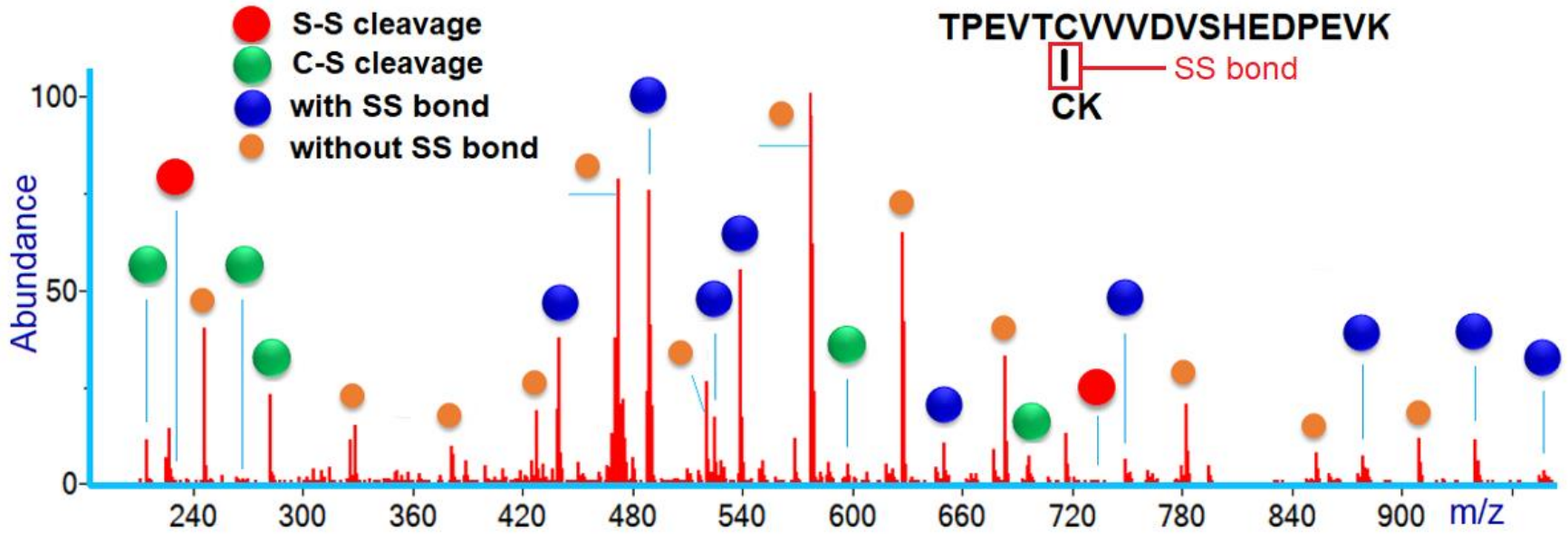
Developing a Mass Spectral Library for the Detailed Analysis of IgG1 Disulfide Bonds (SS) and the Detection of Their Scrambling in LC-MS/MS Experiments



Qian Dong, Xinjian Yan, Yuxue Liang, William E. Wallace, and Stephen E. Stein
Mass Spectrometry Data Center, NIST

CASSS Mass Spec 2021, September 22, 2021

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

OUTLINE

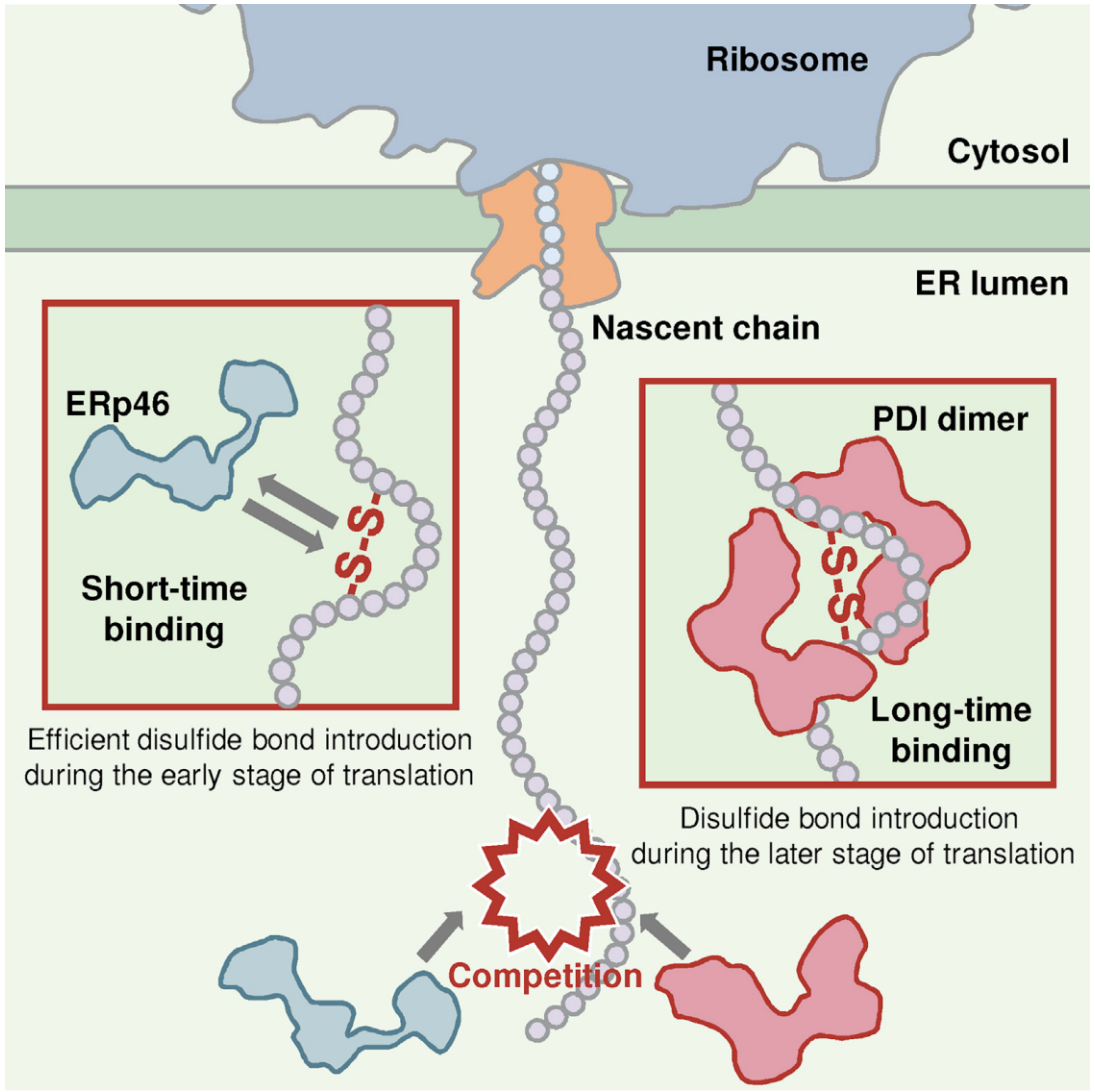
1. Importance of disulfide bonds and their challenges in highly complex disulfide analysis
2. Novel methods for a complete disulfide mapping in the NISTmAb

3. Results
   - Identification of both native and scrambled SS bonds
   - Observation of HCD fragmentation patterns of SS bonds
   - Development of a mass spectral library to assist disulfide analysis

4. Utility of the spectral library

What Are Disulfide Bonds (SS)?

