NEW TOOLS FOR BIOLOGICS ANALYSES AND QC:

The Shredder™ and S-Trap™ Turbo MAM

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INTRODUCTION

Quality control (QC) in biologics manufacturing is mandated and essential for ensuring the safety and efficacy of biological drugs. Comprehensive monitoring of critical quality attributes (CQAs) in biologics, including the analysis of impurities, post-translational modifications (PTMs), and sequence variations, has traditionally relied on methods such as enzymatic and chemical digestion followed by various gas-phase fragmentation techniques.

However, these conventional techniques face several challenges: the presence of contaminants like detergents, lipids, and stabilizers can compromise results; achieving comprehensive sequence coverage can be problematic, especially when peptides produced are too short or when fragmentation fails to yield informative results; and unwanted modifications such as oxidations and deamidations are frequently introduced during sample preparation. As a result, multiple development iterations, including the use of specialized sample cleanup procedures and multiple enzymes, are frequently necessary.

To address these issues, we introduce two new tools. The first, our Multiple Attribute Method (MAM) kit, is specifically engineered to handle contaminants at every stage of biologics manufacturing from cell culture to final formulation, including in automated workflows. It effectively removes surfactants, stabilizers, polymers, buffers, salts, etc. and is compatible with multiple proteases; the MAM kit is specifically engineered to significantly reduce the artificial introduction of chemical artifacts. The second tool, the Shredder, offers a novel approach to bottom-up proteomics sample preparation. It randomly cleaves peptides along the backbone, producing nested sets of peptides that overcome traditional fragmentation challenges and provide enhanced certainty about sequences, including the precise locations of PTMs.

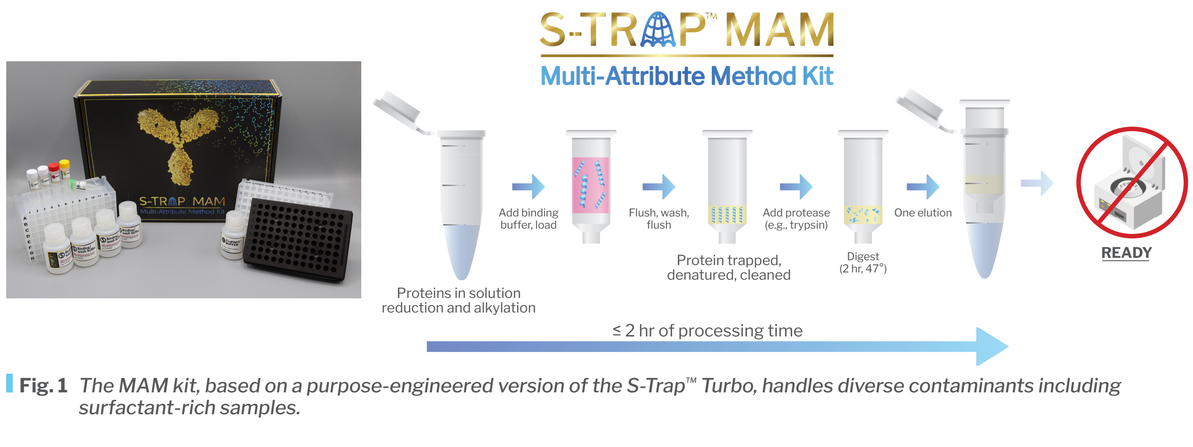


Fig. 1 *The MAM kit, based on a purpose-engineered version of the S-Trap™ Turbo, handles diverse contaminants including surfactant-rich samples.*

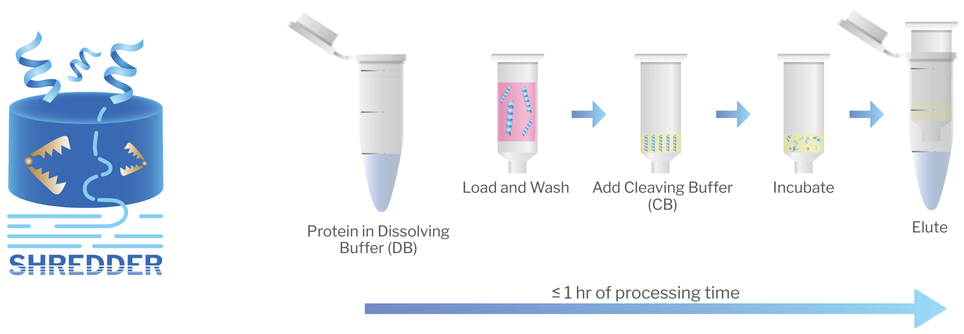


Fig. 2 *The Shredder applies a pseudo-enzymatic exceedingly low-specificity cleavage at all points along a peptide backbone to generate a series of highly overlapping peptides for complete sequence coverage.*

