Protein Therapeutics Interest Group Workshop Report
61st ASMS Conference and Allied Topics, June 10, 2013, Minneapolis, MN

The Protein Therapeutics Interest Group (PTIG) workshop, entitled "Mass Spectrometry-based Characterization of Biotherapeutics", was held from 5:45 PM to 7:00 PM on Mon., Jun. 10, 2013. Approximately 200 chairs were in the room and there was standing room only available. Many, afterwards, mentioned that they were unable to get in to the room for the workshop.

The PTIG also recommended three oral sessions for this year's ASMS conference: "Biotherapeutics, Impurities and Degradants: Structural Characterization", "Biotherapeutics and Biomarkers: Advances in Quantitative Analysis" and, for the first time, "Characterization of Product Variants in Biosimilars". For the second time a short course entitled "Practical Mass Spectrometric Characterization of Protein Therapeutics" was provided for conference attendees on the weekend. The oral sessions, short course and workshop were all well attended and contained considerable discussion on the topic of biotherapeutics. Taken together these show a continued strong interest in the characterization of biotherapeutics within the ASMS community.

Workshop Business Items

1. The PTIG desires to change our name to Biotherapeutics Interest Group. This name enables more topics to be discussed outside of protein-based therapeutics (such as vaccines and oligonucleotide-based therapeutics). The focus of the interest group will not change, namely the qualitative characterization of biotherapeutics in the biopharmaceutical industry. *The PTIG coordinators ask the ASMS Board of Directors to provide input on this matter.*
2. The PTIG desires to establish a LinkedIn group named ASMS Biotherapeutics Interest Group. This LinkedIn group would enable real-time question/answer postings on hot topics and the ability to seek ideas for future workshops. Justin Sperry volunteered to set up and manage the LinkedIn group for the Interest Group. This group would allow networking among scientists throughout the biopharmaceutical industry and would also provide a forum of undergraduate and graduate students to seek contacts within the industry as they are in the midst of graduation. The master list of interest group members would still be managed through the ASMS Member Profile. *The PTIG coordinators ask the ASMS Board of Directors to provide input on this matter.*
3. The PTIG is actively seeking a new coordinator to replace Justin Sperry, who has served his two-year term. Li Tao and Justin Sperry will announce the new coordinator within the next few months to the ASMS Board of Directors.

Workshop Summary

For the first time, the coordinators polled the members of the PTIG prior to the workshop through the use of a SurveyMonkey questionnaire. The ten questions sought answers to the focus area of members (academic, industry, etc.), the types of biotherapeutics of interest, the instrumentation (no brands) used for various biotherapeutic assays, and the specific topics of

interest they would like addressed during the workshop. The survey results are included in the attached presentation.

The PTIG membership that responded to the survey were interested in four main topics:

1. Intact protein analysis
2. Higher-order structure analysis (H/D exchange and covalent labeling)
3. Sequence variant analysis (SVA)
4. Antibody-drug conjugates (ADCs)

The PTIG coordinators focused on topics 1 and 3 from the above list and referred those in attendance to the H/D Exchange and Covalent Labeling and the Pharmaceutical Workshops, respectively, for the other two topics.

The PTIG workshop was organized around two areas of focus in the biotherapeutic industry, the intact mass analysis of proteins (introductory topic) and the analysis of amino acid sequence variants (advanced topic).

(1) **Intact Protein Analysis**: (Discussion led by Justin Sperry or Pfizer)

The workshop was kicked off with a general discussion of intact protein analysis (slides attached). Several literature references of recent biotherapeutic analyses were provided. The audience was polled as to what they desired to discuss regarding the topic: (1) sample preparation, (2) chromatography, (3) mass spectrometry and/or (4) data analysis. An overwhelming majority of the audience wanted to discuss aspects of data analysis, in particular the maximum entropy algorithm for deconvolution of charge-state distributions provided by many vendors. Examples of raw data from an antibody and antibody-drug conjugate were provided along with the deconvoluted spectra. The audience members participated in a fruitful discussion regarding the correct parameters to use to generate the deconvoluted spectra. Several members of the audience mentioned review articles on maximum entropy.

(2) **Sequence Variant Analysis**: (Discussion led by Li Tao, Bristol-Myers Squibb)

The discussion began with a root cause analysis on sequence variant analysis, a current hot topic in the biopharmaceutical industry. A comprehensive set of literature references were provided to the audience (and are present in the attached slides). The audience was particularly interested in commercially available software to detect sequence variants. Several software platforms were discussed, including Mascot's Error Tolerant Search, new software from Protein Metrics called Byonic and Thermo's Sequest platform. There were also several discussion topics regarding the determination of SVA levels in therapeutic proteins and the best approaches for the removal of false positives. It is evident that as mass spectrometry-based technology advances, the ability to detect sequence variants at trace levels is enhanced.

The coordinators plan on utilizing the survey format again in 2014. This aided immensely in the ability to plan the workshop around the current needs of the Interest Group members.

The meeting was adjourned at 7:00 PM with very positive responses!