- Disulfide bonds, also known as disulfide bridges, form in nearly one-third (~7000) of proteins, including:
  - Cell surface proteins
  - Secretory proteins
- Formed during the biosynthesis of the proteins in the mammalian ER
- Occurs co-translationally by 20 enzymes



*Hirayama et al., 2021, **iScience** 24, 102296*
https://doi.org/10.1016/j.isci.2021.102296

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

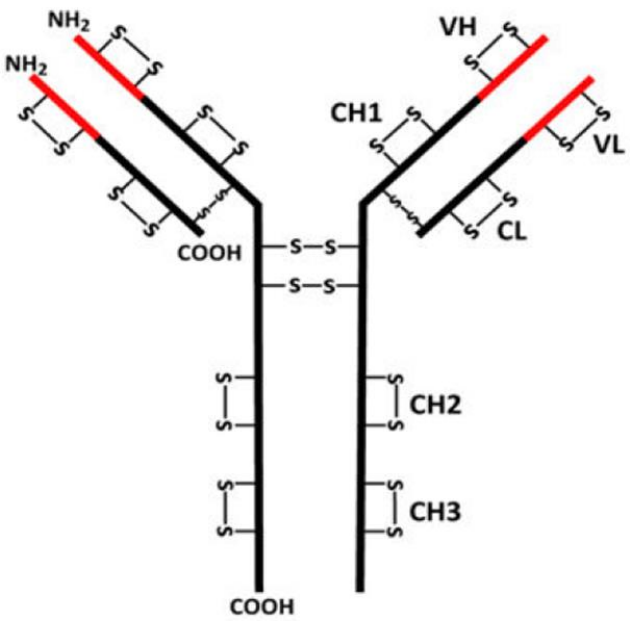
Diverse Functional Roles of Disulfide Bonds and Their Implications in Diseases

Dysregulation of structural disulfide-bond formation involved in neurodegenerative misfolding diseases

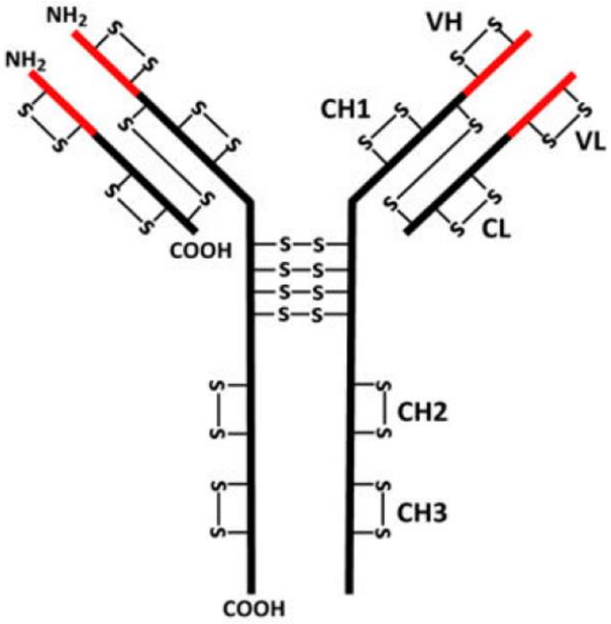
- Alzheimer's disease
- Parkinson's disease
- Huntington's disease
- Spinal and bulbar muscular atrophy X-linked 1
- Spinocerebellar ataxias
- Neurodegenerative disease
- Prion-related disorders
- Amyotrophic lateral sclerosis

All IgGs Are Highly Disulfide-Bonded

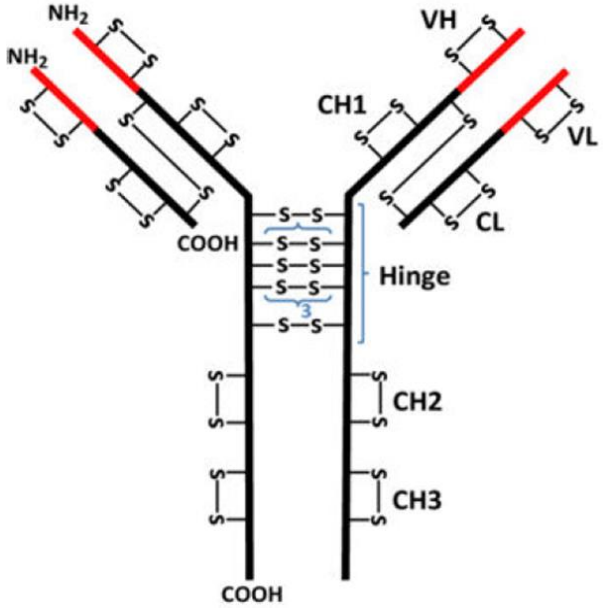
IgG1, 16 SS bonds



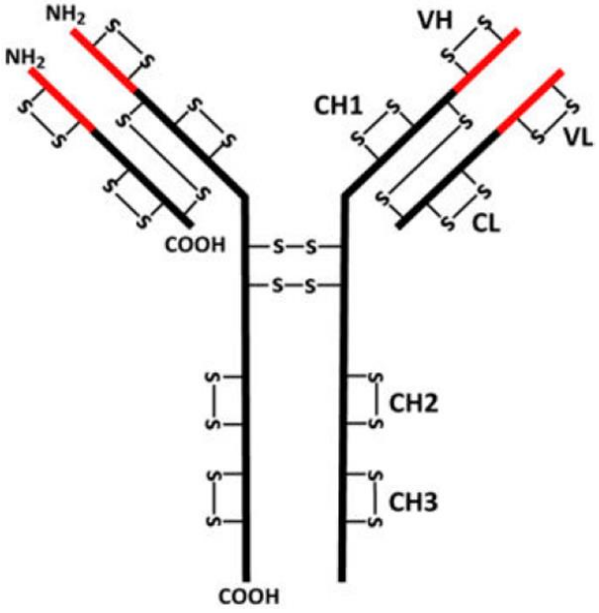
IgG2, 18 SS bonds



IgG3, 25 SS bonds



IgG4, 16 SS bonds



Lakbub, J. C., Shipman, J. T., & Desaire, H. (2018). ***Analytical and bioanalytical chemistry****, 410(10), 2467–2484.*

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

Overview of Available Peptide Mapping Approaches for the Assignment of Disulfide Bonds (SS)

The common strategy

I. Adjust the conditions such as pH before and during protein digestion

II. Digest proteins under non-reduction into peptides containing SS bonds.

III. Separate peptides using electrophoretic or chromatographic methods to isolate SS-bonded peptides

IV. Analyze MS2 spectra to identify SS-linked peptides

The available workflows

A. HPLC peptide profile comparison

B. LC-MS/MS of non-reduced protein

C. LC-MS/MS of partially-reduced protein

D. LC-MS/MS of non-reduced protein/gas phase reduction

Significant Challenges in Disulfide (SS) Analysis

- Spontaneous disulfide shuffling in sample preparation
- Incomplete digestion of non-reduced proteins
- Complex intertwined disulfides
- Large peptide masses, higher charge states, and low ionization efficiency
- Generally, very complex tandem mass spectra of SS linked peptides
- Fragmentation lacks readily identifiable diagnostic fragment ions
- Bioinformatics tools are incapable of recognizing various SS linked ions

