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Aaron Noyes
Pfizer

Ranga Godavarti
Pfizer

Nigel Titchener-Hooker
Pfizer

Jonathan Coffman
Pfizer

Tarit Mukhopadhyay
Pfizer

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Developing a Suite of Analytics to Support Process Development for the Manufacture of Polysaccharides

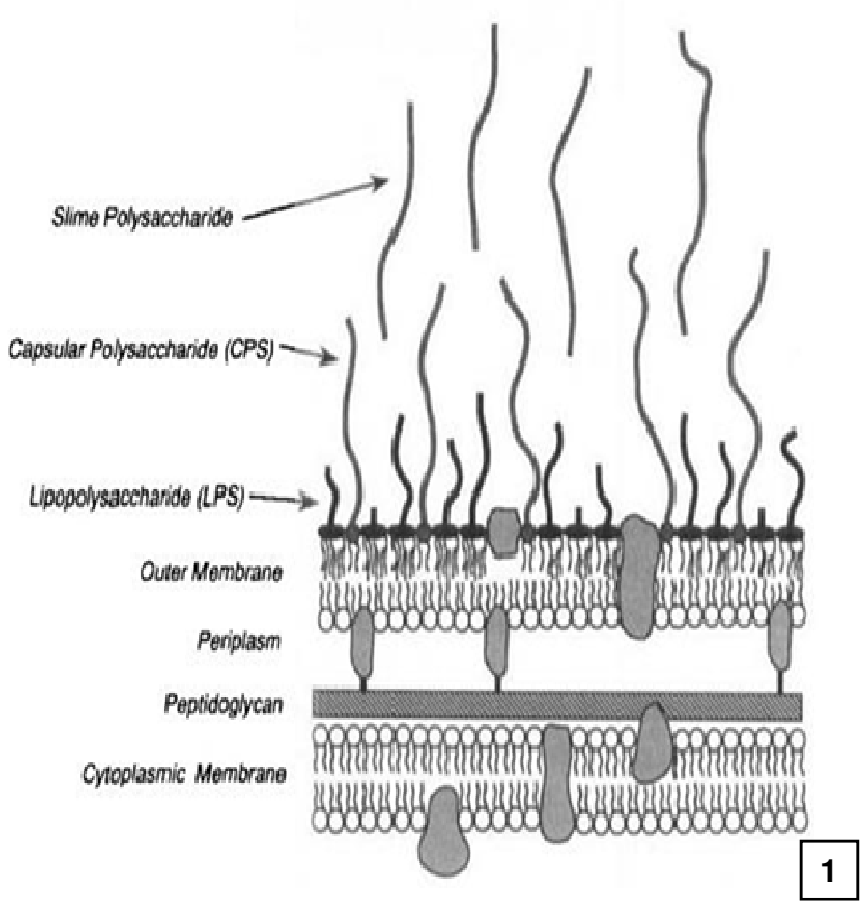
May 23, 2012
Vaccine Technology IV
Albufeira, Portugal

Aaron Noyes, Ranga Godavarti, Nigel Titchener-Hooker, Jonathan Coffman, Tarit Mukhopadhyay

Purification Process Development, Pfizer, Andover, MA, USA
Biochemical Engineering, University College London, London, UK

Glycobiology

- Covalently linked to outer cellular envelope.
- Function
  - Cell hydration
  - Adherence
  - Cloaking
  - Pathogenicity
- Complex and non-uniform
- Size: kDa-mDa



[1]Whitfield, C. & Valvano, M., 1993. Biosynthesis and expression of cell-surface polysaccharides in Gram-negative bacteria. In *Advances in Microbial Physiology*. pp. 135-246.

Processing Context

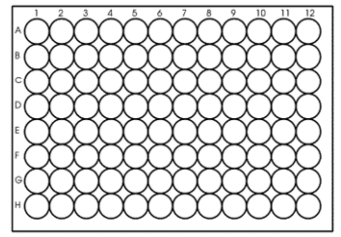
- Historically, polysaccharide processes relied on precipitation, filtration, etc.
- High throughput process development: very limited
- **KEY BOTTLENECK = ANALYTICS**
  - Polysaccharide Titer
  - Impurities
    - proteins, DNA, endotoxin, etc.
  - Polysaccharide Quality
    - size, polydispersity, DP, O-acetylation, lipidation, etc

Methods are low throughput and often complicated

Proposal for HTP Analytics

1 Scientist

Purification Screen



300-600 µL/well
Addition during
shaking/stirring

If multiple stages desired

Shaking/Stirring Phase

Centrifugation/Vacuum

	Endotoxin(Pyrogene)	Sugar(PHS/BCA)	Turbidity (AU)	Protein(BCA)	DNA (Picogreen/A260)
Volume	10 µL/well	100 µL/well	100 µL/well	25 µL/well	50 µL/well
Process [ ]	**0.1-5X10^7 EU/mL**	**0.1-5 mg/mL**		**0.01-10 mg/mL**	**0.01-2 mg/mL**