Respectively submitted,

Justin Sperry, Ph.D.
Li Tao, Ph.D.
Coordinators of Protein Therapeutics Interest Group

Mass Spectrometry-based Characterization of Biotherapeutics

Justin Sperry, PhD – Pfizer
Li Tao, PhD – Bristol-Myers Squibb

ASMS 2013

Sponsored by the Protein Therapeutics Interest Group

ASMS 2013

- Three oral sessions recommended by the Interest Group
  1. MOD am – Biotherapeutics and Biomarkers: Advances in Quantitative Analysis
  2. MOD pm – Biotherapeutics, Impurities and Degradants: Structural Characterization
  3. TOC pm – Characterization of Product Variants in Biosimilars
- Poster sessions
  1. Monday: Protein Therapeutics – Quantitative Analysis (494-521)
  2. Tuesday: Protein Therapeutics – Structural Characterization (225-238)
- Protein Therapeutics Short Course
- Many more related topics: antibodies/ADCs, chemical footprinting, top-down, sequence analysis, etc.

Survey Results

- If you would like a copy of the presentations and survey results, please update your ASMS Member Profile

Other Information

Interest Groups:

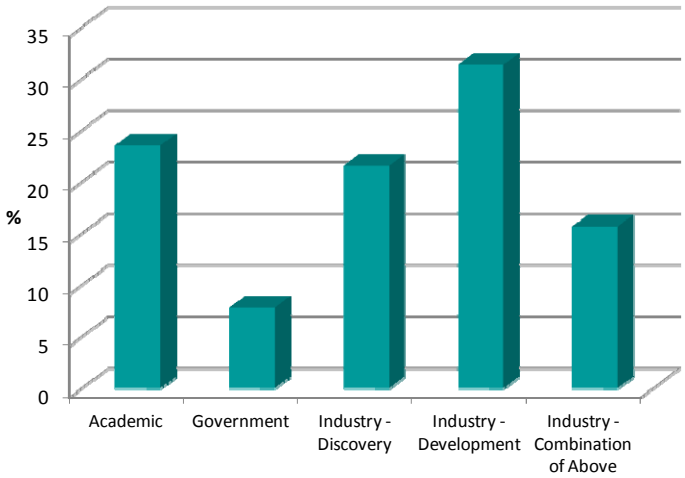
- ☐ Analytical Lab Managers
- ☐ Bioinformatics for MS
- ☐ Clinical Chemistry
- ☐ DNA/RNA
- ☐ Drug Metabolism & Pharmacokinetics
- ☐ Energy, Petroleum & Biofuels
- ☐ Environmental Applications
- ☐ Flavor, Fragrance and Foodstuff
- ☐ Forensics and Homeland Security
- ☐ FTMS
- ☐ Fundamentals
- ☑ H/D Exchange & Covalent Labeling
- ☐ Imaging MS
- ☐ Ion Mobility MS
- ☐ Ion Trap MS
- ☐ LC-MS & Related Topics
- ☐ Metabolomics
- ☐ Metal Ion Coordination Chemistry
- ☐ Peptide Fragmentation
- ☐ Pharmaceuticals
- ☐ Polymeric Materials
- ☑ Protein Therapeutics
- ☐ Quantitative Intact Proteomics
- ☐ Regulated Bioanalysis
- ☐ Undergraduate Research in MS
- ☑ Young Mass Spectrometrists

Test:

Disclaimer
This survey may not accurately reflect the biopharmaceutical industry. It only reflects those that participated.

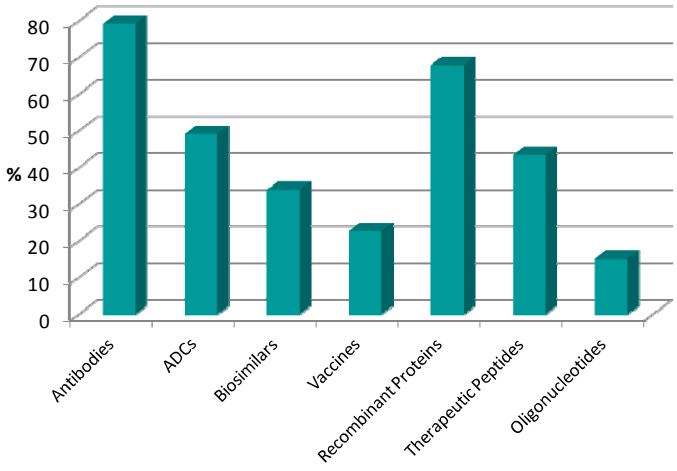
Survey Results

- What is your primary work focus?



