High sensitivity AAV viral protein analysis using CE-SDS-LIF

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Outline

- Applying USP's Mission to gene therapy
- Biophysical characteristics of AAV
- CE-SDS-UV for AAV capsid protein separation
- CE-SDS-LIF for increased sensitivity
- Comparative analysis of VP ratio and purity





Applying USP's Mission to gene therapy

The USP Mission

Mission

To improve people's health through public standards and solutions that help ensure the quality of medicines, dietary supplements, and food ingredients

The USP CGT Trajectory



Before 2003

- Form CGT Expert Committee
- Publish new chapters for: CGT raw materials, cell therapy and gene therapy

2003 - 2020

- Engage CGT stakeholders
- Host AAV focused events
- Collaborate in multiple AAV studies

2021-2024

- Form AAV Expert Panel
- Publish AAV Analytical Guide
- Form LVV Expert Panel

2025

- Launch AAV package of reference materials
- Publish AAV (*PF)* and Plasmid (USP-*NF*) Chapters

2026

- Publish AAV Chapter to *USP-NF*
- Publish LVV Chapter to *PF*
- Revise Gene Therapy and Ancillary Materials Chapters

Our focus on the challenges of AAV stakeholders

Challenges

- Single-dose therapies place extreme pressure on analytical control strategies for safety and efficacy
- Multiple methods in use for key CQAs (e.g., empty/full capsid ratio) with limited harmonization across developers
- Innovative analytical methods not easily standardized or transferred to routine QC environments
- Limited physical reference standards to bridge between orthogonal methods and enable cross-program comparison

USP Activities

2025-2030 Expert Volunteer Committee
BIO4: The diverse group of industry experts that are building the future of USP CGT standards

Physical Reference Standards
Capsid content, capsid and genome titer, residual impurities, raw material qualification

Best Practices Chapter
<1067> *Best Practices for the Manufacture and Quality Control of rAAV Products*
(to be official February 2027)

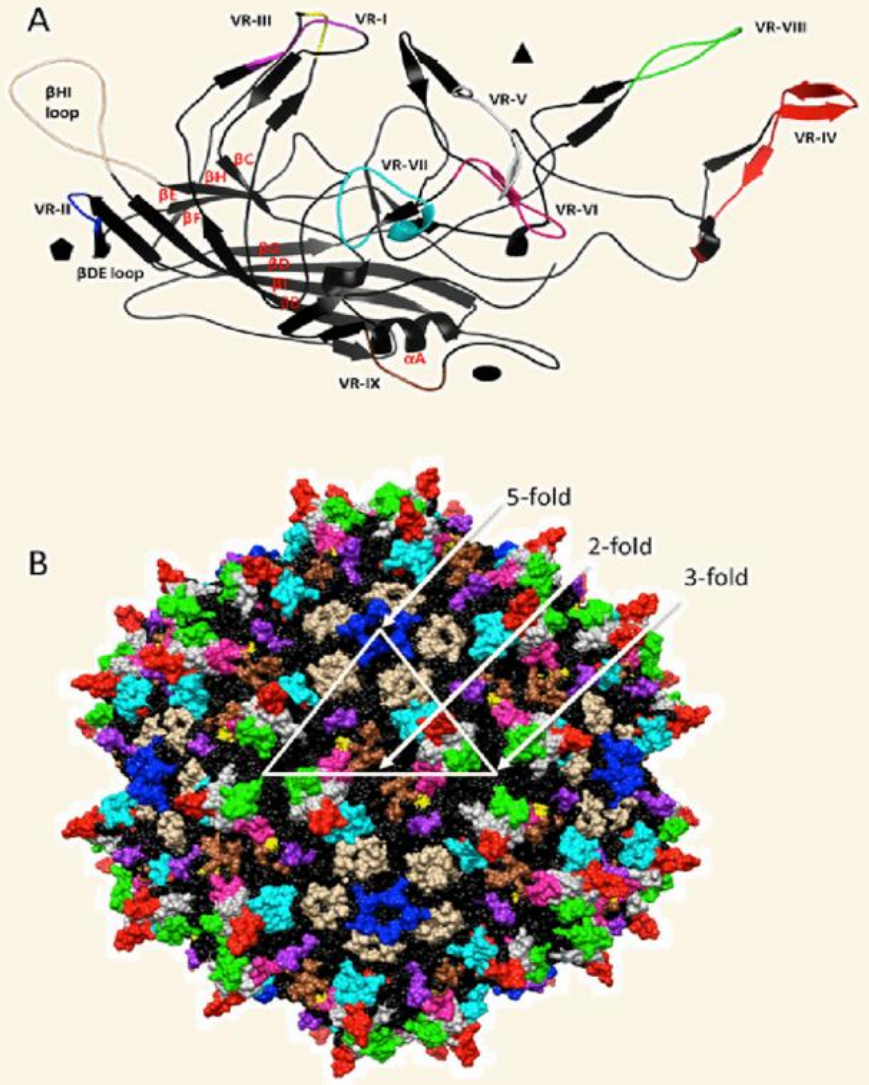
Analytical Resources
Gene Therapy Analytical Guide, application notes, and replicable analytical procedures