Additional Analyses

Total Expmt'l Time
1 day

Total Volume
285 µL/well

Slide 4

10 I think comment is needed on how early you might use this HTP analytics and how 'dirty' the sample can be. Also, division between qualitative and quantiative data that can be taken all the way through to manufacturing.
Tarit Mukhopadhyay, 4/4/2012

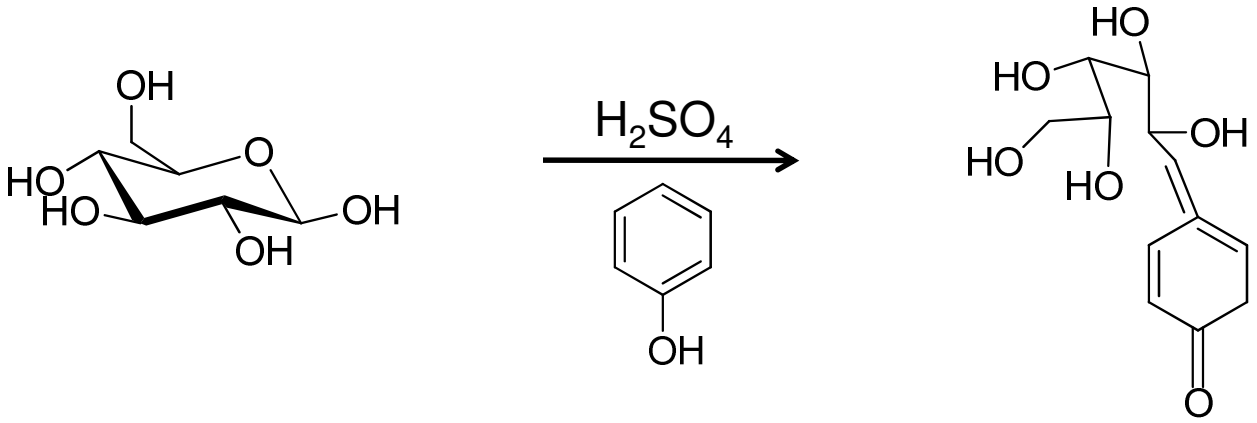
Polysaccharide Assay Objectives

- Linearity
- Accuracy
- Precision
- Universality
- Interference
- Optimization
- Ease of automation

Options

- Phenol sulfuric acid
- Refractive index
- Polarimetry
- Aniline phthalate/ trichloroacetic acid
- 1-napthosulfonate
- Anthrone
- Phenol
- Resorcinol

Phenol Sulfuric Acid (PHS): Reaction Mechanism



- Dependent on structure
- Absorbs strongly 470-490 nm
- Follows Beer's Law

PHS Method Improvements

	Dubois et al	Saha et al	Masuko et al
Year	1951	1994	2005
Total Volume (μL)	8000	3500	230
Sample Volume (μL)	2000	500	50
Assay Range (mg/L)	5-35	10-100	4-585
Vessel covering	yes	yes	no
External Heating	2 water baths	no	2 water baths
Shaking	yes	yes	no
Number of Steps	3	2	3

