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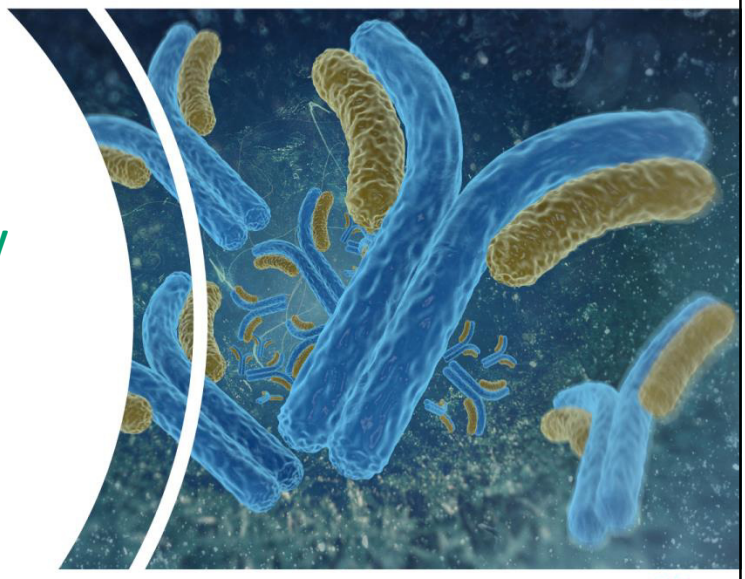
Increasing Productivity for Biopharmaceutical Analysis – Approaches for Chromatography Development



Greg Adams, Ph.D.
Hunter Walker, Ph.D.
Lauren Gilvey, MS.

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Analytical Development

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1

World Leading CDMO

3
Technologies
Microbial
Mammalian
Viral Vaccines / Gene Therapies

Apollo™ MAMMALIAN EXPRESSION PLATFORM
pAVEway™ ADVANCED PROTEIN EXPRESSION

6
LICENSES
For commercial manufacturing

+310
Projects
In process development and/or manufacturing

1,200
Employees
World Wide

SATURN™ mAb solutions

25+
YEARS
Of Biologics CDMO experience.

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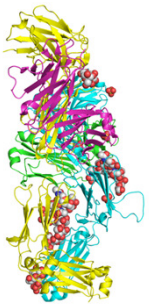
Proteins, they have personalities….

- Proteins are molecules with intricate three-dimensional structures that give each protein has a unique "personality"
- We need to understand the personality
- The analytical methods that evaluate quality attribute of the protein need to be reproducible and robust



3

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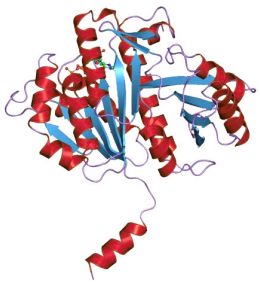
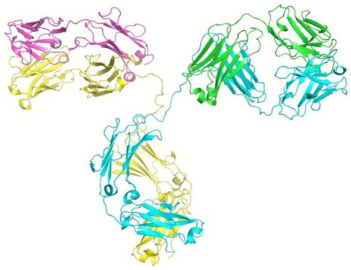
Identify the critical quality attribute(s)….



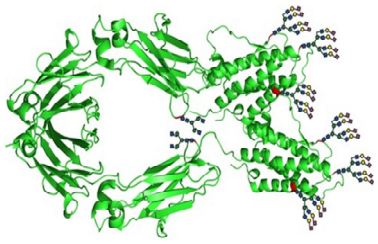
***Escherichia coli* expressed protein**

- Acylation
- Acetylation
- Gluconylation
- Formylation
- Deamidation
- Methylation
- Proteolysis
- Misfolding
- Norleucine, norvaline incorporation
- Des N-terminal methionine



Monoclonal Antibody

- Pyroglutamate formation
- Lysine C-terminal heterogeneity
- N-linked glycosylation
- Sialylation
- Aggregation
- Hinge clipping
- Methionine, cysteine, lysine, histidine, tryptophan oxidation
- Deamidation, succinimide formation
- Glycation



Mammalian cell expressed protein

- N-linked glycosylation
- O-linked glycosylation
- Phosphorylation
- Truncation
- Disulphide scrambling
- Oxidation
- Deamidation, succinimide formation

4

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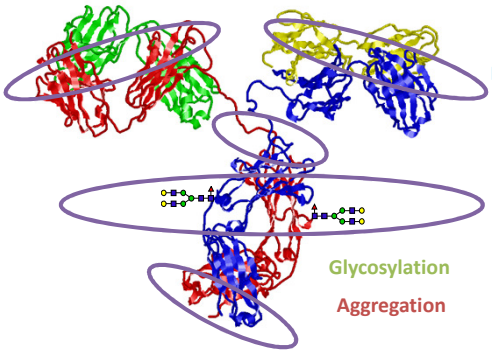
Analytical Tests to Assess mAb Heterogeneity



Titer

Pyroglutamate
Deamidation
Oxidation
Truncation
Hinge Clipping
Half Molecule
Disulfide

Glycosylation

Aggregation

ADC DAR

Size Exclusion UPLC	Reversed Phase UPLC	Affinity HPLC	Ion Exchange HPLC	Hydrophobic Interaction HPLC	Peptide Map UPLC	Glycan HILIC UPLC
• Separation based on size of the molecule • Aggregates, Fragments	• Separate oxidized, fragmented species • Reduced mAb characterization	• Titer by Protein A binding	• Separation based on charge • Monitor deamidation, sialyation, pyroglutamate	• Separate based on hydrophobicity under non denaturing conditions	• mAb Identity • PTM Characterization Disulphides • MAM	• Glycosylation Pattern

5

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Chromatography Requirements

- Columns
  - sizes from 3 – 30 cm
  - UPLC/HPLC
- Buffers
  - Salt/Aqueous
  - Organic
- Detectors
  - TUV
  - Fluorescence
  - ELSD

6

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Managing the modern Laboratory

- Multiple methods require different systems
- 8 potential chromatographic methods for each mAb
- How do we accelerate method development?
- How best to execute the workload of multiple chromatographic methods

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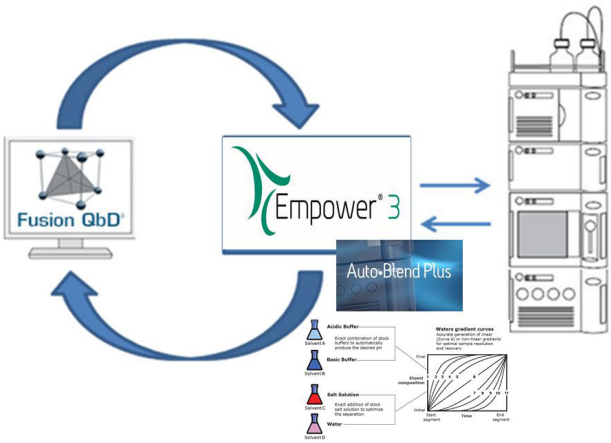
Software and Instrumentation Applications

- Fusion AE™ QBD Software
- AutoBlend Plus™ Technology



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SEC Method Development

Using Fusion AE™ QBD Software with the Acquity H-Class AutoBlend Plus™ Technology

Lauren Gilvey, MS.

