Sample Prep Automation for Biopharmaceutical Analysis

Kenda LJ Evans, PhD
Biopharma Workflow Solutions to Accelerate Biomolecule Characterization

Data Acquisition → MS Spectrum Deconvolution → Zero-charge Mass Determination → Sequence Matching
Determine Post Translational Modifications

Chromatographic Separation

Sample Preparation

Complete Agilent Workflow Solution

Report → Delivery of Results
AssayMAP For Biopharma Characterization Sample Prep

Automated workflows designed for analytical chemists

**Simple User Interface**
Uses customer language - not automation language
- Affinity (protein) Purification
- In-Solution Digestion
- Peptide Cleanup (desalting)
- Protein Cleanup (desalting)
- Phosphopeptide Enrichment
- Normalization
- Fractionation
- N-Glycan Sample Prep

**Positive Displacement Pipetting**
Syringes interface directly with cartridges and enable precise, controlled liquid flow through cartridges with no air bubbles to disrupt binding

**Microchromatography Cartridges**
quantitative binding & elution

Protein purification
- PA-W (protein A)
- PG-W (protein G)
- SA-W (streptavidin)

Reversed-phase cleanup:
- C18 (silica)
- RP-S (polymeric)

Fractionation:
- SCX
- RP-S
- C18

Phosphopeptide enrichment:
- TiO$_2$
- FE(III) NTA
AssayMAP Technology
Complete AssayMAP Automation Portfolio

Antibody Purification

Immunoaffinity Purification

Fractionation

Protein Digestion

Peptide Clean Up

Protein Clean Up

Phosphopeptide Enrichment

N-glycan Sample Prep
AssayMAP Application Portfolio-Affinity Purification

**Antibody Purification**
- Protein A (PA-W) Cartridge
- Protein G (PG-W) Cartridge

**Immunoaffinity Purification**
- Protein A (PA-W) Cartridge
- Protein G (PG-W) Cartridge
- Streptavidin (SA-W) Cartridge

**Immunoaffinity Purification**
- Streptavidin (SA-W) Cartridge
AssayMAP Automation Portfolio-Protein Analysis

- **Protein Cleanup**
  - Reversed-Phase (RP-W) Cartridge

- **Protein Digestion**
  - No Cartridge used

- **Peptide Cleanup**
  - Reversed-Phase (RP-S) Cartridge
  - Reversed-Phase (C18) Cartridge

- **Peptide Fractionation**
  - Strong Cation Exchange (SCX) Cartridge
  - Reversed-Phase (RP-S) Cartridge

- **Phosphopeptide Enrichment**
  - TiO2 Cartridge
  - Fe(III)-NTA Cartridge
User Interface for Each Application

Features
- Designed for bench scientists
- Easy-to-use with minimal inputs required
- Harmonized interfaces across applications

Benefits
- Minimal training required
- Rapid implementation
- Simple protocol transfer between sites
PA-W and PG-W cartridge capacity

- 1 to 100 μg hIgG or hIgG1 spiked into 25 μL CHO cell culture supernatant and loaded onto PG-W or PA-W cartridges (n=3), respectively.
- Cartridges were washed and bound antibodies were eluted and quantified by LC-UV.
- Recovery exceeded 90% of the loaded mass for each cartridge type across the entire range.
PA-W and PG-W elution profiles

5% acetic acid

12 mM HCl/100 mM NaCl
Recovery vs elution volume for PA-W/PG-W

Purification of 50 μg hIgG from cell culture supernatant

> 98% recovery 10 μL of eluate using 5% acetic acid for elution
## Protein quantification and characterization solutions

<table>
<thead>
<tr>
<th>Purify</th>
<th>Digest</th>
<th>Cleanup</th>
<th>Separation</th>
<th>Detect</th>
<th>Analyze</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hardware</td>
<td>AM Bravo</td>
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<td>1290 infinity HPLC</td>
<td>QQQ or QTOF</td>
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<tr>
<td>Software</td>
<td>Protein Sample Prep Workbench</td>
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<td>MassHunter</td>
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<tr>
<td>Consumable</td>
<td>SAW, PAW, or PGW cartridges</td>
<td>Pipette tips</td>
<td>RPS or C18 cartridges</td>
<td>AdvanceBio Peptide column</td>
<td>NA</td>
</tr>
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- **Purify**
- **Digest**
- **Cleanup**
- **Separate**
- **Detect**
- **Analyze**