PHS Method Improvements

Higher linear range

Reduced sample volume

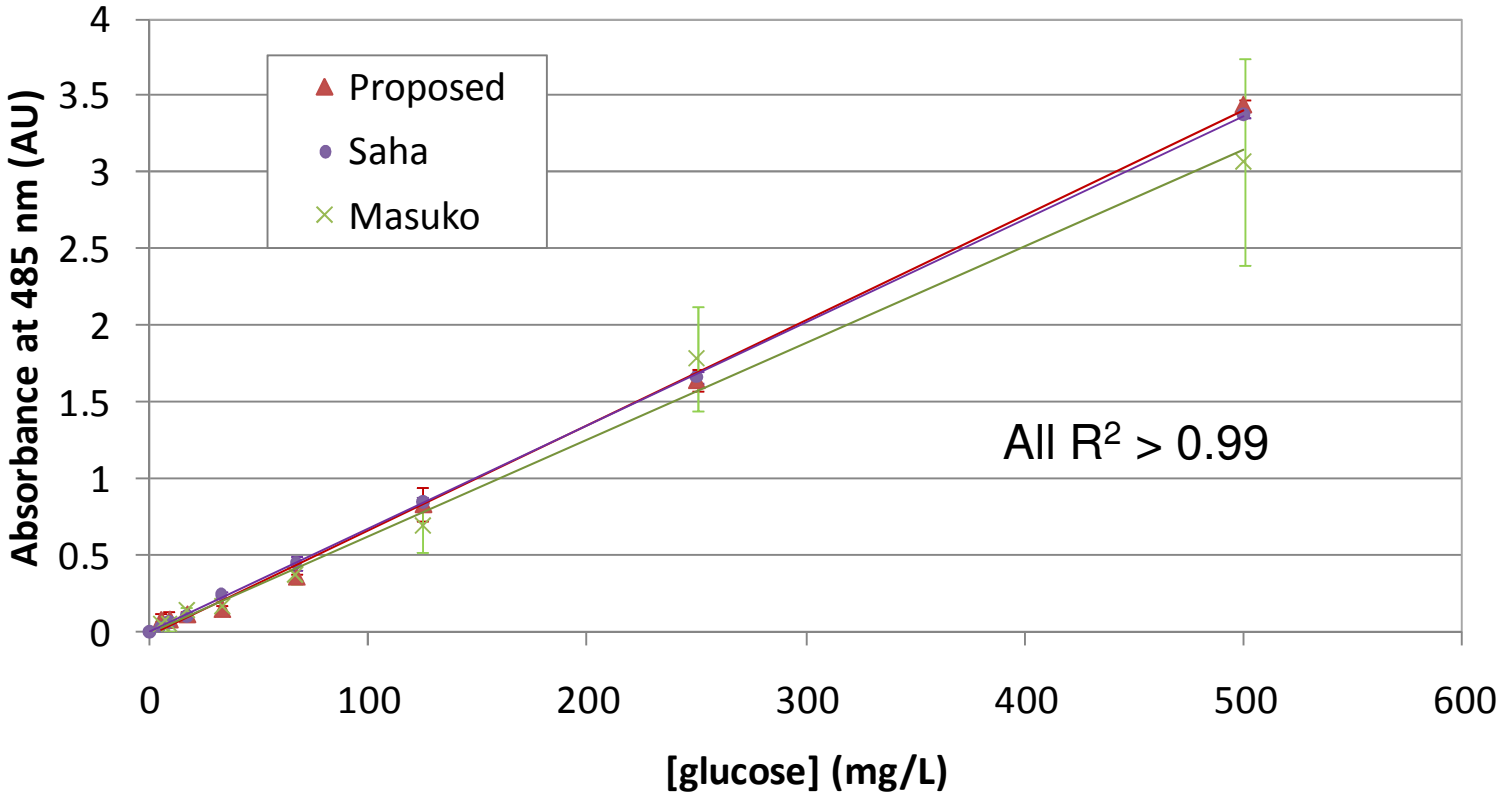
	Dubois et al	Saha et al	Masuko et al	Proposed
Year	1951	1994	2005	2012
Total Volume (µL)	8000	3500	230	175
Sample Volume (µL)	2000	500	50	25
Assay Range (mg/L)	5-35	10-100	4-585	10-1000