LC-MS Results

The LC-MS runs of multiple MAM analyses showed retention time shifts equivalent to technical replicates and good peak alignment across replicates (Fig. 3); LC-MS profiles indicate an efficient protein digestion and a successful clean-up. Adjacent blank prepared samples do not show any trace of peptides or contamination, confirming the absence of cross-contamination between samples. Selected doubly-charged tryptic peptides from the NIST mAb RM 8671 were evaluated for reproducibility ***(Fig. 3, table)***. The variation of the double-charged ions XIC areas were calculated and expressed as % CV. All of the peptides showed an overall CV of ≤ 20%.

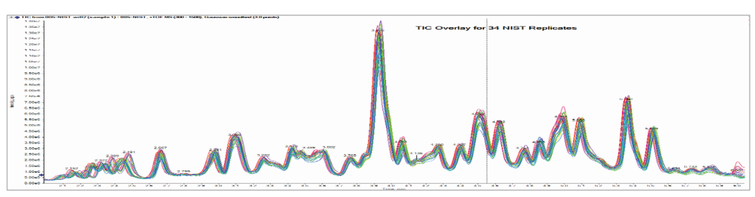


Fig. 3 *Total Ion Current (TIC) overlay for 34 NIST samples.*

Peptide sequence	[M+2H]$^{2+}$	CV (%)	RT (min)
FNWYVDGVEVHNAK	839.4046	14	3.92
ALPAPIEK	419.7553	8.6	3.06
GFYPSDIAVEWESNGQPENNYK	1272.5693	12	4.38
TTPPVLDSDGSFFLYSK	937.4645	16	4.60
LLIYDTSK	476.7711	8.9	3.41
LASGVPSR	393.7271	9.1	2.34
VDNALQSGNSQESVTEQDSK	1068.488	18	2.65
DSTYSLSSTLTLSK	751.8829	20	3.93
VYACEVTHQGLSSPVTK	938.4671	16	4.60

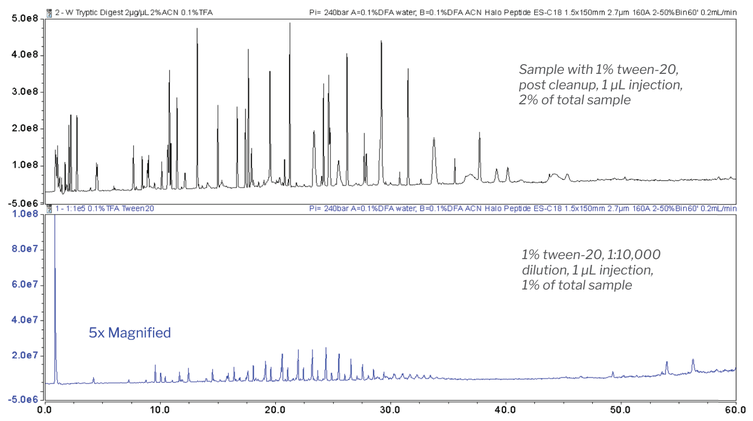


Fig. 4 *S-Trap™ sample processing removes detergents, PEG and other contaminants.*

Contaminant Removal: Example of detergent removal. 100 µL of 1% tween-20 containing 100 µg mAb was processed by S-Trap™. 2% of the sample was then injected ***(Fig. 4, upper panel)***. The lower panel is a 1 µL injection of a 1:10,000 dilution of 1% tween-20 representing a 20,000 fold dilution compared to the upper panel. Assuming a linear response with increasing concentration the equivalent detergent signal would be in the range of 4E12.

Biosimilar Characterization: Data acquired from NIST mAb prepared with the MAM kit was acquired on an Agilent 6546, ZenoTOF, TimsTof Pro and Thermo QE, and analyzed using Protein Metrics Byologic; additional searches on TimsTOF Pro data were performed using Spectronaut. Excellent sequence coverage between 99% - 100%, as well as the expected glycosylations, disulfide bonds, oxidizations and deamidations were observed ***(Figures 5 and 6)***. Digestions were reproducible and over 100 Host Cell Proteins *(HCPs)* were identified from the murine cell line used to generate the mAb RM8671 *(TimsTof analysis)*.

