Best Practices for Aggregate Quantitation of Antibody Therapeutics by Sedimentation Velocity Analytical Ultracentrifugation

Presented by Michael Brenowitz, Ph.D. on behalf of the CASSS Biophysics Working Group (BWG) – Departments of Biochemistry and Molecular Pharmacology, The Albert Einstein College of Medicine, Bronx, New York

The CASSS Biophysics Working Group (BWG) brings together scientists from across public and private sectors to foster collaboration and address unmet challenges in the application of biophysical methods to biologics research and development. The following BWG members participated in this project

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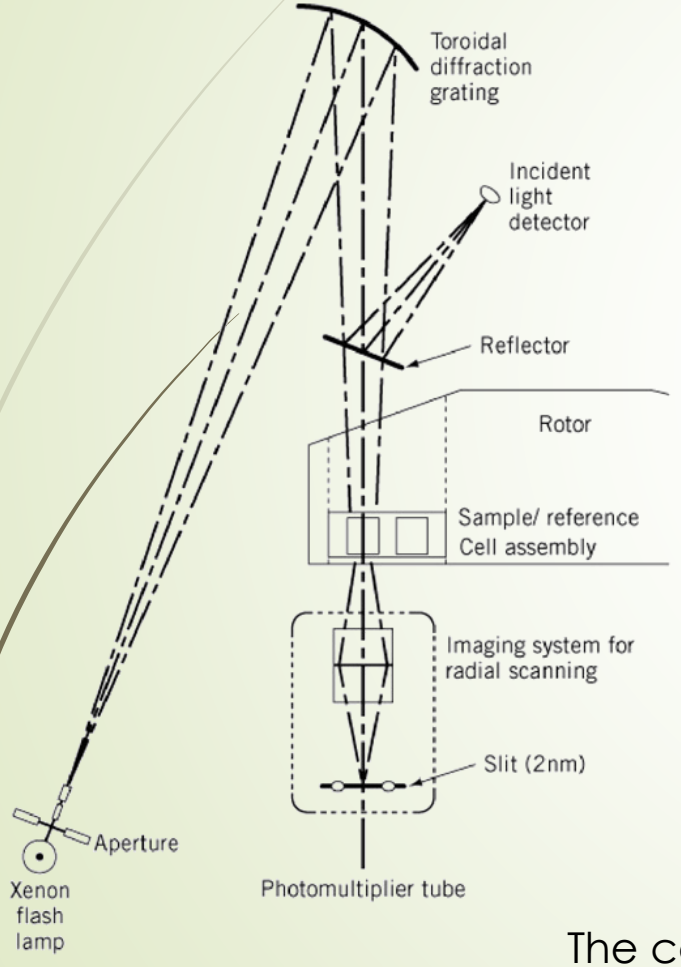
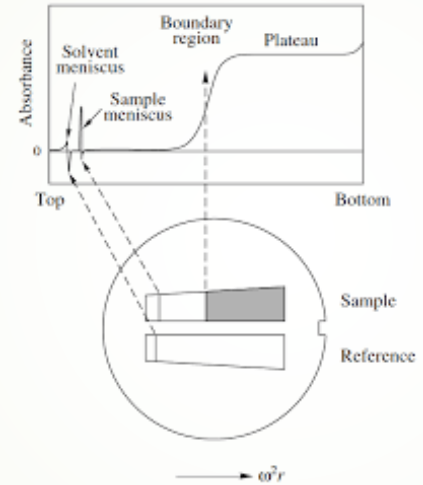
- *Ivan L. Budyak* - Bioproduct Research and Development, Eli Lilly and Company,
- *Michael Brenowitz* - Departments of Biochemistry and Molecular Pharmacology, The Albert Einstein College of Medicine
- *Eric S. Day* - Pharmaceutical Development, Genentech a Member of the Roche Group
- *David Hayes* – Biophysics Consultant, International Solidarity of Scientists
- *John Hill* - Department of Bioengineering, University of Washington
- *Ranajoy Majumdar* - Bioproduct Research and Development, Eli Lilly and Company,
- *Paola Ringhieri* - Analytical Development Biotech Department, Merck Serono S.p.a.
- *Peter Schuck* - Laboratory of Dynamics of Macromolecular Assembly, National Institute of Biomedical Imaging and Bioengineering, National Institutes of Health
- *Jasper C. Lin* - Pharmaceutical Development, Genentech a Member of the Roche Group