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SEC Method Development

- 3 Day Method Development:
  - Day 1: Determine the column load requirements and if sample modifications are required
    - Ideally the sample will contain a Main Peak, a HMW peak and a LMW peak to ensure sufficient resolution
  - Day 2: Buffer Phosphate concentration / pH / Salt concentration
    - Fusion Experiment 1
  - Day 3: Buffer Optimization
    - Fusion experiment 2

10

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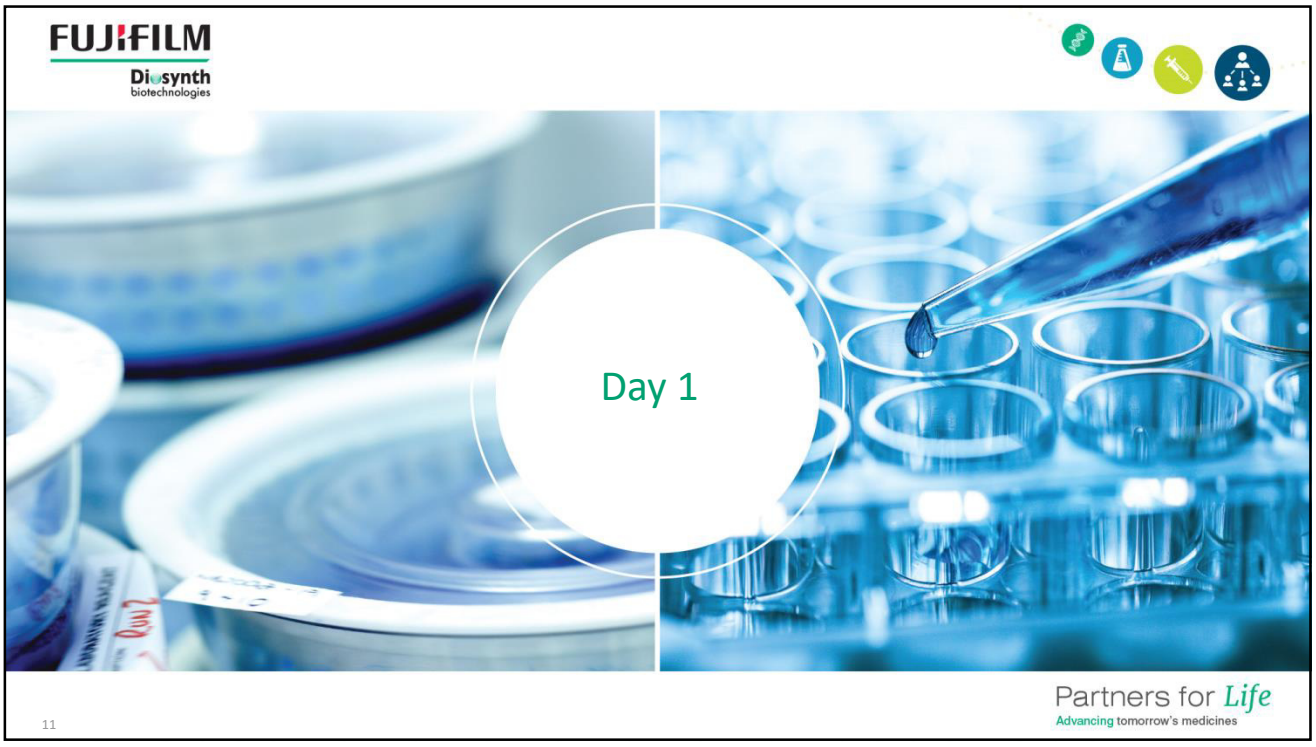

SEC Method Development: Day 1

- Prepare Mobile phases
  - Premade 0.5 M Sodium Phosphate, Monobasic
    - Adjust pH to 2.0 using Phosphoric Acid
  - Premade 0.5 M Sodium Phosphate, Dibasic
  - Premade 1 M Sodium Chloride
- Create 3 Samples
  - Un-spiked
    - If your sample already has a sufficient LMW and HMW content, the spike tests may not be needed
  - Spike 10% LMW
    - LMW: A sample of antibody is treated with FabRICATOR® digestion enzyme (Genovis) to cleave the antibody into Fc and F(ab')2 fragments (~50 kDa and ~100 kDa, respectively)
  - Spike 10% HMW
    - HMW: A sample of antibody is heated at 70°C for 4 hours then vortexed for 2 hours to create aggregates

12

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SEC Method Development: Day 1

- Our “standard” SEC method is used for this testing
  - 200 mm Sodium Phosphate, 100 mM Sodium Chloride, pH 7.2
- Determine the column load requirements
  - Inject the un-spiked sample at various load amounts
    - Typical column load is 15 µg, but this can vary from molecule to molecule Target 5 – 45 µL load.
    - Ideally the injection volume is < 10µL to avoid adding an injection loop

13

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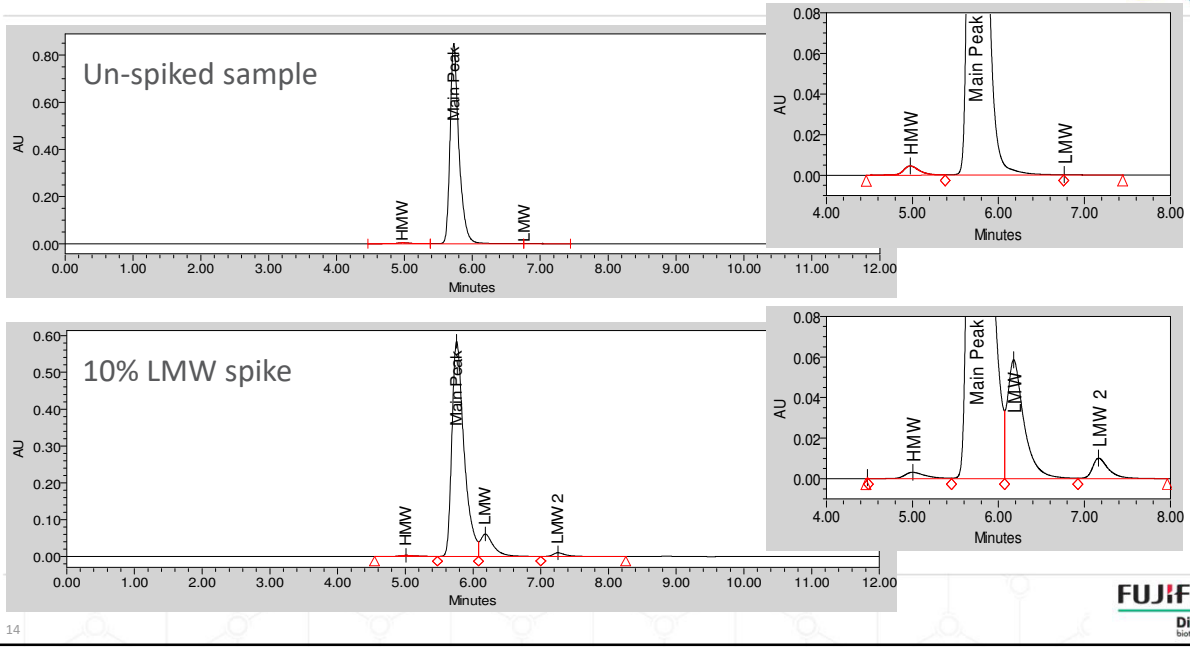
Modification using FabRICATOR®