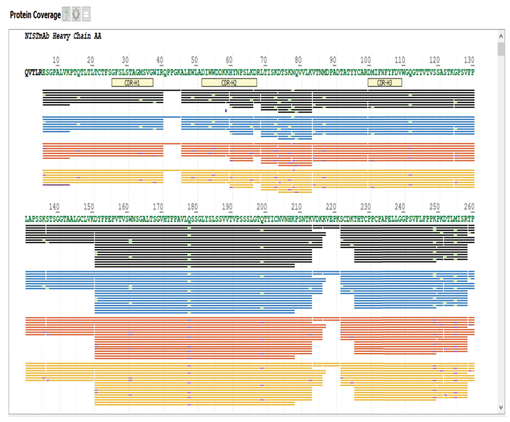


Fig. 5 *Glycopeptide identification (Agilent 6546)*

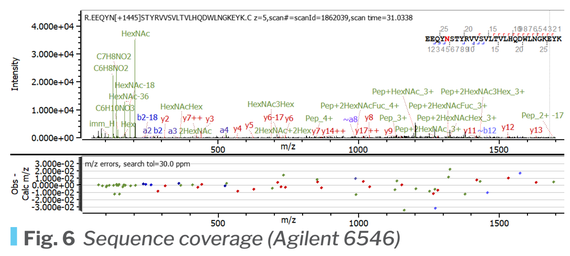


Fig. 6 *Sequence coverage (Agilent 6546)*

Shredder: Single Shot LC-MS Run: The Shredder afforded a 99.7% sequence coverage for Bovine Serum Albumin ***(BSA, Fig. 7)***; as expected, signal- and propeptide which were, as expected, not detectable. The average read depth *("depth of sequencing")* was 62X per amino acid, a concept widespread in genomics but one not yet broadly applied in proteomics. 3436 PSMs were observed containing 2179 unique peptides. Peptide lengths from 7 to 49 amino acids were observed with an average of 16.3 and median of 15. Especially for such a wide range of peptide sizes, the observation of specific peptides is a function of chromatography, mass spec analytical limitations and software search setting; in this case, 7 was the lower limit in the Mascot search settings. Importantly, the resulting peptide with significant overlap holds knowledge of co-occurring PTMs, variants, etc.

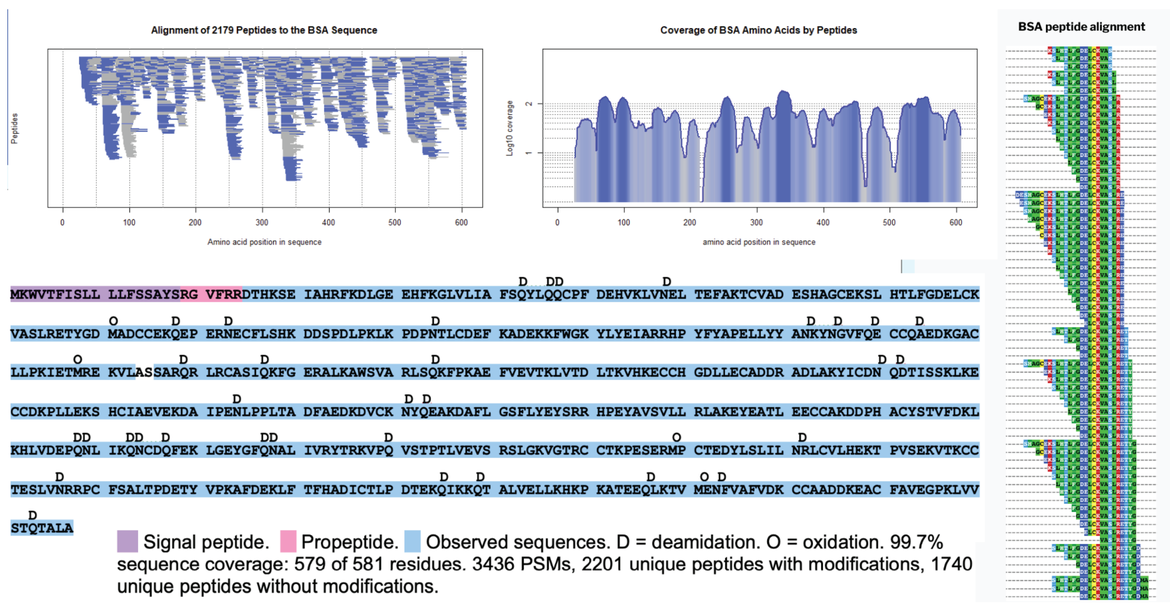


Fig. 7 *Shredder analysis with 100 µg of BSA. Peptide alignment displays an extensive laddering allowing for sequence coverage of >99% in a single shot LC-MS run. The read depth was 62X per amino acid on average.*