Vessel covering	yes	yes	no	no
External Heating	2 water baths	no	2 water baths	no
Shaking	yes	yes	no	no
Number of Steps	3	2	3	1

No covers required

Initial pipette aspirations provide only mixing

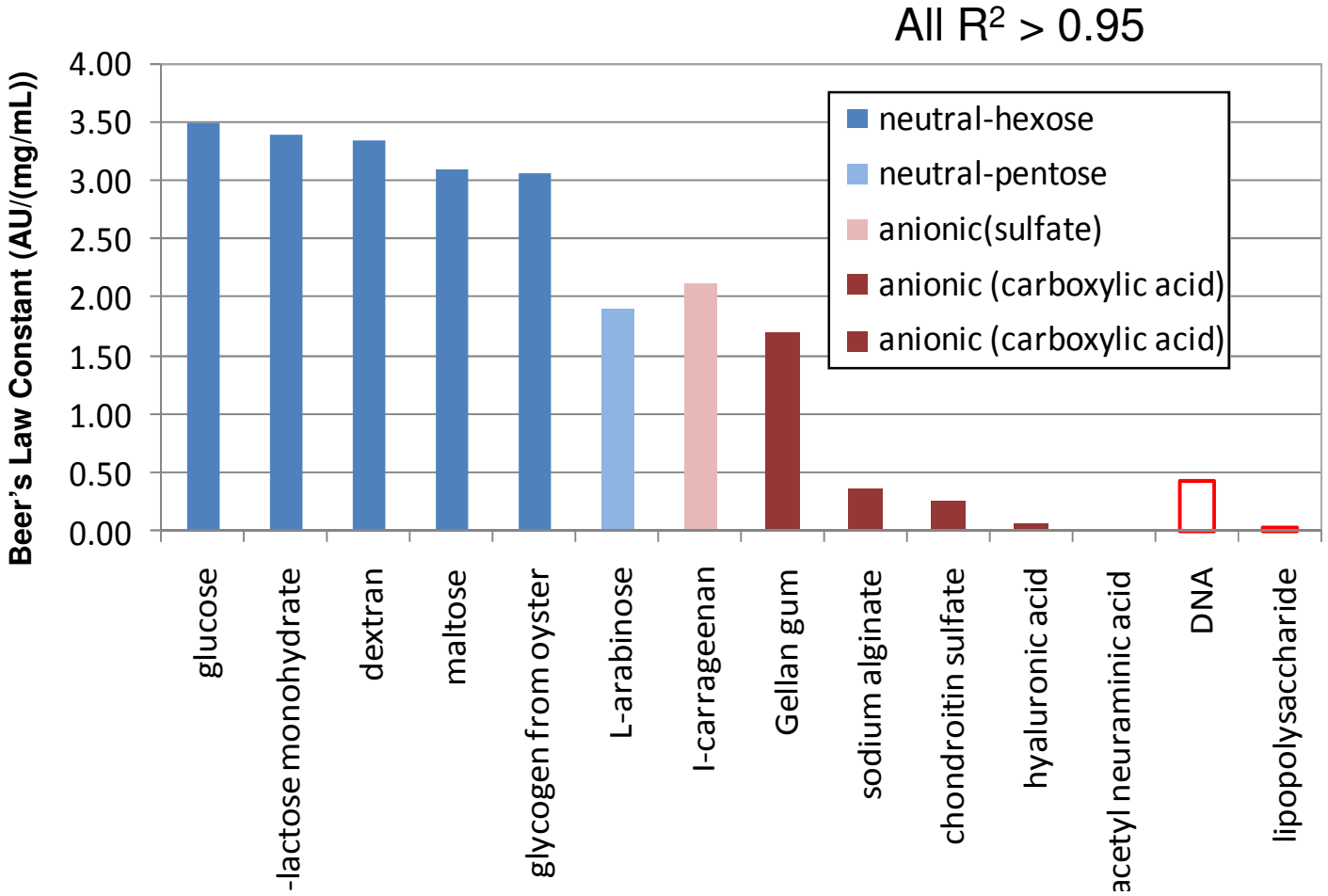
Heating is provided solely by exothermic reaction; polystyrene cp, κ, mass << glass

