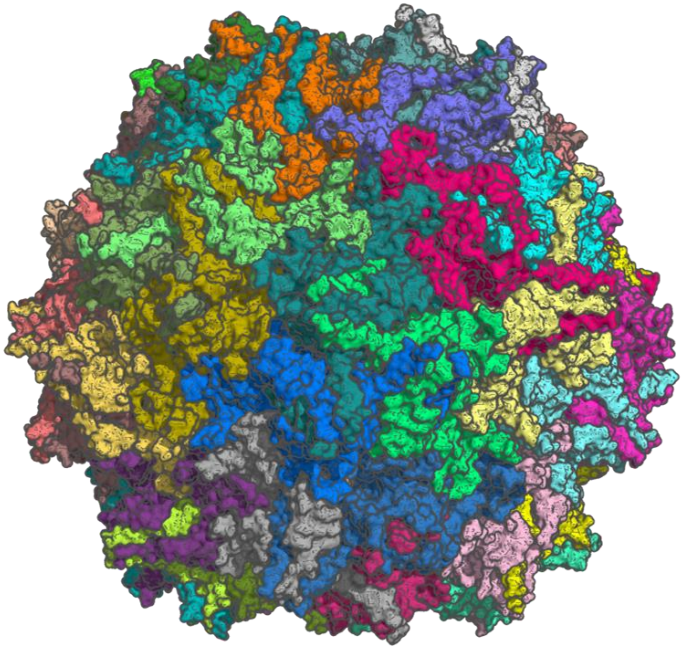
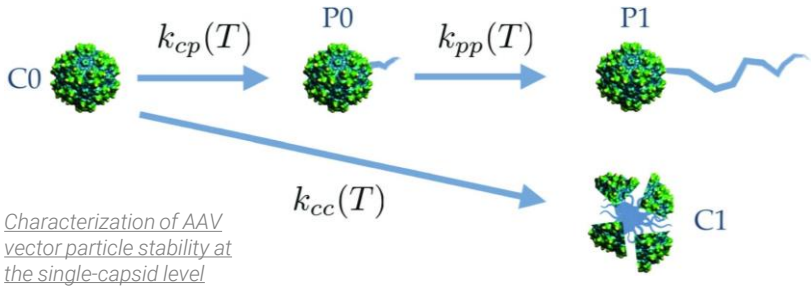
A Comprehensive Degradation Temperature Panel is Vital for AAV Development



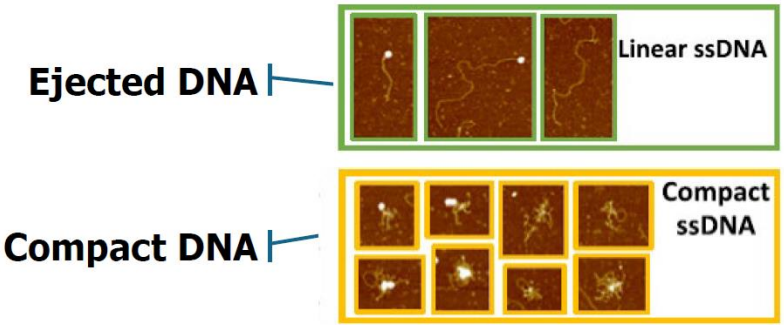
Ronald Toth
Associate Director | Genomic Medicine Characterization

We need to verify that we're concerned about the ***relevant*** major ***degradation pathways***

Major Degradation Pathway for AAVs?



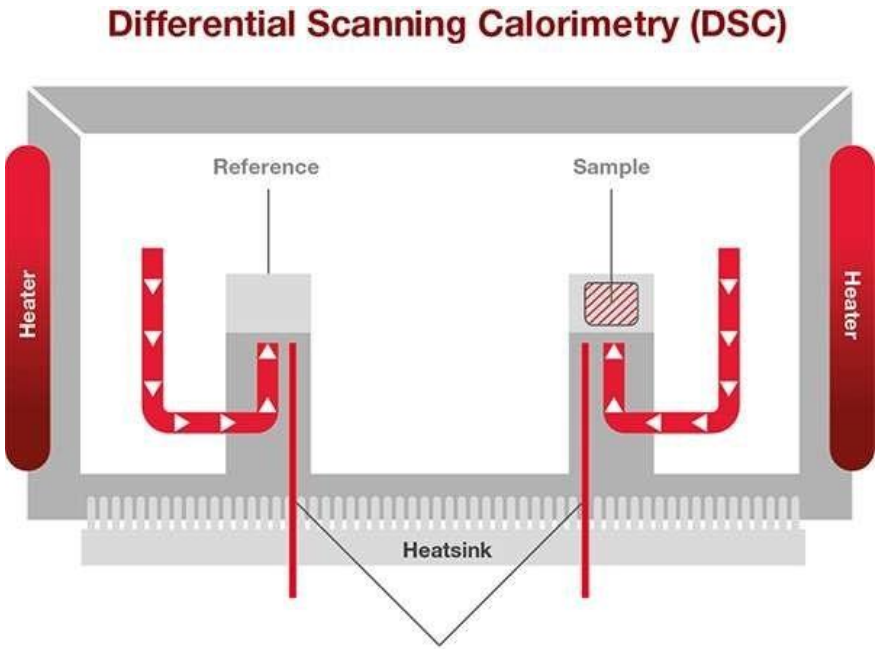
Characterization of AAV vector particle stability at the single-capsid level



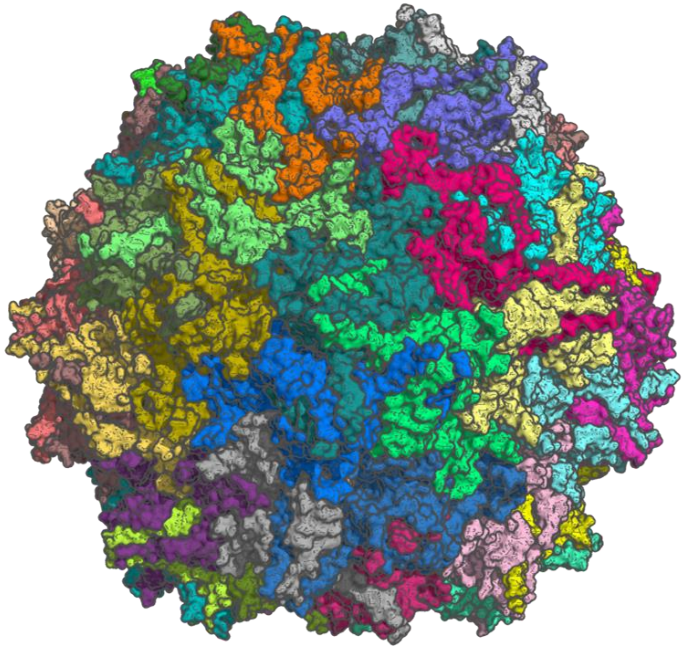
For mAbs, aggregation is the dominant degradation pathway

If we transplant this concern to gene therapies, we risk missing more relevant degradation pathways

Anisothermal should be the opposite of isothermal, not '*Differential Scanning*'



"Differential" refers to the presence of a separate, physical experiment occurring in the instrument with the reference buffer, not the temperature ramp



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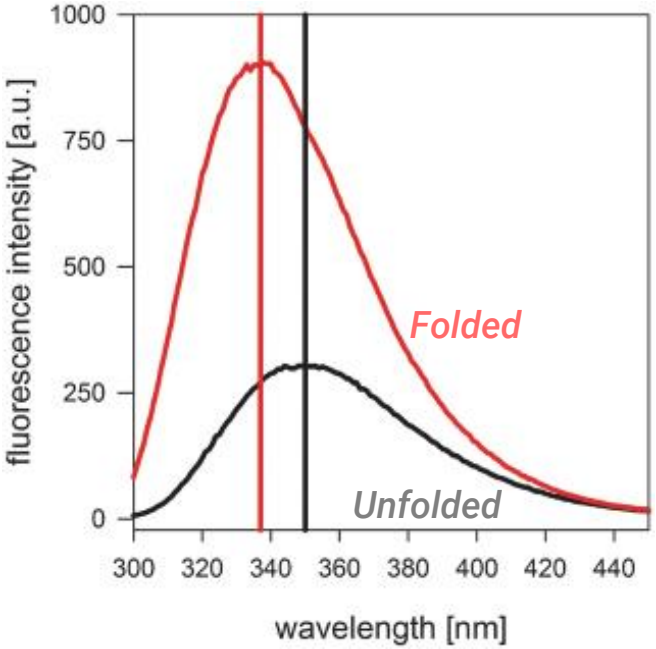
All methods used here reveal information both isothermally and anisothermally