14

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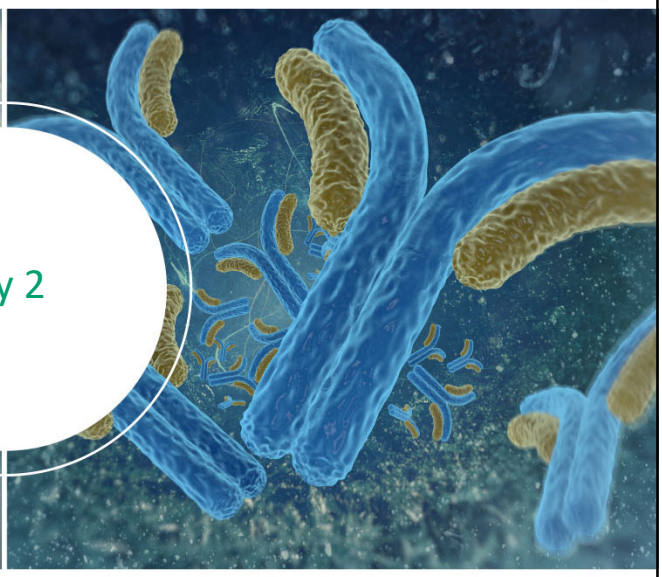
Day 2



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SEC Method Development: Day 2
Fusion Experimental Setup (Page 1)

Project Name	Experiment Name		Instrument Name	Experiment Phase	Experiment Type	Chromatography Type
Buffer, Salt, pH Setup	Experiment 1	Notes...	HPL-0384N	Method Development	General Optimization	Size Exclusion - Gel Filtration

Experiment Setup | Sampling Plan

Method Type: Isocratic

Available Variables: Sample Concentration, Salt Type, Buffer Type, Additive Concentration, Additive Type, Column Type

Included Variables: Pump Flow Rate, Injection Volume, Oven Temperature, Wavelength, pH, Salt Concentration

Activate Online Preparation

Name	Units	Type	Amount
Pump Flow Rate	mL/min	Discrete Numeric	0.200

State: Variable / Constant

Name	Units	Type	Amount
Injection Volume	uL	Discrete Numeric	8.0

State: Variable / Constant

Solvent Settings

No. of Strong Solvents: 1 | No. of Weak Solvents: 1 | Sample Preparation Mode: Online

OK to Blend Strong Solvents | OK to Blend Weak Solvents | Mobile Phase Precision

Mobile Phase Name	Solvent Type	State	Lower Bound	Upper Bound	Reservoir
Salt Solution	Strong	Constant	20.0	---	---
Buffer Solution	Weak	Constant	80.0	---	---

Available Reservoirs: A, B, C, D

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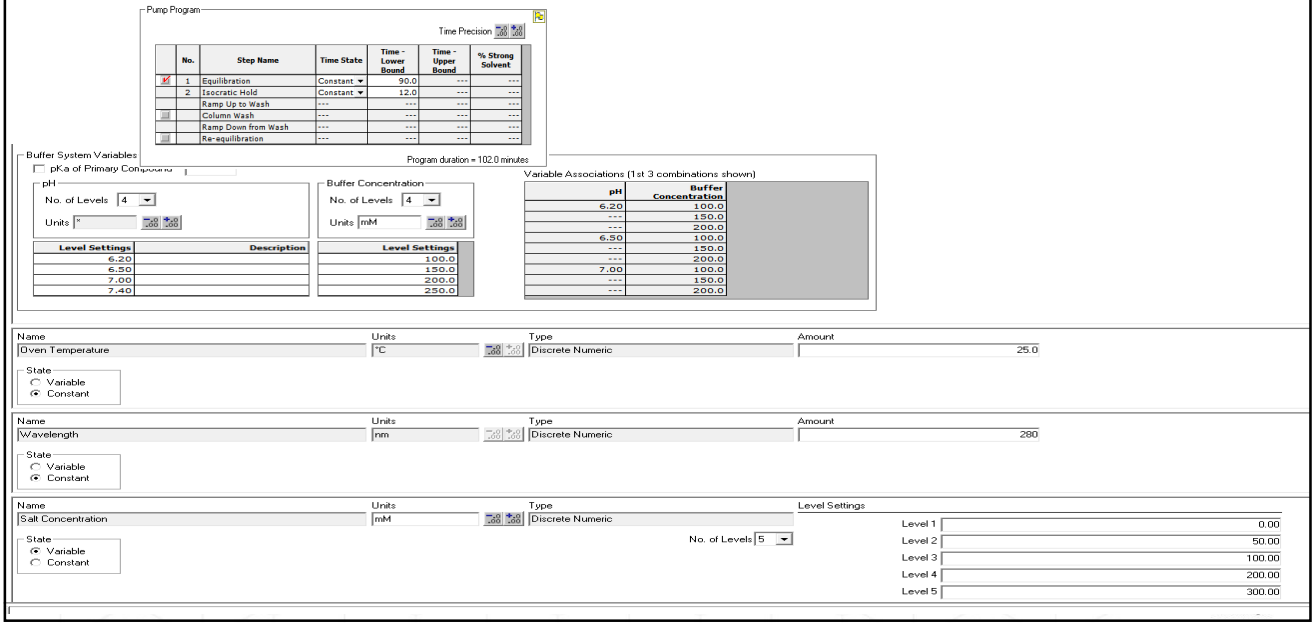
16

SEC Method Development: Day 2
Fusion Experimental Setup (Page 2)