PHS: Glucose Standard Curves



- Similar reactivity with each method

PHS: Standard Curves for Other Sugars

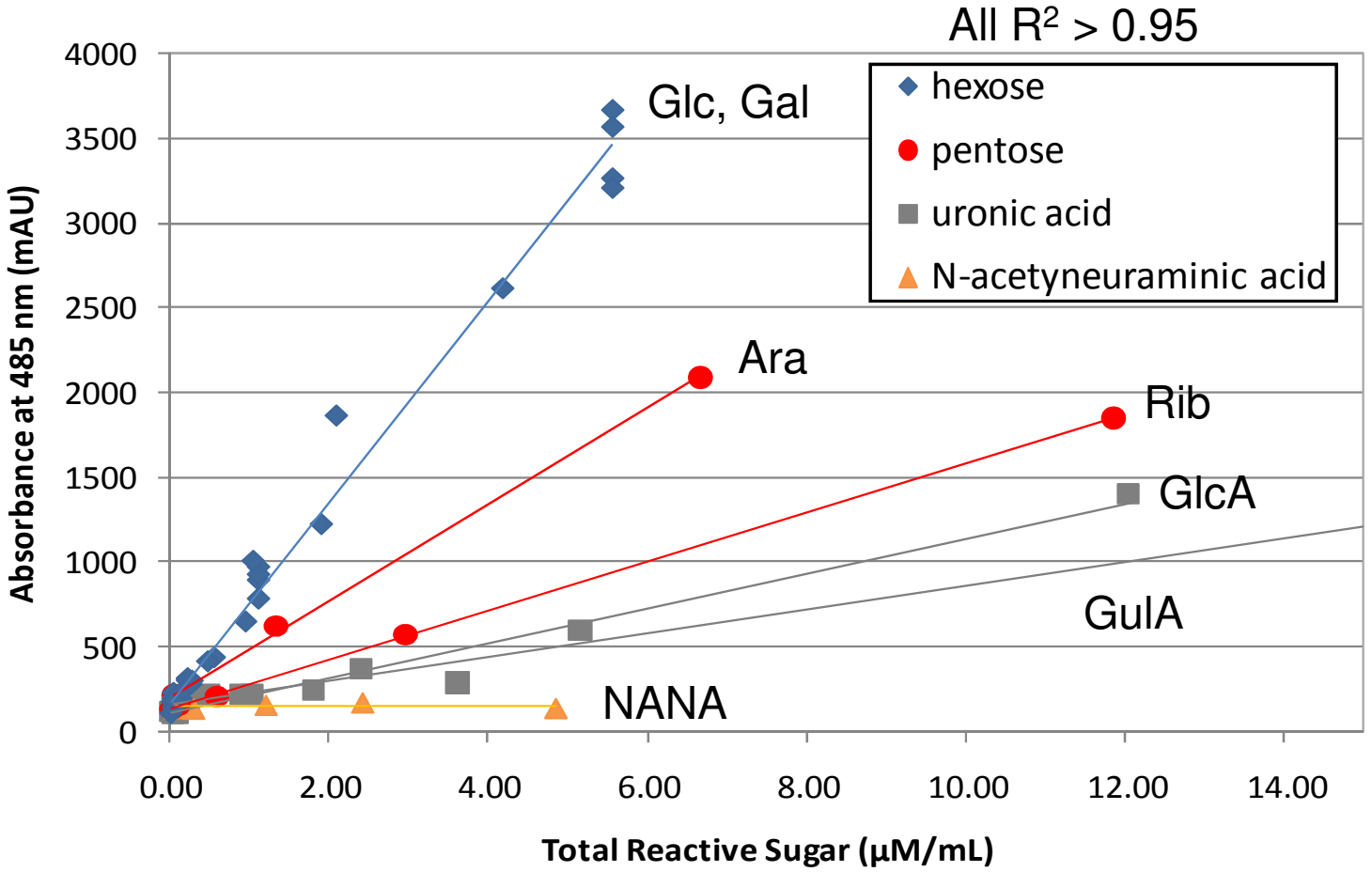


ι-Carrageenan: 1→3)-**α-D-Gal**-6-SO_3-(1→4)-**3-6-β-D-AnGal-2-SO_3**

Gellan gum: [**D-Glc**(β1→4)**D-GlcA**(β1→4)**D-Glc**(β1→4)**L-Rha**(α1→3)]$_n$

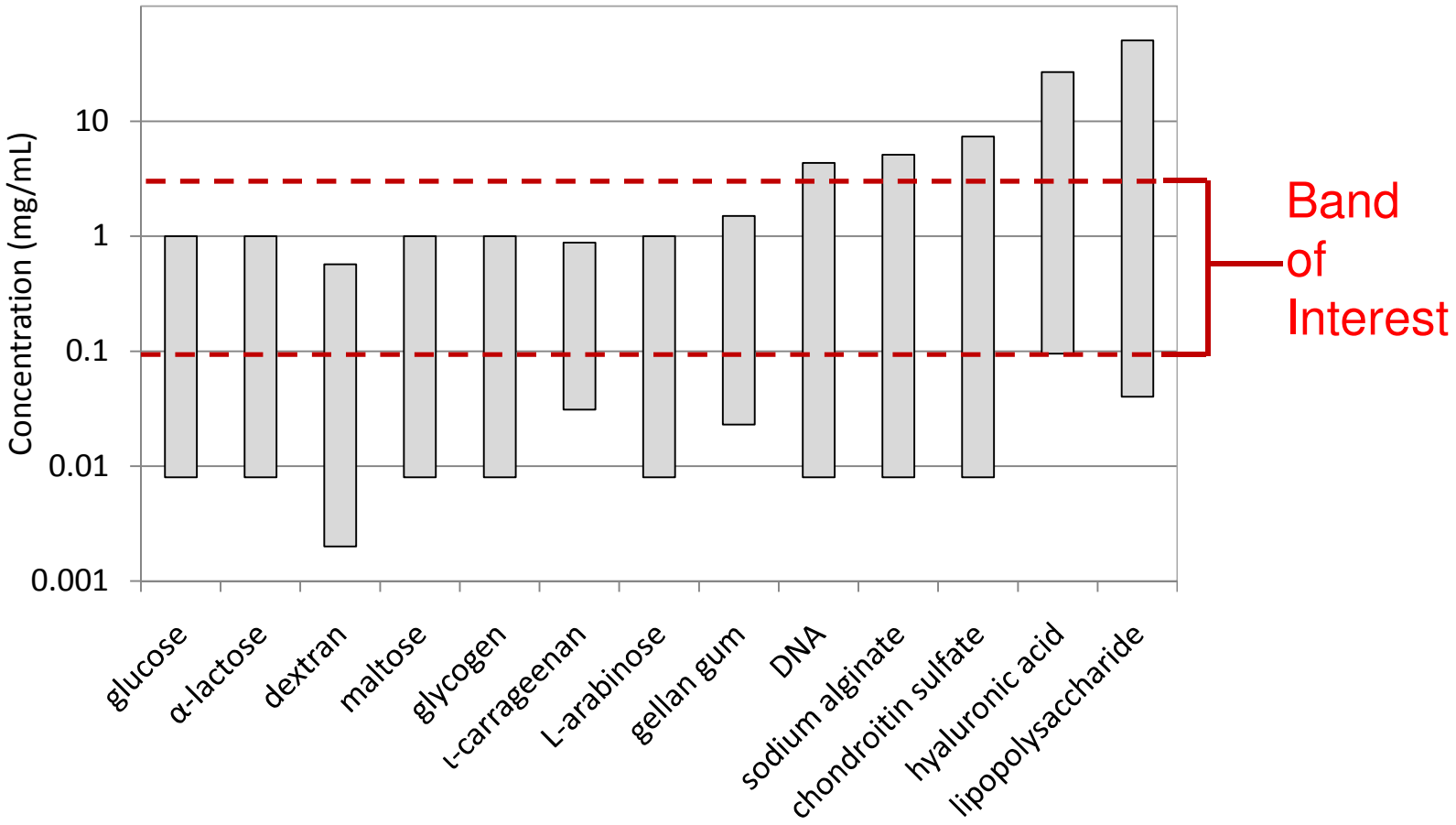
Hyaluronic acid: Ge: **D-GlcA-β1,3-D-GlcNAc-β1,4-]n**

