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

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INTRODUCTION

Multiple Attribute Monitoring (MAM) is an LC-MS based technique designed to simultaneously and directly monitor critical quality attributes (CQAs) of biologics including impurities, PTMs and sequence. MAM's popularity continues to rise in biotherapeutic characterization and in QC and release testing. In general, the highly varied nature of samples generated in biotherapeutic manufacturing presents analytical challenges. Here we present an automated solution for the MAM workflow, that successfully allows for removal of contaminants (e.g. salts, surfactants, excipients, dyes) through a streamlined workflow usable at all stages from bioreactor to final product.

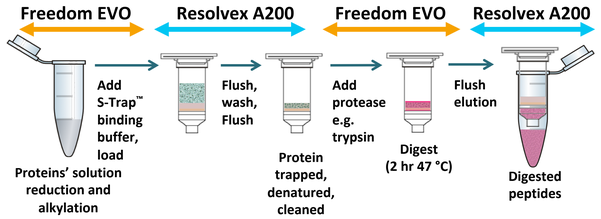


Figure 1. *Workflow for automation of S-Trap™ kit for MAM with Tecan Freedom Evo and Resolvex A200.*

METHODS

Sample Preparation and Reagents

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 H_2O Step 2 - 0.2% FA in H_2O:ACN 1:1	A200

Table 1. *Overview of the tested 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

METHODS

(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

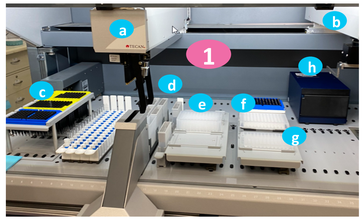


Figure 2. *Tecan Freedom Evo and Resolvex ® A200 system configuration for the MAM workflow.*

Positive Pressure Station

- Buffer dispensing and positive pressure profile was optimized for the S-Trap clean-up protocol

Figure 3. *Tecan Resolvex A200.*

LC-MS/MS Method

- LC Column: Avantor ACE 3 C18-300 2.1x50mm
- Mobile Phase A: 0.1% formic acid in water
- Mobile Phase B: 0.1% formic acid in acetonitrile
- Column temperature: +40°C
- Injection Volume: 2 µl
- LC gradient flow rate: 500 µl/ml

- Mass Spectrometer: SCIEX ZenoTOF 7600
- Ionization: Turbo IonSpray
- Scan Type: Positive TOFMS
- IonSpray Voltage: 5500 V
- Turbo Heater Temperature: +500 °C
- MS1 acquisition mass range (m/z): 300-1500
- Accumulation time (s): 0.25
- Declustering potential (V): 80
- Collision energy (V): 10

Time [min]	B. Conc [%]
0.50	2.5
5.50	40.0
6.50	90.0
7.25	90.0
7.30	5.0
10.00	5.0

Table 2. *LC gradient.*

RESULTS

Liquid Class Optimization and Verification

Liquid handling performances were evaluated spiking yellow dye (Abs 427 nm) in the solutions and measure (n=48) the absorbance (427 nm) with the Tecan Spark® reader (**Table 3**).

Reagent	Pipetting Mode	Pipetted Volume (µL)	Pipetting CV (%)
TCEP 5 mM	Multi-dispense	20	4.0
IAA 40 mM	Multi-dispense	20	5.5
Phosphoric Acid 2.5%	Multi-dispense	20	6.4
S-Trap Binding Buffer	Single-dispense	770	1.7
Trypsin (1:10)	Single-dispense	125	3.6
Digestion Buffer	Multi-dispense	80	1.5

Table 3. *Liquid handling performances.*

RESULTS

LC-MS analysis

The LC-MS runs showed retention time shifts within the acceptable technical performance and good peak alignment across the replicates (**Figure 4**). 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 5**).

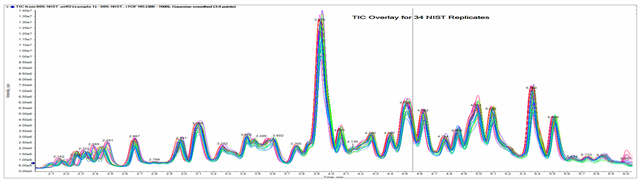


Figure 4. *Total Ion Current (TIC) overlay for 34 NIST samples.*

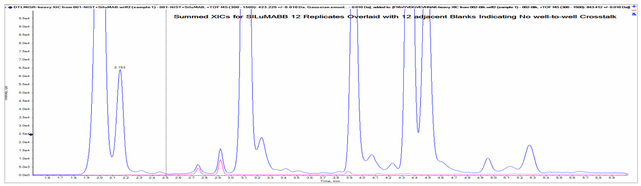


Figure 5. *Summed (12 replicates) Extracted Ion Current (XIC) profiles for SILu™ MAB peptides (purple line) overlaid with 12 adjacent blanks (pink line).*

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

Hc: heavy chain; Lc: light chain **C:** Carbamidomethyl (C); K or R : $^{15}N^{13}C$ labelled stable isotope

Table 4. *List of example peptides.*

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 4**). 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.

A200 Standalone Studies

Biosimilar Characterization

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 Bylogic; 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. 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 6a. Sequence coverage (Agilent 6546).

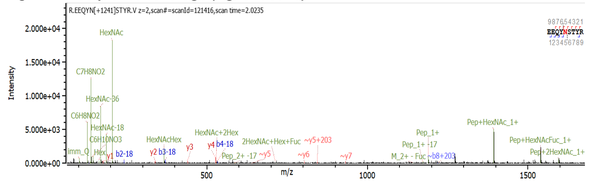
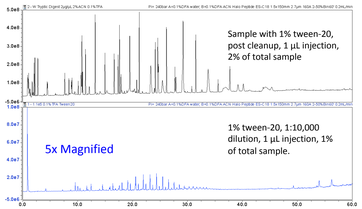


Figure 6b. Glycopeptide identification (Agilent 6546).

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

CONCLUSIONS

The S-Trap™ kit for the MAM protocol has been successfully 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 errors, enhancing the global method performances.

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