2

Biophysical characteristics of AAV capsid proteins relevant to CE-SDS

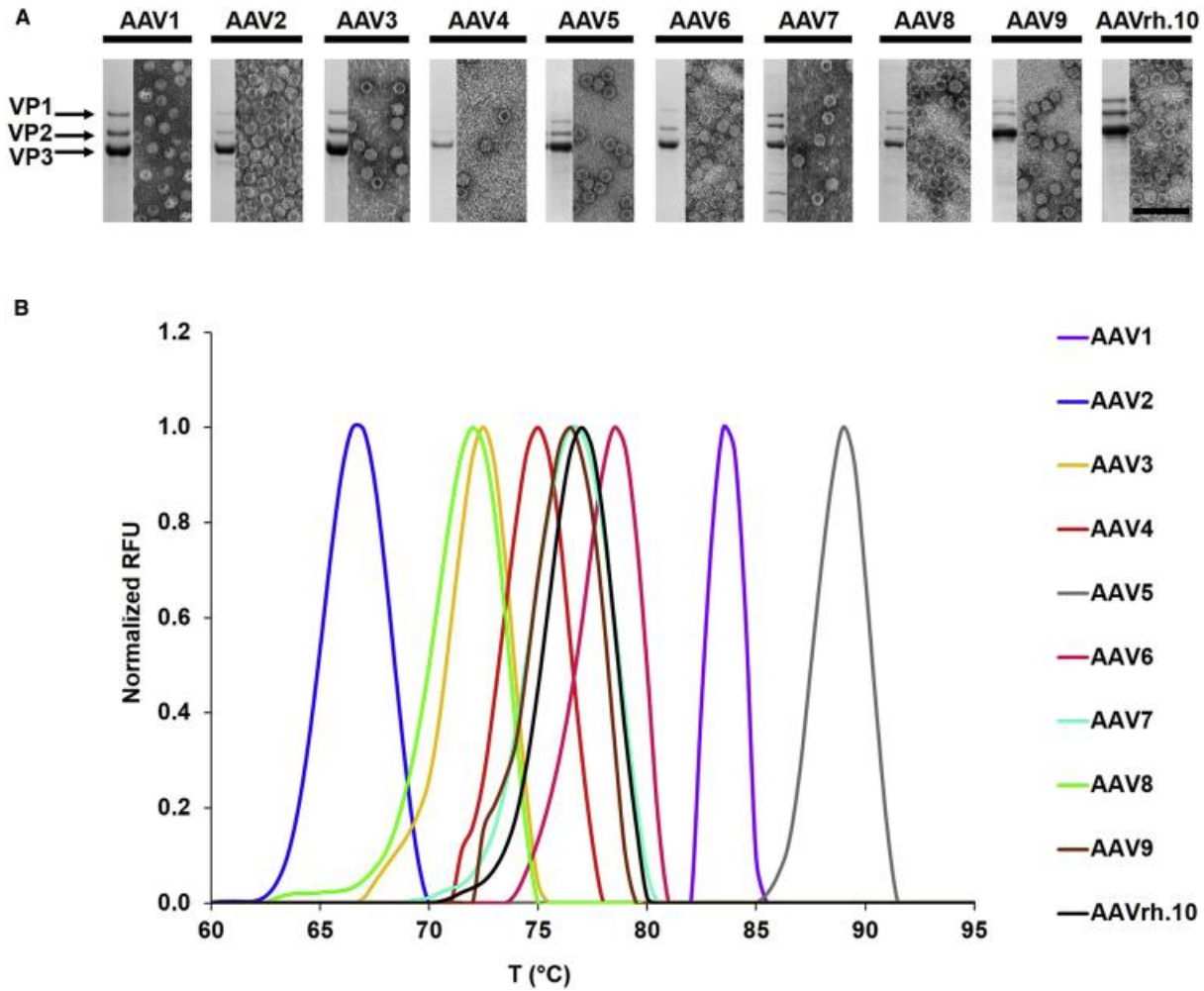
AAV capsid protein composition

- 13 natural AAV serotypes and 100+ variants
- 26 nm in diameter
- Leaky ribosomal scanning allows 1 open reading frame to encode: VP1, VP2, and VP3 with a shared C'-terminus
- 60 subunits in a capsid particle (cp), with variability around the typical of 1:1:10 VP1:VP2:VP3
- High ratios of VP1 and VP2 correlate with high transduction efficiency due to the presence of an N'-terminal phospholipase domain and nuclear localization signal



Structures reproduced from USP <1067>, original source Drouin *Future Virol.*. 2013

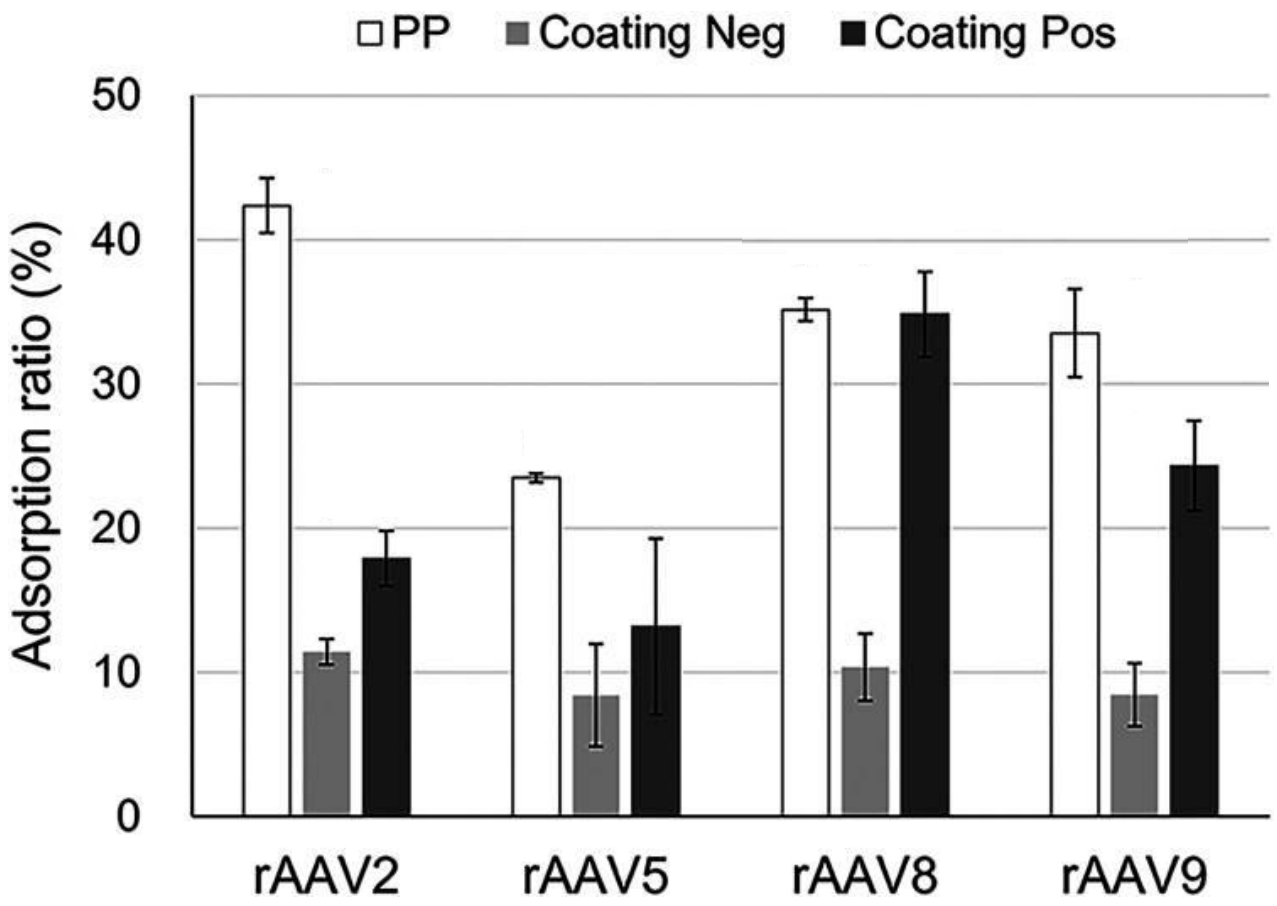
Thermal stability of AAV serotypes



Due to differences in thermal stability, serotypes may require different denaturation conditions

Bennett et al., Thermal Stability as a Determinant of AAV Serotype Identity. Mol Ther Methods Clin Dev. 2017 Jul 24;6:171-182. doi: 10.1016/j.omtm.2017.07.003. PMID: 28828392; PMCID: PMC5552060

Surface properties of AAV serotypes



Due to differences in surface properties, serotypes may require different buffers and methods for concentrating the protein

Adapted from Ueda et al., Quantitative Analysis and Predictive Modeling of Nonspecific Adsorption of Recombinant Adeno-Associated Virus onto Solid Surfaces. Langmuir. 2026 Mar 10;42(9):6657-6666