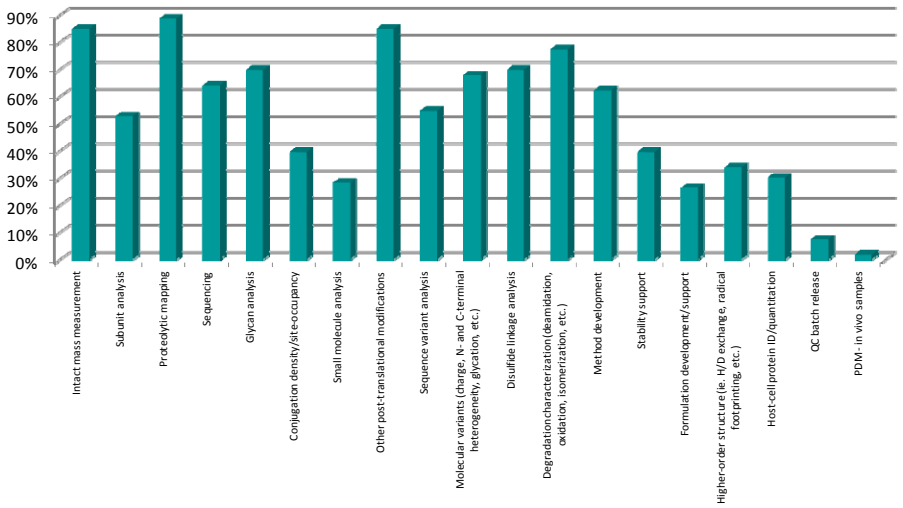
Survey Result

- What are your specific biotherapeutic focus areas?



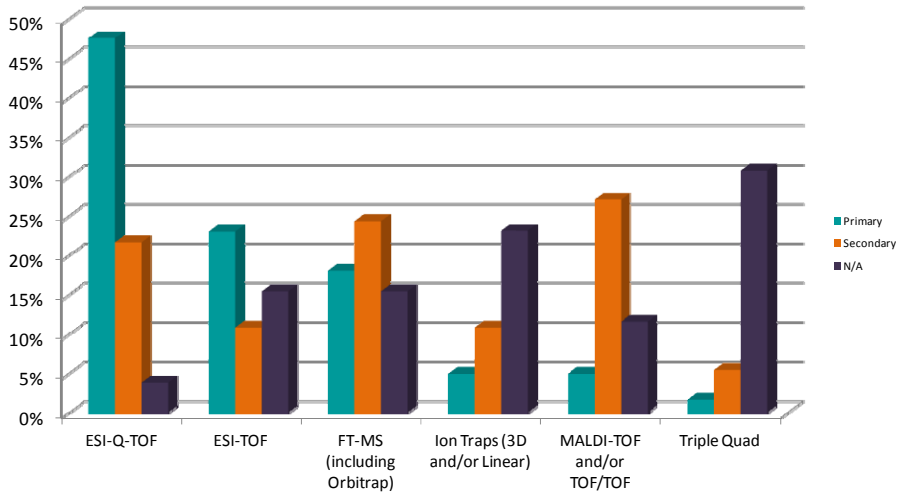
Survey Results

- I/We use mass spectrometry for…



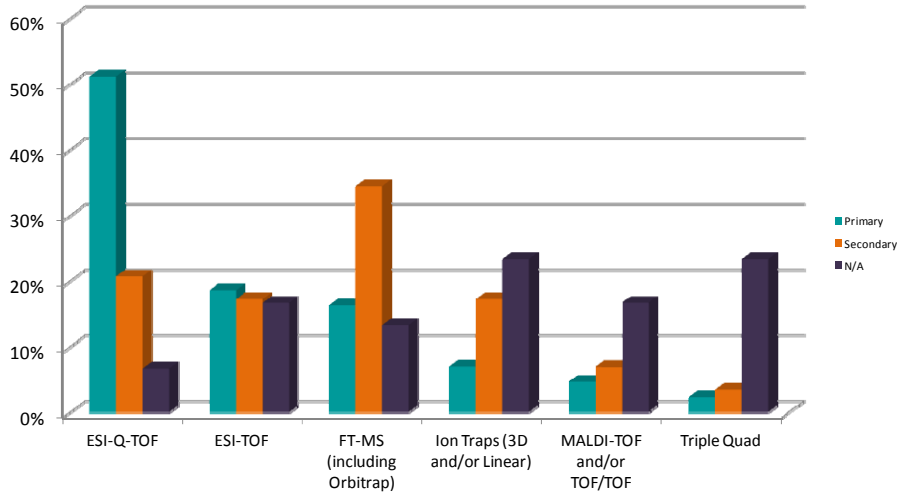
Survey Results

- What your preferred MS system for intact analysis?



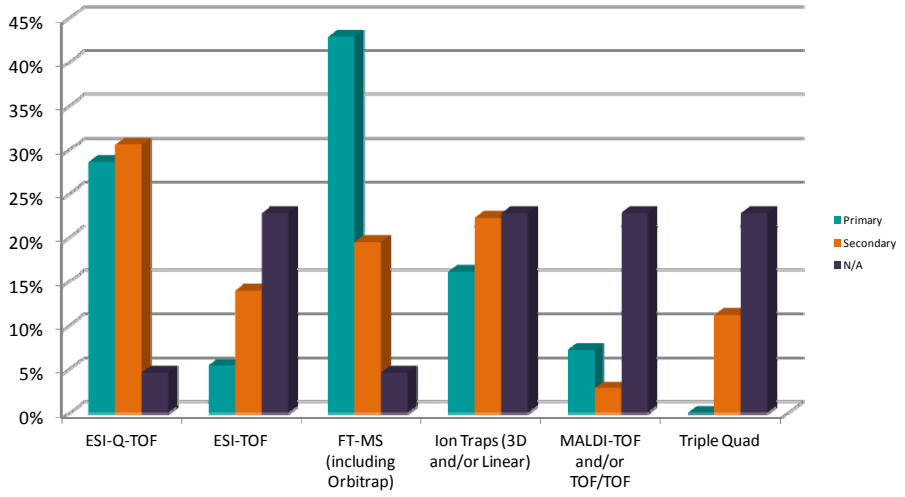
Survey Results

- What your preferred MS system for subunit analysis?