Sedimentation Velocity – Analytical Ultracentrifugation (SV-AUC) is the technique of choice orthogonal to SEC for aggregate characterization and quantitation for biotherapeutics

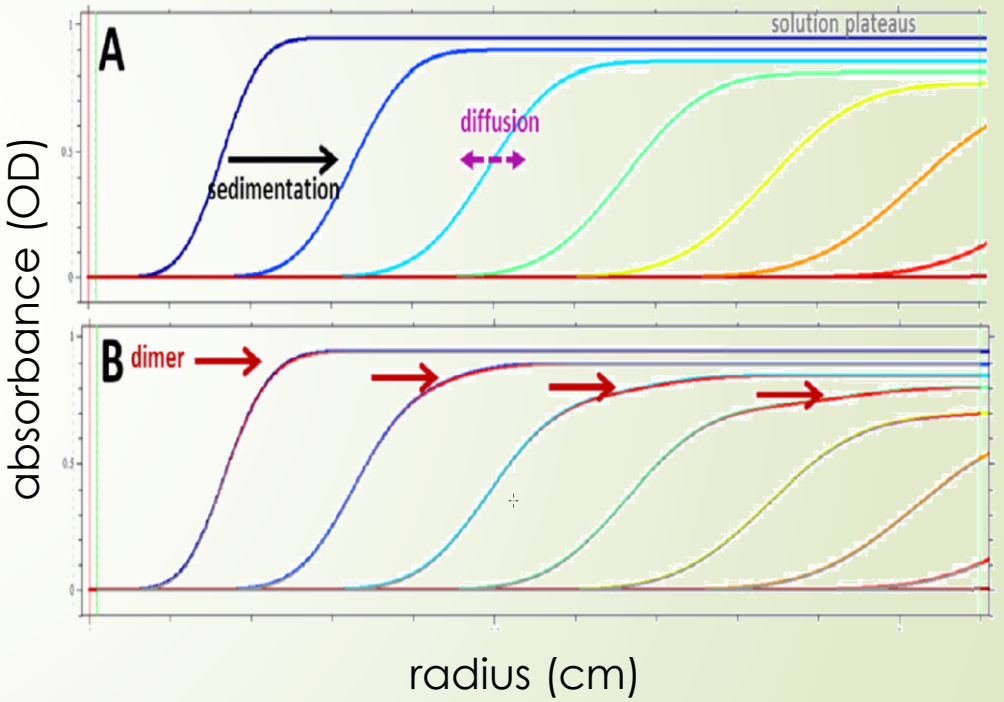
- *SV-AUC is a first-principles solution technique*
  - Differentiates soluble species based on their hydrodynamic properties
  - *Compatible with a range of solution compositions and conditions including* matrices of a final drug substance or product
  - Free from confounding interactions with a stationary phase
- *SV-AUC has limitations*
  - Non-interacting, reversibly associating, and nonideal systems require *different* analysis approaches
  - Relatively low throughput and a limited concentration range (~ 1 O.D.)
  - Excipients can interfere with protein detection and species quantitation
  - Proper experimental design and data analysis are not 'plug and play'

An overview of the analytical ultracentrifuge and the sedimentation velocity experiment





$$s = \frac{v}{\omega^2 r} = \frac{M(1-\bar{\nu}\rho)}{(N_A * f)}$$



The configuration of the absorption optics of the Beckman Coulter ProteomeLab™ and Optima ™ instruments is similar

‘Best practices’ improve the sensitivity and precision and define the limits of detection (LOD) and quantitation (LOQ) for SV-AUC aggregate analysis

- The experimental and instrumental best practices that yield consistent aggregate analysis and maximizes its sensitivity and precision
- How to detect and quantitate aggregate using c(s), continuous distribution, analysis
- Implementing the protocols and best practices articulated by the CASSS Biophysics Working Group

Reliable and reproducible SV-AUC aggregate analysis requires confirmation of system suitability