PHS: Reactivity of Constituent Sugars



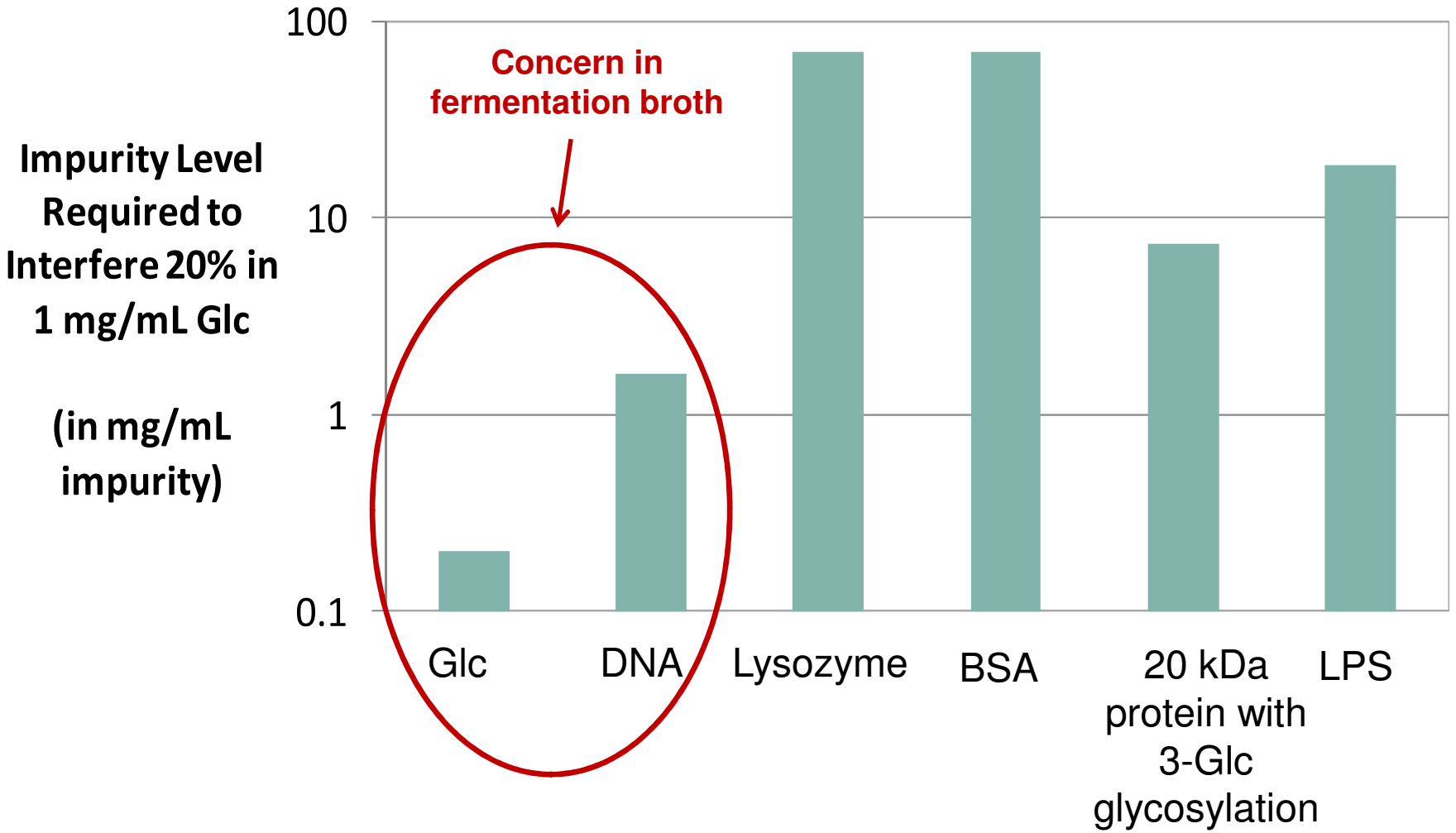
- Reactivity: hexoses > pentoses > uronic acids
- Can use this predicatively….like A_{280} with proteins

PHS: Dynamic Linear Range



- Broader linear range is advantageous for HTP

PHS: [Impurity] Required for Interference



PHS Conclusion

- PHS assay scaled-down to microplate
  - 96 samples in <1 h
  - No separate heating or agitation required
- 10-1000 µg/mL dynamic linear range
  - Appropriate for in-process samples
- Reacts with virtually all polysaccharides
- Basis of reaction verified
- Interference (i.e. DNA, sugars) is manageable

