITY UPLC FLR Detector, **Columns:** SEC Columns, BEH SEC 450 Å, XBridge Protein BEH SEC **Chemistry:** SEC Protein Standard Mix **Informatics:** Empower CDS	**Application Note:** Size Exclusion Chromatography of Adeno Associated Virus (AAV) Preparations Using a 450Å Diol-Bonded BEH Column and a Fluoresence Detection **Complimentary Technologies:** ACQUITY UPLC with RI and MALS detector
 **Protein variants**	Intact protein analysis, Capsid protein ratio Protein variants	**Instrument:** BioAccord LC-MS System incorporating ACQUITY UPLC FLR Detector **Columns:** BEH (Ethylene Bridged Hybrid) Technology, BEH C4 and Peptide BEH C18 300Å **Informatics:** waters_connect with UNIFI intact mass workflow	**Application Note:** Optimizing Adeno-Associated Virus (AAV) Capsid Protein Analysis Using UPLC and UPLC-MS

Transgene Development

Goal: In addition to the delivery vector, the genome also requires characterization and optimization. Molecular biology tools like next generation sequencing, digital, or dPCR are commonly used for identity studies.

Attribute	Description	Technology	Practical Guidance
ID and modifications	Genome identity including quality and length Deep characterization of modifications	**Instrument:** Fragmentation studies and sequencing on HPLC or LC MS/MS, ACQUITY UPLC H-Class PLUS Bio System, ACQUITY UPLC TUV, Xevo G2-XS Qtof MS **Columns:** Oligonucleotide separation columns **Informatics:** MassLynx MS Software	**Application Note:** Developing a Novel, Integrated LC-MS Wrokflow for High-resolution Monitoring and Charcterization of Oligonucleotides **Complimentary Technologies:** Molecular ID via sequencing or specific genomic sequence ID via dPCR or qPCR
Raw material and process impurities	Impurities occuring from both raw materials and degradation products Presence of Linear vs open-circular vs supercoiled. Other considerations related to plasmid purification	**Instrument:** IEX, ACQUITY UPLC H-Class PLUS Bio System, ACQUITY UPLC TUV **Columns:** Protein-Pak Hi Res IEX column, Oligonucleotide separation columns **Informatics:** Empower CDS	**Application Note:** Plasmid Isoform Separation and Quantification by Anion-Exchange Chromatography (AEX) https://www.waters.com/nextgen/us/en/library/application-notes/2021/separation-and-size-assessment-of-dsdna-fragments-by-anion-exchange-chromatography-aex.html

Bioprocess

Goal: Produce the viral vector with an encapsidated transgene. Typical process uses a triple transfection that includes the AAV, transgene, and a helper virus. Process analytical technology is used to monitor both critical process parameters and product quality attributes; LC Optical and LC-MS are both used as PAT and in core labs associated with bioprocess.

	Step	Description	Common Technology
UPSTREAM	**Cell line selection**	Select a cell line with superior transfection efficiency and cell count	Cell analyzers for baseline information like cell titer. Potential use of LC or LC-MS for product attributes
UPSTREAM	**Production**	Bioreactor. Monitor process , critical process parameters and product, critical quality attributes with process analytical technology (PAT)	Bioreactor: stirred-tank, roller bottle, fixed-bedPAT monitoring: examples include pH, temperature, dissolved oxygen, viscosity, LC optical and LC MS. Cell analyzers, nutrient analyzers, concentration and viability assays like ELISA, digital or qPCR and TCID50
DOWNSTREAM	**Harvest and purification**	Cell lysis, purification of insoluble products, filtration	Centrifugation, tangential flow filtration
DOWNSTREAM	**Capture chromatography** **Polish chromatography**	Removes host cell proteins, host cell DNA, empty capsids, and transfection reagents (i.e. polyethylenimine, PEI) Informed by analytical chromatography	Ion exchange chromatography, affinity chromatography, size exclusion chromatography
DOWNSTREAM	**Finish and fill**	Includes the acts of concentrating, filling, labeling, transportation and storage as well as validation of all steps. Quality control for a filled vial is also addresse	Aseptic handling into vials, cartridges, syringes or ampoules. Technology to ensure packaging is in compliance

CMC Product Specification

Goal: Detect and quantify critical quality attributes (CQAs) and critical product attributes for release. AAV products follow the guidance of regulatory agencies. More extensive testing may be required to better ensure that all pre-clinical, clinical, and commercial products are comparable. In addition to those attributes below, raw materials specifications also need to be considered.