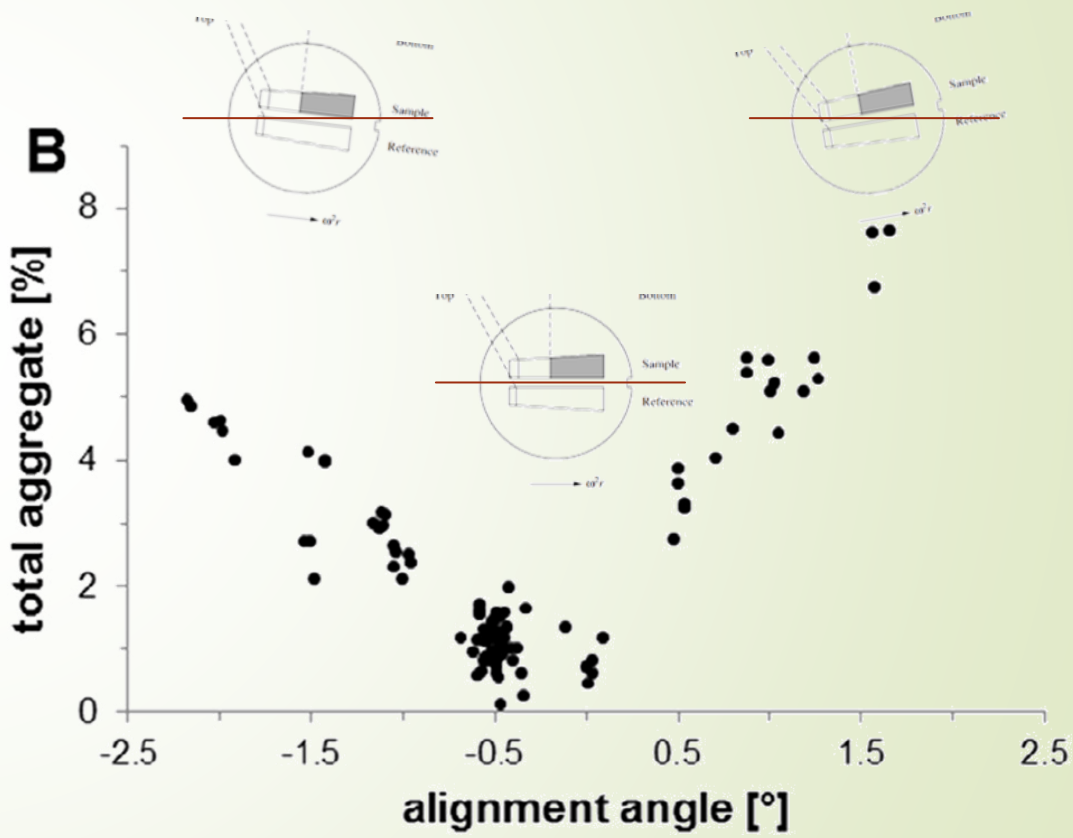
- Confirmation of instrument suitability includes annual preventive maintenance and calibration by a qualified service technician
- User checks include radial calibration, temperature calibration, a test of the detection module(s) functionality, and instrument timestamp
- Confirmation of cell assembly suitability for aggregate analysis includes routine assessment of a protein standard such as BSA or the NIST mAb
  - Best practice is to keep the parts of cell assemblies together and to document their performance over time. Cell assemblies *don't last forever*!
- Atypical data traces and/or large residuals may indicate cell, sample, or instrument problems that require in depth troubleshooting.
- The performance parameters of *every* instrument and *every* cell should be recorded in system charts

Best practice requires careful sample preparation and temperature control

- Optimal mAb sample preparation depends on application and the mode of sample detection. – *Carefully evaluate new samples*
- *Accurate results require accurate values* of the solvent density and viscosity and the mAb extinction coefficient and partial specific volume
  - Special cases that confound SV-AUC experiments include solutes that absorb at 280 nm, nonideality due to low ionic strength or self-buffered formulations, and co-sedimenting solutes that form density gradients
- Triplicate samples account for aggregate quantitation variability
- Temperature affects all aspects of SV-AUC aggregate quantitation
  - Solution density and viscosity as well as mAb stability and oligomerization
  - Sufficient time must be allocated for equilibration at the 20 °C set point. Convection due to temperature gradients can cause aggregate quantitation artifacts