Developing CE-SDS-UV for AAV capsid protein separation

The VP ratio and protein purity CQAs are often assessed by CE-SDS-UV

- CE-SDS (reducing) with UV detection at either 214 nm or 220 nm
- Excellent separation of VP proteins by size and high resolving power
- QC friendly and environmentally friendly
- Fairly high sample concentration requirement of 10^{13} cp/mL (~60 µg protein/mL)
- Buffer exchange is needed as AAV is processed and formulated in high salt to prevent aggregation and surface adhesion



Materials used for method development samples

Commercial AAV (serotypes 2, 5, 8, 9)

- Concentration: varies
- Empty and full capsids

USP *AAV8 (Full Capsids)*

- Intended for **biophysical method** development, validation and assay control
- Concentration: 4.7×10^{12} cp/mL
- Volume: 300 µL
- Full capsids

USP *AAV for Capsid Titer* (serotypes 1, 2, 5, 8, 9)

- Intended for **immunoassay method** development, validation and assay control
- Concentration: varies, target concentration 5×10^{11} cp/mL
- Volume: 100 µL
- Empty capsids

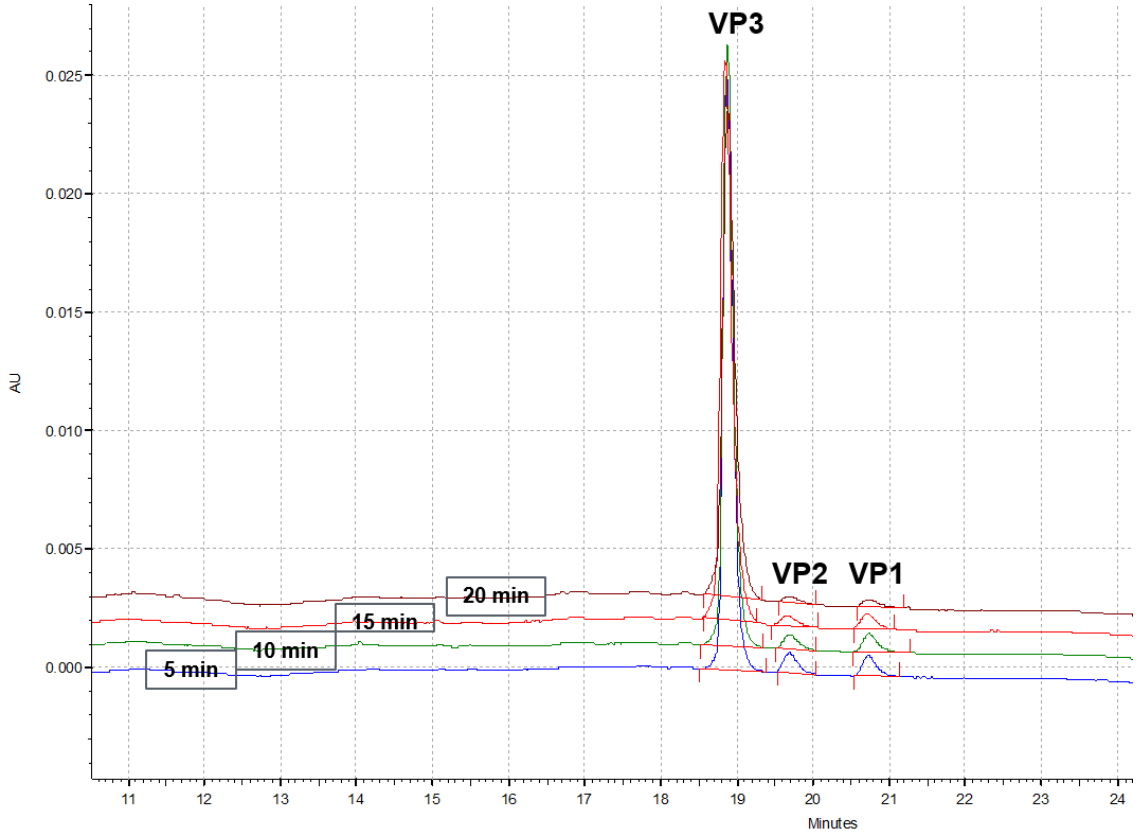
For USP materials, always refer to the current product information on USP's website for values and test descriptions.

CE-SDS-UV method for PA800 Plus

Method parameters

- Commercial AAV serotype 2, 5, 8 and 9: Desalted, buffer exchanged and concentrated by centrifugal filter
- Denatured and reduced in 1% SDS and BME
- **Heating optimized by serotype**
  - Serotype 5 and 8: 70° for 10 minutes
  - Serotype 2 and 9: no suitable condition found
- Detection: UV 214 nm