Protein Assays

Objectives

- Linearity
- Precision
- Universality
- Interference

Mechanisms

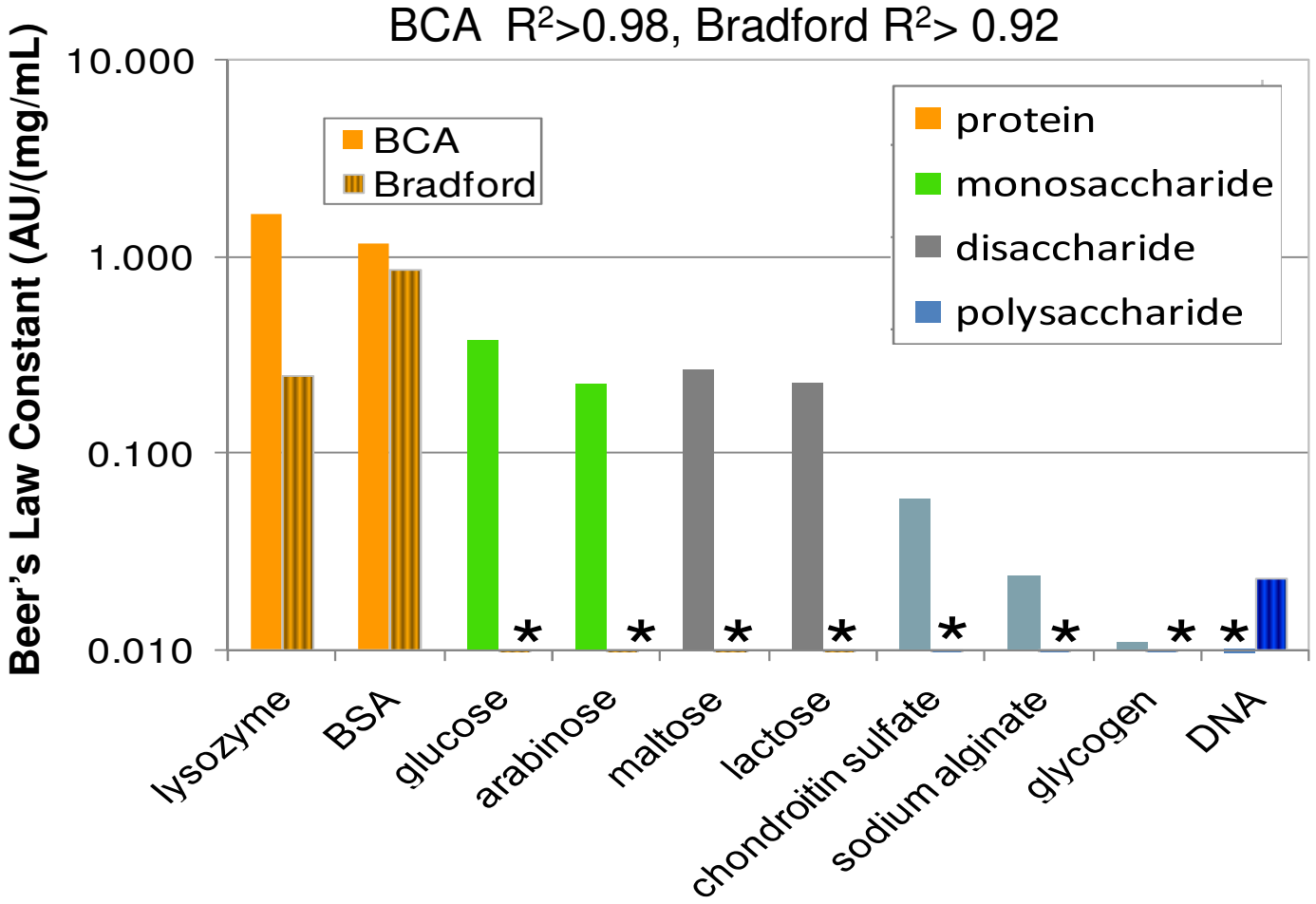
Bicinchoninic Acid (BCA)

- Protein tertiary structure, C, W, Y residues, and peptide bonds determine reactivity
- Dynamic Range: 2 logs
- Interferences: reducing agents, CTAB, thiol, lipids, strong acids/alkalis, reducing sugars

Bradford

- Binds basic and aromatic residues of amino acids
- Dynamic Range: 1 log
- Interferences: detergents, bases

Protein Assay: Reactivity and Interference



- Reducing sugars react in BCA

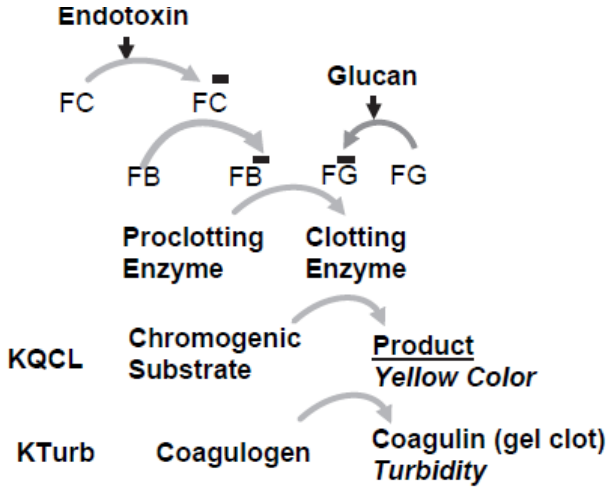
Endotoxin Assay

Objectives

- Linearity
- Precision
- Universality
- Interference

Mechanisms (enzymatic)