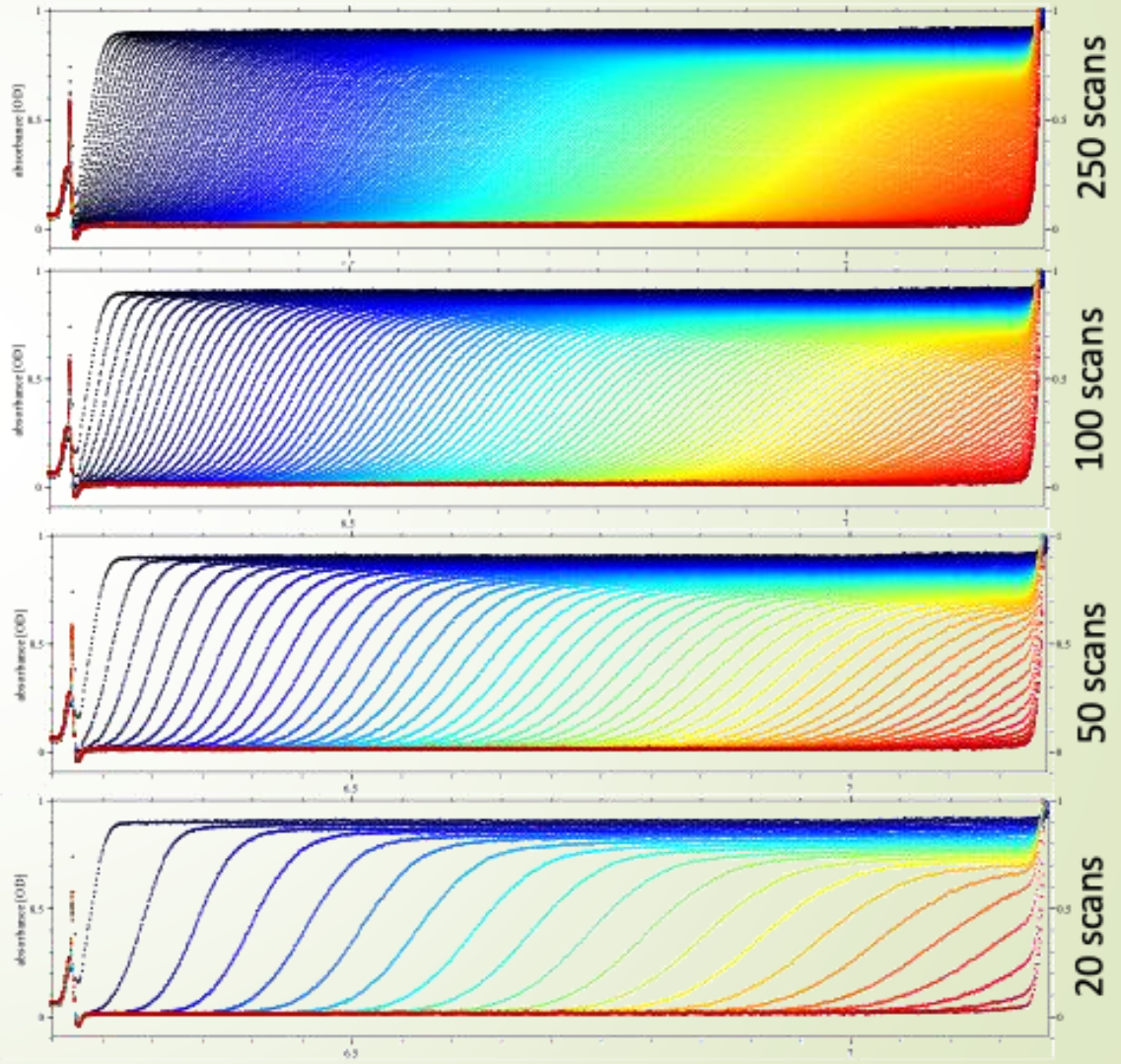
Alignment of the centrifuge cells in the rotor is critical to accurate aggregate quantitation