Electropherograms for AAV5 at 70°

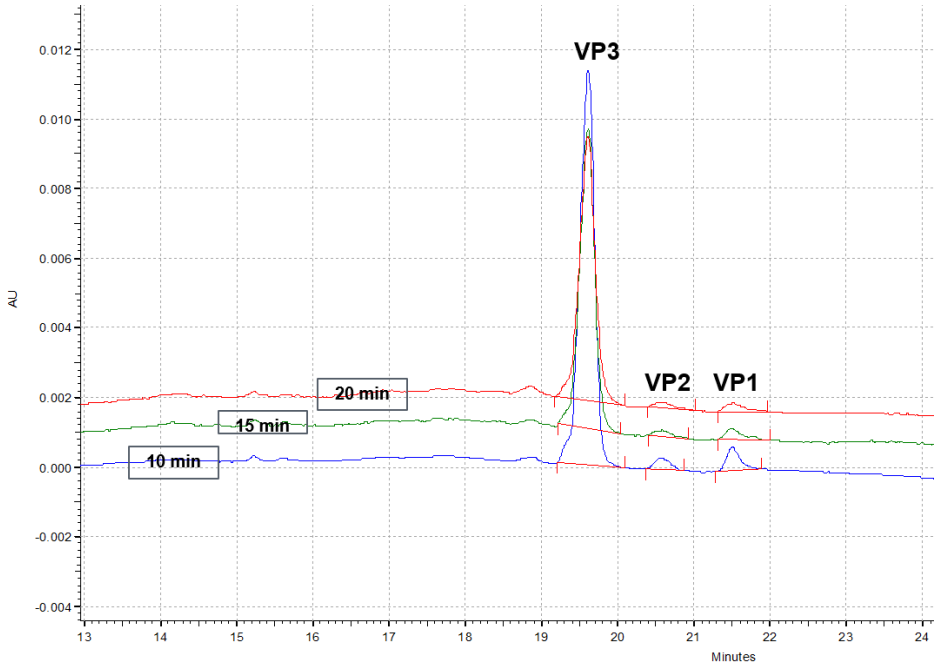


CE-SDS-UV method development for PA800 Plus

Temperature optimization

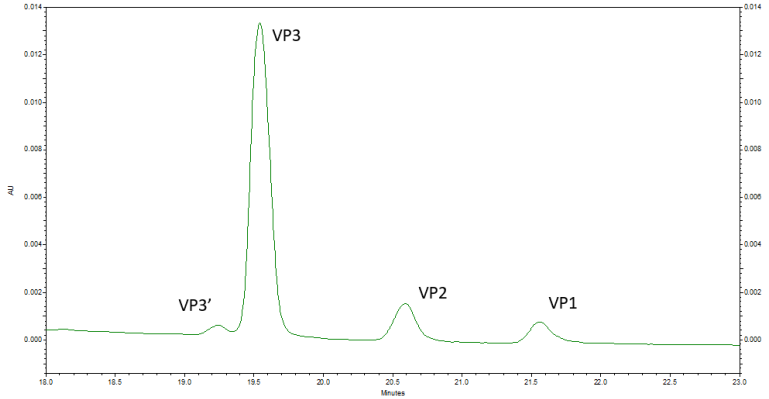
- At 50° for 10 minutes
  - ↓ AAV5: very low signal and only one peak
  - ↑ AAV8: Good signal and good VP peak ratio
- At 70° for 5 minutes – variable performance
  - ↓ AAV2: very low signal and only VP3 peak
  - – AAV5: good signal but low VP1 peak
  - ↑ AAV8: good signal and good VP peak ratio
  - ↓ AAV9: low signal and low VP2 and VP1 peaks
- At 70° for 5, 10, 15 and 20 minutes
  - ↑ AAV5: Highest signal at 10 minutes; declining VP1 and VP2 at longer duration
  - ↑ AAV8: Highest signal at 10 minutes; declining VP1 and VP2 at longer duration

AAV8 at 70°

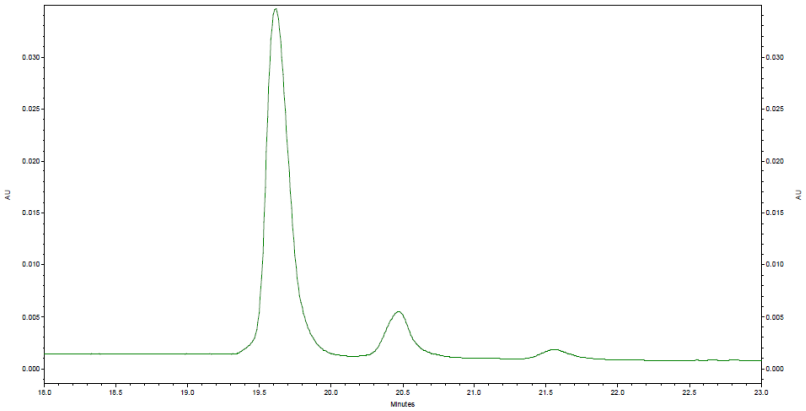


CE-SDS-UV with low concentration samples for PA800 Plus

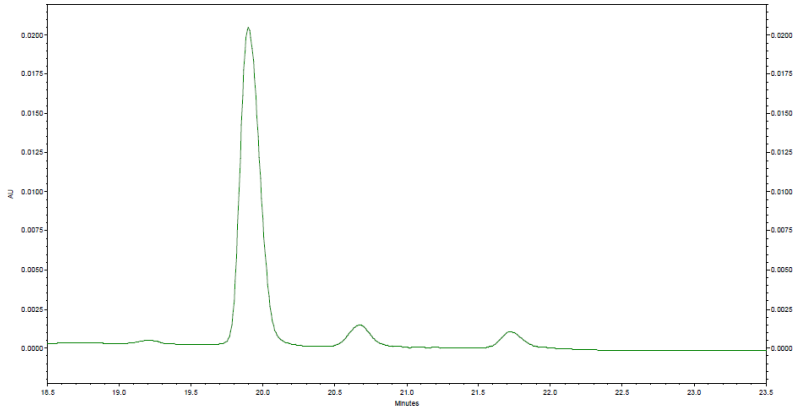
AAV1 (4.3×10^{11} cp/mL)



AAV5 (1.1×10^{12} cp/mL)



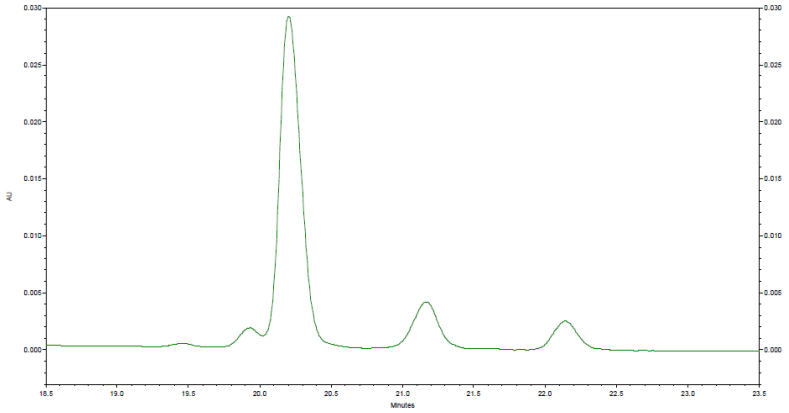
AAV9 (2.3×10^{11} cp/mL)



AAV2 (1.4×10^{11} cp/mL)

Multiple failed attempts to concentrate the material

AAV8 (4.6×10^{11} cp/mL)