	Isothermal (*IT*)	*Anisothermal* (*AT*)
Intrinsic Fluorescence	• Structural Integrity	• Temperature of protein structure melting (T_{PMELT})
Extrinsic Fluorescence	• [free nucleic acid]	• Temperature of Genome Ejection (T_{EJC})
Dynamic/Static Light Scattering	• Average Size • Heterogeneity • Mass% High-Diameter • [particle] (#/mL)	• Temperature of Aggregation (T_{AGG}) • Temperature of Genome Ejection (T_{EJC}) • In cases where these effects can't be deconvoluted, we report $T_{DLS\ DEG}$, a combination of T_{AGG} and T_{EJC}

Ratio of intrinsic fluorescence at 350:330 nm reports on protein unfolding



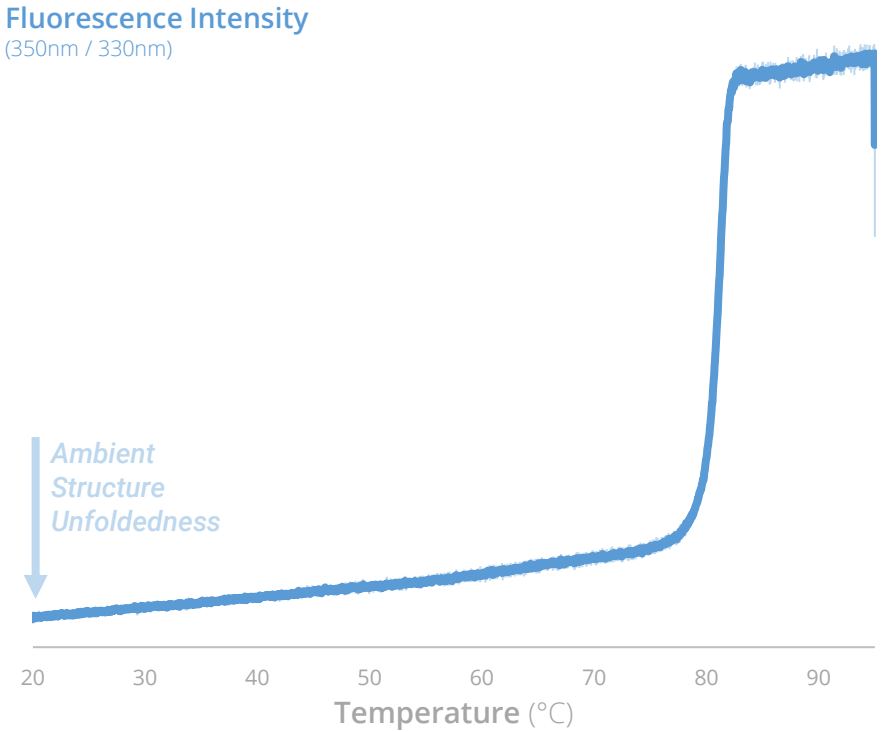
As proteins unfold, their tryptophan emission spectra become more redshifted

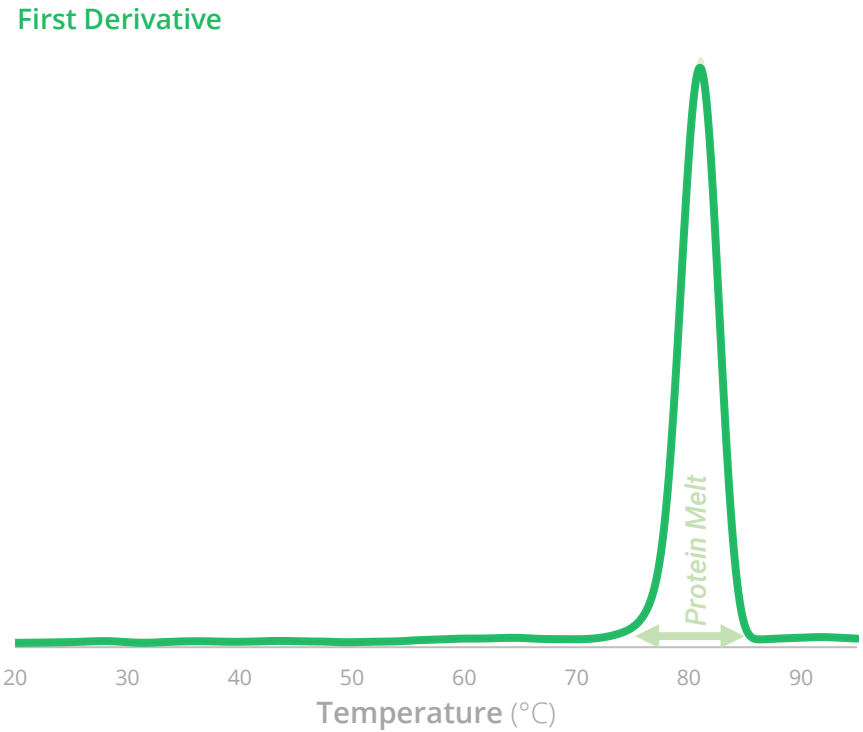
The ratio of fluorescence at 350:330 nm reports on the amount of redshift

Lower | less redshift | less unfolded
Higher | more redshift | more unfolded

Anisothermal Intrinsic Fluorescence

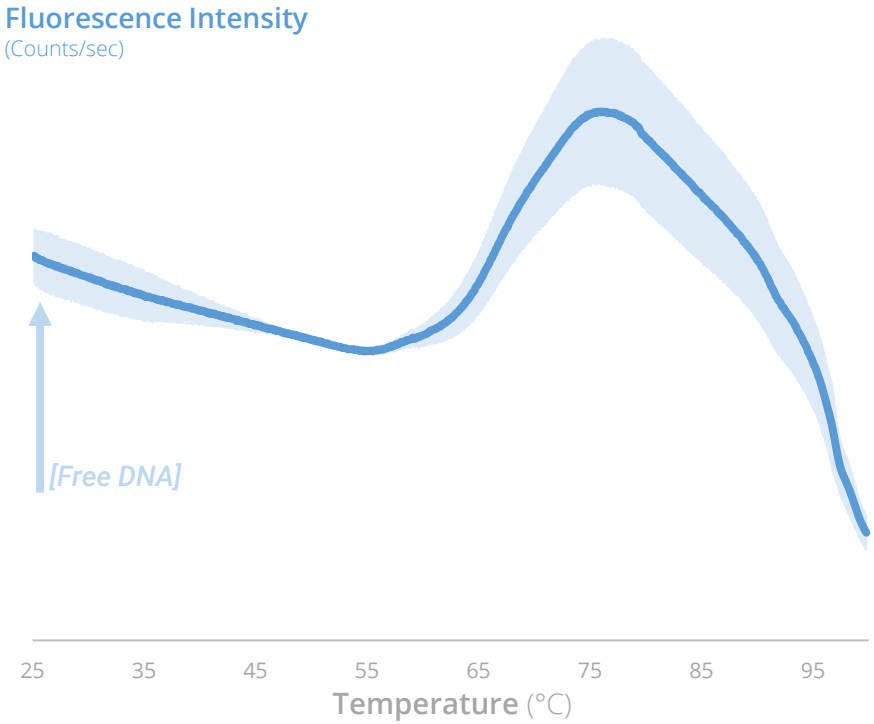
ambient structure and temperature of protein melting