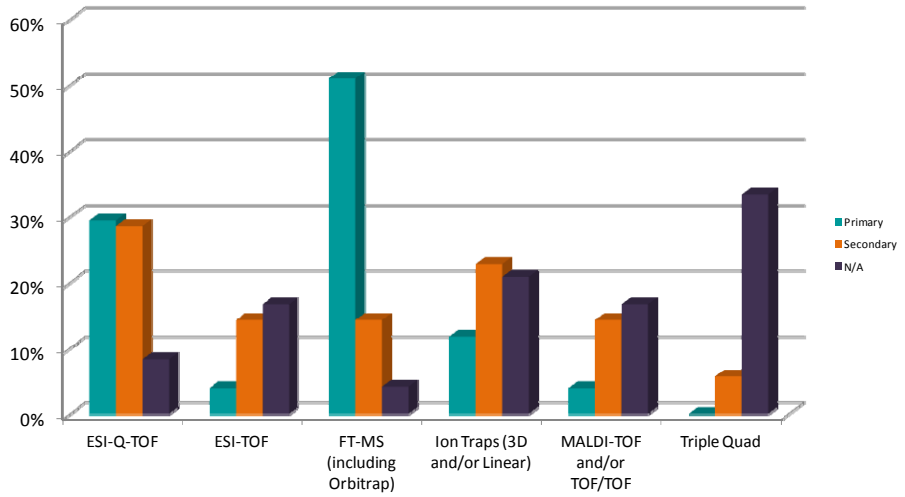
Survey Results

- What your preferred MS system for peptide mapping?



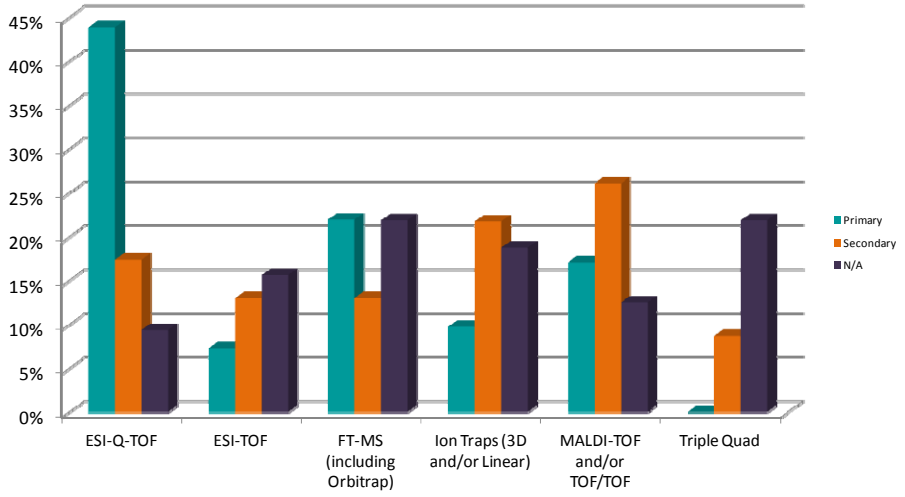
Survey Results

- What your preferred MS system for PTMs?



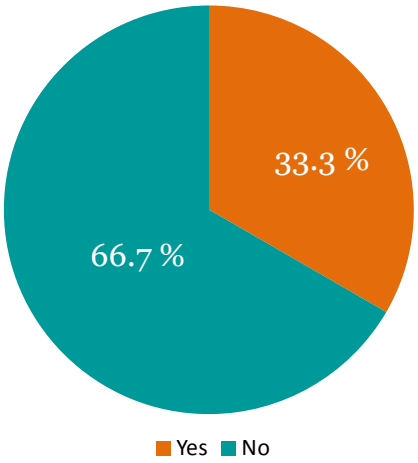
Survey Results

- What your preferred MS system for glycan analysis?



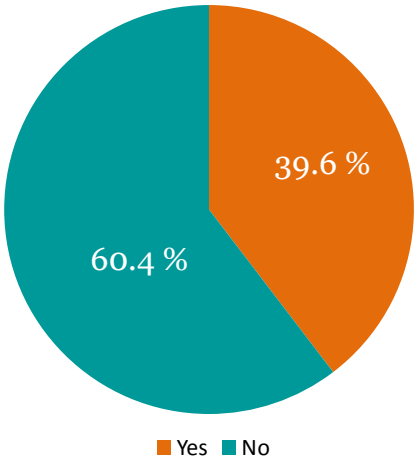
Survey Results

- Would you be willing to actively participate in the workshop?



Survey Results

- Have you attended the workshop in the past?