- Convective flow due to cell misalignment ultimately leads to artifactually high apparent aggregate content
- Visual alignment should be supplanted by either mechanical or optical alignment
- Mechanical alignment requires a tool and compatible cell housings
- The optical method aligns the septum which separates the centerpiece sectors parallel to the centrifugal force. This method is the most accurate and is the recommended best practice



Optimally setting up SV-AUC aggregate analysis

- The sedimentation behavior of each species in a sample depends on its hydrodynamic properties that are function of everything I have just presented!
- Maximal resolution of the migration velocity of discreet species is obtained by acquiring scans follow them from the top to the bottom of the longest possible sample column and maximizing the time-interval between the first and last scan
- *In practice, the optimal rotor speed for a given sample balances species heterogeneity, sedimentation rate, diffusion rate, and the centrifuge scan rate*



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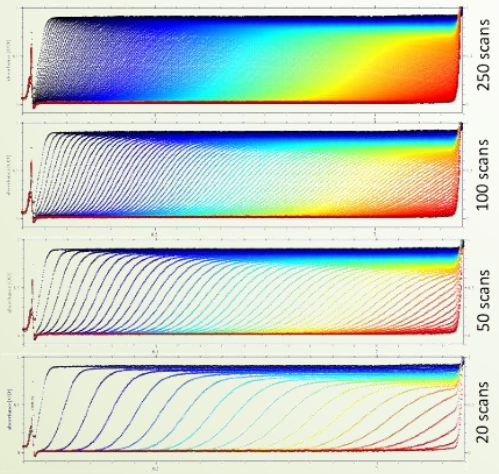
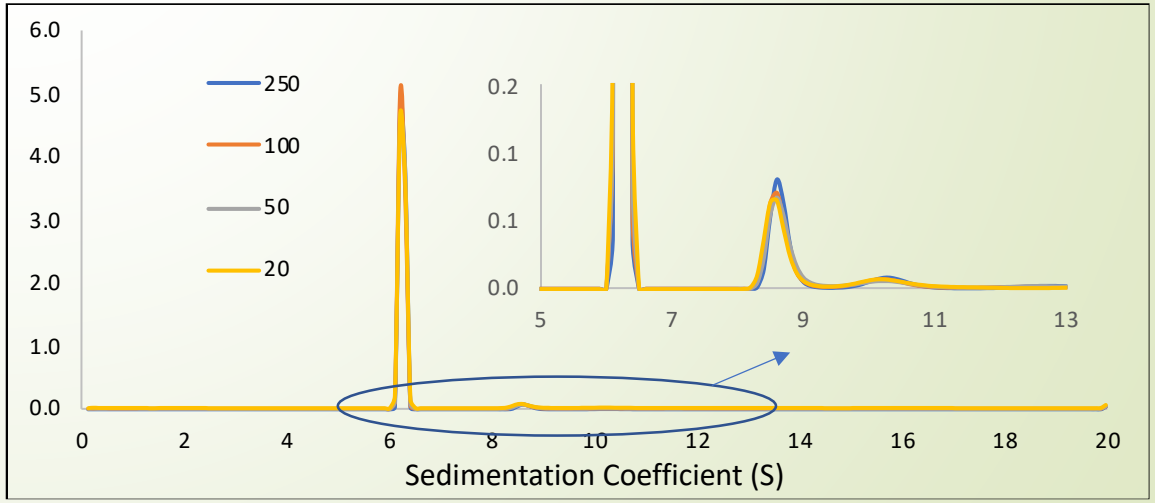
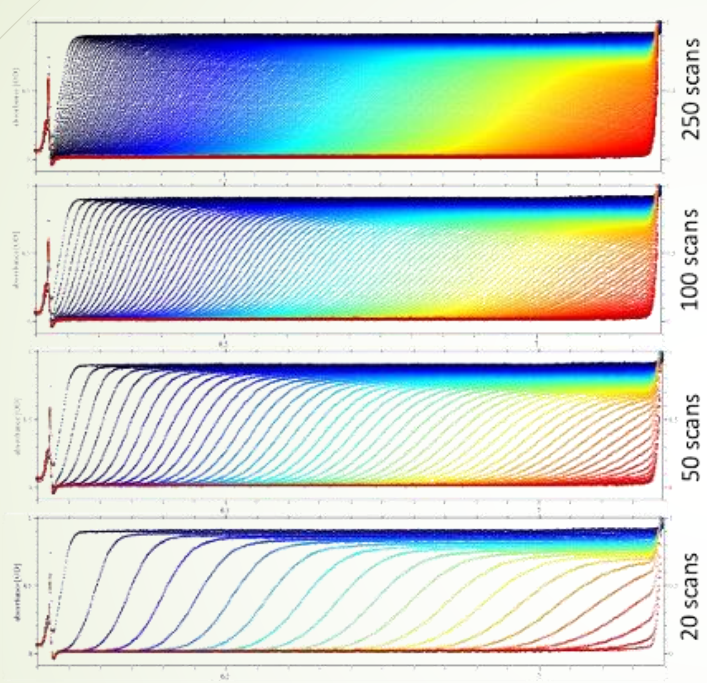
The continuous c(s) distribution model, implemented in SEDFIT, is widely used for aggregate quantitation