A JPR Paper and Disulfide Spectral Library

Journal of proteome research

pubs.acs.org/jpr

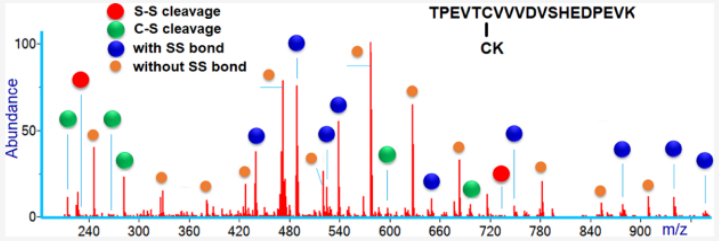
Article

Comprehensive Analysis of Tryptic Peptides Arising from Disulfide Linkages in NISTmAb and Their Use for Developing a Mass Spectral Library

Qian Dong,* Xinjian Yan, Yuxue Liang, Sanford P. Markey, Sergey L. Sheetlin, Concepcion A. Remoroza, William E. Wallace, and Stephen E. Stein

Cite This: *J. Proteome Res.* 2021, 20, 1612–1629 Read Online

ACCESS | Metrics & More | Article Recommendations | Supporting Information



ABSTRACT: This work presents methods for identifying and then creating a mass spectral library for disulfide-linked peptides originating from the NISTmAb, a reference material of the humanized IgG1k monoclonal antibody (RM 8671). Analyses involved both partially reduced and non-reduced samples under neutral and weakly basic conditions followed by nanoflow liquid chromatography tandem mass spectrometry (LC–MS/MS). Spectra of peptides containing disulfide bonds are identified by both MS1 ion and MS2 fragment ion data in order to completely map all the disulfide linkages in the NISTmAb. This led to the detection of 383 distinct disulfide-linked peptide ions, arising from fully tryptic cleavage, missed cleavage, irregular cleavage, complex Met/Trp oxidation mixtures, and metal adducts. Fragmentation features of disulfide bonds under low-energy collision dissociation were examined. These include (1) peptide bond cleavage leaving disulfide bonds intact; (2) disulfide bond cleavage, often leading to extensive fragmentation; and (3) double cleavage products resulting from breakages of two peptide bonds or both peptide and

NISTmAb Mass Spectral Library of Disulfide-Linked Peptides

Paper: https://pubs.acs.org/doi/10.1021/acs.jproteome.0c00823
Library: https://chemdata.nist.gov/dokuwiki/doku.php?id=peptidew:lib:disulfidepeptides

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

Our Goal: Build an Advanced Spectral Library for Analyzing Disulfide Bonds in All Biologic Drugs

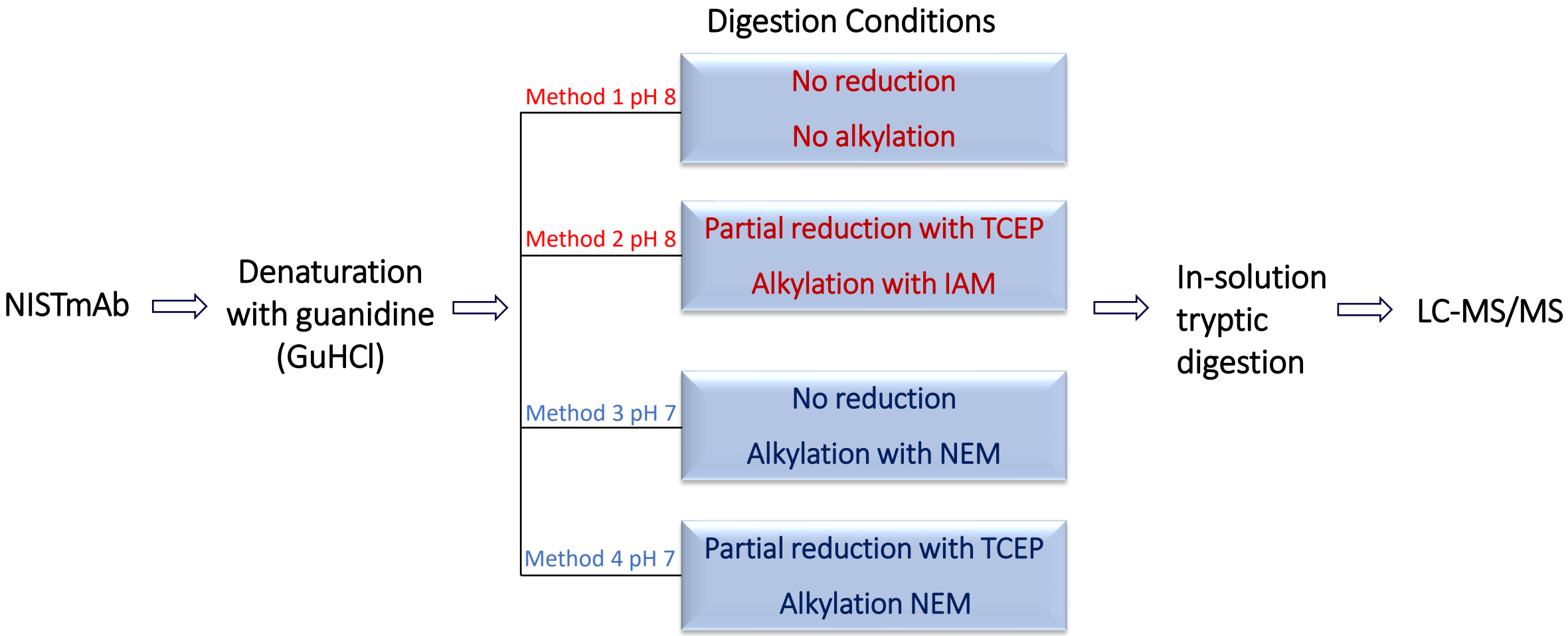
Current objectives:

1. Develop methods for identifying as many disulfide-bonded peptides and their product ions as possible in LC-MS/MS
2. Enable automated profiling and identification of the disulfide linkages of IgG1 antibodies
3. Build a reference spectral library to assist routine disulfide analysis in the BioPharma industry

