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PROTIFI™ Bringing precision omics to life!™

Kitted universal MAM: Automatable Sample Processing for all Stages of Biological Drugs

S-TRAP MAM Multi-Attribute Method Kit

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Multiple Attribute Monitoring (MAM)

Multiple Attribute Monitoring (MAM), an LC-MS-based technique, enables the simultaneous direct monitoring of critical quality attributes (CQAs) in biologics, including impurities, post-translational modifications (PTMs), and sequence variations. FDA mandated comprehensive analysis of biological drug characterization, including precise PTM quantification. Unfortunately, artificial PTMs like deamidation and oxidation can occur during biologics production and sample preparation, with potential extents of up to 90%[1], necessitating their minimization. The automated MAM workflow reduces artifactual peptide deamidation by ~4- to 5-fold and simultaneously eliminates contaminants like salts, surfactants, excipients, and dyes. This streamlined process is applicable throughout the production pipeline, from the bioreactor to the final product.

[1]Tajiri-Tsukada et al. Bioengineered. 2020, doi: 10.1080/21655979.2020.1814683

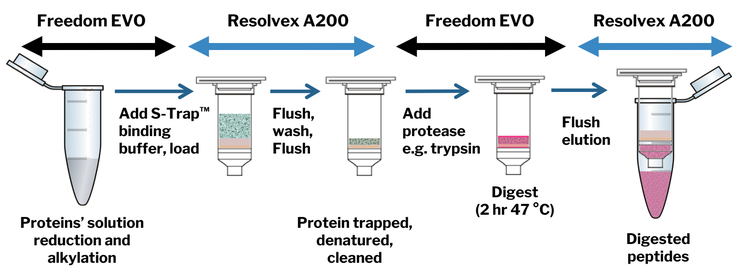


Figure 1. Workflow for automation of S-Trap™ kit for MAM with Tecan Freedom Evo and Resolvex A200.

Evaluation of the automated protocol

The automated sample preparation protocol was tested (**Table 1**) using 48 wells with 100 µg of NISTmAb RM 8671 dissolved in 5% SDS, 50 mM TEAB pH 7.55 to a final concentration of 2 µg/µl. In 12 out of 48 samples, 8 µg of SILu™ MAB heavy labelled antibody were spiked in 12 out of the 48 processed samples. 48 wells were left blank. After the elution, the samples were dried under nitrogen flow and resuspended with 200 µl of a solution containing 24 fmol/µl of the Pierce™ Peptide Retention Time Calibration Mixture in mobile phase A.

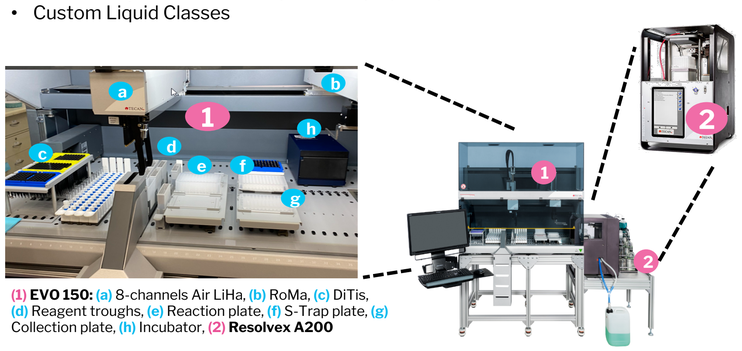
Protocol steps	Protocol reagents	Device
Reduction	TCEP 5 mM	EVO
Alkylation	IAA 40 mM	EVO
Acidification	Phosphoric Acid 2.5%	EVO
Dilution	S-Trap Binding Buffer	EVO
Wash	S-Trap Binding Buffer	A200
Protease	Trypsin (1:10)	EVO
Digestion	Digestion Buffer	EVO
Elution	Step 1 - 0.2% FA in H2O, Step 2 - 0.2% FA in H2O:ACN 1:1	A200

Table 1. Overview of the protocol

Liquid handling system

Tecan EVO 150 with 8 channels Air Liquid Handling Arm™ (Air LiHa), Robotic Manipulator Arm™ (RoMa), with integrated Resolvex® A200

- 200/1000 µL conductive disposable tips (DiTis)
- Custom Liquid Classes



(1) **EVO 150:** (a) 8-channels Air LiHa, (b) RoMa, (c) DiTis, (d) Reagent troughs, (e) Reaction plate, (f) S-Trap plate, (g) Collection plate, (h) Incubator, (2) **Resolvex A200**

Figure 2. Tecan Freedom Evo and Resolvex ® A200 system configuration for the MAM workflow.