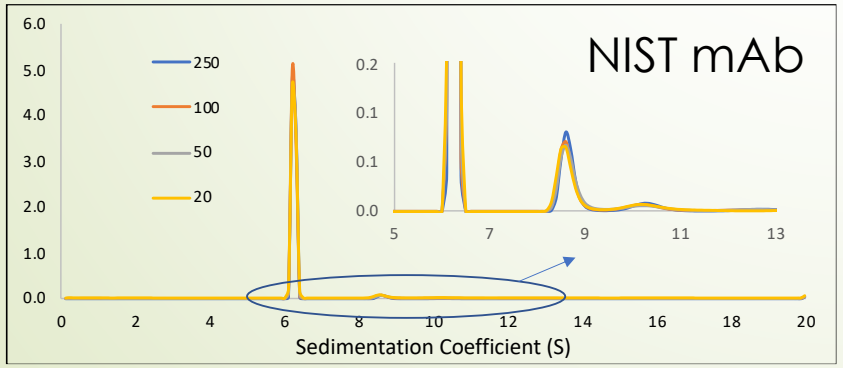
- The first step in analyzing *any* SV-AUC run is to examine the raw scans to evaluate of the data and diagnose aberrant behavior
- The c(s) model is only valid for modeling *non-interacting* particles.
- By including diffusional boundary broadening in the sedimentation model, and globally fitting scans covering the entire sedimentation process, sharp peaks are obtained, and baseline-resolution of features become evident that are not visually recognizable in the raw data.





More scans do not necessarily yield ‘better’ results





- Individual species will generally yield separate peaks in a c(s) distribution whose areas can be integrated to determine relative concentrations
- *The c(s) distributions are calculated curves derived by curve-fitting the raw data and are thus model-dependent*
- Identification and quantitation of small aggregate peaks is not nearly as robust as quantitation of the total aggregate

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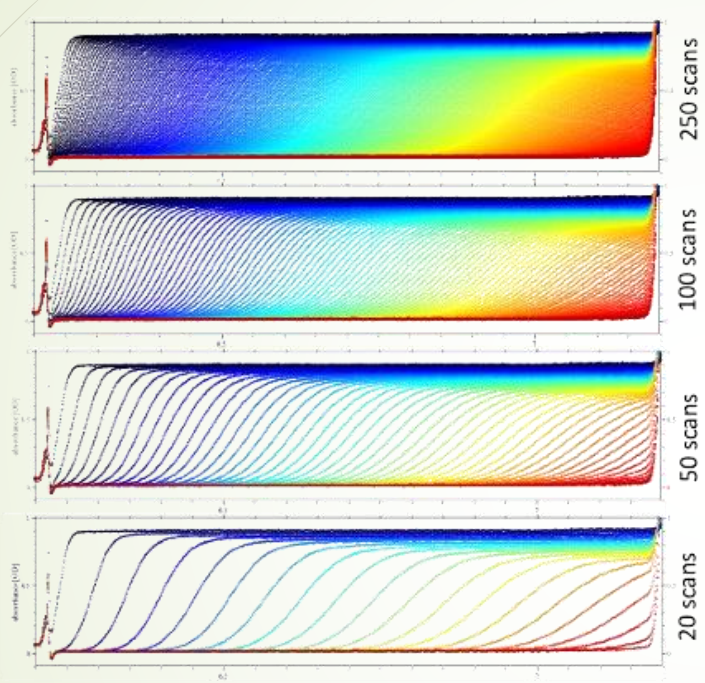