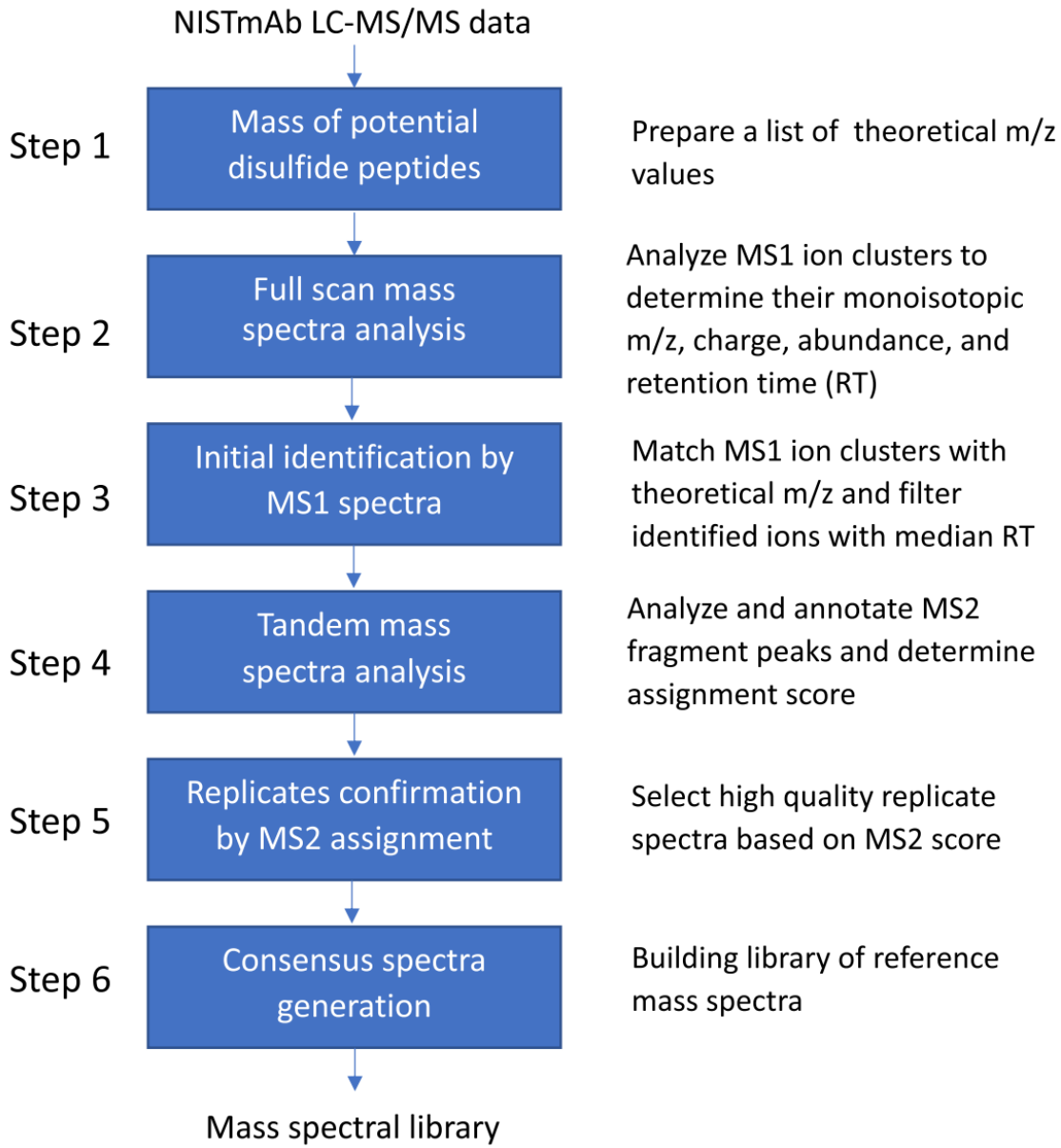
Rationale for these objectives:

1. Provide an easy-to-use analytical platform for automated disulfide analysis in the biopharma industry
2. Facilitate difficult MS2 spectra interpretation for disulfide-bonded peptides

Protocols for Sample Preparation Under pH 8 and 7



Methods of Integrating MS1 and MS2 Data for Disulfide Analysis and Spectral Library Creation



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

144 *Native* Disulfide-Linked Peptide Ions Identified From Non/Partial Reduction Conditions at pH 8

A complete SS bonds mapping in the NISTmAb

# bond	disulfide linkage	Peptide ions
(1) VH	**Cys22 - Cys97**	**34**
(2) CH1	Cys147- Cys203	34
(3) CH2	Cys264 - Cys324	30
(4) CH3	Cys370 - Cys428	12
(5) VL	Cys23 - Cys87	3
(6) CL	Cys133 - Cys193	13
(7) H-L	Cys223 - Cys213	9
(8 & 9) Hinge	Cys229 - Cys229 Cys232 - Cys232	20

Using multiple classes of SS-linked peptides for disulfide mapping

(a) Tryptic peptide

ESGPALVKPTQTLTLTCTFSGFSLSTAGMSVGWIR

VTNMDPADTATYYCAR

(b) Miscleaved peptide

ESGPALVKPTQTLTLTCTFSGFSLSTAGMSVGWIR

VTNMDPADTATYYCARDMIFNFYFDVWGQGTTVTVSSASTK

(c) Semitryptic peptide

ESGPALVKPTQTLTLTCTFSGF~~SLSTAGMSVGWIR~~

VTNMDPADTATYYCAR

(d) Oxidized peptide

ESGPALVKPTQTLTLTCTFSGFSLSTAGM(oxid)SVGW(Oxid)IR

VTNM(Oxid)DPADTATYYCAR

(e) Metallated peptide

ESGPALVKPTQTLTLTCTFSGFSLSTAGMSVGWIR

VTNMD(Ca[II])PAD(Ca[II])TATYYCAR

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

144 *Native* Disulfide-Linked Peptides Identified From Non/Partial Reduction Conditions at pH 8

Complete SS bonds mapping in the NISTmAb

# bond	disulfide linkage	Peptide ions
(1) VH	Cys22 - Cys97	34
(2) CH1	Cys147- Cys203	34
(3) CH2	Cys264 - Cys324	30
(4) CH3	Cys370 - Cys428	12
(5) VL	Cys23 - Cys87	3
(6) CL	Cys133 - Cys193	13
(7) H-L	**Cys223 - Cys213**	**9**
(8 & 9) Hinge	Cys229 - Cys229 Cys232 - Cys232	20