SEC Method Development: Day 2
Instrument & Sample set method modifications

- Instrument & Sample set method modifications
  - The fusion experiment is exported into empower. This creates all of the Instrument methods, method sets, and sample sets.
  - The sample sets can be shortened to remove extra conditioning, washes and shut-downs
  - The instrument methods are modified to deliver the correct percentages of Mobile phases A, B, C and D for each targeted condition

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Modify Sample sets

	Plate/Well	Inj Vol (uL)	# of Injs	Label	SampleName	Level	Function	Method Set / Report Method	Run Time (Minutes)	Column Position
1							Condition Column	Exp1_ 001_035	10.00	No Change
2							Equilibrate	Exp1_ 001_001	90.00	No Change
3	1:A,1	0.1	1	Unk-001-001	01_100mM Phos_0mM Salt_pH6.2		Inject Samples	Exp1_ 001_001	12.00	
4							Condition Column			
5							Equilibrate			
6	1:A,1	0.1	1	Unk-001-002	02_100mM Phos_0mM Salt_pH7.2		Inject Samples			
7							Equilibrate	Exp1_ 001_003	90.00	No Change
8	1:A,1	0.1	1	Unk-001-003	03_100mM Phos_0mM Salt_pH7.2		Inject Samples	Exp1_ 001_003	12.00	
9							Condition Column	Exp1_ 001_037	2.00	No Change
10							Equilibrate	Exp1_ 001_004	90.00	No Change
11	1:A,1	0.1	1	Unk-001-004	04_150mM Phos_0mM Salt_pH6.2		Inject Samples	Exp1_ 001_004	12.00	
12							Condition Column	Exp1_ 001_038	2.00	No Change
							Condition Column	Shutdown	10.00	No Change

Remove all of the Column Condition lines (Leave the equilibrate steps)

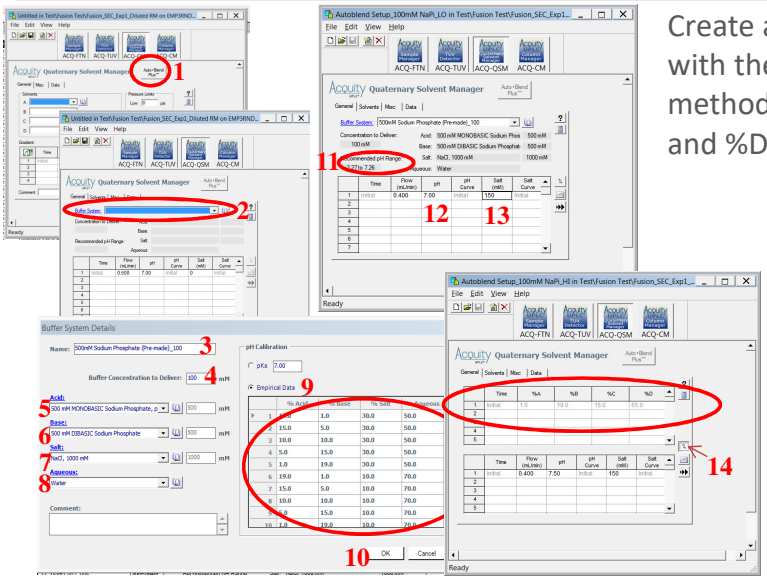
Change the Vial to 1A1 in all the sample sets

Remove shutdown from each sample set

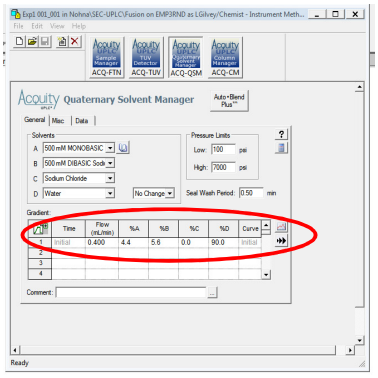
	Plate/Well	Inj Vol (uL)	# of Injs	Label	SampleName	Level	Function	Method Set / Report Method	Run Time (Minutes)	Column Position
1							Equilibrate	Exp1_ 001_001	90.00	No Change
2	1:A,1	0.1	1	Unk-001-001	01_100mM Phos_0mM Salt_pH6.2		Inject Samples	Exp1_ 001_001	12.00	
3							Equilibrate	Exp1_ 001_002	90.00	No Change
4	1:A,1	0.1	1	Unk-001-002	02_100mM Phos_0mM Salt_pH7.2		Inject Samples	Exp1_ 001_002	12.00	
5							Equilibrate	Exp1_ 001_003	90.00	No Change
6	1:A,1	0.1	1	Unk-001-003	03_100mM Phos_0mM Salt_pH7.2		Inject Samples	Exp1_ 001_003	12.00	
7							Equilibrate	Exp1_ 001_004	90.00	No Change
8	1:A,1	0.1	1	Unk-001-004	04_150mM Phos_0mM Salt_pH6.2		Inject Samples	Exp1_ 001_004	12.00	

19

Modify Instrument methods



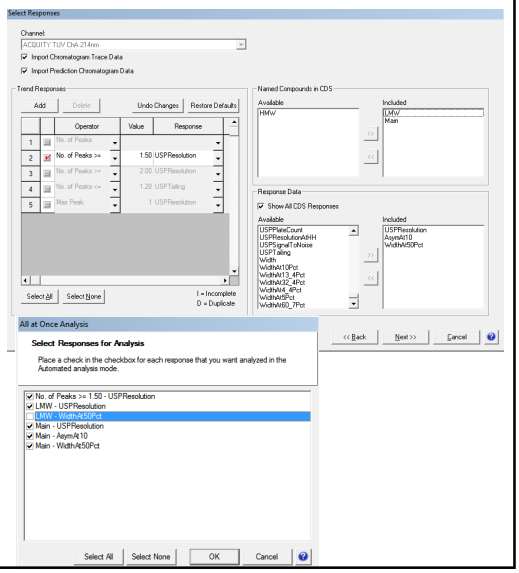
Create an Auto-Blend Plus method with the empirical tables. Use this method to determine the % A, %B, %C and %D for each fusion method