LC-MS results

The LC-MS runs showed retention time shifts within the acceptable technical performance and good peak alignment across the replicates (**Figure 3**). The LC-MS profiles indicate an efficient protein digestion and a successful clean-up. Moreover, the adjacent blank prepared samples do not show any trace of peptides, confirming the absence of cross-contamination between samples (**Figure 4**). Selected tryptic peptides and their doubly-charged ions for the NISTmAb RM 8671, SILuMAB™ and of the Pierce™ Peptide Retention Time Calibration Mixture were chosen in order to evaluate the method reproducibility (**Table 3**). 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%. The CV obtained for the calibration mix peptides indicates the contribution to the variability (CV ~ 10%) of only the LC-MS analysis conditions used for these experiments.

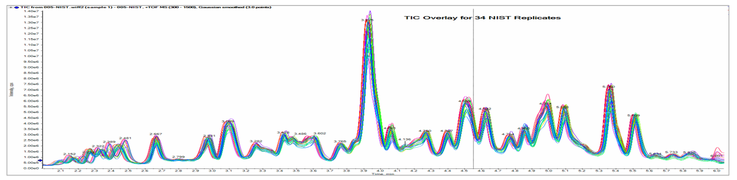


Figure 3. Total Ion Current (TIC) overlay for 34 NIST samples.

Protein	Peptide sequence	[M+2H]2+	CV (%)	RT (min)
NIST Hc	FNWYVDGVEVHNAK	839.4046	14	3.92
NIST Hc	ALPAPIEK	419.7553	8.6	3.06
NIST Hc	GFYPSDIAVEWESNGQPENNYK	1272.5693	12	4.38
NIST Hc	TTPPVLDSDGSFFLYSK	937.4645	16	4.60
NIST Lc	LLIYDTSK	476.7711	8.9	3.41
NIST Lc	LASGVPSR	393.7271	9.1	2.34
NIST Lc	VDNALQSGNSQESVTEQDSK	1068.488	18	2.65
NIST Lc	DSTYSLSSTLTLSK	751.8829	20	3.93
NIST Lc	VYACEVTHQGLSSPVTK	938.4671	16	4.60
SILuMAB Hc	DTLMISR	423.2249	4.6	3.10
SILuMAB Hc	FNWYVDGVEVHNAK	843.4117	6.7	3.92
SILuMAB Lc	AGVETTTPSK	499.7658	6.8	1.99
SILuMAB Lc	YAASSYLSLTPEQWK	876.4402	9.1	4.34
Cal Mix	SSAAPPPPPR	493.7683	7.4	2.27
Cal Mix	HVLTSIGEK	496.2867	8.3	2.69
Cal Mix	IGDYAGIK	422.7363	8.6	2.91
Cal Mix	SAAGAFGPELSR	586.8003	11	3.34
Cal Mix	SFANQPLEVVYSK	745.3924	10	3.94
Cal Mix	LTILEELR	498.8018	7.4	4.36

Table 4. List of example peptides. Hc: heavy chain, Lc: light chain **C:** Carbamidomethyl (C), **K** or **R** : $^{15}N^{13}C$ labelled stable isotope

Reduction of artificial deamidation

The optimized MAM digestion buffer effectively mitigates unspecific deamidation on both glutamine (Q) and asparagine (N) residues, reducing deamidation. By carefully optimizing the composition, undesired chemical modifications occurring during the digestion process are minimized to yield greater accuracy in the final quantifications.