Using ss-linked peptides with missed cleavage for disulfide mapping

1. Tryptic peptide not detectable:

SCDK
|
GEC

2. Miscleaved peptides

(a) SCDKTHTC(CAM)PPC(CAM)PAPELLGGPSVFLFPPKPK
|
GEC

(b) SCDKTHTC(CAM)PPC(CAM)PAPELLGGPSVFLFPPKPK
|
SFNRGEC

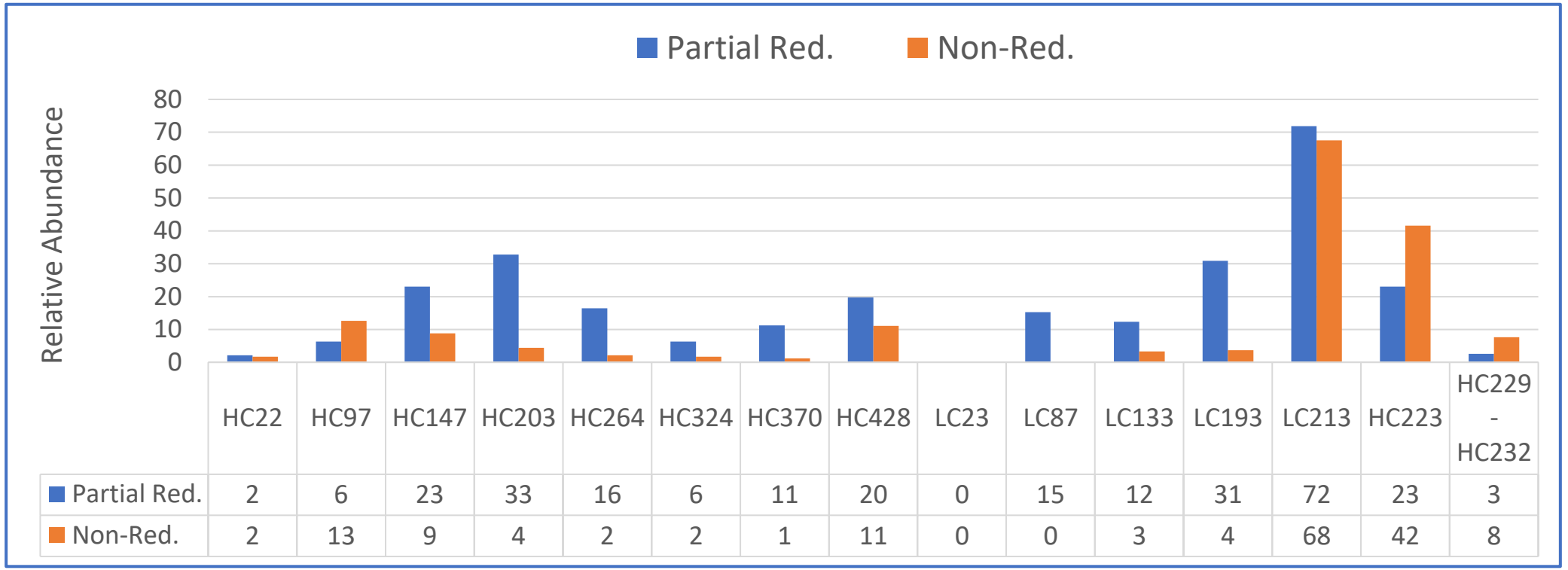
(c) SCDKTHTC(CAM)PPC(CAM)PAPELLGGPSVFLFPPKPKDTLMISR
|
SFNRGEC

(d) SCDK
|
SFNRGEC

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

86 *Non-Native* Disulfide Bonds Identified From Non/Partial Reduction Conditions at pH 8 (63% of 136 possible linkages)

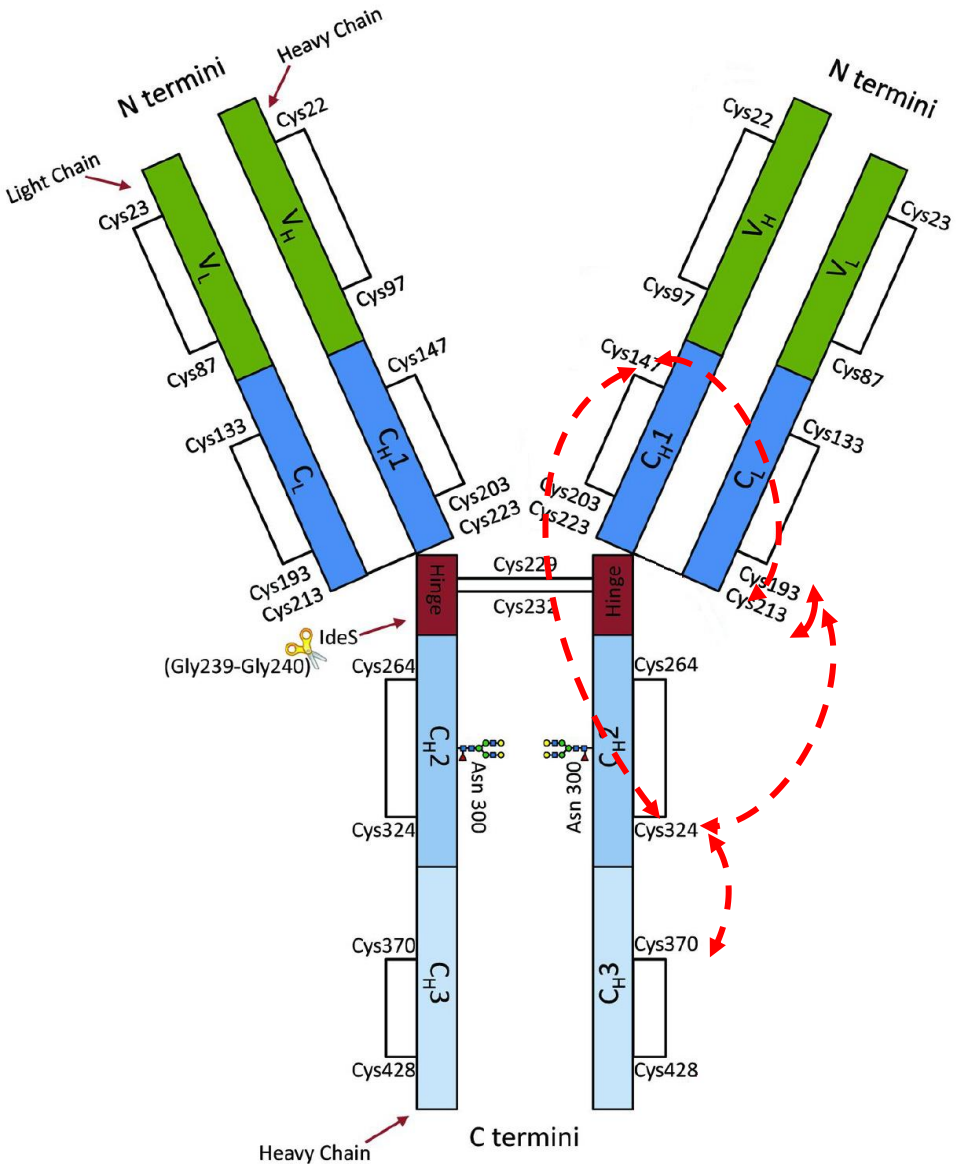
Various Levels of Scrambling Occurring at Cys Residues in 18 h Tryptic Digest (pH 8)



	HC22	HC97	HC147	HC203	HC264	HC324	HC370	HC428	LC23	LC87	LC133	LC193	LC213	HC223	HC229 - HC232
Partial Red.	2	6	23	33	16	6	11	20	0	15	12	31	72	23	3
Non-Red.	2	13	9	4	2	2	1	11	0	0	3	4	68	42	8

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

Five Very Low-Level Scrambled SS Bonds Identified From Non-Reduction Conditions at pH 7

