The NISTmAb Reference Material

A Global Partnership to Advance Biopharmaceutical Analytics

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Glycan

IBBR
INSTITUTE FOR BIOSCIENCE & BIOTECHNOLOGY RESEARCH

NIST
National Institute of Standards and Technology
U.S. Department of Commerce

MATERIAL MEASUREMENT LABORATORY
Higher-order Physicochemical and Biophysical characterization

- Product-related substances: Intrinsic heterogeneity expected from manufacture process
- Product-related impurities: Undesirable degradation products
DEVELOPMENT OF BIOLOGICS IS ASSOCIATED WITH A BROAD RANGE OF TECHNICAL CHALLENGES DRIVEN BY THEIR INHERENT STRUCTURAL COMPLEXITY

**Key challenges:**

- Complexity of characterizing biological molecules (larger, complex and dynamic structures and consist of diverse populations of molecules)
- Expanding ability to fully characterize biologics using current analytical/biophysical methods
- Understanding critical quality attributes of a biologic, identifying relevant process parameters and defining acceptable manufacturing process ranges to ensure safety and efficacy

**Atorvastatin (Lipitor)**
Small chemical molecule
800-1000 Da
Well established chemical synthesis

**Calcitonin**
“Simple” Biologic
3455 Da, ~32 Amino acids
Produced in yeast, bacteria

**Monoclonal Antibody (IgG)**
Complex Biologic
150,000 Da, ~1300 Amino acids
(with host cell modifications)
Produced in mammalian cells

*Note: relative scale is illustrative*
National Institute of Standards and Technology

- Non-regulatory agency within U.S. Department of Commerce
- Founded in 1901 as National Bureau of Standards
- NIST responsible for US physical standards, test methods, & calibrations

**Unique Mission within the Federal Government**

to promote innovation and industrial competitiveness by advancing measurement science, standards, and technology in ways that enhance economic security and improve our quality of life
NIST Program in Biomanufacturing

Measurement science, tools & standards to support development, manufacturing & regulatory approval of biologic drugs

Program Areas:
1. Measurements & standards for protein stability, aggregation, & particles
2. Protein structure: higher-order structure, post-translational mods (glycosylation)
3. Measurement tools & science to understand production cell variability

• Well characterized and certified standard is an ideal means to:
  • Assess precision and accuracy across methods and labs.
  • Identify potential gaps and develop new technologies to fill them.
  • Adoption of new technology by correlating existing data with that from new technologies.
  • Assist reviewers and sponsors by allowing them to provide/assess methods and historical data for the standard.
NIST mAb Attributes:
- Humanized mAb (IgG1κ) expressed in murine suspension culture
- Frozen bulk “Drug-like substance”
  - 10 mg/mL, ≥ 98% purity
  - 12.5 mM L-His, 12.5 mM L-His HCl (pH 6.0)

Definitions:
- In-House Standard: Manufacturer-specific drug substance
- Reference Material: Issued under NIST trademark and established to be fit for intended use in measurement of nominal property values.
- Standard Reference Material: Issued under NIST trademark and assigned one or more specified property values with associated uncertainties and traceability.

Approach for IgG SRM:
- Complete rigorous interlaboratory characterization
  - Results used for book compilation
- Compile reference data, methods, etc.
- Certify for concentration traceable to the kg
- RM 8671 available early 2016
  - 10 mg/mL, 800 μL per vial

Peptide Mapping by MS
- Primary Sequence
- S-S Bridge Analysis
- PTM analysis
- Abs. Quant.

Intact, Middle down M
Glycosylation Analysis
SDS-PAGE
HOS: NMR, HDX, XRD
Biophysical
  - CD, FTIR, DSC, DLS, AUC, SLS, DSF

LC: SEC, RP, IEX, HIC
Protein Particulates
CE: cIEF, cSDS
mAb Structure

Light Chain

Heavy Chain

Disulfide Bonds

Antigen Binding

Complex molecule = Complex Assays

Fab

Glycan

Fc

Heavy Chain

1. VQTLXXXVXWEPQTYTLYLTCTFVGFLSXXXWXXXWQPFGKALWAKXXXLXXXWXXXRLXXDDGXXXKWXXX 90
2. DTAATYCARXXXWXXGKTVSTVXXASTGPSVPLAFFSKSTSGCTAALGCVKYFFPVTWVNGALTSGVHTFPVLQSS 180
3. GLYSLSLVCTVFSLLGQTYICNVRHPSTTVKDRIFVPRSCD6THTCXXXLXLGFSVLFPPFPPFDSTMLFSRTFTTCTCTVS 270
4. BGDFVYKPVNYVCGSVGNAKTFREQSVQRTSYTVFVSVGVLGQCMGSHYXCNVSXKALPAFIEXTISAKAEJKPSQPQTVTLPPRGE 360
5. MTKQXXXXKXXXXXXFPSCIAVEWESNQGFEHNYKVTFFVLDGGDSSFLYSLKTVISGRWQGQVFSCKVHVEALIBHYTQKSLSLSGK 450