Samples: USP AAV for Capsid Titer

Instrument: PA800 Plus

Heating: 70°, 5 minutes

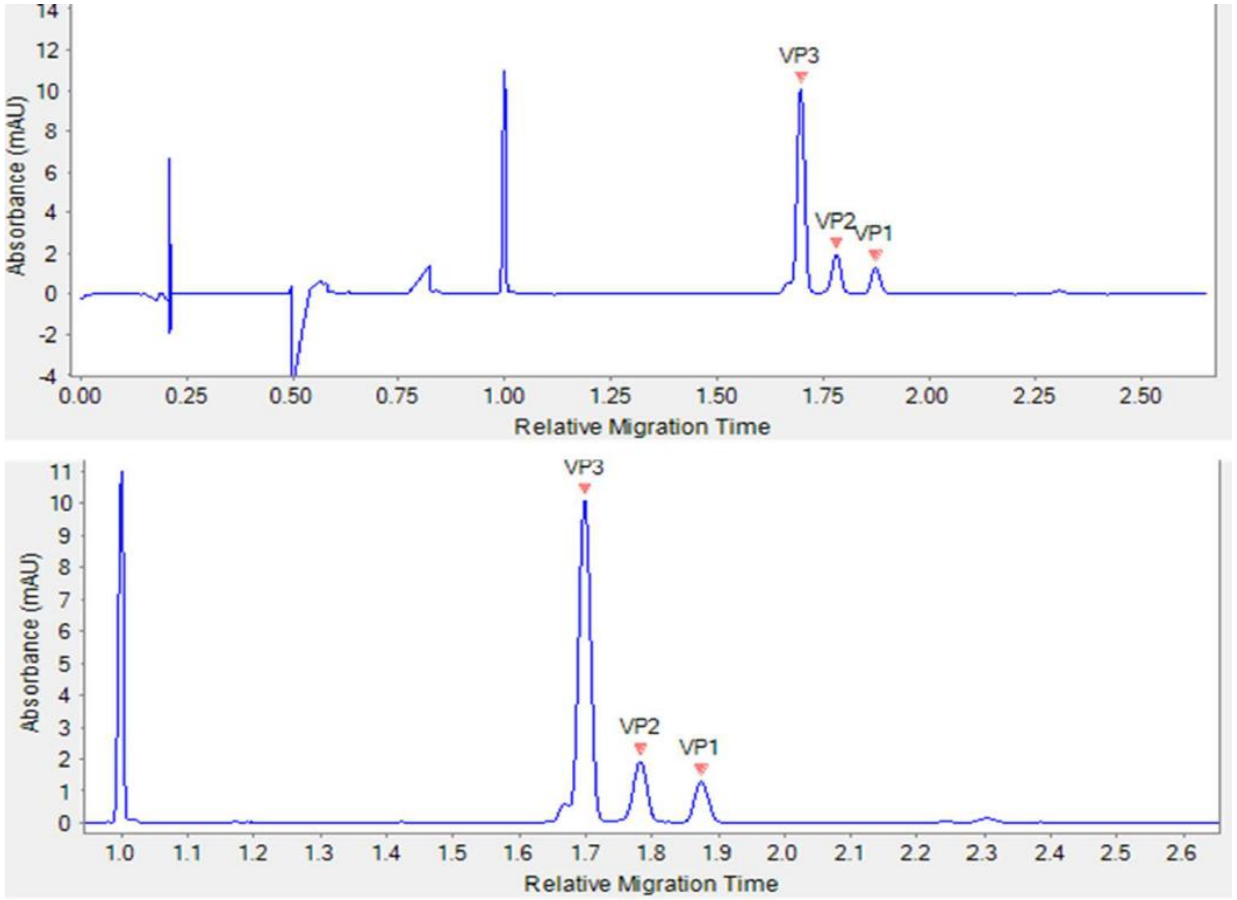
All cp/mL values are before buffer exchange and concentration

CE-SDS-UV method development for Maurice

Method parameters

- Maurice CE-SDS PLUS
- USP AAV8 (Full Capsids) and AAV8 (Empty Capsids) desalted, buffer exchanged and concentrated to $1x10^{14}$ VP/mL by centrifugal filter
- 8 µL sample, 1 µL Internal Standard, 2 µL BME. Final volume adjusted to 40 µL with 1x SDS Sample Buffer
- Heated at 70° for 10 minutes.
- Injection: 4600V, 20 seconds
- Detection: UV 220 nm

Representative Electropherogram AAV8 (Full Capsids)

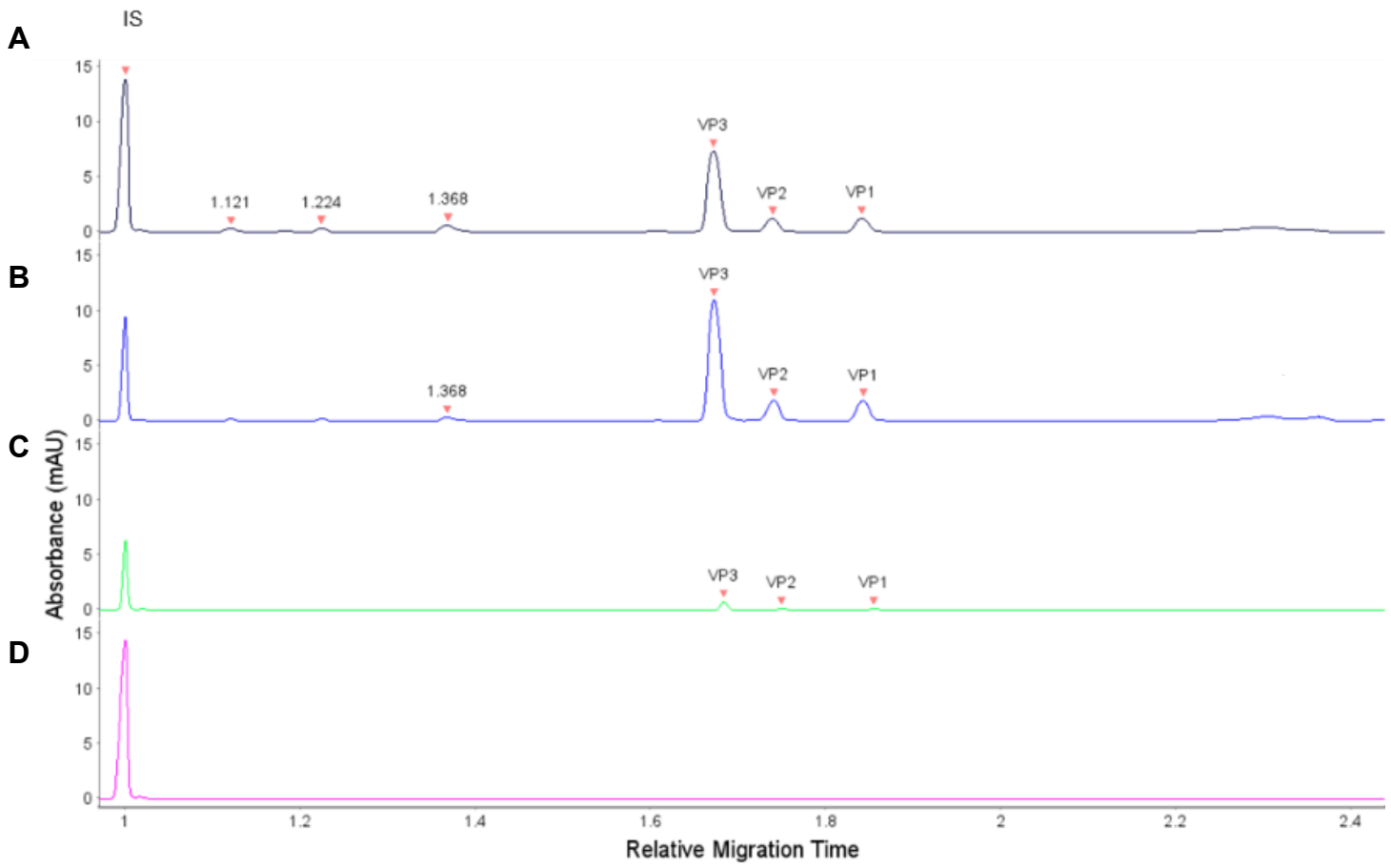


CE-SDS-UV buffer exchange/desalting methods

A. Buffer exchange in 0.25x PBS + 0.001% Pluronic F-68 using centrifugal filter tubes
B. Cold acetone precipitation
C. Untreated
D. Blank

- Buffer exchange selected
  - Best impurity resolution
  - Good peak shape
  - Cold acetone precipitation has equivalent signal