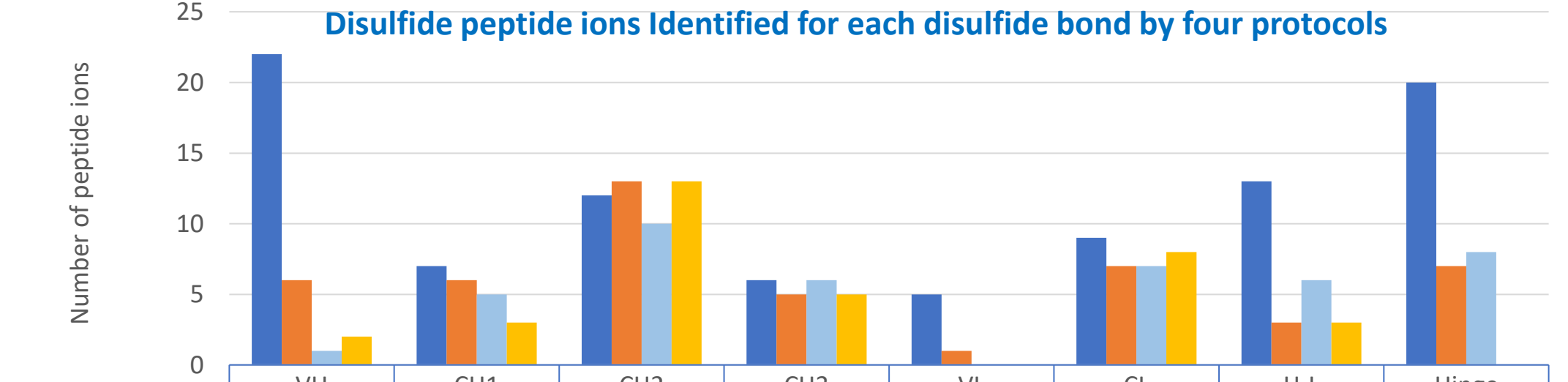


Scrambled disulfide bond	MASS	SS-linked peptide	*z*	RT	Log Int
HC147-LC213	1568.718	STSGGTAALGCLVK_SS_GEC	2,3	91.3	8.7
	2072.962	STSGGTAALGCLVK_SS_SFNRGEC	4	91.7	7.9
HC147-HC324	1510.748	STSGGTAALGCLVK_SS_CK	2,3	83.0	8.6
HC324-HC370	1350.700	CK_SS_NQVSLTCLVK	2,3	86.1	8.7
HC324-LC193	2064.997	CK_SS_VYACEVTHQGLSSPVTK	2,3,4	82.1	8.1
LC193-LC213	2122.966	VYACEVTHQGLSSPVTK_SS_GEC	3	88.9	6.8

Note
Z: charge state
Log Int: log 10 peptide ion intensity values

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

Comparison of Four Digestion Protocols Using Native SS-Linked Peptide Analysis



	VH	CH1	CH2	CH3	VL	CL	H-L	Hinge
pH 8 Partial-Red.	22	7	12	6	5	9	13	20
pH 8 Non-Red.	6	6	13	5	1	7	3	7
pH 7 Partial Red.	1	5	10	6	0	7	6	8
pH 7 Non-Red.	2	3	13	5	0	8	3	0

144

55

Observations:

- More IDs by the partial reduction at pH 8 than other methods
- 114 disulfide-linked peptides by pH 8 were obtained, whereas 55 IDs by pH 7
- Control experiment at pH7 did not detect two linkages

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

What are the Product Ions of Disulfide-Linked Peptides?

Example peptide: Contains a single disulfide bond in the Light Chain constant region (CL bond)

(1) SGTASVV**C**LLNNFYPR P1 (LC133)

|

VYA**C**EVTHQGLSSPVTK P2 (LC193)

(2) MS/MS spectrum =

b, y, and other ion series from "SGTASVV**C(P2-2H)**LLNNFYPR" +

b(2), y(2), and other ions from "VYA**C(P1-2H)**EVTHQGLSSPVTK"

(3) Three groups of product ions:

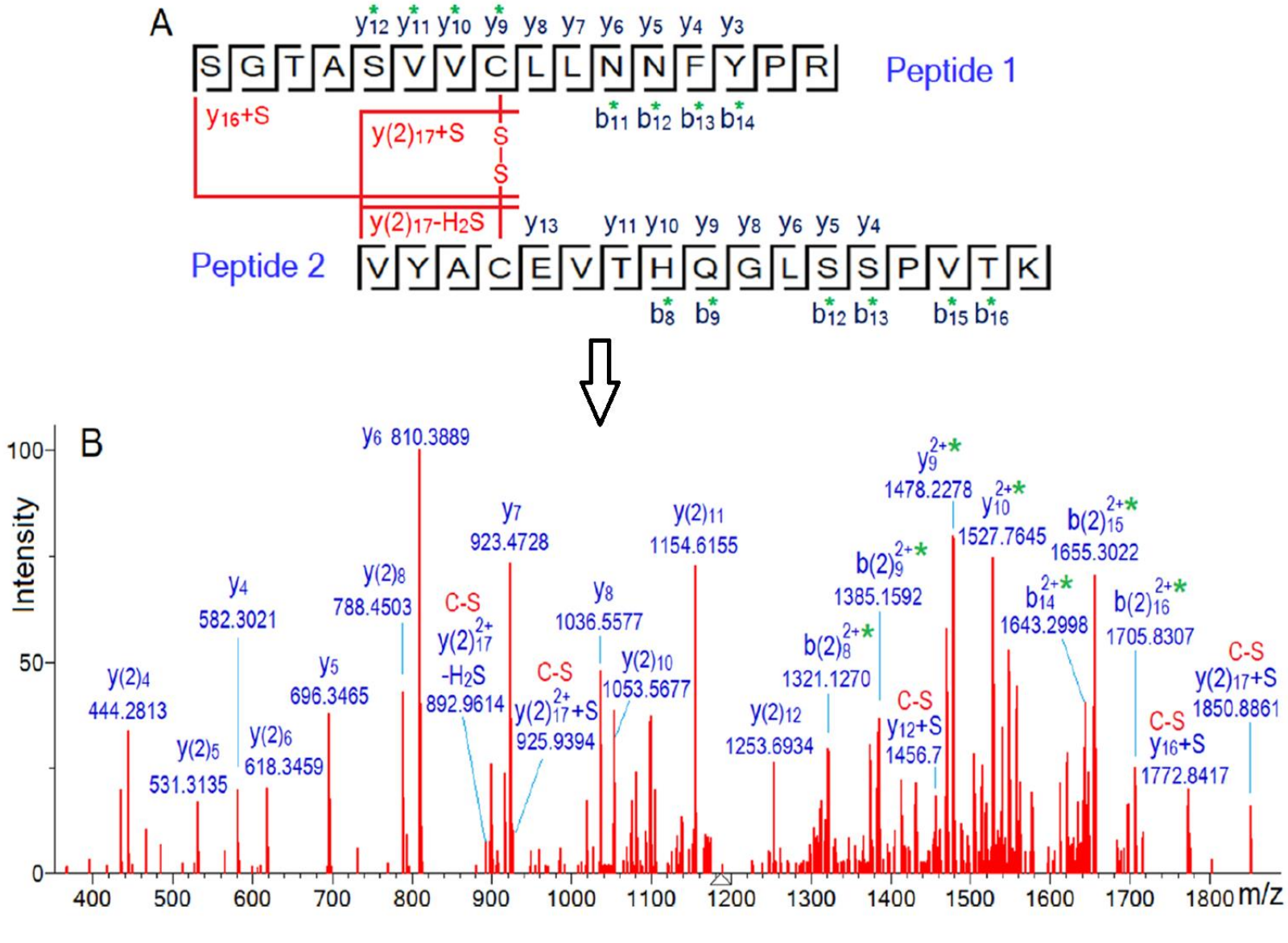
Group 1. Peptide backbone cleavage products with an intact disulfide bond

