AUTOMATED SAMPLE PREPARATION FOR HYBRID LC-MS/MS PROTEIN QUANTIFICATION

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INTRODUCTION

Developing highly sensitive and accurate LC-MS methods for large molecule therapeutic quantification is quite challenging. This is especially true for protein quantification workflows that commonly employ the bottom up approach, or surrogate peptide approach, using enzymatic digestion to break down the proteins into smaller peptides. Method development of this entire process is time consuming and complex, containing many steps and various reagents which often require high levels of expert scientists for optimization. Adding to the complexity, for extreme sensitivity and selectivity needs, protein level clean up of the complex biological matrices can be employed at different specificities to capture certain species and remove many or most endogenous components. Thus, there is a strong need for a more simplified and standardized sample preparation workflow. A generic, kitted approach including universal protocols, the necessary reagents, and even automation methods would increase lab productivity, reduce analytical variability, and streamline the sample preparation.

This work demonstrates the comparability of fully automated surrogate peptide workflows to manual preparations, including affinity capture, protein digestion, and solid phase extraction (SPE). Additionally, this work exhibits automated, accurate and reproducible quantification of proteins, a direct digestion of whole plasma with SPE clean-up and affinity purification of plasma before digestion for high sensitivity. Proving automation achieves the same, if not arguably better, statistics than manually prepared samples. Having an automated system perform these time consuming workflows increases productivity of high level scientists and busy labs.

METHODS

Sample Preparation

Whole Plasma Samples

Using the Waters ProteinWorks™ (ProteinWorks) Auto-eXpress Direct Digest and µElution SPE Clean-Up Kits with their included protocols, rat plasma samples (35 µL) containing various concentrations of the therapeutic mAbs, infliximab and cetuximab, sample digestion and subsequent peptide level clean-up was performed using the Hamilton Microlab® STAR (STAR).

Affinity Purified Plasma Samples

Performed by a Hamilton MicroLab STAR, 25 µL of streptavidin beads were charged with 33 µL (comparability study) or 50 µL (quantification study) Goat Anti-Human Biotinylated IgG antibodies for 2 hours. 50 µL spiked or blank rat plasma were allowed to capture with 50 µL spiked internal standard overnight at room temperature. Eluted with 90 µL 0.1% formic acid and neutralized with 9 µL of 500 mM ammonium bicarbonate, the samples were further digested using the Waters ProteinWorks™ Auto-eXpress Low 5 Digest Kit[9] and the included protocol. Subsequent peptides were injected directly without further clean up.

LC-MS/MS Conditions

LC-MS/MS quantification of resulting peptides was performed using a Waters Xevo® TQ-XS quadrupole MS (ESI+). Chromatographic separation was achieved using an ACQUITY UPLC® Peptide BEH C18 Column, 300Å, 1.7 µm, 2.1 mm x 150 mm column , at a flow rate of 0.3 mL/min using a linear gradient with 0.1% formic acid in water and acetonitrile on an ACQUITY UPLC I-Class PLUS. Signature tryptic peptides and MS conditions used for etanercept, trastuzumab, and infliximab are summarized in Table 1.

Protein	Peptide	MRM Transition	Collision Energy (eV)
Infliximab	SINSATHYASESVK	469.6 > 603.8	13
	LEESGGGLVQPGGSMK	773.4 > 576.3	24
	DILLTSSSPAILSVSPGER	632.7 > 545.3	16
	YASESMSGIPSR	642.8 > 359.2	19
Cetuximab	SQVFFK	378.2 > 244.2	19
	YASESISGIPSR	564.8 > 347.2	17
	DILLTQSPVILSVSPGER	568.8 > 716.4	11
Etanercept	IC*TC*RPGWYC*ALSK	591.3 > 749.9	16
	C*SSDQVETQAC*TR	771.3 > 865.4	25
Trastuzumab	GLEWVAR	415.7 > 660.4	14
	FTISADTSK	485.3 > 721.4	20
	IYPTNGYTR	542.8 > 404.7	16
	DTYIHWVR	545.3 > 710.4	24
	LSCAASGFNIK	584.3 > 665.4	16

*Denotes a carbamilation of the cysteine residue contributing +57 amu

Table 1. MRM conditions for infliximab, cetuximab, etanercept, and trastuzumab.

RESULTS

I. COMPARABLE AUTOMATED VS. MANUAL PROTEIN DIGESTION PERFORMANCE

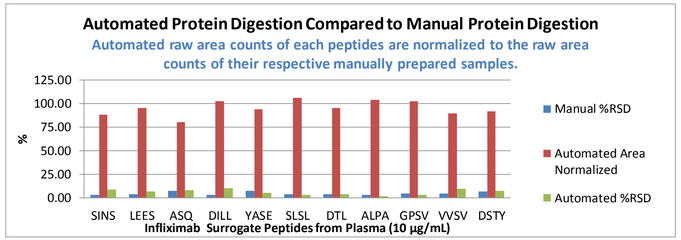

Figure 1. Comparable automated (STAR) vs. manual digestion performances using the ProteinWorks Auto-eXpress Digest Kits and LC-MS/MS analysis of signature tryptic peptides from infliximab.