Your Top-4 Requested Topics

1. Intact Protein Analysis
   - Topic for Discussion Today (~20-30 min)
2. Higher-Order Structure (HDX and covalent labeling)
   - HOS Workshop on Wed.
3. Sequence Variant Analysis (SVA)
   - Topic for Discussion Today (~20-30 min)
4. Antibody-Drug Conjugates (ADCs)
   - Pharmaceutical Workshop on Wed.

Future

- For future proposals regarding oral sessions, the workshop and/or interest group activities, please contact
  - Justin Sperry – justin.sperry@pfizer.com
  - Li Tao – Li.Tao@bms.com
- We will be looking for a new coordinator next year, please contact us if you are interested
  - 2 year terms

Intact Protein Analysis

Recent References

Electrospray Ionization Quadrupole Ion-Mobility Time-of-Flight Mass Spectrometry as a Tool to Distinguish the Lot-to-Lot Heterogeneity in N-Glycosylation Profile of the Therapeutic Monoclonal Antibody Trastuzumab

Carola W. N. Damen,[a] Weibin Chen,[b] Asish B. Chakraborty,[b] Mike van Oosterhout,[c] Jeffrey R. Mazzeo,[b] John C. Gebler,[b] Jan H. M. Schellens,[d,e] Hilde Rosing,[a] and Jos H. Beijnen[a,d]

http://rd.springer.com/article/10.1016%2Fj.jasms.2009.07.017

FOCUS: THE ORBITRAP

Mass Measurement and Top-Down HPLC/MS Analysis of Intact Monoclonal Antibodies on a Hybrid Linear Quadrupole Ion Trap–Orbitrap Mass Spectrometer

Pavel V. Bondarenko,[a] Tonya P. Second,[b] Vlad Zabrouskov,[b] Alexander A. Makarov,[c] and Zhongqi Zhang[a]

[a] Amgen Incorporated, Thousand Oaks, California, USA
[b] Thermo Fisher, San Jose, California, USA
[c] Thermo Fisher, Bremen, Germany

http://rd.springer.com/article/10.1016%2Fj.jasms.2009.03.020

Protein Mass Spectrometry

DOI: 10.1002/anie.201206745

Exploring an Orbitrap Analyzer for the Characterization of Intact Antibodies by Native Mass Spectrometry**

*Sara Rosati, Rebecca J. Rose, Natalie J. Thompson, Esther van Duijn, Eugen Damoc, Eduard Denisov, Alexander Makarov, and Albert J. R. Heck**

http://onlinelibrary.wiley.com/doi/10.1002/anie.201206745

The impact of mass spectrometry on the study of intact antibodies: from post-translational modifications to structural analysis

Natalie J. Thompson,[ab] Sara Rosati,[ab] Rebecca J. Rose[ab] and Albert J. R. Heck*[ab]

http://pubs.rsc.org/en/Content/ArticleLanding/2013/CC/c2cc36755f

Development of a Native Nanoelectrospray Mass Spectrometry Method for Determination of the Drug-to-Antibody Ratio of Antibody–Drug Conjugates

Jia Chen, Sheng Yin, Yongjian Wu, and Jun Ouyang*

Protein Analytical Chemistry, Genentech, Inc., South San Francisco, California 94080, United States

http://pubs.acs.org/doi/abs/10.1021/ac302959p

http://www.sciencedirect.com/science/article/pii/S0731708511000562

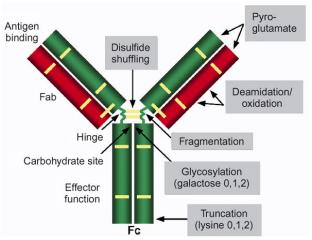
Review

Intact protein analysis in the biopharmaceutical field

Aline Staub[a,b], Davy Guillarme[a,b], Julie Schappler[a,b], Jean-Luc Veuthey[a,b], Serge Rudaz[a,b,*]

[a] School of pharmaceutical sciences, University of Geneva, University of Lausanne, Bd d'Yvoy 20, 1211 Geneva 4, Switzerland
[b] Swiss Centre for Applied Human Toxicology (SCAHT), University of Geneva, CMU, Rue Michel-Servet 1, 1211 Geneva 4, Switzerland

What are the gaps? What needs addressed?