Group 2. Disulfide bond cleavage products (C-S, S-S)

Group 3. Peptide backbone cleavage products without an intact disulfide bond

Note: Both single and double cleavage occurring for all above groups

MS/MS Spectrum of 3+ Ion at m/z 1186.257 By FT-CID Fragmentation at NCE of 35%



Group 1
contains 35 peptide backbone fragments all with an intact disulfide linkage

Group 2
contains 5 product ions arising from disulfide cleavage

Group 3
includes 15 standard peptide fragments without a disulfide bond

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

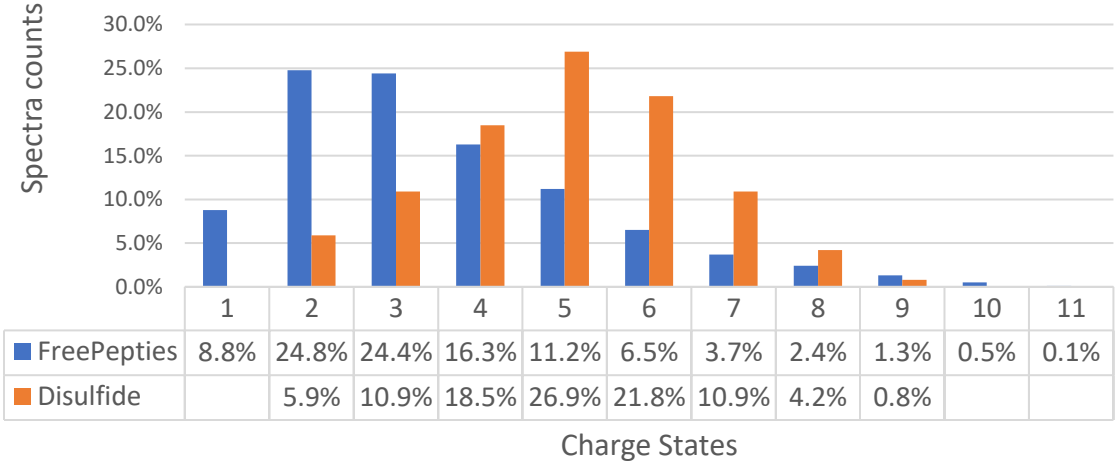
NISTmAb Disulfide-Linked Peptide Library v21.1

Library Features

1) 1,228 Reference Spectra (478 Consensus and 596 "Best" Selected Spectra)
2) 144 different peptides from native SS bonds
3) 109 different peptides from 88 scrambled SS bonds
4) Multiple collisional energies
5) 82 LC–MS/MS runs under non/partial reduction
6) Orbitrap Fusion Lumos and Q Exactive
7) Reference spectra for peptides with large masses, high charge states, and long peptides

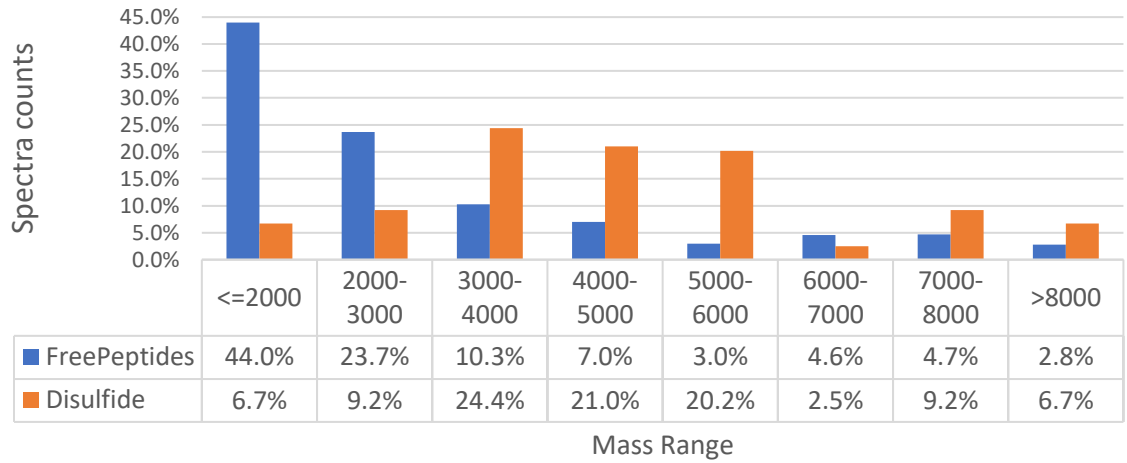
https://chemdata.nist.gov/dokuwiki/doku.php?id=peptidew:lib:disulfidepeptides

Figure A Charge states of free and disulfide peptides



	1	2	3	4	5	6	7	8	9	10	11
FreePepties	8.8%	24.8%	24.4%	16.3%	11.2%	6.5%	3.7%	2.4%	1.3%	0.5%	0.1%
Disulfide		5.9%	10.9%	18.5%	26.9%	21.8%	10.9%	4.2%	0.8%		

Figure B Mass distribution of free and disulfide peptides



	<=2000	2000-3000	3000-4000	4000-5000	5000-6000	6000-7000	7000-8000	>8000
FreePeptides	44.0%	23.7%	10.3%	7.0%	3.0%	4.6%	4.7%	2.8%
Disulfide	6.7%	9.2%	24.4%	21.0%	20.2%	2.5%	9.2%	6.7%

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

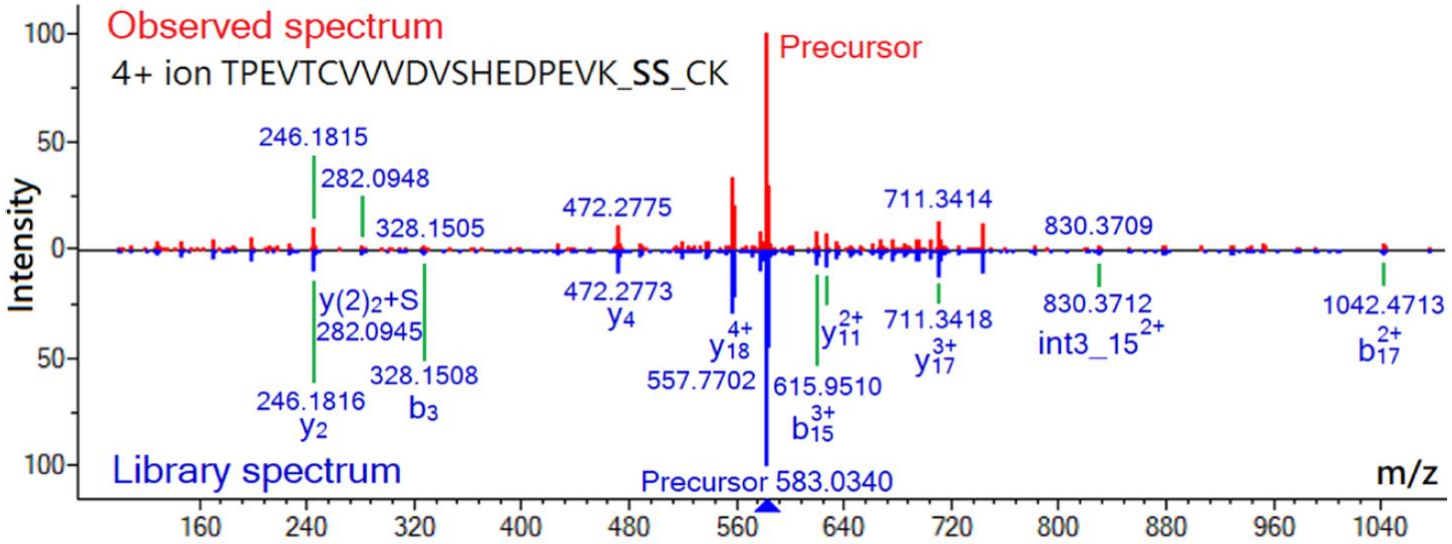
Use of the Reference Library in Evaluating Effectiveness of Reduction in Sample Preparation