Light Chain

1. DXXXQPSXXLSXGXRWYXXXCTXXXXXZXQKPGKQXPLXXLXYXXXXXGXXVFFSSGSSGXTXELTISXXDDFTTVCQX 90
2. XXXXXXGXTXGXE1SXVXTAQVQFIFPSRDQLTSXATVCTLNFFPR8ARVQMVAPAALSXDISPLAYTEDSXDGTYSLSTLTL 180
3. SKADYKSGKVLDSVTLQGSLSPFTFSNNEZEC 213
LC-UV-MS/MS Peptide Map

Consistent with Expected Primary Structure

- Intact MS
- Middle down MS
- LC-UV-MS/MS
- Multiple Enzyme Maps
- Digest, MS, LC critical
- Guanidine/EDTA
- Long gradient
- MS/MS
- Peptide level coverage
  - 98/96 % coverage trypsin
  - 100% coverage multi-enzyme

Heavy Chain

Trypsin = 98.67%
Chymotrypsin = 91.33%
GluC = 64.44%

*N: N-glycosylation site
*Q: pyro-glutamination
*K: Lysine-clipping

QVTLXXXXXXLVKPTQTLTLTCTFSGFSLSTAGMSVGWIRQPPGKALEWLAXXXXXXXXXXXXXXXXXXXXXXXRLTXXKDTSKXXXXXKXXXXXXX

DTATYYCARXXXXXXXXXXWGQGTTVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSS

GLYSLSGVTYVSSSLTQTFTYICNTHKPSNKVKRQDEKSCLDLXXXXXXVXXXXXXXLPFFKRDTLMSISSRTPEVTCVVDVSS

HEDPEVKFNWYVDGVHEKRTKPREEQGNSVYVRVSVTTLTVLHODWINGHYKQYSHNLPLLMSHAKGQPREPQVYLPSREE

MTKINXXXXXXFXFPSLWHEESNGDXYNTKPTPVLDSGSPFLSGLTVDSDKRQGQNVFSCSVMEALHNHTQKSLSLPGK

Time (min)
Post Translational Modifications

- PTMs found in low abundance in native mAb
- Product-related substances
- PTMs intrinsic to protein’s and should be characterized
  - Manual validation of MS/MS
  - Accelerated stability to identify susceptible sites
  - Peptide map and XIC most common, followed by more precise MRM assays when necessary

NISTmAb useful external control material

Note: disulfide structures not shown for clarity
UV Quantification 29%
XIC 36%
  Summed charge state monoisotopic
Co-eluting species contribute
Variety of inter-laboratory factors
  Digest conditions
  Method conditions
  Data interpretation/Quant methods
    Monoisotope vs. most abundant vs. summed
    Summed charge states
    Missed cleavages
Relative Value!

UV-based Quantification
Glycosylation

Proteolysis

Glycan Sequence
Glycan Position
Relative Quantification

Glycan Release

Label

Permethylate
Exoglycosidase

Glycan Sequence
Relative Quantification
Linkage

MATERIAL MEASUREMENT LABORATORY
Method Pre-Qualification

- Example: Evaluation of new platform
- Accurate m/z identified Glycamine byproduct
  - C2 Epimers
- Only detected when use MS
  - Monitor labeling efficiency
  - Unwanted byproducts
  - Co-eluting glycans
  - Process impurities
- MS useful during early process/method development

![GlcNAC and ManNAC structures]

**2AB**
- Top = MS$^1$ Base Peak
- Bottom = Fluorescence
- nG0F nG0F$^+$
- nG1F nG1F$^+$
- nG2F nG2F$^+$

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**2AB C18 BIG**
- Top = MS$^1$ Base Peak
- Bottom = Fluorescence

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![Graphs showing relative fluorescence units over time](data:image/png;base64,iVBORw0KGgoAAAANSUhEUgAAAAEAAABCAQMAAACAAAAACgAAAAHdElNB8AAAB1JREFUeNvgn7h_RDAMhZMzGmQgUAAAABJRU5ErkJggg==)
Differentiate Sample from Method Artifact