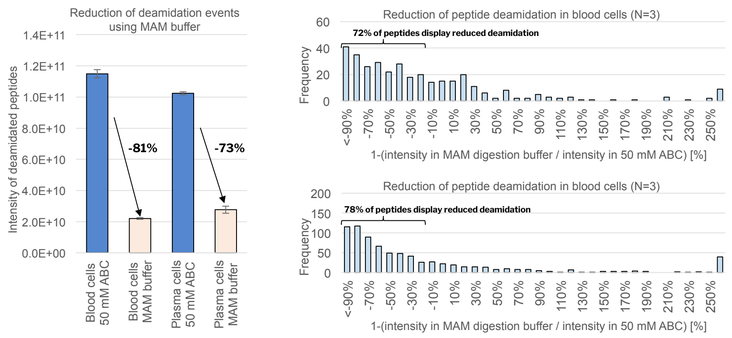


Figure 6. Reduction of artificial deamidation using MAM digestion buffer. In comparison to a standard digestion (50 mM ABC buffer, 37 C, overnight), occurrence of deamidation is reduced 4- to 5X.

Biosimilar characterization

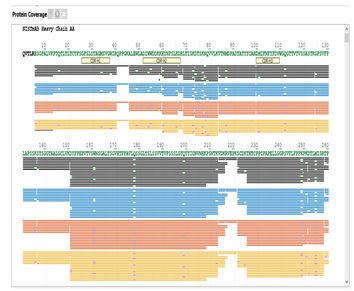


Figure 5. Sequence coverage (Agilent 6546).

In addition to the targeted runs acquired on the ZenoTOF, data acquired from samples prepared with 96-well S-Trap plates was acquired on an Agilent 6546 and Thermo QE and analyzed using Protein Metrics Byologic, additional data was acquired on a Bruker TimsTOF Pro and searched using Spectronaut. Excellent sequence coverage between 99% - 100% (at left), as well as the expected glycosylations (below), disulfide bonds, oxidizations and deamidations were observed **(Figure 5, 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).

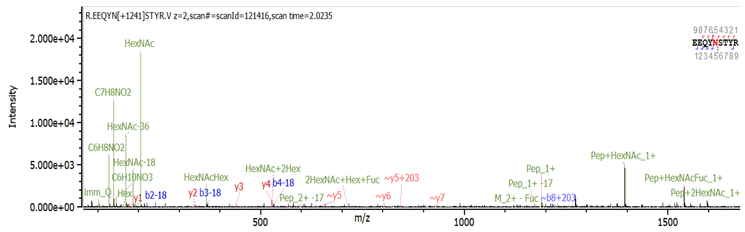


Figure 6. Glycopeptide identification (Agilent 6546).

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 (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 (**Figure 7**).

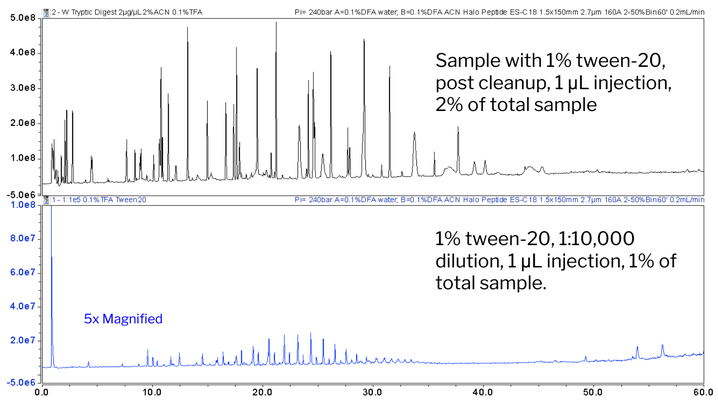


Figure 7. S-Trap sample processing removes detergents, PEG and other contaminants

Summary

The S-Trap™ kit for the MAM kit is automated on the fully integrated Tecan Freedom EVO liquid handling-Resolvex™ A200 workstation. The workflow shows efficient sample clean-up, good peptide recovery and high reproducibility, exhibits high throughput (up to 96 samples per run), minimizes sample processing times and removes potential operator and sample preparation errors, in particular artifactual introduced chemical PTMs, to yield reliable quantifications of biologics.

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