(1) Library search using the NISTmAb disulfide-linked peptide library v21.1

Three LC-MS/MS datasets	Spectra	SS bond
1. Humira, commercial mAb drug	5	2 native disulfide bonds
2. NIST reference material SRM 1950	11	2 native and 2 scrambled bonds
3. NISTmAb prepared without second reduction	29	5 native and 4 scrambled bonds

(2) Head-to-tail plot of experimental and reference HCD tandem mass spectra

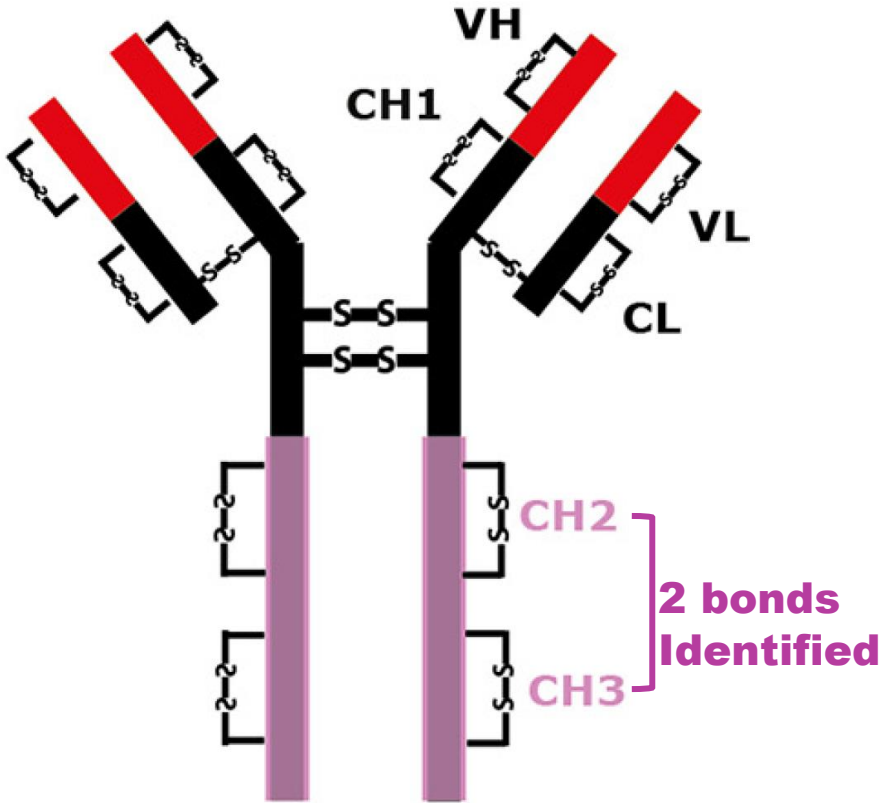
An example of a 4+ peptide ion containing CH2 disulfide linkage found in the 2D LC–MS/MS analysis of reduced human plasma reference material (NIST SRM 1950)



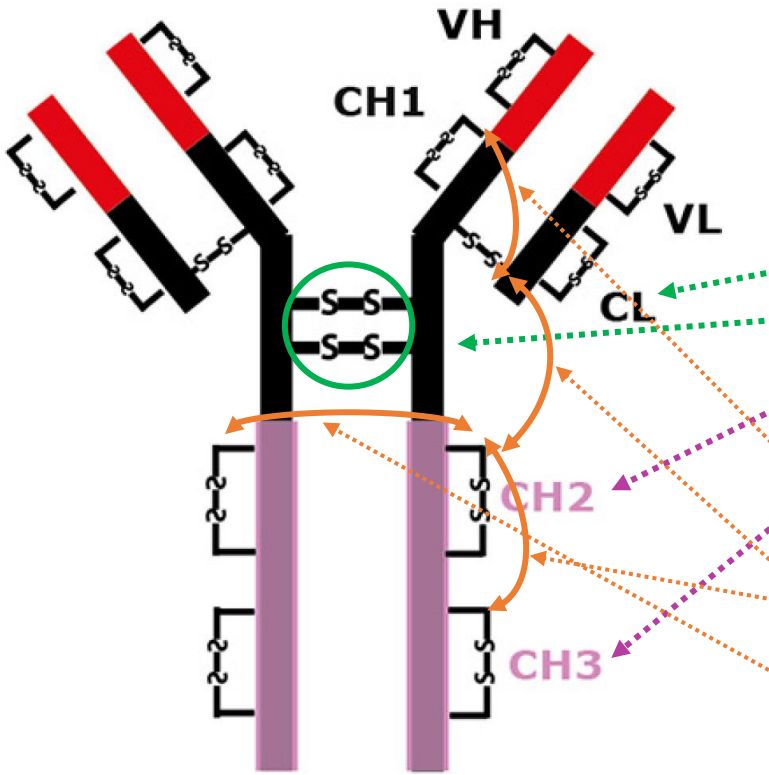
National Institute of Standards and Technology
U.S. Department of Commerce

Use of Spectral Library in Reanalysis of a Public Dataset of Non-reducing SS Bond Mapping Experiment to Improve Disulfide Identification

Original NISTmAb SS bond mapping



NISTmAb SS bond reanalysis by the Spectral Library



29 disulfide peptides

Native SS bonds:

- CL
- Hinge
- CH2
- CH3

Scrambled SS bonds:

- HC147-LC213
- HC264-HC370
- HC264-LC213
- HC264-HC264

NISTmAb Disulfide-Linked Peptide Library v21.1

Samodova D et al, ProAlanase is an Effective Alternative to Trypsin for Proteomics Applications and Disulfide Bond Mapping. Mol Cell Proteomics. 2020 Dec;19(12):2139-2157

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

Key Points/Takeaways

I. Disulfide linkages are one of the most critical post-translational modifications due to their direct impact on the higher-order structure of mAbs, and hence their potency, safety and efficacy

II. The availability of an easy-to-use, automated bioinformatics platform for routine disulfide analysis is the greatest challenge in the analytics required for biotherapeutics development and production.

III. Spectral library-based analysis of SS bonds is advantageous over database search tools for facile identification of not only native but also scrambled SS bonds in antibody drugs or in experiments. As such, spectral library has great potential to be a pivotal tool in S-S bond analysis in current biologics development.

Acknowledgement

Stephen Stein
Yuxue Liang
Eric Yan
Oleg Toropov
Sanford Markey
William Wallace
Sergey Sheetlin
Concepcion Remoroza

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