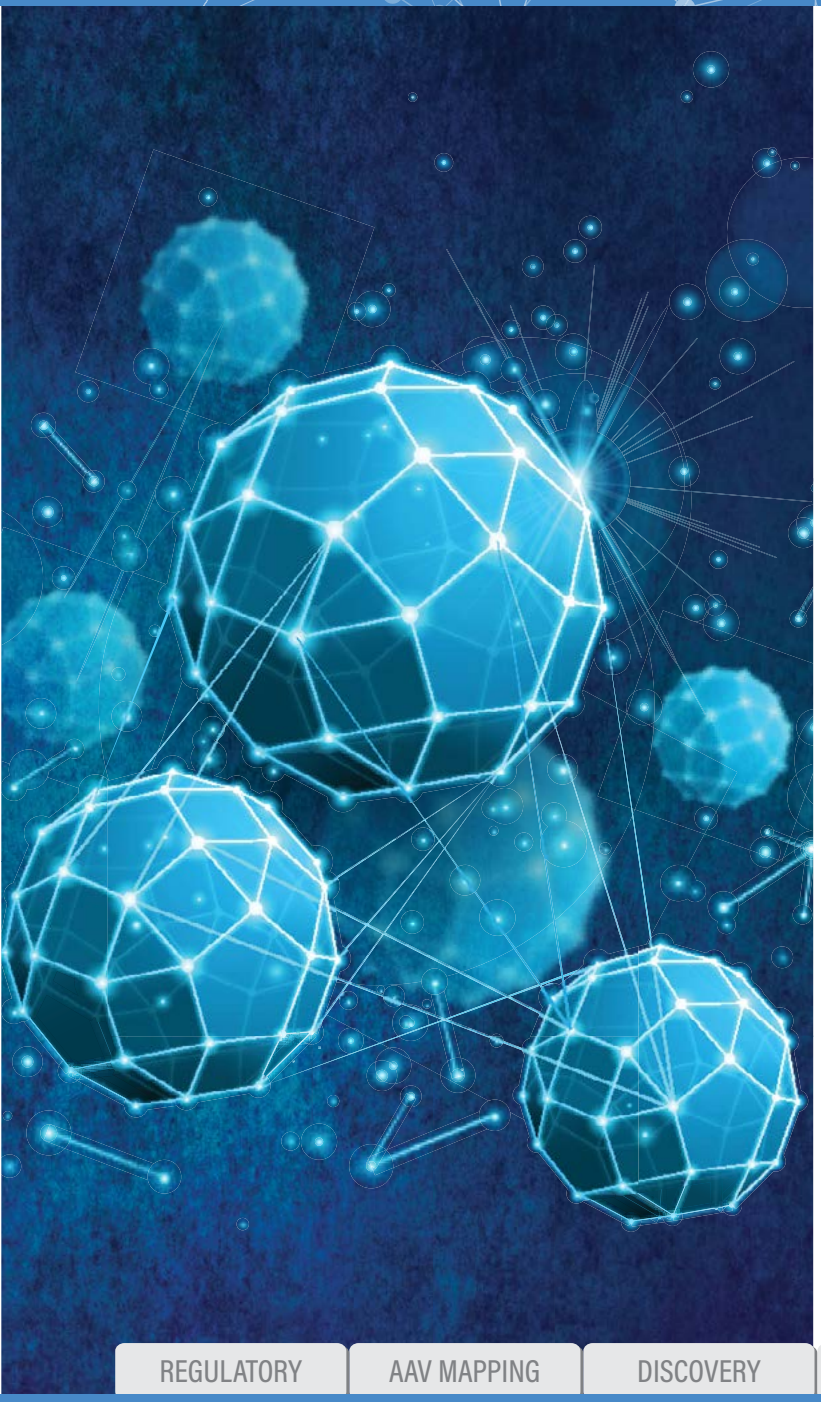
Attribute	Description	Common Technologies – Non exhaustive	Acceptable Level[3]
Identity	Genome identity for correct, capsid identity	Molecular ID via sequencing or specific genomic sequence ID via dPCR or qPCR; restriction digest/gel electrophoresis. Immunoassay for expressed gene	N/A
Content	AAV titer and infectious/non-infectious AAV levels	Titer:Next Generation Sequencing, dPCR, qPCR. total protein	Therapy specific. Ophthalmologic therapies tend to require lower concentrations than systemics therapies
Purity	Product-Related: empty particles, non-functional vector, unwanted genetic sequences, aggregation Process Related: host cell DNA, host cell protein, adventitious agents, and other process related impurities	Purity: SDS PAGE; Empty v Full (IEX), visual inspection, LAL assay, BCA protein assay, Analytical HPLC, RT-PCR	Residual bacterial chromosomal DNA: < 2 µg/mg DNA Residual RNA: < 0.2 µg/mg DNA Residual bacterial protein: < 3 µg/mg DNA Endotoxin: < 10 EU/mg
Infectivity	Determine the concentration at which 50% of the infected cells display a pathological change	TCID50 – median tissue culture infectious dose. FLR assay	Therapy specific
Potency	Labeled dose. Cell assays. Function of expressed gene. Often secondary effect	In vitro, ELISA, FACS, RT-PCR, Light absorbance (A260), transgene expression; gene editing efficiency NGS	Transgene specific