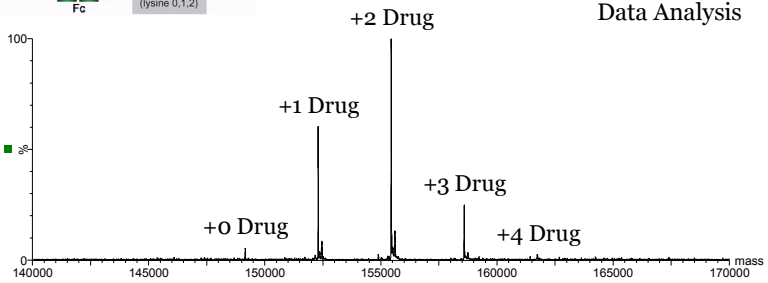


Sample Preparation

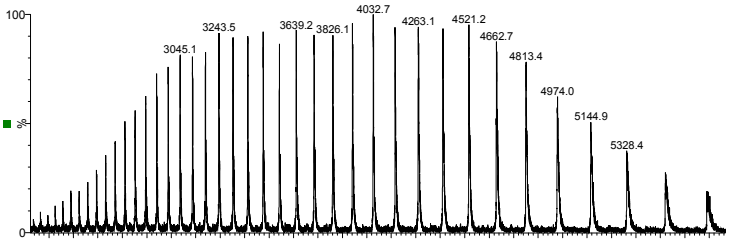
Chromatography

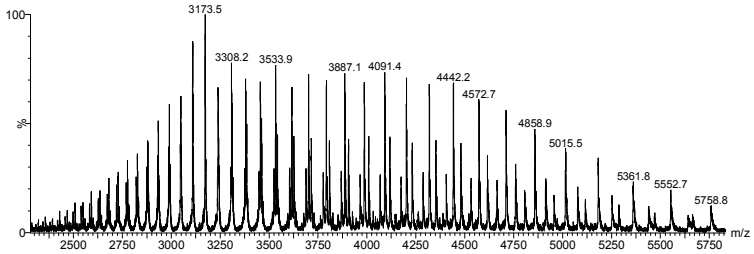
Mass Spectrometry

Data Analysis



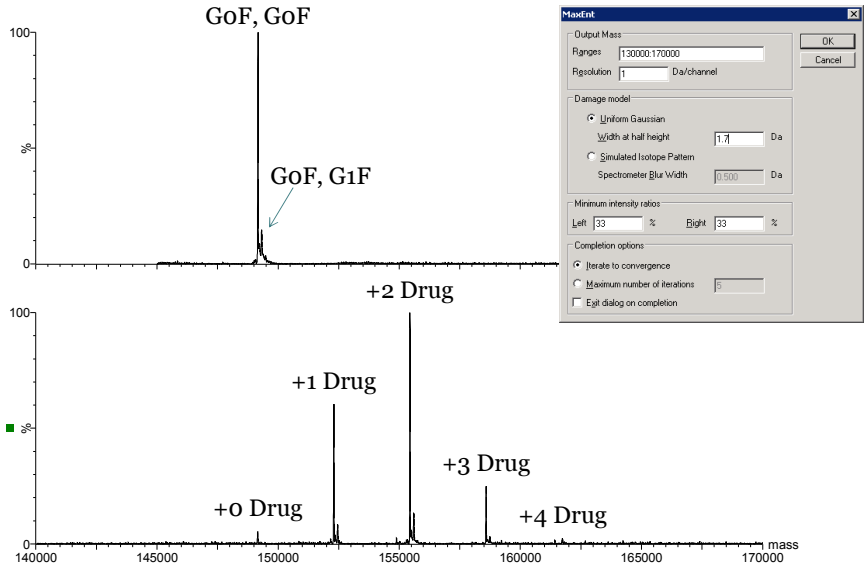
Antibody and ADC – Raw Data





- Caution regarding the *m/z* range selection for MaxEnt1

Antibody and ADC – MaxEnt1 Deconvolution



Sequence Variants

Li Tao and Justin Sperry

ASMS'13 Work Shop

Sequence Variants (SV)

- A critical issue in biologics development
- Safety and efficacy concerns
- SV can be evaluated separately-only theoretically
- More complications down the road during development if SV is at a "significant level"
- Why SV exists?

Sequence Variants (SV)

- Caused by errors during the complex process of protein biosynthesis
  - DNA replication
  - mRNA transcription
  - Protein translation
    - mRNA codon-anticodon reading
    - aminoacyl-tRNA synthesis and proof reading

Errors Rates at Each Step

Steps	Error Rate
DNA replication	~10^{-8}
mRNA transcription	~10^{-5}
Protein translation	~10^{-3}-10^{-4}

Sequence Variants - Causes

- When SVs are detected, it is critical to examine their causes
- Certain amount of ambiguity in genetic coding for protein may have evolutionary significance *(Curr. Opinion Microbio. 2009, 12, 631-637)*
- SVs resulting from mistranslation is not unusual and has been known by the health authorities
  - Low level (<0.5%), Common type (e.g. Ser<->Asn)

Bristol-Myers Squibb 5

Sequence Variants - Impact

Significant issues may arise if SVs are caused by heterogeneous cell lines

- Significant impact on process control, specification setting, molecular properties, safety and efficacy profiles, etc.
- Costly and time consuming if restarting cell line development

- What's your experience on SV?
- Comments on homogeneity of cell lines?

Bristol-Myers Squibb 6

Sequence Variants - Challenges

Challenges in assuring cell lines are homogeneous

- Detecting low levels of sequence variants in the presence of overwhelmingly correct forms
- Limited amount of samples during cell line development
- Comments?

What can be done to reduce SVs?

- During DNA replication---little?
- During mRNA transcription
  - ---codon optimization, others?
- During protein translation
  - ---codon optimization, concurrent expression of tRNA, slow down expression rate, etc.
- Comment?