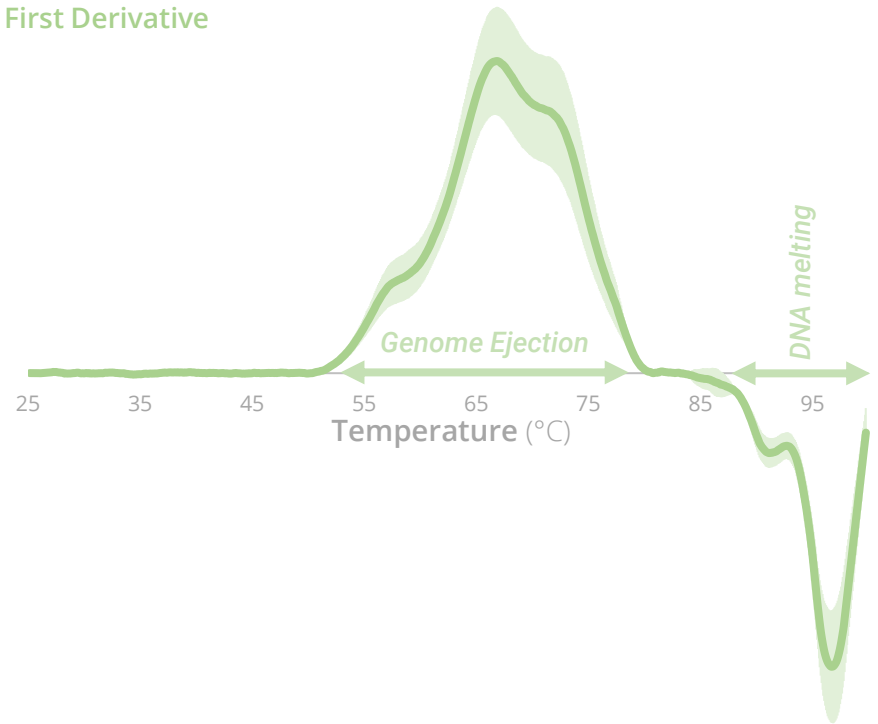




Anisothermal Extrinsic Fluorescence

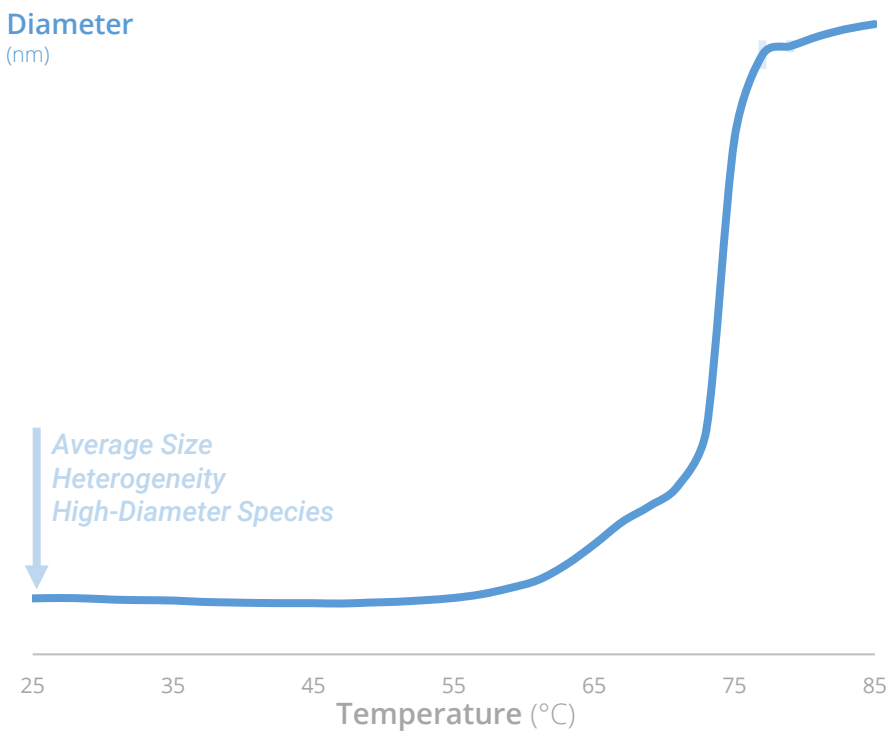
free [DNA] and temperature of genome ejection