- Peptide mapping
- Quantify
- PTM analysis

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**Agilent Technologies**
In-Solution Digestion

Input:
- Particulate-free, solutions of proteins
- Up to 384 samples (four) 96-well plates
- For example, the eluates from the Affinity Purification protocol
- Sample volume dependent on chemistry conditions – Reagent Calculator helps reconcile volumes compatible with the protocol

Output:
- Sample plates contain digested proteins = peptides
- Samples now ready for peptide cleanup
AssayMAP tool for Peptide Cleanup: C18 (silica) or RP-S (polymeric)

Input:
- Particulate-free, aqueous solutions of peptides
- Sample plates from In-Solution Digestion protocol
- Up to 96 samples (1 plate)
- Up to 1000 µL sample load

Output:
- Salt-free, solutions of peptides in organic solvent
- Elution volume as little as 10 µL
- Up to 100x concentration factor
BSA digestion and cleanup

Low %CVs for both intra- and inter-day digestion and cleanup for samples prepared using urea or guanidine-based denaturation.

Day 1 cleanup = C18
Day 2 cleanup = RP-S

<table>
<thead>
<tr>
<th></th>
<th>Urea</th>
<th>Guanidine HCl</th>
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<tbody>
<tr>
<td></td>
<td>Day 1</td>
<td>Day 2</td>
</tr>
<tr>
<td># of samples</td>
<td>64</td>
<td>62</td>
</tr>
<tr>
<td># of peptides monitored</td>
<td>25</td>
<td>25</td>
</tr>
<tr>
<td>Avg. peak area %CV</td>
<td>3.3</td>
<td>3.7</td>
</tr>
<tr>
<td># Peptides %CV &lt;5</td>
<td>23</td>
<td>21</td>
</tr>
<tr>
<td># Peptides 5&gt;%CV&lt;10</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td># Peptides %CV &gt;10</td>
<td>0</td>
<td>1</td>
</tr>
</tbody>
</table>
Antibody quantification -

<table>
<thead>
<tr>
<th>Peptide</th>
<th>%CV</th>
<th>Chain</th>
</tr>
</thead>
<tbody>
<tr>
<td>1) SLSHSPG</td>
<td>7.8</td>
<td>Heavy</td>
</tr>
<tr>
<td>2) TSTSPIVK</td>
<td>5.6</td>
<td>Light</td>
</tr>
<tr>
<td>3) SQVFLLK</td>
<td>8.0</td>
<td>Heavy</td>
</tr>
<tr>
<td>4) FTGSGGTQFSLK</td>
<td>6.6</td>
<td>Heavy</td>
</tr>
<tr>
<td>5) VNSAAFPAPIEK</td>
<td>5.0</td>
<td>Heavy</td>
</tr>
<tr>
<td>6) DVLTTTLPK</td>
<td>7.2</td>
<td>Light</td>
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Across 96 sample replicates

EIC overlays of mAb peptides from a single row of samples (n = 12)
AssayMAP Power Users

Daniel Spellman (Merck, West Point, USA)
Field: large molecule pharmacokinetics
AssayMAP Application: enrichment, digestion, peptide cleanup
Dan has made AssayMAP a critical part of a workflow used at Merck. AssayMAP provides significant reductions in both consumable cost and time to results. No more messy mag beads!