Factors Inducing SVs

Factors	Misincorporation Causes	Reference
Selecting reagent (MTX)	Ser>Arg, DNA mutation Ser>Asn, mistranslation	Biotechnol. Bioeng, 2010, 107,163–171.
Reactive oxygen species	Ser>Asn, editing defect and missense suppression	PNAS, 2010, 107, 4028-4033.
AA starvation	His>Gln, Asn>Lys, Asn>Ser, etc, misreading	PNAS, 1978, 75, 1091-1095. Biotech. Bioengr. 2010, 107, 116-123.
High expression system	Cys>Phe, etc, missense	Nucleic Acids Research, 1991,19, 3511-3516.
Certain vectors, genes, expression systems	Tyr>Gln, transfection, etc	Mol. Cellular Biology, 1984, 1951-1960. Nat. Biotech. 1993, 11, 1293-1297.

SVs-Codon Effect

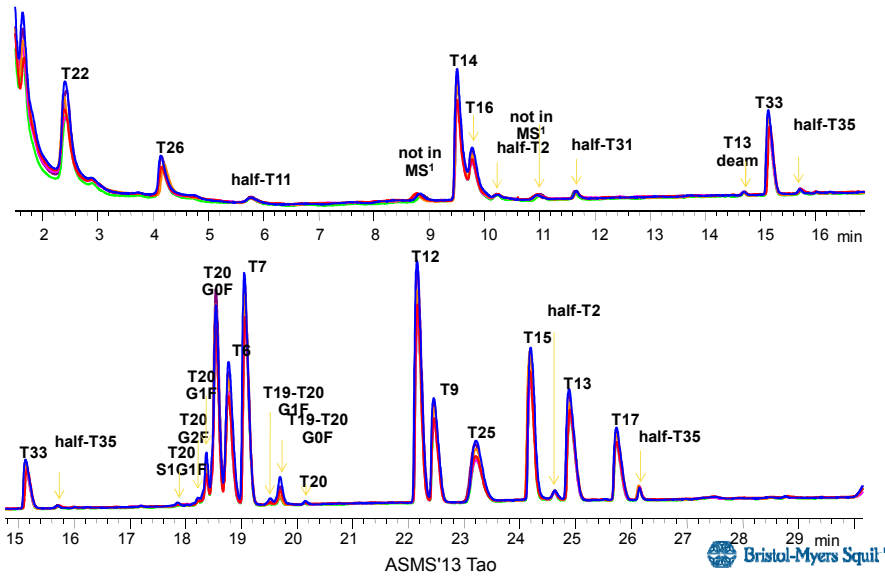
Pair	Host	Codons Involved	Reference
Arg>Lys	E. coli	AGA	Biochem. Biophys. Res. Commun, 1988, 155, 518-523.
Arg> Glu		CGG	Protein Expr. Puri. 2003, 27, 365-374.
Ser>Asn	CHO	AGC	Anal. Chem. 2009, 81, 9282-9290. Biotech. Bioengr. 107, 163–171.
Stop>Gln Stop TAA>Glu, Stop UGA>Trp			mAbs, 2012, 4, 694-700.
Gly>Glu	E. coli	GGA	Protein Sci., 2012, 12, 625-632.

SV Detection

- DNA
- mRNA
- Protein
- Comment?

Bristol-Myers Squibb

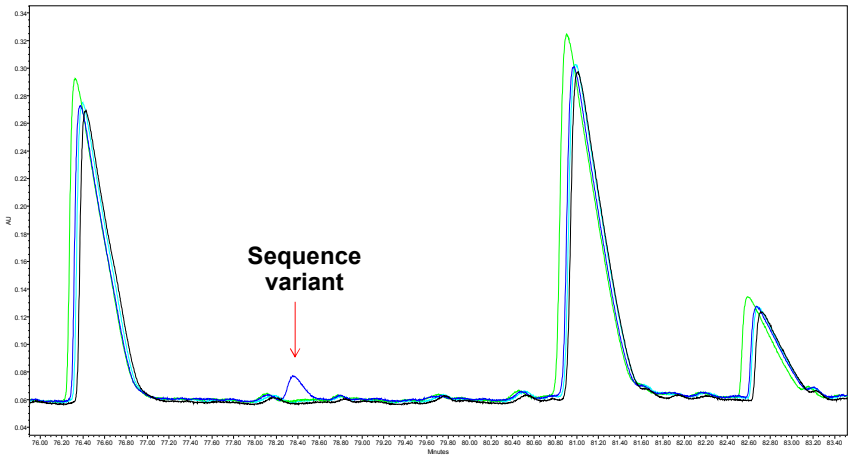
Peptide Mapping – Overlay of Multiple Clones