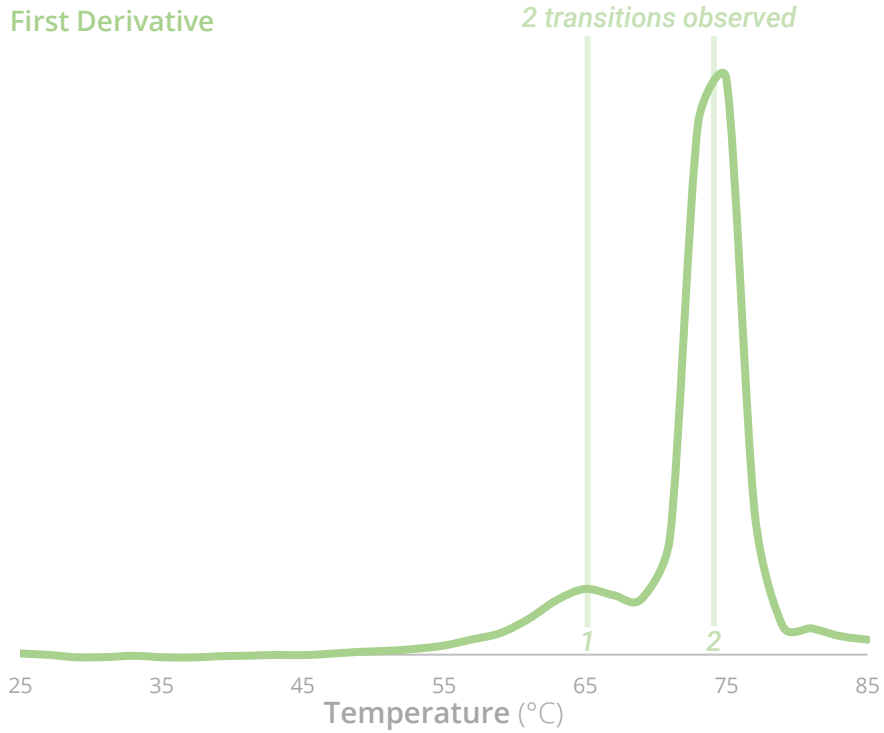




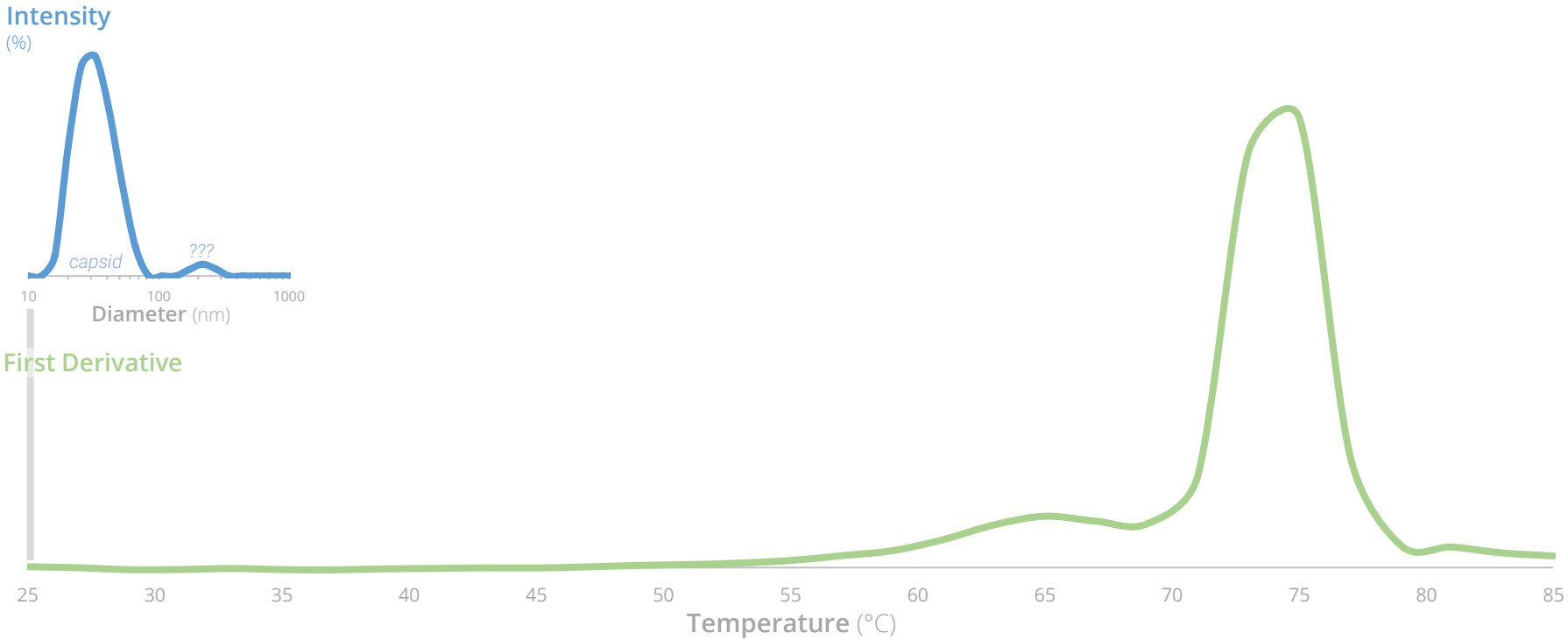
Anisothermal Dynamic Light Scattering

ambient size, temperature of aggregation and genome ejection

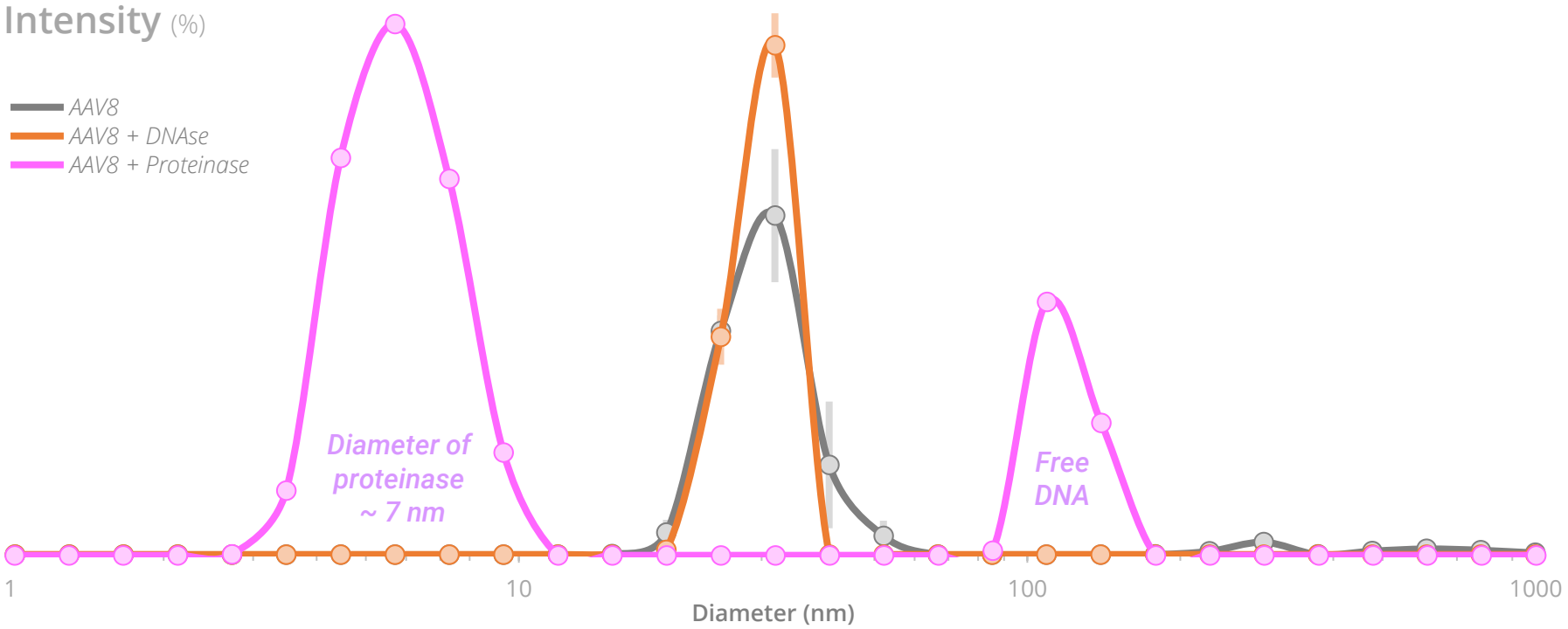




Examining size distributions can help deconvolute AT-DLS transitions



Capsid disruption results in ~100nm DLS peak attributable to free DNA



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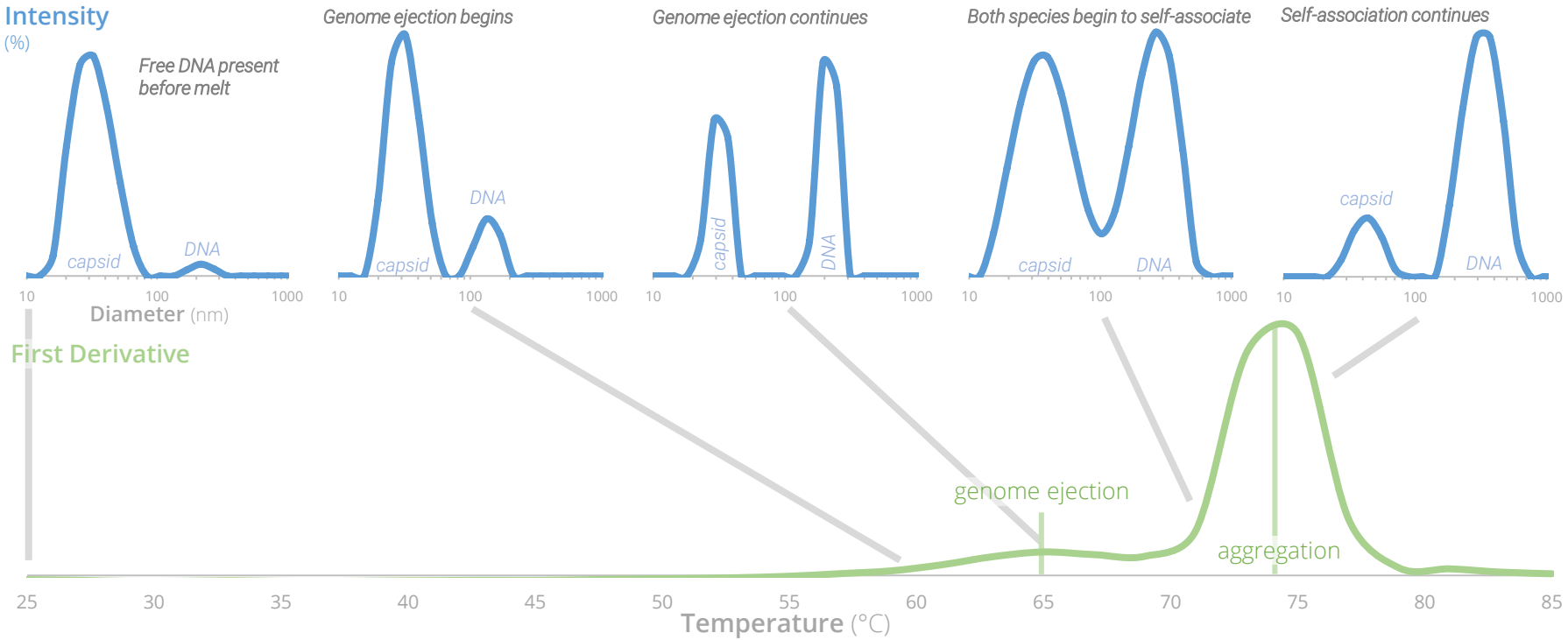
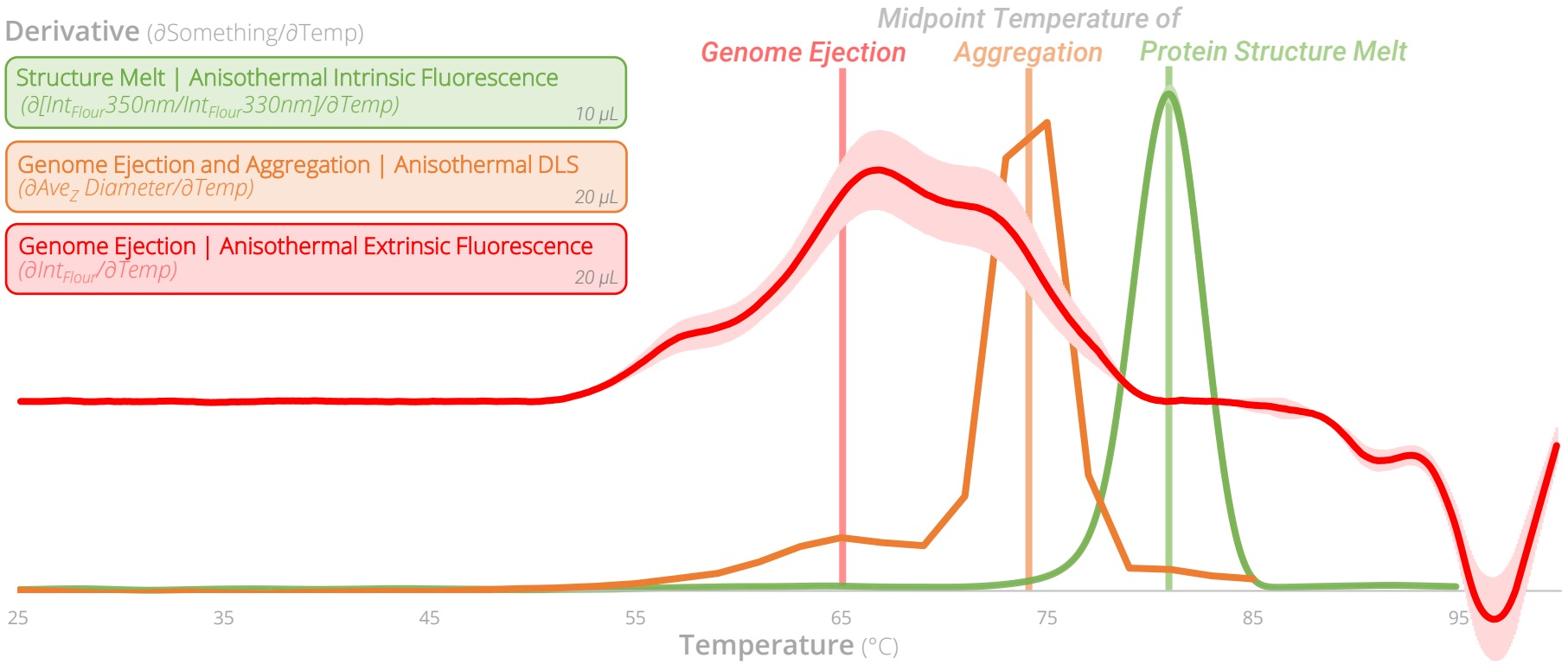


A high-throughput, comprehensive T_m panel for AAV!