Stacked Electropherogram
AAV9



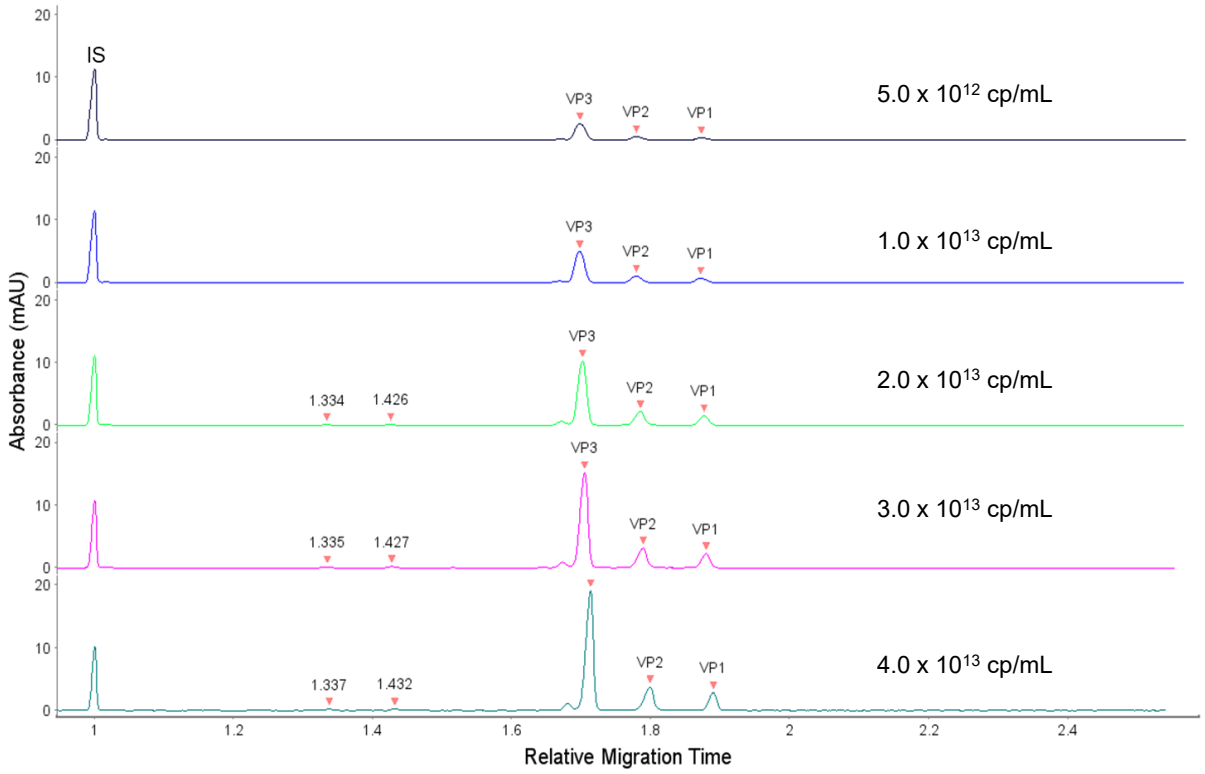
CE-SDS-UV linearity analysis

Peak area and capsid peak ratio (AAV8)

	% of Total Peak Area				Capsid Peak Ratio		
Concentration (cp/mL)	VP1	VP2	VP3	% Total	VP1	VP2	VP3
5E12	10.5	15.8	73.8	100.0	1.0	1.5	7.1
1E13	10.0	15.9	73.8	99.6	1.0	1.6	7.4
2E13	9.9	16.1	72.0	98.0	1.0	1.6	7.3
3E13	10.0	16.1	71.9	98.0	1.0	1.6	7.2
4E13	10.0	16.2	71.8	97.9	1.0	1.6	7.2
Average	10.1	16.0	72.6	98.7	1.0	1.6	7.2
% RSD	2.2	1.0	1.4	1.0	0.0	2.9	1.6

- Better impurity detection at higher concentration
- Consistent VP ratio

Representative Electropherogram AAV8 (Empty Capsids)





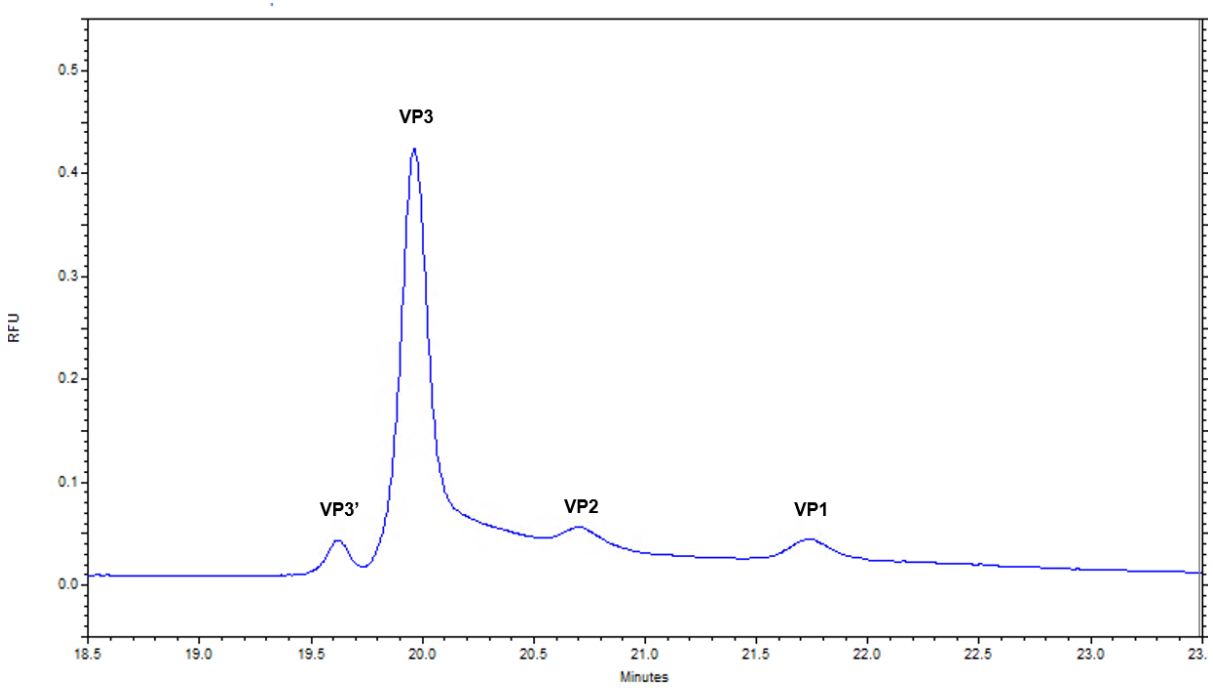
Developing CE-SDS-LIF for high sensitivity AAV capsid protein separation

CE-SDS-LIF method development for PA800 Plus

Optimized method parameters

- Untreated samples: USP AAV for Capsid Titer, serotypes 1, 2, 5, 8, and 9
- Denatured and reduced in 50% sample buffer with 90 mM DTT
- Heating optimized by serotype:
  - Serotype 1, 2, 8 and 9: 70° for 10 minutes
  - Serotype 5: 90° for 10 minutes
- Labeling: 45 µg/mL Chromeo P503 dye at 70° for 10 minutes, then diluted 1:3 with water
- Injection time optimized by concentration:
  - ≥ 2.3 x 10^{11} cp/mL: 20 seconds
  - < 2.3 x 10^{11} cp/mL (AAV2): 80 seconds
- Detection: Ex 488 nm, Em 600 nm