- C18 after HILIC
  - Improved MS² low abundance
  - Decrease in sialic
- Larger inj. quantity
  - No effect on resolution
- Apparent glycoprofile method-dependent
  - Biosimilar implications
  - Regulatory review
- Class-specific historical data useful in evaluating method suitability
Interlaboratory Comparison

- Diff. sample prep and method
  - Different labels and method
  - Retention and/or MS ID
- Unique ID’s less than 1% total glycan
- Quantitative comparability good
  - Minor differences due to chromatographic selectivity and ID

Harmonized method leads to robust, transferrable glycoprofile

Representative Methods

- SEC and cSDS
  - size/ monomeric purity
- WCX and cIEF
  - Charge variants/purity
- Qualify with challenge material

Fraction Collect

- Product variants for detailed analysis
- Forced degraded material
  - Challenge Analytical Methods
Non-Reduced cSDS

Integration Limits for Triplicate Injections on Three Days

<table>
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<tr>
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<th>Average</th>
<th>CV (%)</th>
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<tr>
<td>Corrected Area</td>
<td>11204</td>
<td>7</td>
</tr>
<tr>
<td>Purity (%)</td>
<td>99.0</td>
<td>0.2</td>
</tr>
<tr>
<td>Migration Time (min)</td>
<td>28.3</td>
<td>0.1</td>
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<tr>
<td>H (µm)</td>
<td>87</td>
<td>16</td>
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Capillary Isoelectric Focusing

- pH gradient within gel-filled capillary
- Main peak pI = 9.1 in 1.5 M Urea
- Method Optimization includes ampholyte composition
  - Tested different ampholyte ratios
    - pH 3-10 (broad range = BR)
    - pH 8-10.5 (narrow range = NR)
  - Control is 12 BR:0 NR
- Robust evaluation of charge heterogeneity
  - Consistent with CEX
- Acidic variant character. ongoing
  - Sialic Acid
  - Deamidation

**Diagram:**
- Detection Window
- Anolyte pH 1.4
- Catholyte pH 13
- Anodic Stabilizer
- Cathodic Stabilizer

**Graph:**
- BR:NR
  - 0:12
  - 3:9
  - 9:3
  - 12:0

- Acidic Varients
  - +2K
  - +K
# NIST Characterization

## Bionalytical Science Group
John Schiel, Abigail Turner, Trina Formolo, Katharina Yandrofski, 
Kerry Bauer, Larik Turko, Karen Phinney
- **Separation Science**
  - cSDS, cIEF, SEC, RP, HIC, CEX, WAX
- **Mass Spectrometry and LC-MS**
  - Peptide mapping, middle down, and intact
  - PTM and SVA
  - Glycoanalysis
  - Host Cell Proteins

## Bionalytical Science and Bioassay Methods Groups
John Schiel, Trina Formolo, Mark Lowenthal, Ashley Beasley, Karen Phinney, Steven Choquette, John Travis
- **Certification of Concentration**
  - AAA
  - Peptide IDMS
  - UV-Vis

## Biomolecular Structure and Function Group
John Marino, Rob Brinson, Luke Argoblast, Jane Ladner, Travis Gallagher, Jeff Hudgens, Elyssia Gallagher, Ioannis Karageorgos
- **Higher Order Structure**
  - NMR, XRD, HDX

## Mass Spectrometry Data Center
Steve Stein, Qian Dong, Yuxue Liang, Jeri Roth, M. Lorna De Leoz, Yuri Mirokhin, Dimitri Tchekhoxskoi, Sara Yang, Pedatsur Neta, Eric Yan
- **Mass Spectral Database**
  - Peptide MS/MS
  - Glycan MS/MS

## Biomolecular Structure and Function Group and Biosystems and Biomaterials Group
John Marino, Curt Meuse, Ken Cole, Brian Lang
- **Biophysical Measurements**

## Bioprocess Measurements Group
Dean Ripple, Richard Cavicchi, Srivali Telikepalli
- **Protein Particles**

## NCNR
Joseph Curtis, Dan Neuman, Susan Krueger, Ronald Jones
- **Neutron-Based Measurements**

## Polymers and Complex Fluids Group
Steve Hudson
- **Rheology**

## Applied Genetics Group
Peter Vallone
- **Residual DNA Testing**
## ACS Book Project

“State-of-the-Art and Emerging Technologies for Therapeutic Monoclonal Antibody Characterization”