Summary of temperature transitions for AAV8



These methods would anticipate and prevent development issues if performed early

We tend to prefer platform approaches for both process development and analytical development

However, susceptibility to stresses varies greatly among serotypes

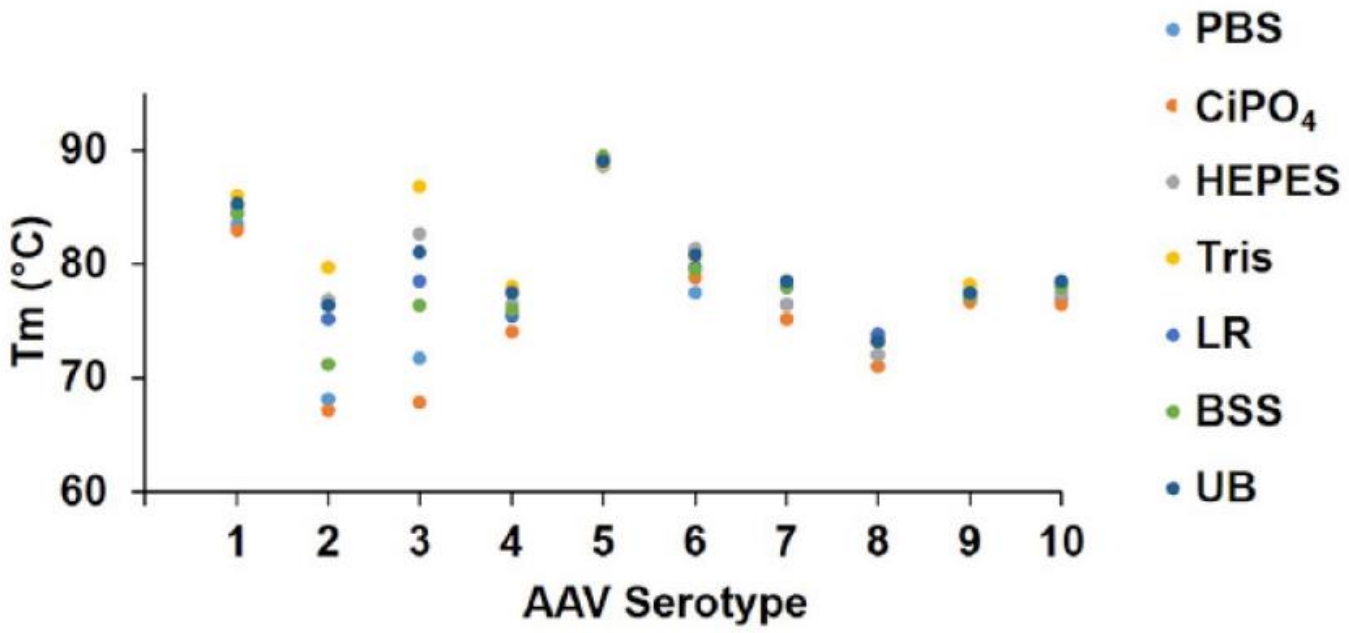
We tend to charge ahead without stopping to evaluate how well things are going

Are AAVs amenable to platform approaches?

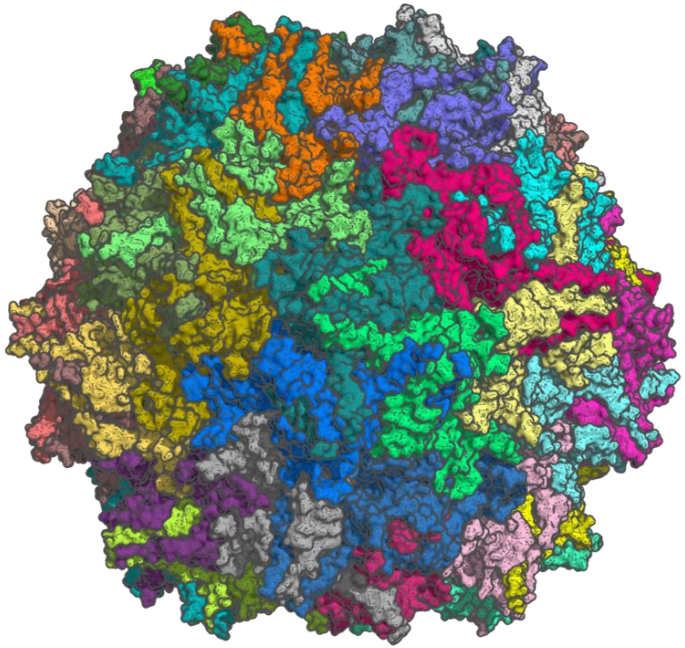
Oddities were observed in titers of AAV2 during analytical development