20

SEC Method Development: Day 2 Experimental Results

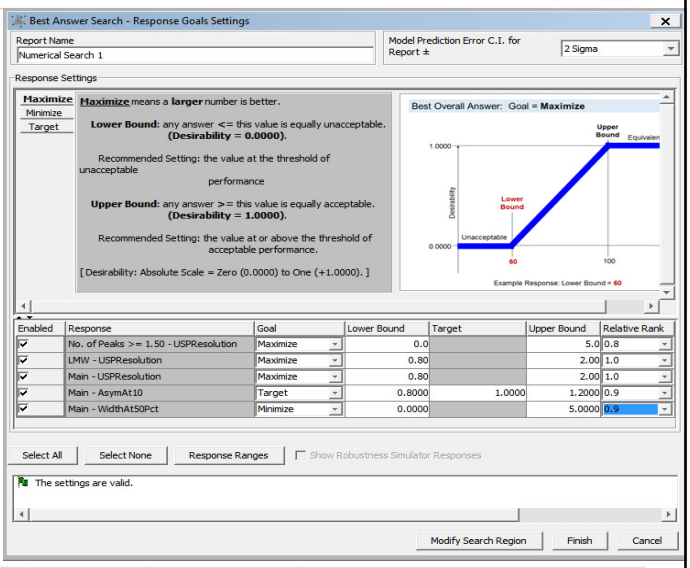
- Results processed in Empower, and imported into Fusion Software
- Select the criteria important to the assay
  - For Example:
    - Maximize the number of peaks with a USP Resolution ≥ 1.50
    - Maximize the USP Resolution between the peaks
    - Minimize the main peak width at 50% (minimizes tailing)
    - Target an asymmetry of 1.0 at 10% on the Main peak (minimizes tailing)



21

SEC Method Development: Day 2 Experimental Results

- Set the boundaries for your search criteria
- Rank which criteria are most important …
  - 1 = Most important
  - 0.1 = Least important

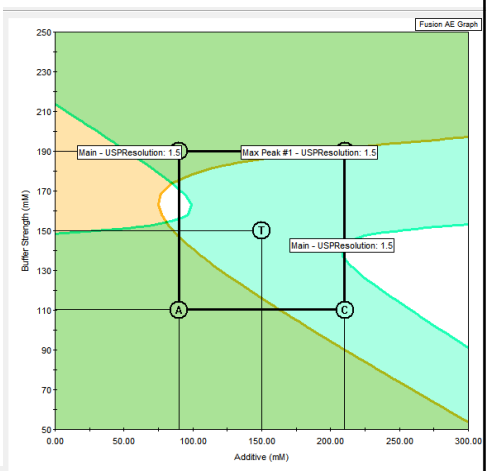


22

Acceptable Performance Region
EXAMPLE: Non-Robust Region

- "White Space" or operating space is determined where all criteria are met via statistical calculation.
  - Red = Maximize the number of peaks (minimum of 2)
  - Blue = Maximize the number of peaks with a USP Resolution ≥ 1.50
  - Green = Maximize the number of peaks with a USP Resolution ≥ 2.00
  - Orange = Maximize the USP Resolution of Peak #1 (minimum of 1.5)
  - Teal = Maximize the USP Resolution of the Main Peak (minimum of 1.5)
  - Purple = Minimize the width at 50% of the Main peak (Reduce tailing) < 0.16

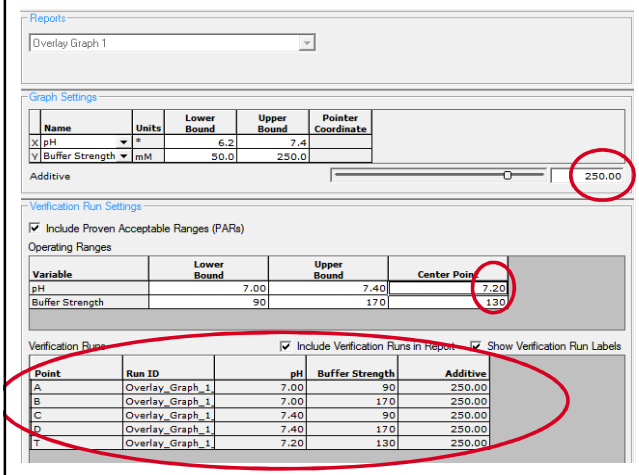
- The small white space in this image indicates a limited operating range under these conditions



Response Settings

Name	Goal	Lower Bound	Upper Bound	Target Predictions	Pointer Predictions	Contour Label	Color
No. of Peaks	Maximize	2.0		7.7		☑	Red
No. of Peaks >= 1.50 - USPResolution	Maximize	0.0		1.7		☑	Blue
No. of Peaks >= 2.00 - USPResolution	Maximize	0.0		0.4		☑	Green
Max Peak #1 - USPResolution	Maximize	1.500000000		1.583746555		☑	Orange
Main - USPResolution	Maximize	1.50		1.50		☑	Teal
Main - WidthAt50Pct	Minimize		0.163833367	0.126924419		☑	Purple

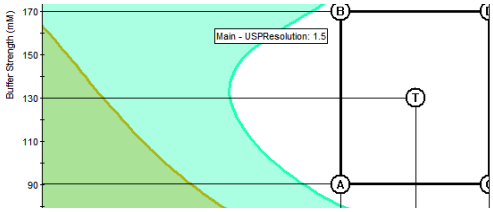
Acceptable Performance Region
EXAMPLE: Robust Region



The large white region in this graph indicates a greater area of assay robustness.

In this example, the targeted (T) optimum operating conditions are:

130mM Sodium Phosphate pH 7.2
250mM Sodium Chloride



In this example, the Robust Range is (A,B,C,D):

90 – 170 mM Sodium Phosphate
pH 7.00 – 7.40
250mM Sodium Chloride

Main - USPResolution	Maximize	1.50		1.72		☑	Teal
Main - WidthAt50Pct	Minimize		0.163833367	0.139180580		☑	Purple

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Day 3

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SEC Method Development: Day 3
Test the Robust Range

- The next step in the Fusion Software, creates the Robustness Testing Sample set / Method sets
- In this example the following conditions will be tested:

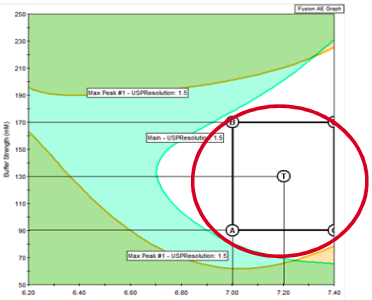
(A): pH 7.00, 90 mM Sodium Phosphate, 250 mM Sodium Chloride

(B): pH 7.00, 170 mM Sodium Phosphate, 250 mM Sodium Chloride

(C): pH 7.40, 90 mM Sodium Phosphate, 250 mM Sodium Chloride

(D): pH 7.40, 170 mM Sodium Phosphate, 250 mM Sodium Chloride

(T): pH 7.20, 130 mM Sodium Phosphate, 250 mM Sodium Chloride



Reports

Overlay Graph 1

Graph Settings

	Name	Units	Lower Bound	Upper Bound	Pointer Coordinate
X	pH	*	6.2	7.4	
Y	Buffer Strength	mM	50.0	250.0	

Additive 250.00

Verification Run Settings

Include Proven Acceptable Ranges (PARs)