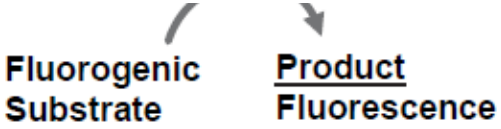
Kinetic QCL (LAL)



Dynamic Range: 0.005-50 EU/mL

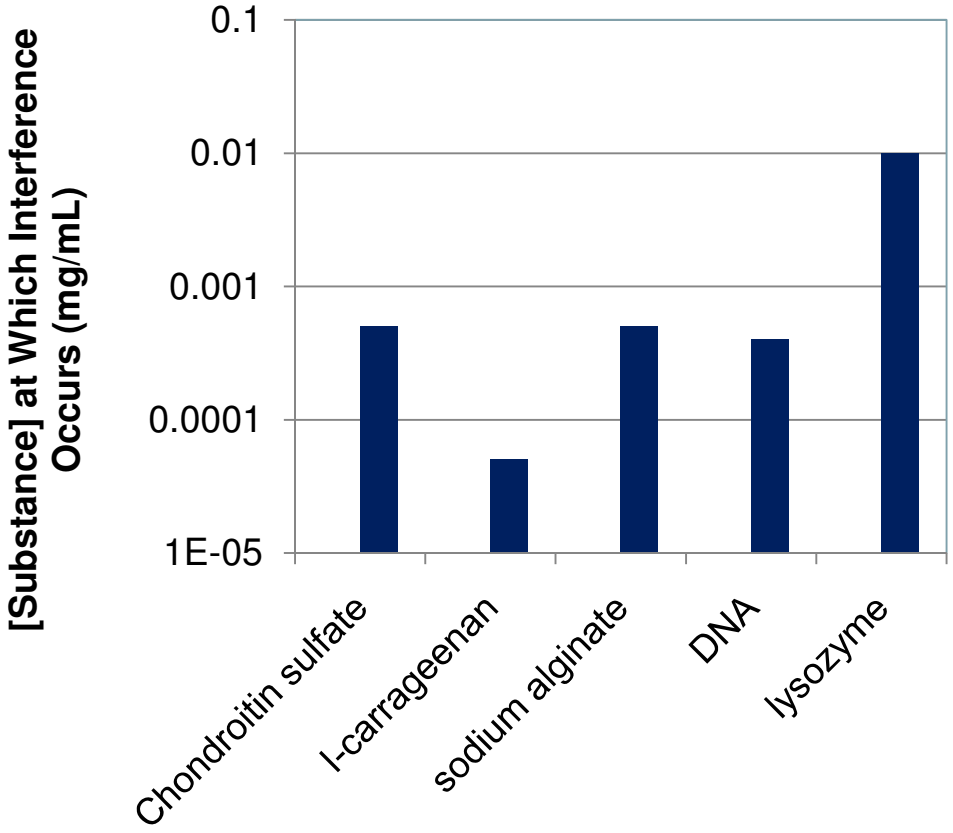
Pyrogene Recombinant Factor C

Modified to single point, room temperature measurement



Dynamic Range: 0.01-10 EU/mL

Endotoxin: Interference



- Several compounds inhibit assay but manageable for typical in-process polysaccharide: endotoxin ratios

	[Endotoxin] *(EU/mL)*	**[Endotoxin] with Assay Dilution** *(EU/mL)*	**Available Log Removal Value** *(LRV)*
Post-harvest	> 20,000,000	2,000-20,000	~5-6
Post-primary recovery	20,000	2-20	~2-3

Overall Improvements and Conclusion

	Sugar		Protein		Endotoxin	
	Previous	Proposed	Previous	Proposed	Previous	Proposed
# of sample	10	80	10	80	80	80
Time (min)	180	45	180	90	180	45
Time/sample (min/sample)	18	0.6	18	1.1	2.3	0.6
% Throughput Improvement	**30-fold**		**16-fold**		**4-fold**	
Heating Steps	Y	N	Y	Y	Y	N
Automatable	N	Y	N	Y	Y?	Y

- Interference, linearity, versatility, precision of assays verified in polysaccharide context
- Automatable analytical suite developed to support high throughput process development

Acknowledgments

- Pfizer
- UCL
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- Dr. Sa V. Ho (Pfizer)
- Thomas Emmons (Pfizer)
- Dr. Khurram Sunasara (Pfizer)
- Dr. Dave Brunner (Pfizer)

OBRIGADO!

THANK YOU!

QUESTIONS?