Jacob Jaffe, The Broad Institute in Cambridge and MIT, USA
Field: High-Throughput Proteomics
AssayMap Application: mAb & phosphopeptide enrichment, digestion, cleanup
"Using the combination of extremely consistent, parallelized digestion with automated reverse-phase cleanup via AssayMAP, at a scale appropriate for ultrasensitive proteomics applications, has enabled us to contemplate collaborative studies of previously unheard-of scales and throughput."
Reversed-Phase Protein Cleanup Enabling Faster Trypsin Digestion

Problem

- Slow time to results

Day 1
- Purify: ~1 hr
- Digest: ~6-18 hr

Day 2
- Cleanup: ~1 hr
- Separate: ~12 hrs

Day 3
- Analyze: ~8 hrs

Decision

Waiting, waiting, waiting
# Rapid Antibody Digestion w/ RP Protein Cleanup

## Sample Prep < ½ Day

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RP-W Protein Desalting

- **Graph 1:**
  - X-axis: Load, µg
  - Y-axis: Recovery, µg
  - Points represent Eluate and Flowthrough.

- **Graph 2:**
  - X-axis: Cumulative Elution Volume, µL
  - Y-axis: µg per fraction
  - Bar chart showing distribution.

- **Graph 3:**
  - X-axis: Sample Number
  - Y-axis: Eluate, µg
  - Scatter plot showing eluate distribution.

- **Table:**
  - | Samples   | Load, µg | % Recovery | % CV |
  - |-----------|----------|------------|------|
  - | 1 - 12    | 10       | 104        | 2.6  |
  - | 13 - 24   | 25       | 103        | 1.8  |
  - | 25 - 36   | 50       | 103        | 1.3  |
  - | 37 - 48   | 75       | 99         | 1.4  |
Peptide fractionation

Input:
- Particulate-free, solutions of peptides
- Sample plates from Peptide Cleanup protocol
- Up to 96 samples (1 plate)
- Up to 250 µL sample load

Output:
- Solutions of peptides in various solvents/salts
- As many as 6 fractions can be collected
- Can collect small volume fractions (~ 5 µL)
Host Cell Proteins (HCPs) in Protein Therapeutics

Host cells

HCPs

Therapeutic antibodies

Harvest supernatant

Downstream processing (e.g. Affinity purification)

Wash + Elution

Residual HCPs could:
- Reduce drug efficacy
- Induce adverse patient immunoreactions
General HCP Identification Workflow

Sample Preparation
- Sample Extraction
- Peptide digestion
- Peptide fractionation

LC/MS Analysis
- 1D or 2D LC separation
- MS analysis (Iterative directed MSMS, 6550)

Data Processing
- Database search and identification (Spectrum Mill)
Data-dependent vs. Directed MS, 2D-LC/MS

**Data-dependent method**

A. Total HCPs identified (≥ 2 peptides): 19

B. Total HCP peptides identified (Protein IDs ≥ 2 peptides): 54

**Directed MS method**

C. Total HCPs identified (≥ 2 peptides): 67

D. Total HCP peptides identified (Protein IDs ≥ 2 peptides): 242
From Identification to Quantitation

Discovery

Method Validation/Quantitation

Purified Sample
Heavy-labeled peptides
Trypsin digestion
Peptides
Analyze by 6490 iFunnel QQQ

Retention time

TIC
Alifasek
Lateradolipin
Noveltoxin

Overlayed MRM

Agilent Technologies
GlykoPrep® N-Glycan Sample Preparation Platform

Purify Antibody (30 - 60 min)
Denature & immobilize (30 - 60 min)
Digest with N-Glycanase (15 - 60 min)
Fluorescent labels (0-1 hour)
Cleanup & elute in water (15 - 30 min)

Microfuge Format
- Up to 24 samples
- Microfuge tubes
- Microcentrifuge
- ~4-5 hours
- Attended

Plate Format
- Up to 4 x 96 samples
- 96-well microplates
- Centrifuge
- ~4-5 hours
- Attended

Automation Format
- n x 96 samples
- 96-well microplates
- AssayMAP Bravo
- ~4-5 hours/plate
- Walk-away