Glossary for Viral Vectors

Full Name	Description & Key Takeaways
Vector	The transgene's delivery vehicle. Many vectors can exhibit tissue tropism, that is, specificity for a particular cell. Vector selection determines the size of the genomic information that can be delivered.
Capsid	The protein shell of a virus.
Adeno associated virus (AAV)	A non-replicating viral vector. Relatively innocuous and comparatively small. 20 nm diameter. Episomal transgene delivery. Its capsid is composed of 60 proteins in a 1:1:10 ratio of three different viral proteins (VP1, VP2, VP3). Packaging capacity of approximately 4.7 kb.
Adenovirus (AdV)	A viral vector that has been used for DNA based vaccines, including COVID-19 vaccines. 90-100 nm diameter. Episomal transgene delivery. Packaging capacity of approximately 7.5 kb.
Lentiviral vector (LVV)	A retrovirus that is used to incorporate a gene into a cell's genome. A critical reagent used in cell therapy. 90 nm diameter. Surrounded by a lipid coat. LVV's have the potential to integrate DNA into the genome. Packaging capacity of approximately 9 kb.
Transgene	Gene sequence that is being transferred into a cell.
Plasmid	Extrachromosomal DNA. Plasmids are a way of delivering a healthy and functional gene to a cell with minimal concern of integrating the new DNA into a chromosome and risking new mutations. Different forms: relaxed or nicked, linear, or supercoiled.
Episomal DNA	DNA stored outside the chromosome.
Cytokine storm	Severe immune reaction when the body releases too many cytokines in response to a foreign body.
Quiescent cells	Non-dividing cells. AAV, LVV, AdV all work in quiescent and dividing cells.
TCID50	Median tissue culture Infectious Dose required for 50% of infected cells exhibit a cytopathic effect. Method of determining the viral titer in units of plaque forming units (PFUs).

Glossary of Common Analytical Technologies

Technology	Abbreviation	Full Name	Description
Waters Technology	LC Opt or FLR	Liquid Chromatography with optical UV or fluoresence detector	Separation and detection. Used in development and QA/QC for AAV.
	SEC	Size exclusion chromatography	Separation and detection of aggregates. Paired with detectors like PDA (photodiode array) and MALS. Heavily used in development and QA/QC for AAV.
	LC-MS	LC with mass spectrometer detector	Separation and detection by mass to charge ratio. Preferred for AAV post-translational modification, mapping and sequencing.
TA Technologies	DSC	Differential Scanning Calorimeter	Stability assay. Alternative: DSF. Thermal analysis for release.
Other Technologies	CDMS	Charge Detection Mass Spectrometry	Top-down view of product heterogeneity, including empty/partial/full.
	MALS/DLS	Multi-Angle /Dynamic Light Scattering	In-solution sizing of diameter, monomers/aggregates; mass/size approximation. Commonly use with SEC.
	CE	Capillary Electrophoresis	Separation. Often paired with laser-induced fluorescence for AAV studies.
	Part Analyzer	Particle Analyzer	Subvisible and visible particle detection. Critical for immunogenicity. Standard QA/QC assay.
	AF4	Asymmetric flow field flow fractionation	Separation and characterization of large, fragile modalities.
	DSF	Differential Scanning Fluorimetry	Stability assay. Typically used with fluorescent dye. Alternative: DSC.
	AUC	Analytical Ultracentrifugation	Quantitative instrument for determination of mass and stoichiometry. Detects empty/partial/full viral vectors.
	Cell Analyzer		A cell analyzer provides multiple parameters to characterize the cells. Often used interchangeably with flow cytometry.
	ELISA	Enzyme Linked Immunosorbent Assay	Specific affinity assay. Protein ID and titer. Alternative is enhanced chemiluminescence.
	q/RT PCR	Quantitative/digital polymerase chain reaction	Quantification of genes & expression.
	Cell Counter		Viable cell counts. Used to normalize flow cytometry data.
	NGS	Next Generation Sequencing	Nucleic acid ID. Qualitative assessment of gene editing / plasmids.
	Flow Cyt.	Flow Cytometry	Detects and measures cell ID, counting, markers. Optical detection; Fluorescence option (FACS).



Further Reading and References

To learn more about Cell and Gene Therapy, visit our website at www.waters.com/cgt. Learn more with the Further Reading resources below.

Further Reading:

- The clinical landscape for AAV gene therapies. https://www.nature.com/articles/d41573-021-00017-7
- Adeno-associated virus vector as a platform for gene therapy delivery. https://www.nature.com/articles/s41573-019-0012-9
- AAV-mediated gene ttherapy for research and therapeutic purposes. http://www.hixonparvo.info/AAV%20Review_2014.pdf
- Adeno-associated virus (AAV) as a vector for gene therapy. https://link.springer.com/article/10.1007/s40259-017-0234-5
- Advances with the use of bio-inspired vectors towards creation of artificial viruses. 2010. Expert Opinion on Drug Delivery 7(4):497-512
- AAV vector manufacturing platform selection and product development. bioProcess international. 2019.

References:

1. Novartis website STR1VE study
2. Luxturna Multi-Luminance Mobility Test (MLMT) Results
3. Levels listed are those set by the FDA and World Health Organization, downloaded 2021. These levels are for clinical CMC product specification.

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