Electropherogram for AAV2



- AAV2 peaks were resolved due to increased sensitivity, reduced sample handling and extended injection

CE-SDS-LIF method development for PA800 Plus

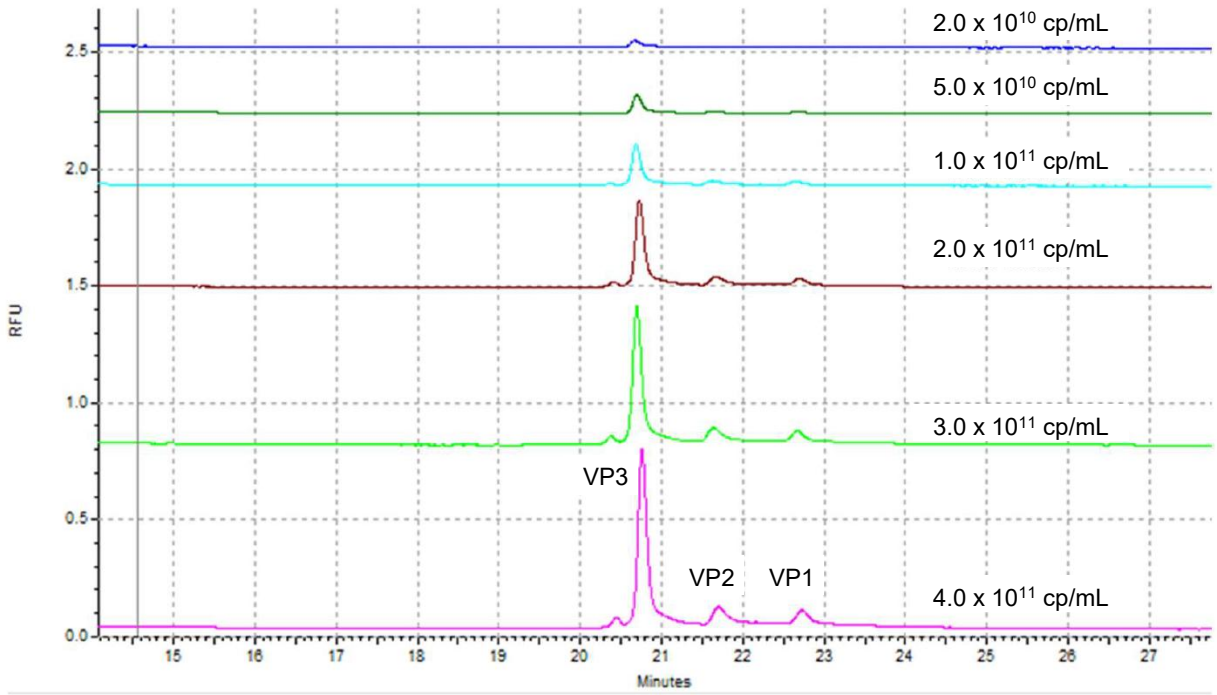
Repeatability

- %RSD for VP3 is below 1%
- Most variability is in VP2 and VP1 peak area

Sample Name	Total Peak Area %RSD		
	VP3	VP2	VP1
AAV1 for Capsid Titer	0.9	3.4	4.4
AAV2 for Capsid Titer	0.6	2.6	1.5
AAV5 for Capsid Titer	0.6	2.8	6.6
AAV8 for Capsid Titer	0.3	2.3	1.3
AAV9 for Capsid Titer	0.5	4.8	3.2

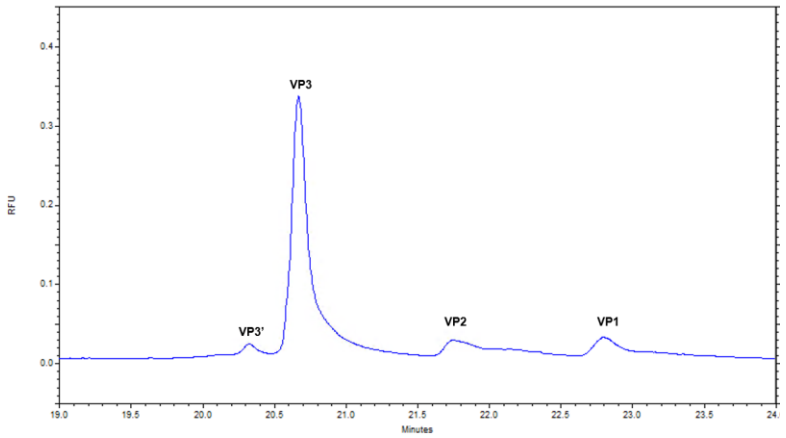
Linearity (AAV8)

- Total peak area R^2=0.9993

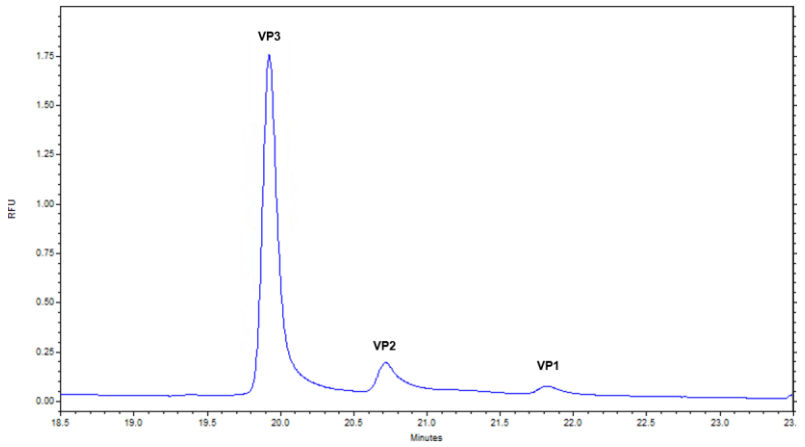


CE-SDS-LIF with low concentration samples for PA800 Plus

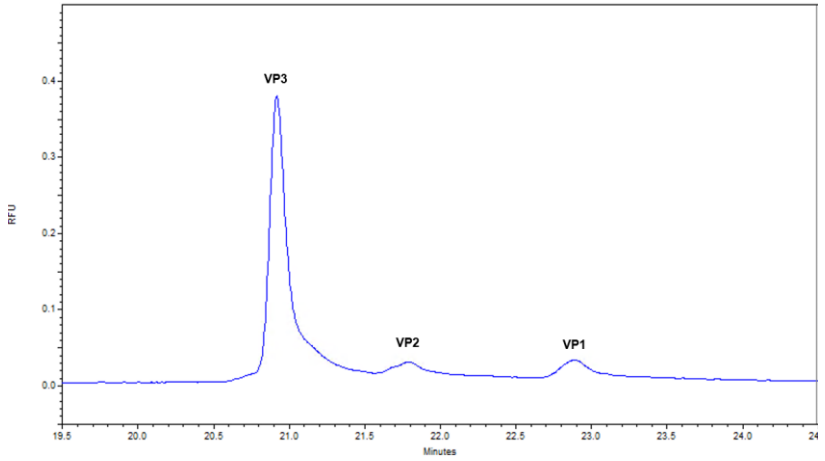
AAV1 (4.3×10^{11} cp/mL)