Operating Ranges

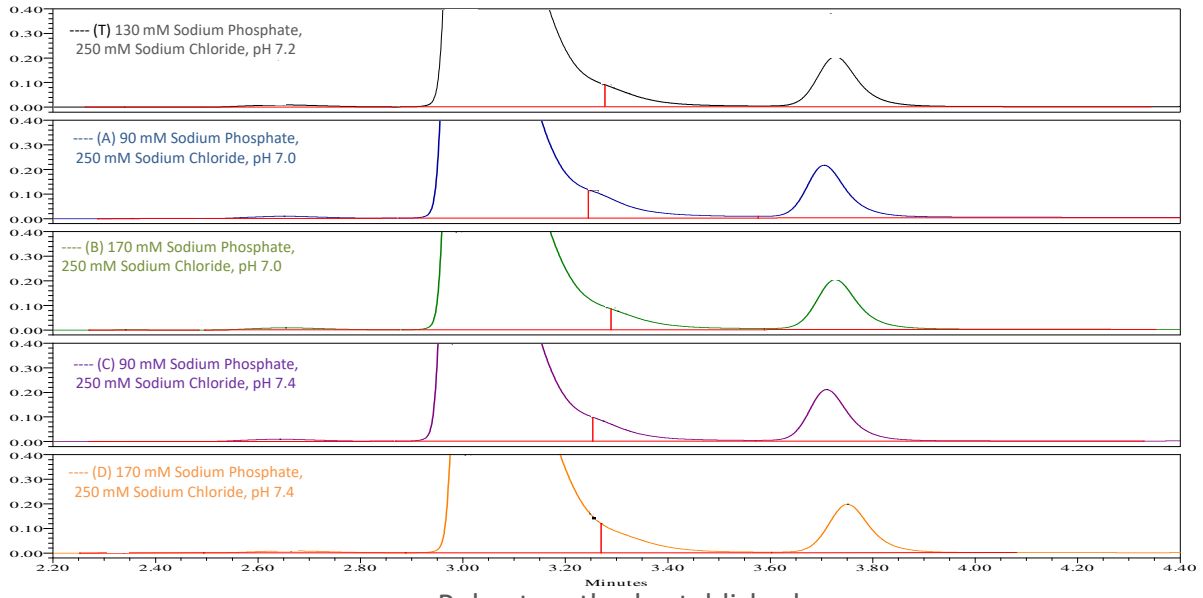
Variable	Lower Bound	Upper Bound	Center Point
pH	7.00	7.40	7.20
Buffer Strength	90	170	130

Verification Runs — Include Verification Runs in Report — Show Verification Run Labels

Point	Run ID	pH	Buffer Strength	Additive
A	Overlay_Graph_1	7.00	90	250.00
B	Overlay_Graph_1	7.00	170	250.00
C	Overlay_Graph_1	7.40	90	250.00
D	Overlay_Graph_1	7.40	170	250.00
T	Overlay_Graph_1	7.20	130	250.00

26

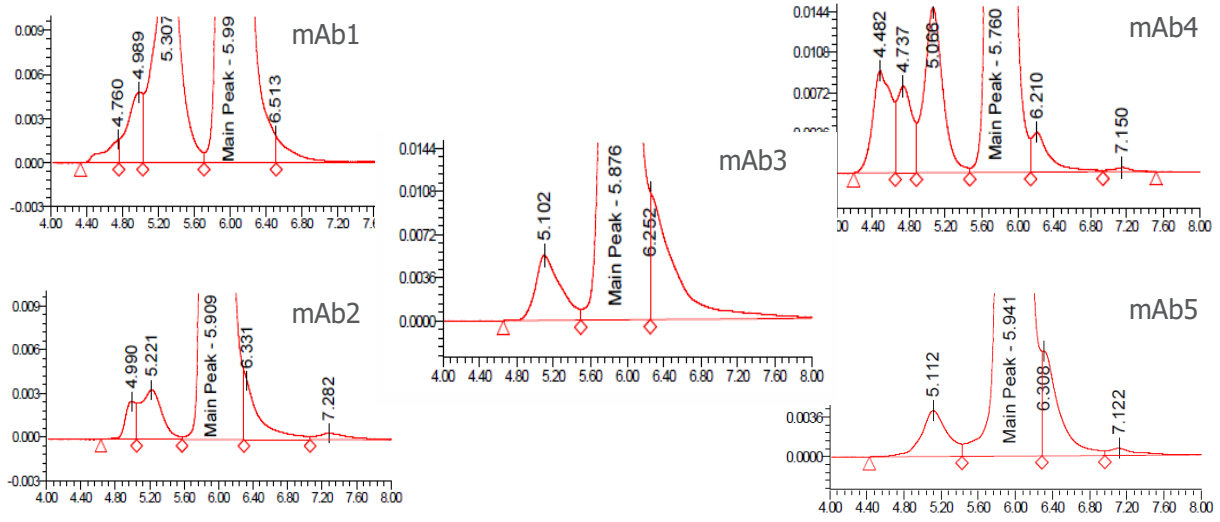
SEC Method Development: Day 3
Test the Robust Range



27

Robust method established

Applied Across Multiple mAbs



28

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Fusion + Autoblend Benefits

- SEC development is accomplished in 3 days – huge time saver
- Using Autoblend Plus we only have to prepare 4 solutions
  - Using premade buffers we only have to adjust the pH of the monobasic
- Using Fusion AE QBD software we can be confident the method is robust

30

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Protein A Titer and SEC Combination Platform mAb Analysis Strategy

Hunter Walker, Ph.D.

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Protein A Titer/SEC Combination Platform

- **Purpose:** Use Waters Autoblend feature to harmonize Protein A titer and SEC mAb analyses into a single instrument platform.
- **Strategy:** Generate a Phosphate/NaCl buffer set that accommodates buffer systems for both Protein A Titer and SEC
  - pH range from ~2.8-7.0.
  - NaCl concentration range from 0-300 mM.
  - Phosphate concentration range from 0-250 mM.
- **GOAL**: Perform Protein A titer and SEC using the same instrument, buffers, and samples in the same day.