ASMS'13 Tao

Bristol-Myers Squibb

Peptide Mapping – Overlay of Multiple Clones



SV Detection on Proteins - Mass Shift

			G	A	S	P	V	T	C	L/I	N	D	Q/K	E	M	H	F	R	Y	W
			57	71	87	97	99	101	103	113	114	115	128	129	131	137	147	156	163	186
G	57			14	30	40	42	44	46	56	57	58	71	72	74	80	90	99	106	129
A	71		-14		16	26	28	30	32	42	43	44	57	58	60	66	76	85	92	115
S	87		-30	-16		10	12	14	16	26	27	28	41	42	44	50	60	69	76	99
P	97		-40	-26	-10		2	4	6	16	17	18	31	32	34	40	50	59	66	89
V	99		-42	-28	-12	-2		2	4	14	15	16	29	30	32	38	48	57	64	87
T	101		-44	-30	-14	-4	-2		2	12	13	14	27	28	30	36	46	55	62	85
C	103		-46	-32	-16	-6	-4	-2		10	11	12	25	26	28	34	44	53	60	83
L/I	113		-56	-42	-26	-16	-14	-12	-10		1	2	15	16	18	24	34	43	50	73
N	114		-57	-43	-27	-17	-15	-13	-11	-1		1	14	15	17	23	33	42	49	72
D	115		-58	-44	-28	-18	-16	-14	-12	-2	-1		13	14	16	22	32	41	48	71
Q/K	128		-71	-57	-41	-31	-29	-27	-25	-15	-14	-13		1	3	9	19	28	35	58
E	129		-72	-58	-42	-32	-30	-28	-26	-16	-15	-14	-1		2	8	18	27	34	57
M	131		-74	-60	-44	-34	-32	-30	-28	-18	-17	-16	-3	-2		6	16	25	32	55
H	137		-80	-66	-50	-40	-38	-36	-34	-24	-23	-22	-9	-8	-6		10	19	26	49
F	147		-90	-76	-60	-50	-48	-46	-44	-34	-33	-32	-19	-18	-16	-10		9	16	39
R	156		-99	-85	-69	-59	-57	-55	-53	-43	-42	-41	-28	-27	-25	-19	-9		7	30
Y	163		-106	-92	-76	-66	-64	-62	-60	-50	-49	-48	-35	-34	-32	-26	-16	-7		23
W	186		-129	-115	-99	-89	-87	-85	-83	-73	-72	-71	-58	-57	-55	-49	-39	-30	-23	

- AAMs in red have been reported (list may be complete)

Detecting Sequence Variants by MS -False Negatives

Peptide #1

% spiked into mAb #1	10.0%	5.0%	2.5%	1.3%	0.6%	0%
Observed in mass spectra?	yes	yes	yes	yes	yes	no
Identified by SEQUEST and homebuilt scripts?	yes	yes	yes	yes	yes	no
Identified by Mascot ETS?	yes	yes	yes	yes	no	no

Peptide #2

% spiked into mAb #1	10.0%	5.0%	2.5%	1.3%	0.6%	0%
Observed in mass spectra?	yes	yes	yes	no	no	no
Identified by SEQUEST and homebuilt scripts?	yes	yes	yes	no	no	no
Identified by Mascot ETS?	no	no	no	no	no	no

Bristol-Myers Squibb

Detecting Sequence Variants by MS -False Negatives

Peptide #3

% spiked into mAb #1	5.0%	1.0%	0.2%	0%
Observed in mass spectra?	yes	yes	no	no
Identified in SEQUEST and homebuilt scripts?	yes	no	no	no
Identified in Mascot ETS?	yes	no	no	no

Peptide #4

% spiked into mAb #1	5.0%	1.0%	0.2%	0%
Observed in mass spectra?	yes	no	no	no
Identified in SEQUEST and homebuilt scripts?	yes	no	no	no
Identified in Mascot ETS?	yes	no	no	no

Bristol-Myers Squibb

Detecting Sequence Variants by MS -False Positives (FPs)

mAb#2 spiked into mAb#1

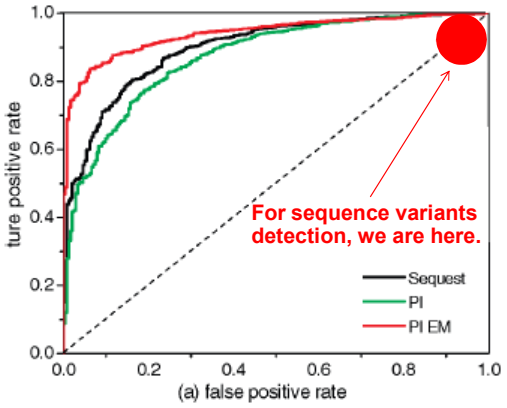
Spiked molar percentage	10.0%	5.0%	2.5%	1.3%	0.6%	0%
FPs by SEQUEST and homebuilt scripts, mass shift only	65	48	58	54	54	53
FPs by SEQUEST and homebuilt scripts, mass shift & position	116	79	110	113	122	111
FPs by Mascot ETS, mass shift only	67	68	62	62	56	55
FPs by Mascot ETS, mass shift & position	73	70	65	67	57	57

Synthetic peptide spiked into mAb#1

Spiked molar percentage	5.0%	1.0%	0.2%	0%
FPs by SEQUEST and homebuilt scripts, mass shift only	48	49	54	55
FPs by SEQUEST and homebuilt scripts, mass shift & position	110	82	93	106
FPs by Mascot ETS, mass shift only	62	58	57	67
FPs by Mascot ETS, mass shift & position	63	59	60	68

Bristol-Myers Squibb

Sensitivity vs False Positive



Sun, et al. J. Proteo. Res. 2008, 7, 202–208.

Bristol-Myers Squibb