II. PROTEIN QUANTIFICATION USING DIGESTION KITS AND AUTOMATED LIQUID HANDLER

LINEAR & ACCURATE CETUXIMAB QUANTIFICATION

Peptide	Linear Dynamic Range (ng/mL)	Weighting	Linear Fit (r^2)
SQVFFK	100-250,000	1/x	0.9989
YASESISGIPSR	500-250,000		0.9957
DILLTQSPVILSVSPGER	200-250,000		0.9977

Table 2. Representative standard curves for signature peptides used to quantify cetuximab, automatically digested and extracted using ProteinWorks Auto-eXpress Digest Kit and ProteinWorks µElution SPE Clean-up Kit and respective protocols on the Hamilton STAR.

Peptide	QC Conc. (ng/mL)	Mean Calc. Conc. (ng/mL)	%RSDs	Mean % Accuracy
SQV	250.0	230.00	7.06	92.35
	4000.0	4140.00	3.27	103.47
	40000.0	40130.00	4.37	100.30
	200000.0	192960.00	6.07	96.47
YASE	4000.0	3750.00	0.82	93.80
	40000.0	40780.00	1.34	101.97
	200000.0	184990.00	4.90	92.50
DILL	250.0	240.00	13.13	89.80
	4000.0	4030.00	10.51	100.65
	40000.0	40120.00	2.40	100.30
	200000.0	186890.00	4.15	93.43

Table 3. QC sample statistics for tryptic peptides used to quantify cetuximab from rat plasma. Sample digestion and SPE was performed using the Hamilton STAR.

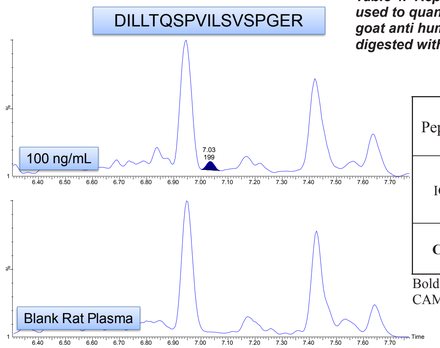

Figure 2. Example chromatogram of cetuximab surrogate peptide, DILL, LLOQ at 100 ng/mL compared to the blank plasma sample. Both samples were automatically digested and SPE extracted using the Hamilton STAR.

III. COMPARABLE AUTOMATED VS. MANUAL AFFINITY PURIFICATION PERFORMANCE USING MAGNETIC BEADS

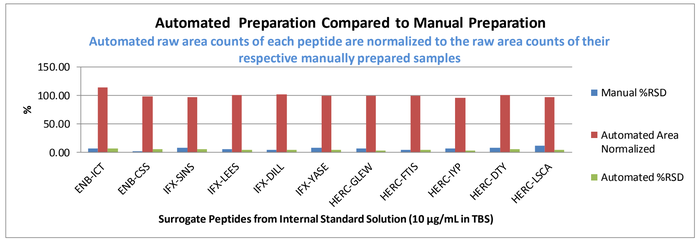

Figure 3. Comparable automated (STAR) vs. manual sample capture performance (peak areas and %RSDs) of surrogate peptides representing etanercept, infliximab, and trastuzumab. The Automated Area Normalized bar represents the raw area counts of the automated samples normalized to the raw area counts of the manually prepared samples.

IV. PROTEIN QUANTIFICATION USING MAGNETIC BEADS, DIGESTION KITS, AND AUTOMATED LIQUID HANDLER

LINEAR & ACCURATE ETANERCEPT QUANTIFICATION

Peptide	Linear Dynamic Range (ng/mL)	Weighting	Linear Fit (r2)
ICTCPGWYCALSK	1.0-10,000	1/x	0.9976
CSSDQVETQACTR	5.0-10,000		0.9991

Bolded cysteine residue denotes the addition for 57 amu due to CAM modifi-

Table 4. Representative standard curves for signature peptides used to quantify etanercept, automatically affinity purified using goat anti human IgG magnetic beads and automatically digested with ProteinWorks Auto-eXpress Low Digest kit.

Peptide	QC Conc. (ng/mL)	Mean Calculated Conc. (ng/mL)	%RSD	Mean % Accuracy
ICT	3.0	3.03	10.07	102.03
	30.0	28.90	4.20	96.33
	300.0	300.43	2.13	100.13
	3000.0	2,944.30	3.16	98.13
CSS	30.0	31.93	6.64	106.47
	300.0	321.767	1.75	107.23
	3000.0	3,043.53	1.28	101.43

Bolded cysteine residue denotes the addition for 57 amu due to CAM modification from alkylation.

Table 5. QC sample statistics for tryptic peptides used to quantify etanercept from purified rat plasma. Sample affinity purification and digestion were performed using the Hamilton STAR.

APPLICATION HIGHLIGHTS

- A comparability test using infliximab was executed to assess the performance on the automated protein digestion method (Figure 1). The automated method exhibited comparable results to manually prepared samples.
- Fully automated digestion and SPE of cetuximab, performed by the Hamilton STAR, was highly linear reproducible down to 100 ng/mL (Table 2, Table 3, Figure 2).
- A comparability test using etanercept, infliximab, and trastuzumab was used to assess the performance of automated affinity capture (Figure 3).
- Fully automated affinity capture of etanercept using streptavidin magnetic beads biotinylated to anti human IgG antibodies and subsequent digestion resulted in sensitive and reproducible surrogate peptides (Table 4 and Table 5).

CONCLUSION

Automating the complex and time consuming sample preparation for protein quantification workflows streamlines the process, maximizes productivity, reduces errors, and ensures analytical method performance, while maximizing productivity, eliminating the costly time spent of high level scientists at the lab bench.

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