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Phosphate and Sodium Chloride Stock Buffers

- **Components:**
  - Phosphate Monobasic, Phosphate Dibasic, NaCl, Water
  - Each component must be concentrated enough to allow for range of possible Phosphate and NaCl concentrations for molecule-specific optimization.
- **Platform Buffers:**
  - Mobile Phase A: 400 mM Sodium Phosphate Monobasic, pH 2.0.
  - Mobile Phase B: 400 mM Sodium Phosphate Dibasic
  - Mobile Phase C: 1000 mM Sodium Chloride
  - Mobile Phase D: Water

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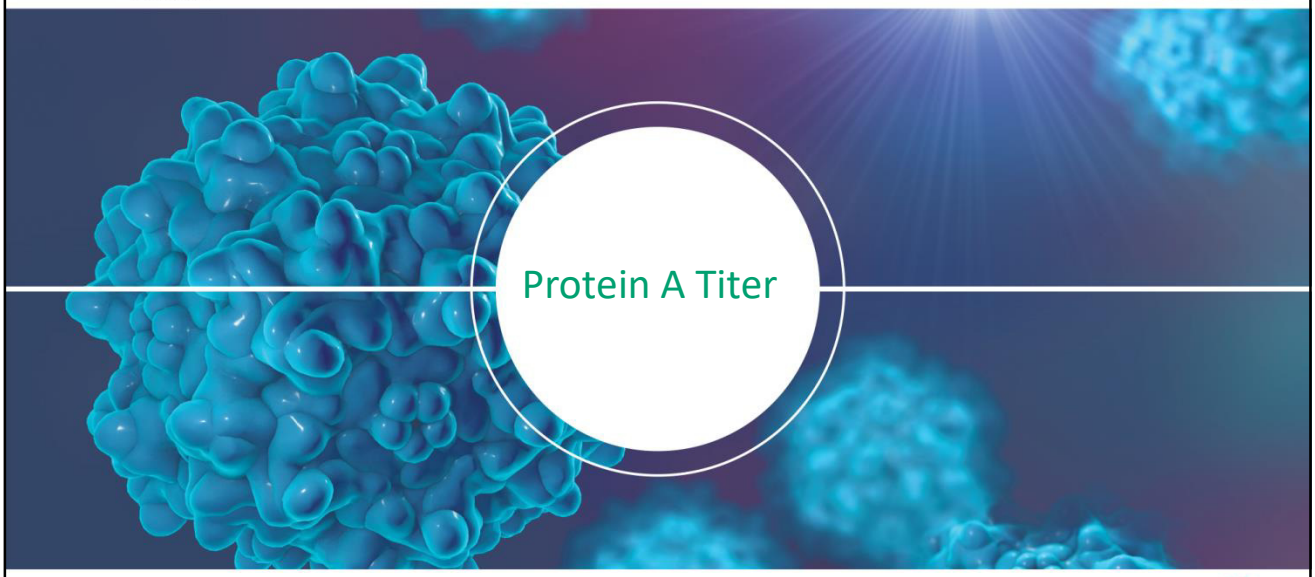

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Protein A Titer Buffer Table

- **HPLC Titer Method:**
  - Equilibration Buffer: pH 7.0, 300 mM NaCl, 20 mM Phosphate
  - Elution Buffer: pH 2.8, 300 mM NaCl, 20 mM Phosphate

Covers entire range of both elution and equilibration buffers of conventional method.

Autoblend Buffer Table

Final Concentration of $NaPO_4$	Final Concentration of NaCl	% 400 mM Monobasic, pH 2.0	% 400 mM Dibasic	% 1000 mM NaCl	% Water	pH
20	0	4.5	0.5	0	95	2.55
20	0	2.5	2.5	0	95	6.44
20	0	0.5	4.5	0	95	7.72
20	150	4.5	0.5	15	80	2.39
20	150	2.5	2.5	15	80	6.18
20	150	0.5	4.5	15	80	7.45
20	300	4.5	0.5	30	65	2.46
20	300	2.5	2.5	30	65	6.04
20	300	0.5	4.5	30	65	7.24

36

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Protein A Titer Method

- Use Autoblend in combination with the buffer/pH tables to generate the Protein A Titer equilibration and elution conditions:

Mobile Phase A: 400 mM Sodium Phosphate Monobasic, pH 2.0
Mobile Phase B: 400 mM Sodium Phosphate Dibasic
Mobile Phase C: 1000 mM Sodium Chloride
Mobile Phase D: Water

Gradient Table

Time	Flow (mL/min)	% A	% B	% C	% D
Initial	1.5	0.9	4.1	30.0	65.0
0.5	1.5	0.9	4.1	30.0	65.0
0.7	1.5	4.3	0.7	30.0	65.0
2.5	1.5	4.3	0.7	30.0	65.0
2.6	1.5	0.9	4.1	30.0	65.0
3.0	1.5	0.9	4.1	30.0	65.0
3.1	1.5	4.3	0.7	30.0	65.0
3.6	1.5	4.3	0.7	30.0	65.0
3.8	1.5	0.9	4.1	30.0	65.0
5.0	1.5	0.9	4.1	30.0	65.0

37

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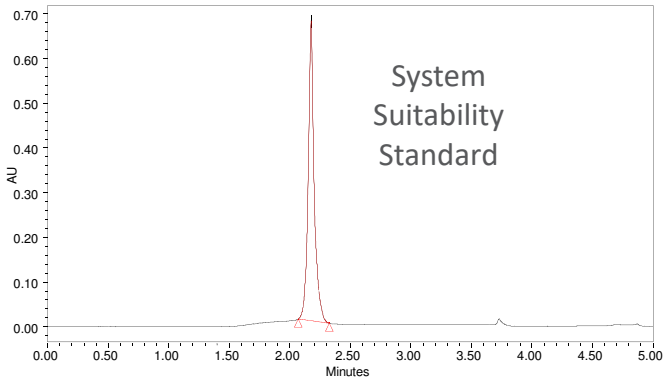
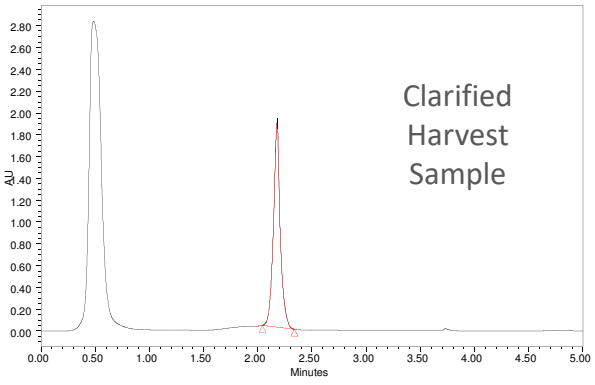
Protein A Titer Method





38

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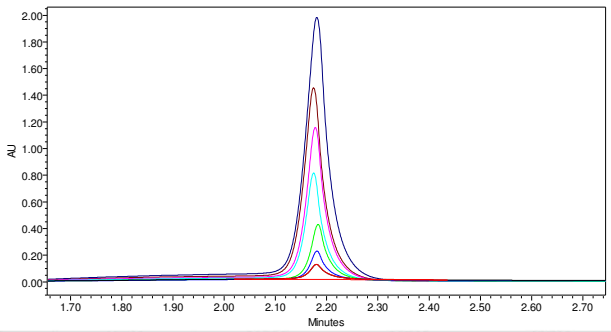
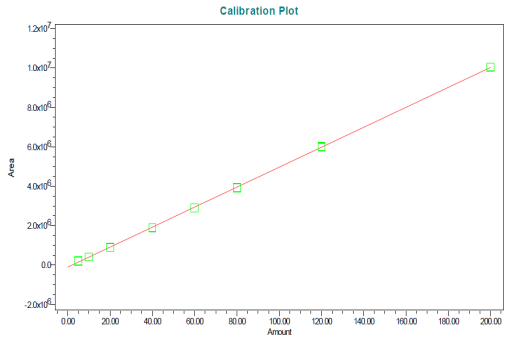
Protein A Titer Method