Technique	AAV8	AAV2
PCR	1e13 VG/mL	1e13 VG/mL
UV-Vis	**1.2e13 VG/mL**	**2.5e13 VG/mL**
SV-AUC	8.2e12 CP/mL Full 1.4e13 CP/mL Total	2.0e13 VG/mL Full 2.6e13 VG/mL Total
SLS (DLS)	1.2e13 particles/mL	2.7e13 particles/mL

Literature suggest we should be especially careful with AAV2 development



Bennett, et al., Mol Ther Methods Clin Dev (2017) 6:171-182



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Let's (statistically) **design a set of experiments to evaluate the effect of common stresses during analytical development**

[AAV]

Almost all methods require dilution

[NaCl] and pH

Many methods require altering these in test article

Freeze/Thaw

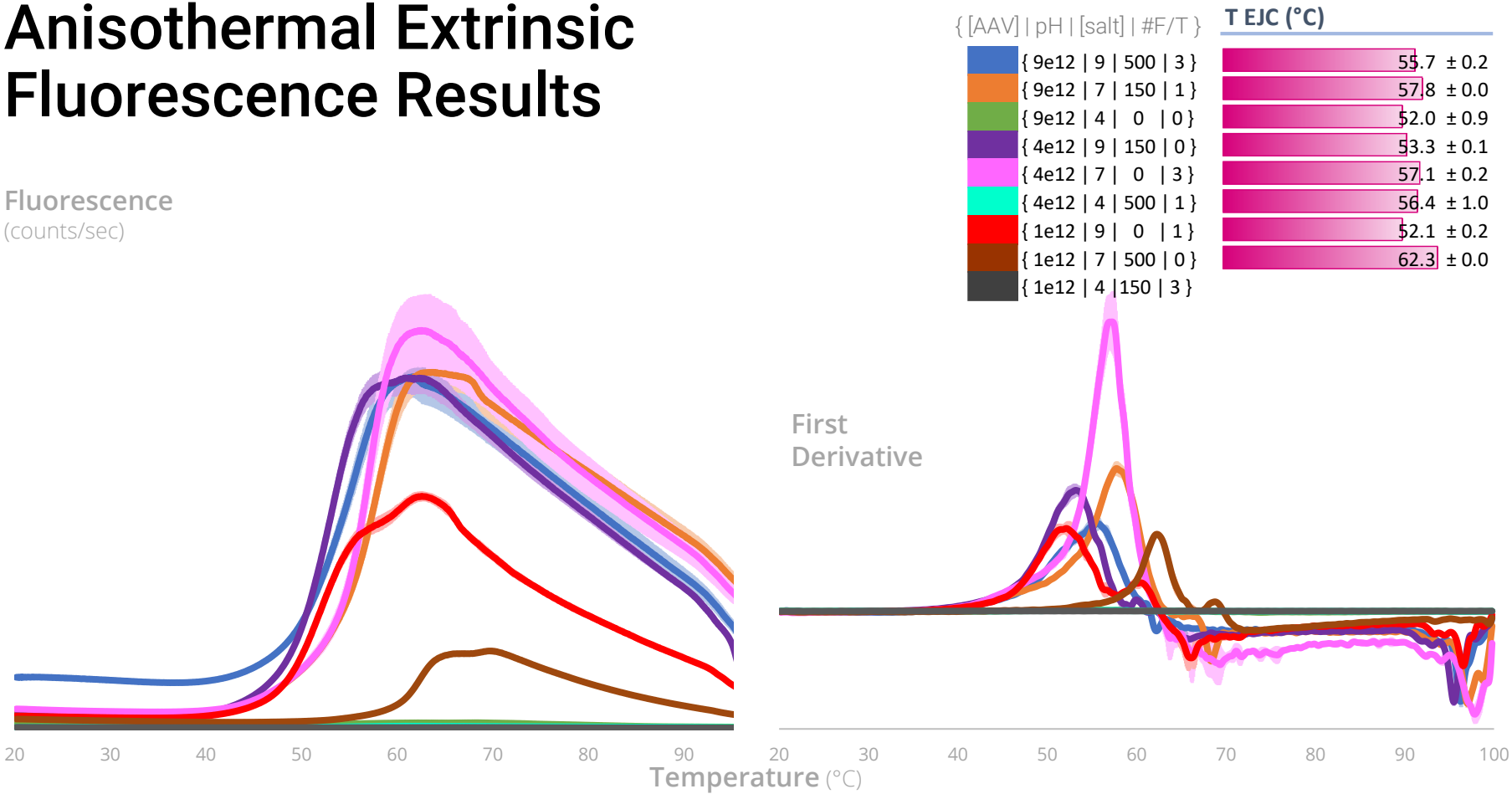
Needed to store and transfer samples

The Taguchi L9, a $3^{(4-2)}$ fractional factorial design, tests 4 factors at 3 levels in only 9 conditions

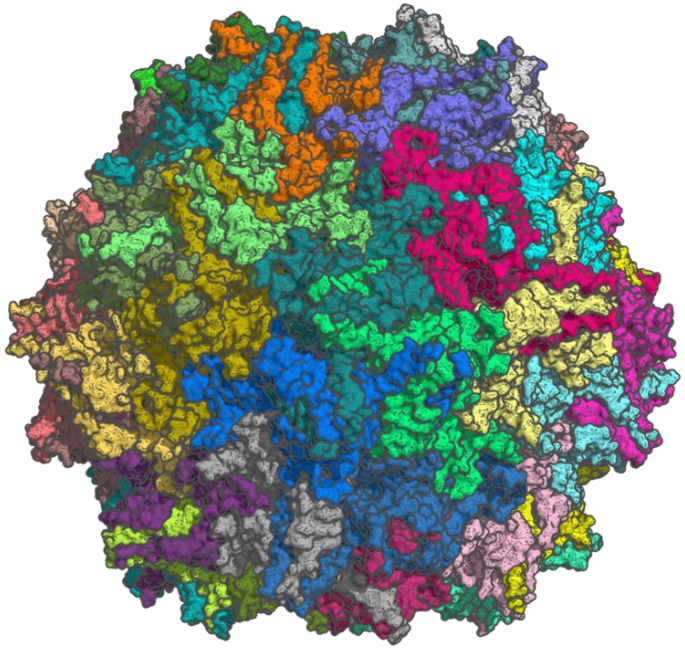
[AAV]	pH	[salt] (mM)	Freeze/Thaw Cycles
9E+12	9	500	3
9E+12	7	150	1
9E+12	4	0	0
4E+12	9	150	0
4E+12	7	0	3
4E+12	4	500	1
1E+12	9	0	1
1E+12	7	500	0
1E+12	4	150	3

Replicating design 3x only consumes ~400 µL of ~2e13 VG/mL stock!

Anisothermal Extrinsic Fluorescence Results



Stop! Statistics Time!



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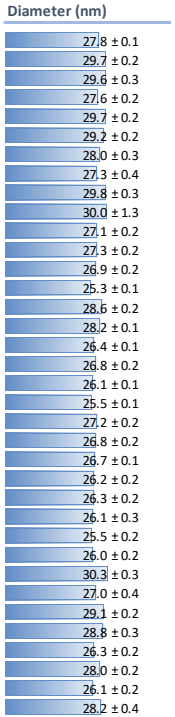
We are tempted to look at small changes in data and say ‘no trends’ without any analysis