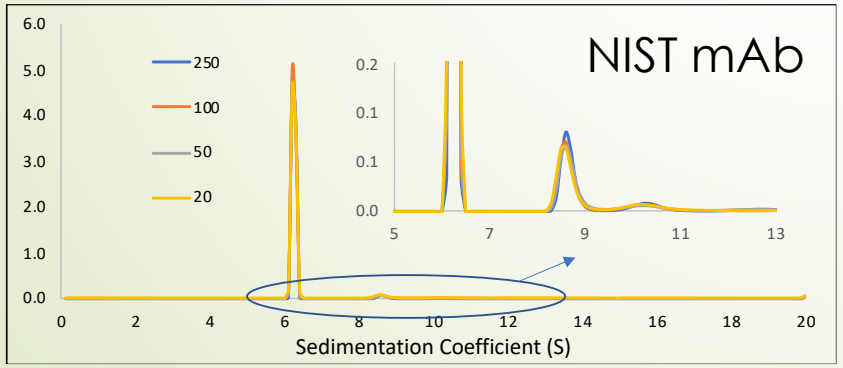

- Statistical noise is reduced to a level below the ultimately unavoidable adventitious systematic errors of SV-AUC experiments at 50 – 100 scans
- Deconvolution of diffusion rates depend on both boundary position (a function of the sedimentation coefficient and time) and change in boundary shape (dependent on diffusion and the square root of time and the resolution between species
- Deconvolution of time independent (TI) noise is optimal when at least 10 – 20 ‘baseline’ scans that are free of any sedimenting species) are included in the analysis

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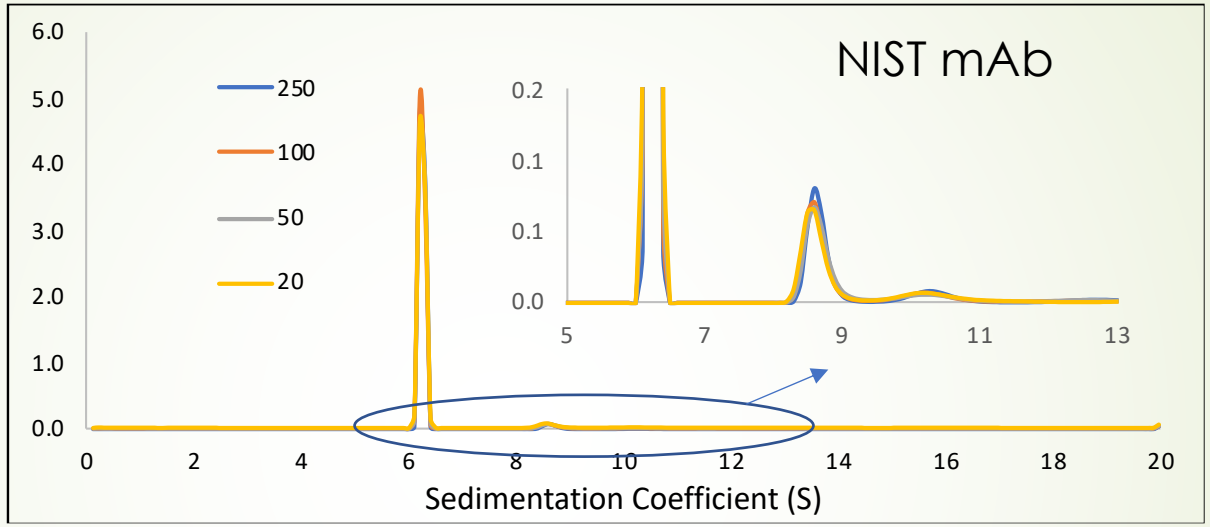

Identification and quantitation of NIST mAb dimer ‘aggregate’

Scans	Monomer	Dimer	f/f0	RMSD
250	6.24 S, 95.3%	8.65 S, 3.0%	1.67	0.00232
100	6.24 S, 95.5%	8.63 S, 3.0%	1.67	0.00232
50	6.24 S, 95.3%	8.66 S, 3.0%	1.67	0.00233
20	6.24 S, 95.0%	8.61 S, 3.0%	1.67	0.00231