- Linearity Standards prepared at 0.5 and 4.0 mg/mL and injected at the following levels:
  - 5, 10, 20, 40, 60, 80, 120, and 200 µg loads.
- The assay is shown to be linear ($R^2 \geq 0.999$) at all levels from 5-200 µg loads





39

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Size Exclusion Chromatography

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Size Exclusion Chromatography

- **Fusion DOE Buffer Screening:**
  - Parameters: Varied pH (6.2-7.4), phosphate concentration (50-250 mM), and NaCl concentration (0.0-300 mM)
  - Optimized Buffer: 150 mM Sodium Phosphate, 150 mM NaCl, pH 6.8

Autoblend Buffer Table

Final Concentration of $NaPO_4$	Final Concentration of NaCl	% 400 mM Monobasic, pH 2.0	% 400 mM Dibasic	% 1000 mM NaCl	% Water	pH
150	0	18.7	18.8	0.0	62.5	6.29
150	0	12.5	25.0	0.0	62.5	6.72
150	0	5.0	32.5	0.0	62.5	7.33
150	150	18.7	18.8	15.0	47.5	6.10
150	150	12.5	25.0	15.0	47.5	6.56
150	150	5.0	32.5	15.0	47.5	7.20
150	300	18.7	18.8	30.0	32.5	6.00
150	300	12.5	25.0	30.0	32.5	6.50
150	300	5.0	32.5	30.0	32.5	7.10

41

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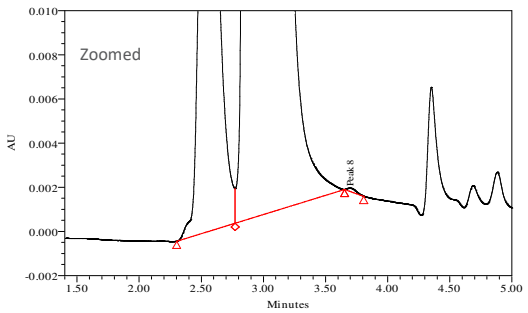
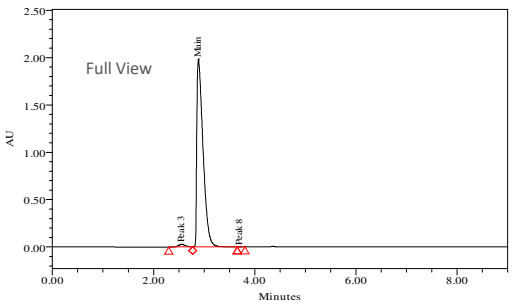
Size Exclusion Chromatography

Gradient Table:

Time	Flow (mL/min)	% A	% B	% C	% D
Initial	0.400	9.7	27.8	15.0	47.5
9.00	0.400	9.7	27.8	15.0	47.5

Mobile Phase A: 400 mM Sodium Phosphate Monobasic, pH 2.0
Mobile Phase B: 400 mM Sodium Phosphate Dibasic
Mobile Phase C: 1000 mM Sodium Chloride
Mobile Phase D: Water





42

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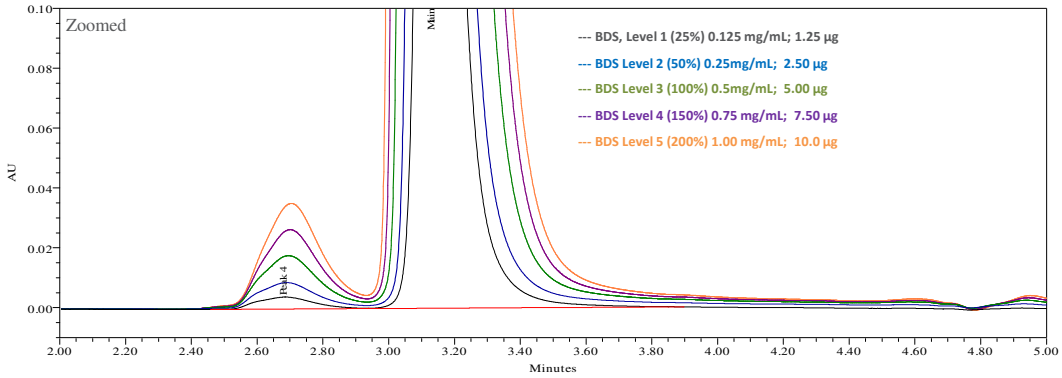
Size Exclusion Chromatography

- Reference material injected at 5 different levels from 1.25 µg to 10 µg loaded on column

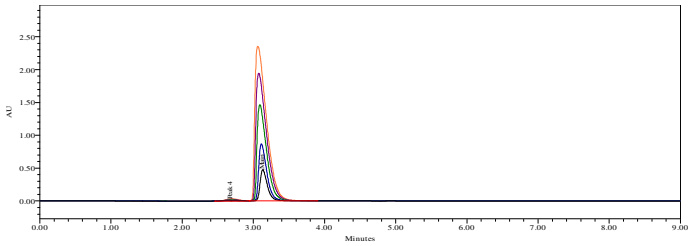


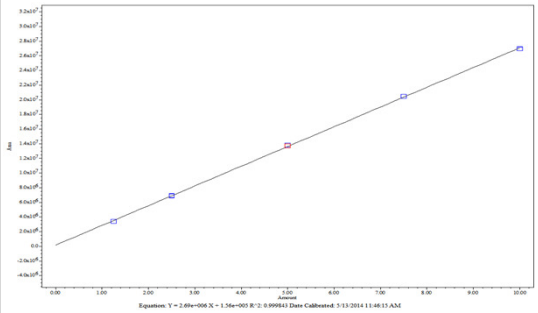
43

FUJIFILM Diosynth biotechnologies

Size Exclusion Chromatography

- Reference material injected at 5 different levels from 1.25 µg to 10 µg loaded on column





- Linearity demonstrated from 1.25 µg to 10 µg loaded on column ($R^2 \geq 0.999$)

44

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Platform Harmonization Benefits

Minimize Time, Cost, and Supplies

- Consolidate mobile phase preparation and instrument set-up time.
- Two-column setup allows for both Protein A Titer and SEC samples to be analyzed on the same day using the same instrument.
  - 60 minute equilibration used between each method.
- Stock Buffers easily prepared (i.e. All buffers are a single component, and only Mobile Phase A needs pH adjustment).
- Often, samples can be analyzed neat by both methods, minimizing sample preparation time.

46

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Acknowledgments

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