John Schiel (NIST), Oleg Borisov (Novavax), Darryl Davis (Janssen)

Co-editors

Structure overview – three volumes
- mAb Structure, Function, and Regulatory Space
- Characterization: The NIST mAb Case Study
- Defining the Next Generation of Analytical and Biophysical Techniques

## Industry Collaboration

**Contributors**
- 100+ Industry, Regulatory, and Academic participants confirmed
  - Global Biopharma Participation
  - Multiple NIST and FDA Chapters
  - Academia and OEM contributions
- 33 Chapters

**Progress and Activity**
- Complete physicochemical and biophysical characterization
- Vol. 1 Released December 2014,
- Vol. 2 and 3 Complete, in production

## Approach

- Characterization of NIST mAb as book topic
- Engage industry scientists to collaboratively demonstrate best practices round robin characterization NIST mAb
- Establish NIST mAb as Industry Standard
Published December 2014

*Volume 1: Monoclonal Antibody Therapeutics: Structure, Function, and Regulatory Space*

Ch1. On the Need for Reference Material for Biopharma

Ch2. Monoclonal Antibodies: Mechanism of Action

Ch3. Heterogeneity of IgGs: Structure Function as related to process parameters.

Ch4. What Constitutes a Well-Characterized Biological Protein

Ch5. Using QbD Principles in setting a control strategy for product quality attributes

[http://pubs.acs.org/isbn/9780841230262](http://pubs.acs.org/isbn/9780841230262)
Global Partnership

- Characterization of NISTmAb as book topic along with methods, data, and discussion of best practices
- 100+ industry, academic, and government contributors

Publication Sept. 2015

Volume 2: Biopharmaceutical Characterization: The NIST mAb Case

Preface Book 2
Ch1. Determination of The Primary Sequence/Structure
Ch2. Sequence Variant Analysis
Ch3. Structural Elucidation of Co and Post-Translational Modifications
Ch4. Glycan Analysis
Ch5. Separation Assays and Orthogonal Methods
Ch6. Biophysical
Ch7. Formulation/ Developability
Ch8. Protein Particulates
Ch9. Process Related impurities

Volume 3: Defining the Next Generation of Analytical and Biophysical Techniques

Preface Book 3
Ch 1. Higher Order Structure
Ch 2. Covalent Labeling Techniques for Higher Order Structure
Ch 3. Ion mobility
Ch 4. Aggregation
Ch. 5. Simultaneous Multi-cell SLS
Ch 6. Bioinformatics
Ch 7. Adventitious Agent Testing of Biologicals; Changing to a new frontier of technology.

OEM Section of Book 3
Ch 8. Microfluidics
Ch 9. Intact Protein Mass Spectrometry
Ch 10. Rapid Middle Down sequence determination of antibodies by MALDI-ISD-MS
Ch 11. Online Automation
Ch 12. Recent developments in analytical methods for host cell protein analysis
Ch 13. Targeted Informatics
Ch 14. Summary and Outlook
Future Directions: Reference Materials

- Explore Additional Modalities as Potential RMs
  - IgG classes, degraded products, aggregates
  - ADCs, Bispecifics, G-CSF, etc.
- Expand Identity, Quality, and Stability Laboratory
  - Method Qualification
  - Accelerated Stability
  - Novel Characterization Platforms
- Continued Development of HOS Methods
- Round Robin Studies
  - HDX of Fab (Jeff Hudgens)
  - NMR of Fab (John Marino, Rob Brinson)
  - Glycoanalysis (Maria Lorna deLeoz)

Fab from NIST mAb
Conclusion

• RM with Established “Baseline” Historical Data
• RM to Supplement In-House Reference Standard Program
  • Streamline implementation of new technology
  • Assist method qualification
  • System suitability
  • Harmonize approaches to well characterize
  • Assess method variability, utility, etc.
  • Differentiate method vs. product related artifacts
• High Level of Industry Support
  • ACS Book Series
• Expected Impact
  • Underpin regulatory decisions
  • Higher-order characterization
    o Method accuracy, precision, comparability
    o Translate to product safety and efficacy
• Opportunity to Establish Unprecedented Open Innovation through Biotherapeutics Consortium
Acknowledgements

- ACS Book Co-Editors
  - Darryl Davis, Janssen
  - Oleg Borisov, Novavax

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  - Abigail Turner
  - Katharina Yandrofski

- NBRC Core
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  - John Marino
  - John Kerwin
  - Bill Bentley