Agilent AssayMAP Bravo Liquid Handling Platform
GlykoPrep® Label Choices

Glycoprotein

N-Glycanase

InstantDyes

Rapid-Reductive-Amination™

InstantAB™
• InstantDye for LC-FLR
• Similar HILIC elution profile to 2-AB

InstantPC™
• InstantDye for LC-MS

2-AB
• Standard dye for LC-FLR
• Introduces negative charge for CE

APTS
• Introduces negative charge for CE
Rituxan, 2-AB label

AM Bravo Methods and Materials

- 4 lots of Rituxan, 24 replicates each
- GlykoPrep-\textit{plus} Rapid N-Glycan Sample Preparation with 2-AB
- AssayMAP Bravo
- Analysis by UHPLC
- ~20 nanograms of N-glycan sample in aqueous buffer (1 µl)
- ProZyme’s 10-Minute Screening Method
New Instant PC Dye Run Time and Resolution Comparison

InstantPC-glycans
5-minute method (AdvanceBio column)

RapiFluor-MS-labeled Enbrel N-glycans
55-minute method (BEH column)

Highest fluorescence of any glycan label tested
High MS response
Flexible workflows in development
Phosphopeptide Enrichment Portfolio

Fe(III)-NTA

Bind

Wash

Elute

TiO₂

Bind

Wash

Elute

Motif mAb’s

Immobilize

Bind

Wash

Elute
Fe(III)-NTA Phosphopeptide Enrichment

Sample Input

Not Bound

Cartridge Eluate

*T = Phosphopeptide

TIC Counts (x10^7) vs. Acquisition Time (min)
Phosphopeptide Enrichment From α-Casein using Fe(III)-NTA Cartridges

Elution with 1% aqueous NH$_3$ (≈ pH 11)
Complete Sample Prep Workflow For Intact mAb Analysis

Affinity Capture

On-cartridge Deglycosylation/Limited digestion

Elution

In-solution Reduction

- No Enzyme
- PNGaseF
- Flow through
- IdeS
- Flow through

- mAb Eluate
- Deglyco-mAb Eluate
- F(ab')2 Eluate

- Reduced Eluate
- Reduced Eluate LC
- Reduced Eluate

Agilent Technologies
Introduction

- Antibody-drug conjugate (ADC) are an emerging class of biotherapeutics
- DAR is a critical attribute
- Serum ADC DAR determination needed for PK modeling
- An AssayMAP solution for ADC DAR determination
DAR Calculation Workflow

Sample Preparation → LC/MS → DAR Calculation

Agilent Restricted - For Research Use Only. Not for use in diagnostic procedures.
ADC DAR Determination for Samples in Complex Matrices

A 100 ng control ADC

B 100 ng purified ADC

C DAR=3.5

D DAR=3.5

E

F
ADC DAR Characterization Across Various Concentrations in Serum

A  
\[ \text{CADC} = 20 \mu\text{g/mL} \]
\[ \text{DAR}=3.5 \]

B  
\[ \text{CADC} = 10 \mu\text{g/mL} \]
\[ \text{DAR}=3.5 \]

C  
\[ \text{CADC} = 5 \mu\text{g/mL} \]
\[ \text{DAR}=3.5 \]

D  
\[ \text{CADC} = 2.5 \mu\text{g/mL} \]
\[ \text{DAR}=3.5 \]

E  
\[ \text{CADC} = 1.25 \mu\text{g/mL} \]
\[ \text{DAR}=3.5 \]

F  
\[ \text{CADC} = 0.625 \mu\text{g/mL} \]
\[ \text{DAR}=3.5 \]
Summary

• AssayMAP DAR solution
  • Increases reproducibility and scalability
  • Purifies ADCs from serum with high yield and purity.
  • Generates high resolution spectra.
  • Provides easy, consistent and accurate DAR calculation.
AssayMAP Bravo Platform

Great Reproducibility
• Increase reproducibility for total workflow by reducing sample prep variability
• Reduce number of replicates to increase throughput

Increased Walk-Away Time: More Time to Do More!
• Minimize hands-on time
• Allow scientist to do more value-added work

Easy-to-use software control
• Designed for non-automation experts
• Minimal training required enabling rapid adoption
• Simple person to person or site to site transfers

Single platform for various sample prep needs