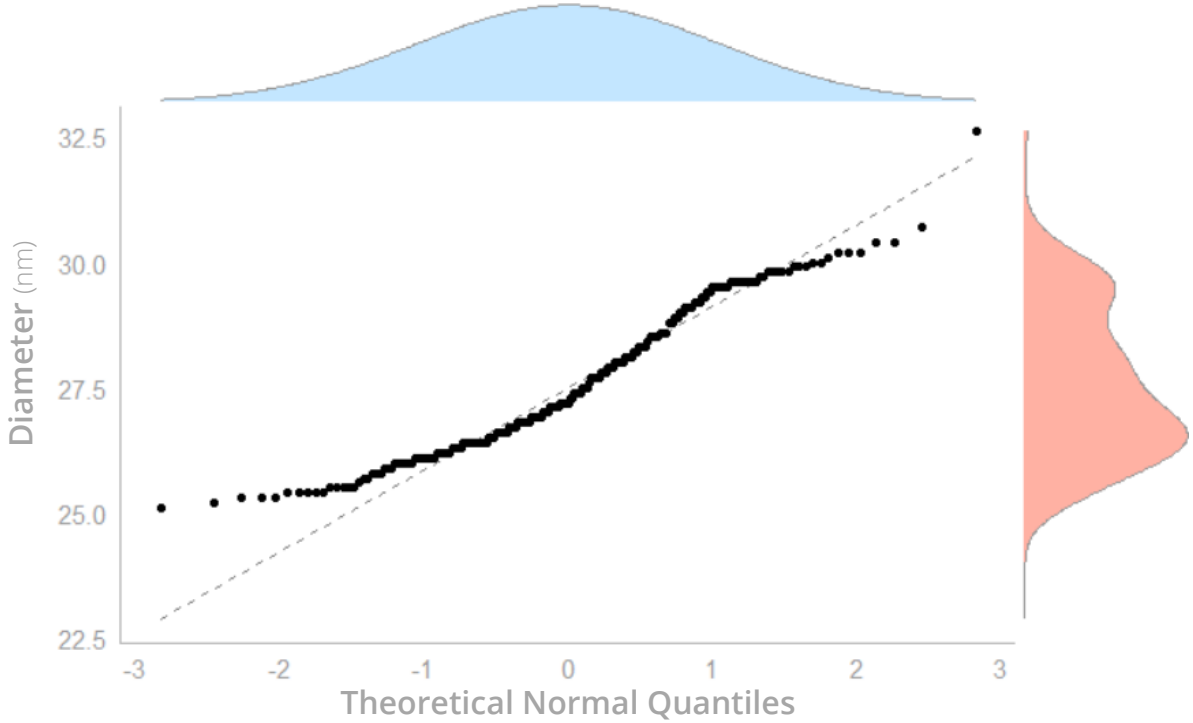
Imagine a dataset where DLS diameter is varying more subtlety by only a few nm

An Analysis of Variance will almost always find some things are significant, how do we know we’re not reading into noise?

One way to tell: does the variance exceed that expected of the assay?

- ~0.5 nm standard deviation observed during DLS qualification testing
- Here we observe ~1.5 nm standard deviation

If there are no trends in data, we would expect a *normal* distribution of the results



An Analysis of Variance (ANOVA) allows all effects and interactions to be summarized in one table

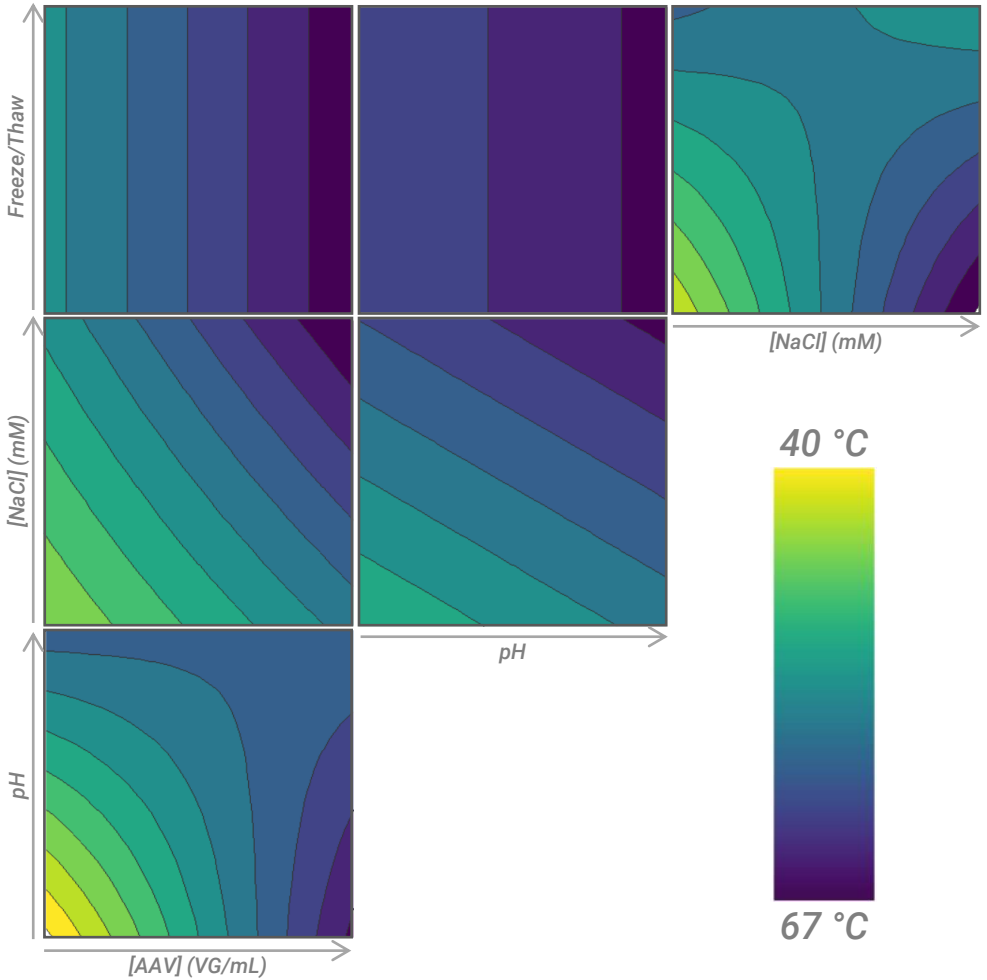
Summary of ANOVA Results for AAV2 Developability DoE

	T_{EJC} (°C)		Diameter (nm)		T_{AGG} (°C)		% Ambient Unfolding		T_{PMELT} (°C)	
[AAV] (per 1e12)	0.39	***	0.29	*			-1.1	***	0.14	***
pH	0.28	***	-18.62	***	0.46	**			-0.24	***
[Salt] (per 100 mM)	0.49	***								
F/T Cycles										
[AAV]*pH	-0.042	***	-1.931	***					-0.008	**
[AAV]*[Salt]	0.0053	*							-0.0056	*
pH*[Salt]			2.1391	***			0.072	**	0.0186	***
[Salt]*F/T	-0.12	***	1.53	**					-0.06	***
R^2	0.98		0.96		0.74		0.86		0.98	

P-value	code
< 0.001	***
0.001 – 0.01	**
0.01 – 0.05	*

Midpoint Temperature of Genome Ejection (°C)

	T_{EJC} (°C)	
[AAV] (per 1e12 VG/mL)	0.39	***
pH	0.28	***
[Salt] (per 100 mM)	0.49	***
F/T Cycles		
[AAV]*pH	-0.042	***
[AAV]*[Salt]	0.0053	*
pH*[Salt]		
[Salt]*F/T	-0.12	***
R^2	0.98	