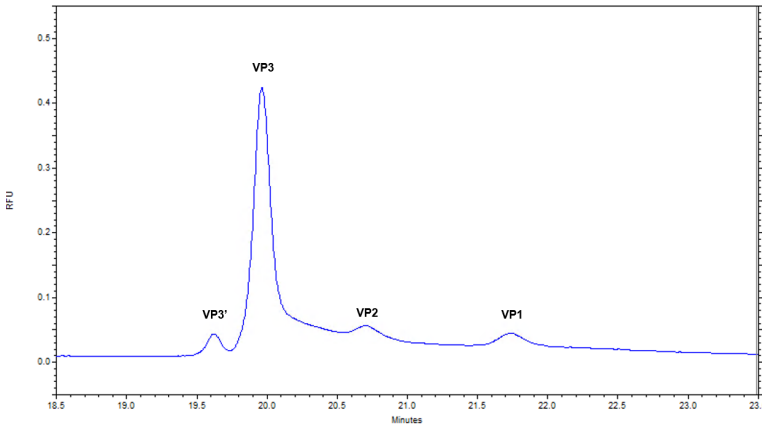
AAV5 (1.1×10^{12} cp/mL)



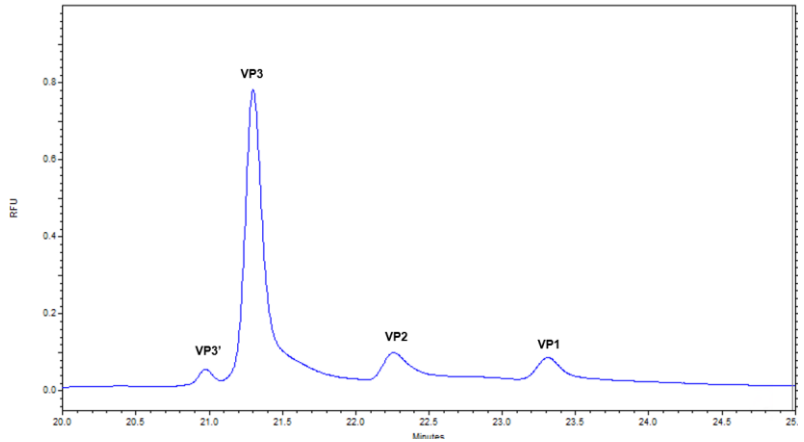
AAV9 (2.3×10^{11} cp/mL)



AAV2 (1.4×10^{11} cp/mL)



AAV8 (4.6×10^{11} cp/mL)



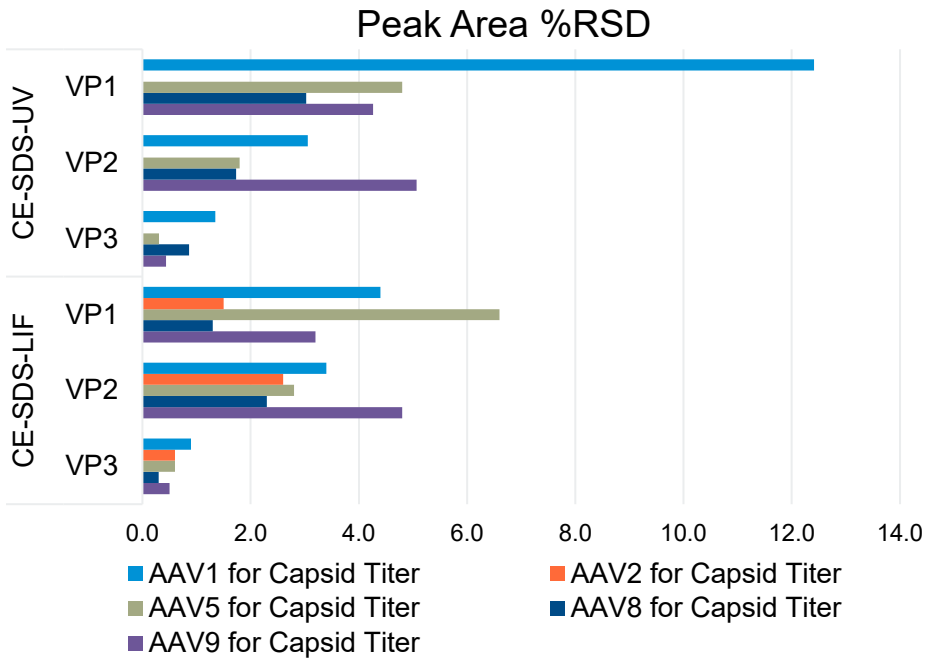
Samples: USP AAV for Capsid Titer

Instrument: PA800 Plus with LIF

Comparison of CE-SDS-LIF to CE-SDS-UV with low concentration samples

Repeatability

- Slight improvement



Sensitivity and Peak Ratio

Sample	CE-SDS-UV			CE-SDS-LIF		
	VP3	VP2	VP1	VP3	VP2	VP1
AAV1 for Capsid Titer	15.0	1.8	1.0	10.0	1.3	1.0
AAV2 for Capsid Titer	Below detection limit			10.2	2.2	1.0
AAV5 for Capsid Titer	32.8	4.6	1.0	18.3	2.8	1.0
AAV8 for Capsid Titer	11.2	1.8	1.0	11.6	1.3	1.0
AAV9 for Capsid Titer	18.7	1.3	1.0	12.7	1.4	1.0

- AAV2 shows the benefit of better sensitivity, reduced sample handling and extended injection
- AAV5 shows benefit of optimize denaturation temperature

Alternative high sensitivity methods

Methods

- Microfluidic gel electrophoresis with fluorescent labeling
- Capillary Western – Excellent sensitivity but antibody specificity should be confirmed
- UHPLC with fluorescent labeling
- Electrophoresis coupled to Mass Spectrometry – Rich data
- UHPLC coupled to Mass Spectrometry – Rich data
- SDS-PAGE with silver stain or fluorescent labeling – Semi-quantitative

Summary and next steps

- A new CE-SDS method with fluorescence detection has been developed and evaluated against a set of five low concentration AAV samples
  - The fluorescence detection method outperforms two CE-SDS methods that were developed with UV detection
  - Improved sensitivity
  - Reduced sample handling
  - Better serotype-specific optimization of denaturation conditions
- CE-SDS with fluorescence will continue to support the quality of USP AAV standards by providing purity and VP ratio values
- A new set of high concentration AAV reference standards are in development that will be characterized by CE-SDS with fluorescence detection
- USP welcomes collaboration with stakeholders to better understand the method's performance, evaluate its potential as a platform method, and assess its comparability with orthogonal methods

Thank you



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