Considerations for recording and reporting SV-AUC aggregate quantitation results

- Plots of c(s) distribution functions and a tabulation of the primary reportable results (sedimentation rate, percent species, f/f_o, and RMSD) are particularly informative
- Reports should document all relevant experimental and analysis parameters. Values below the LOD (generally, 1-2% of the total signal) should not be individually identified
- Careful tracking of results allows setting limits for acceptable ranges in subsequent studies.
  - Experience is a powerful teacher…

The goal of SV-AUC method validation is to demonstrate that the analytical procedure is a quantitative test for total soluble aggregate

- The most critical aspects of an SV-AUC method validation are determination of precision, LOD, LOQ, and linearity
  - The recommended approach determines the standard deviation (s) in the response of a given sample and the slope (a) of a standard curve generated using known sample concentrations.
  - LOD and LOQ are calculated from these data as 3.3s/a and as 10s/a, respectively.
  - This approach does not depend on the isolation of aggregate species. Linearity and range along with the LOD and LOQ are determined in a single set of experiments

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Summary of recommendations for the quantitation of total aggregate level in mAb samples.

Stage	Description	First-to-try recommendation	Further options / Alternatives
Sample Prep	General	Load sample at approx. 1 OD (l=1.2 cm) for UV	Dialysis is required for interference
	Absorbing excipient in buffer	Dialysis	
	Low ionic strength or self-buffered formulations	Add ~25 mM salt	
	Co-solute	Dialyze to remove co-solute	Run with co-solute but use inhomogeneous c(s) model
	Replicates	3	2
Cell Alignment	Method for cell alignment	Optical (preferred) or mechanical	Manual
Temperature	Temperature	20 °C	Other temperatures within the instrument range (4 – 37 °C)
	Temperature equilibration	1-2 hours	Longer for extreme temperatures
System suitability	Instrument check: Radial calibration	Once every time a rotor and/or counterbalance is changed	Before every run
	Cell suitability	Keep cell components together, monitor performance, perform a check at 3,000 rpm	
SV-AUC run setup	Rotor speed	50,000 rpm	Up to 60,000 rpm for Ti-60 rotor
	Detection mode	Absorbance-based at 280 nm (intensity or absorbance)	Absorbance-based at other wavelengths (*e.g.*, 230 nm) or interference
Fitting parameters	Resolution	Start with 37, increase to 181 (0.1 s)	Start directly with 181 (0.1 s)
	s-values range	0.1 to 20	2 to 20. Increase upper range for samples containing larger oligomers, in extreme cases using log-scaled division to cover very large range efficiently
	Frictional ratio	Start at 1.5 and float	Input prior knowledge value and float
	Baseline	Set to 0 and float	
	TI noise	Floated	
	RI noise	Fixed	Floated (especially beneficial for data collected on Beckman Optima™)
	Meniscus	User defined and floated	
	Meniscus fitting range	± 0.2 mm around meniscus	
	Bottom	7.2 cm and fixed	Floated in presence of small MW contaminants or other species presenting back-diffusion
	Fitting range		Meniscus + 0.1 cm to Bottom - 0.1 (or - 0.15 cm)
	F-ratio (confidence interval)	0.68	none
	Regularization algorithm	Alternate between Simplex and Marquart Levenberg	Simplex only
	Partial specific volume	Calculated or measured	0.73 mL/g as a reasonable approximation[65]
	Viscosity	User defined	
	Density	User defined	
Data reporting	What should be reported		c(s) distribution, table with major species
	Sedimentation coefficients		only for major species up to 2 decimal places
	Amounts in %		only for major species up to 1 decimal place

Best practices are not the only practices

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Lessons Learned

Best Practices for Aggregate Quantitation of Antibody Therapeutics by Sedimentation Velocity Analytical Ultracentrifugation

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- Our article distills the experimental and analysis procedures used by the authors for SV-AUC with the goal of harmonizing aggregate quantitation approaches across the industry and providing an entrée for new AUC practitioners
- Information about the Biophysics Working Group can be found at CASSS.org