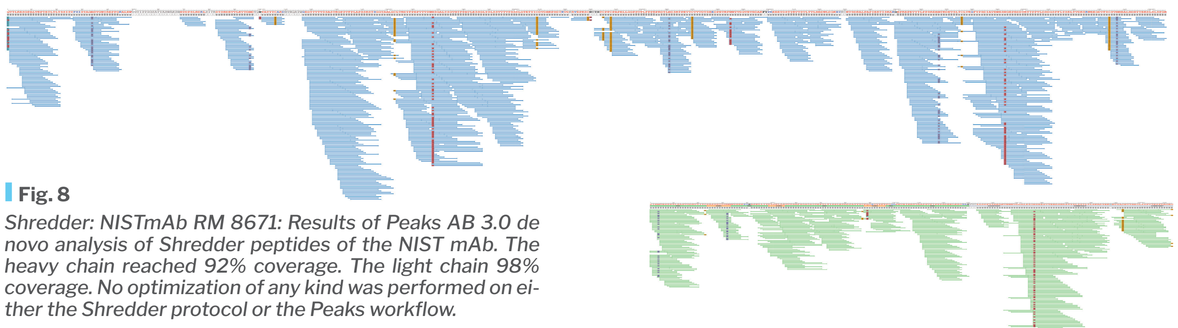
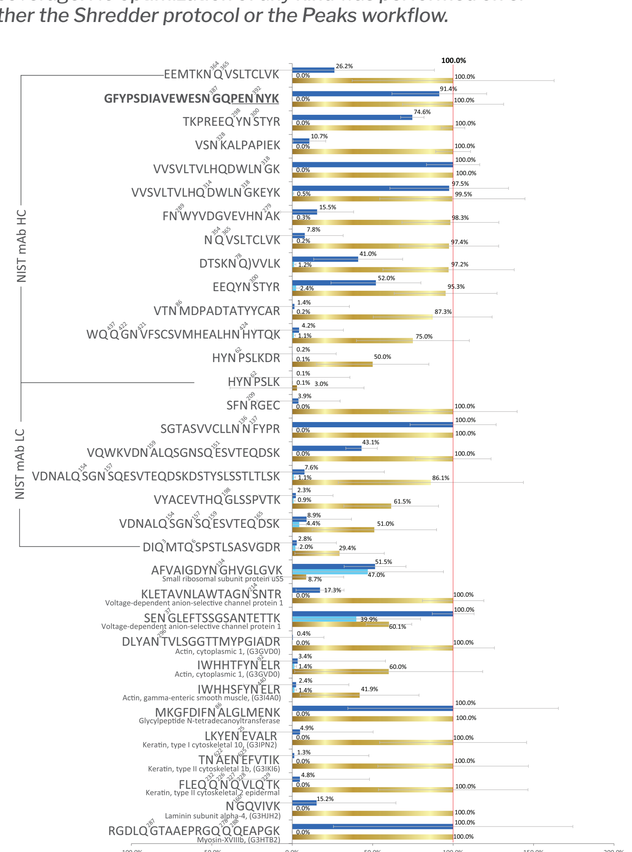


Fig. 8

Shredder: NISTmAb RM 8671: Results of Peaks AB 3.0 de novo analysis of Shredder peptides of the NIST mAb. The heavy chain reached 92% coverage. The light chain 98% coverage. No optimization of any kind was performed on either the Shredder protocol or the Peaks workflow.



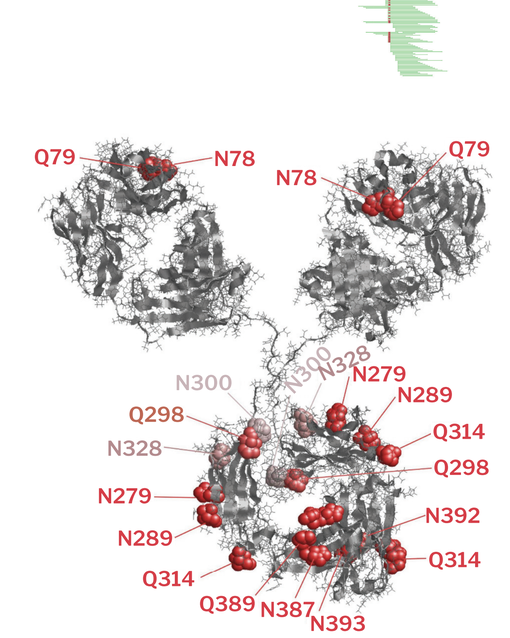


Fig. 9

Reduction of artificial deamidation: use of the MAM kit drastically reduced deamidation of N/Q when processing NISTmAb antibody in comparison to a standard workflow. The optimized MAM digestion buffer effectively mitigates unspecific deamidation on both glutamine (Q) and asparagine (N) residues, yielding greater accuracy in the final quantifications.

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