Conclusions

Dilution and low salt pull the temperature of genome ejection close to 40°C

PCR methods dilute into low salt buffer

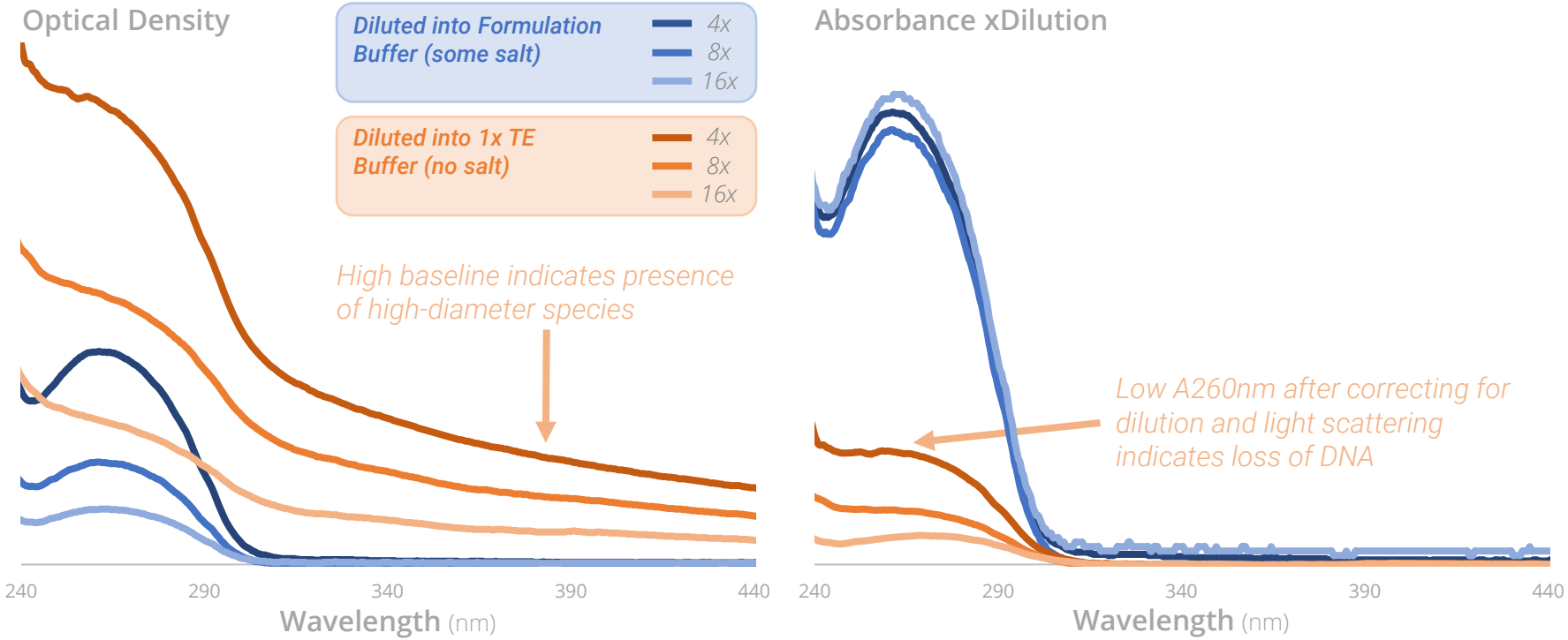
- Much lower [AAV] than can be tested via these methods, likely T_{EJC} under these conditions is below 40°C

DNAse inactivation is performed at 40°C

Provides a possible explanation for method-induced artifact under-reporting titer

It’s important to validate conclusions from DoEs

*UV-Vis testing for AAV2 **heated** for **10m** at **40 °C***



Perspectives

This information would have been useful to gather before proceeding with a platform approach for PCR

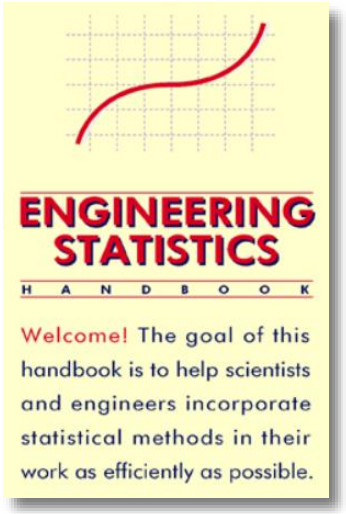
Leveraging statistics combined with these high-throughput methods allowed us to, in triplicate

- monitor 3 distinct degradation pathways
- in response to 4 distinct factors
  - with all 2-factor interactions
- with only 400 µL of stock
- with only 3 experiments performed in one day

Leveraging statistics more routinely would allow us to do more with less

A Special Thanks to the Following:

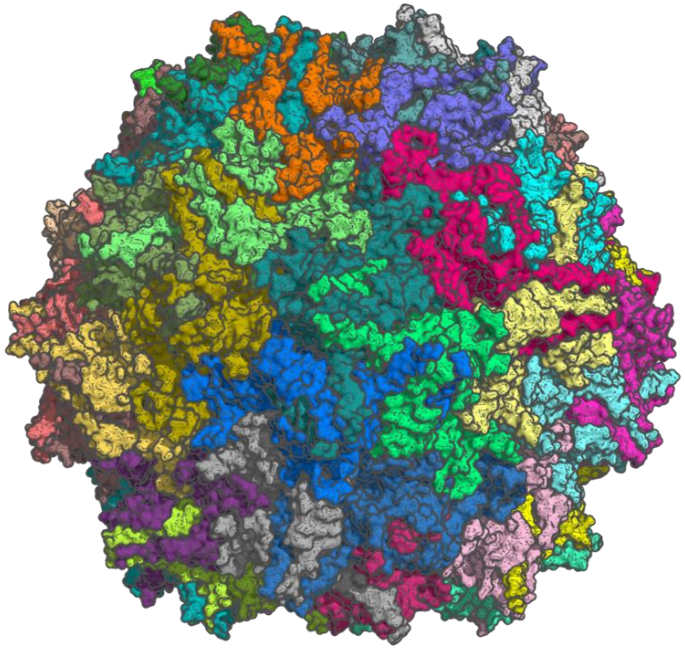
Xiaoying Jin



The NIST/SEMATECH e-Handbook of Statistical Methods

- 3. Choosing an Experimental Design
- 4. Analysis of DOE Data
- 5.3.3.4.7. Summary tables of useful fractional factorial designs

Factor #	Design Specification	Runs
3	2_{III}^{3-1}	4
4	2_{IV}^{4-1}	8
5	2_{V}^{5-1}	16
5	2_{III}^{5-2}	8
6	2_{VI}^{6-1}	32
6	2_{IV}^{6-2}	16
6	2_{III}^{6-3}	8
7	2_{VII}^{7-1}	64
7	2_{IV}^{7-2}	32
7	2_{IV}^{7-3}	16
7	2_{III}^{7-4}	8
8	2_{VIII}^{8-1}	128
8	2_{V}^{8-2}	64
8	2_{IV}^{8-3}	32
8	2_{IV}^{8-4}	16
9	2_{VI}^{9-2}	128
9	2_{IV}^{9-3}	64
9	2_{IV}^{9-4}	32
9	2_{III}^{9-5}	16
10	2_{V}^{10-3}	128
10	2_{IV}^{10-4}	64
10	2_{IV}^{10-5}	32
10	2_{III}^{10-6}	16
11	2_{V}^{11-4}	128
11	2_{IV}^{11-5}	64
11	2_{IV}^{11-6}	32
11	2_{III}^{11-7}	16
15	2_{III}^{15-11}	16
31	2_{III}^{31-26}	32



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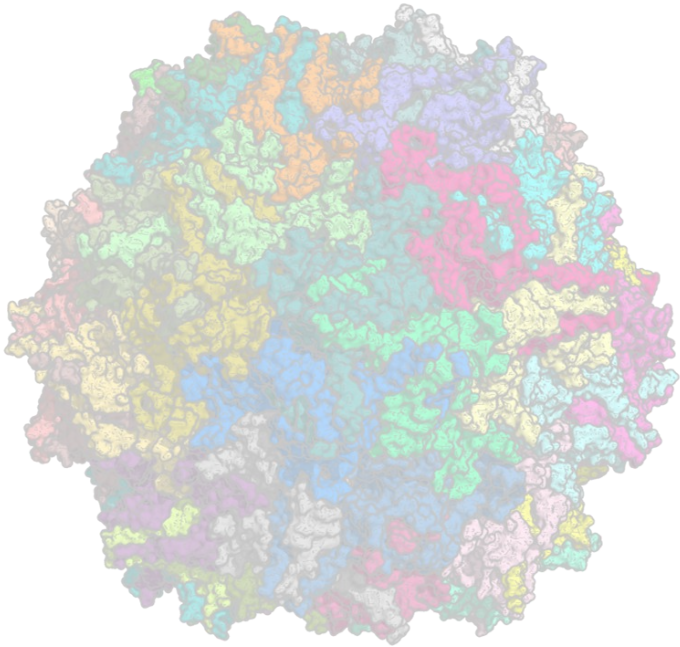

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