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Automated and Standardized, Kit-Based Workflow for Protein Quantification

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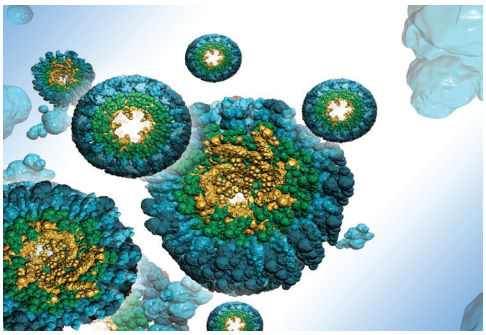

GOAL

Demonstrate accurate and reproducible protein quantification using a fully automated kit-based sample preparation approach with ProteinWorks™ Auto-eXpress Digest and µElution SPE Clean-up Kits for digestion and peptide purification.

BACKGROUND

In any bioanalytical or clinical research assay, one of the greatest sources of variability arises from the sample preparation. This is especially true for protein quantification workflows, which commonly employ the bottom-up (surrogate peptide) approach using enzymatic digestion and analysis of resulting peptides. This multi-step workflow is time consuming, complex and often introduces variability. The multitude of possible options within this workflow typically require a skilled and experienced scientist and, even still, method development times can be lengthy. A simpler, standardized workflow could alleviate the current burden of method development and sample preparation. An automated and generic, kitted approach that includes a protocol and the reagents necessary, can streamline this process and minimize the sample preparation complexity. The implementation

Bottom-up, protein quantification workflows are time consuming and complex. A need exists for simpler and standardized workflows which facilitate accurate and robust protein quantification.

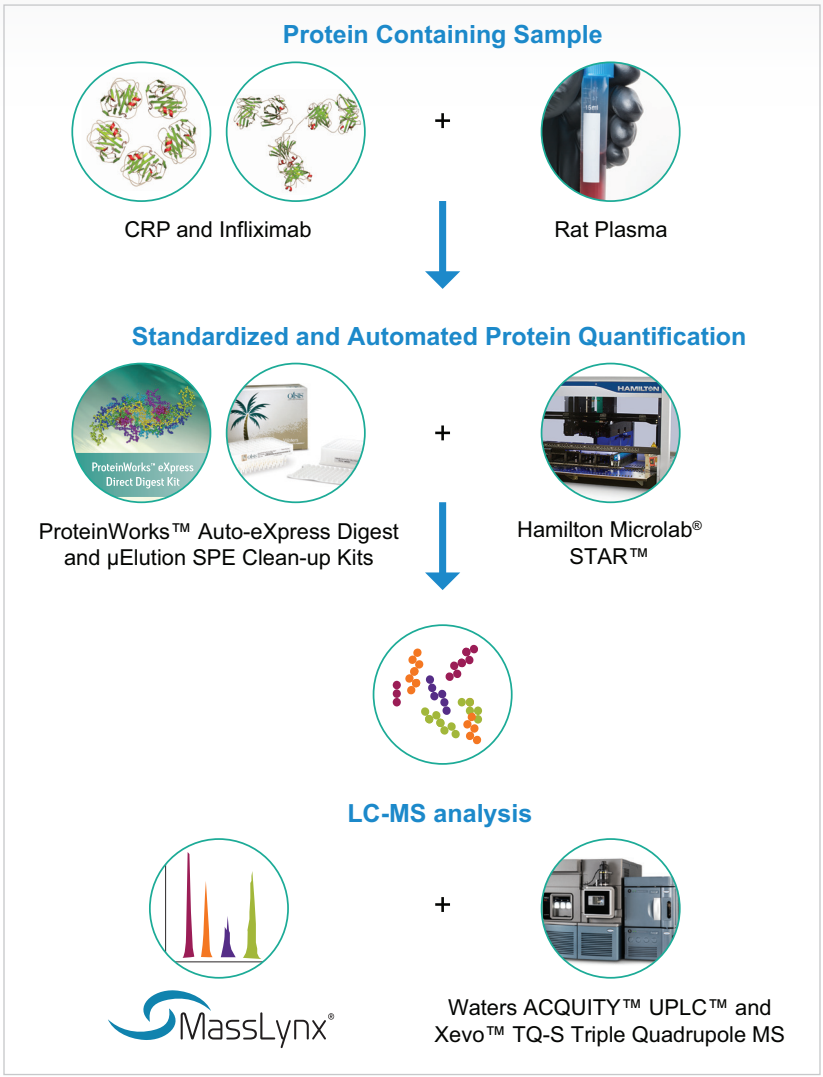

Figure 1. Standardized and automated LC-MS/MS protein quantification workflow using ProteinWorks Auto-eXpress Digest and µElution SPE Clean-Up Kits with the Hamilton Microlab STAR liquid handler to streamline the process, maximize productivity, reduce errors, and improve analytical method performance.

of an automated liquid handler can maximize productivity, reduce human error, and improve analytical method performance even further.

This work aims to provide a practical, broadly applicable strategy to simplify and streamline the protein bioanalysis workflow (Figure 1), using an automated, kit-based approach with pre-measured reagents and a universal protocol, to accurately and reproducibly quantify proteins in plasma.

THE SOLUTION

ProteinWorks Auto-eXpress Digest Kits are flexible, broadly applicable sample preparation kits designed for manual and automated use on liquid handling instruments, enabling accurate and robust LC-MS quantification of proteins via the surrogate peptide approach. The kits contain pre-measured, lot traceable reagents and universal protocol to streamline the protein sample preparation workflow and facilitate implementation by less experienced users.

The goals of this work were to demonstrate comparable automated versus manual sample digestion performance and accurate protein quantification using the ProteinWorks Auto-eXpress Digest Kits performed on the Hamilton Microlab® STAR™ liquid handler (STAR). Using the ProteinWorks Auto-eXpress High 5 Digest Kit and universal protocol, a direct digestion of human C-reactive protein (CRP) and infliximab in rat plasma (28 µL) was performed manually and with automation using the STAR. Several unique tryptic peptides resulting from the digestion of each of the proteins were evaluated and analyzed by LC-MS/MS (ACQUITY™ UPLC™/Xevo™ TQ-S MS). Raw area counts for multiple tryptic peptides from the aforementioned proteins were used to assess comparability and the coefficient of variation (CV), as high CV values are indicative of poor reproducibility and precision. Comparable automated versus manual sample digestion performance using the ProteinWorks Auto-eXpress Digest Kits and LC-MS/MS analysis of signature tryptic peptides from CRP and infliximab can be seen in Figure 2. Mean peptide area counts from digestion performed on the STAR were within 25% of those manually digested (Figure 2A) and intra-assay reproducibility for all tryptic peptides was excellent with CVs ≤15% for samples prepared on the STAR and manually (Figure 2B). Using this standardized and automated approach with ProteinWorks Auto-eXpress Digest and µElution SPE Clean-Up Kit performed on the STAR, yields excellent quantification performance. Standard curve and QC statistics for CRP and infliximab quantification are highlighted in Tables 1 and 2, respectively.

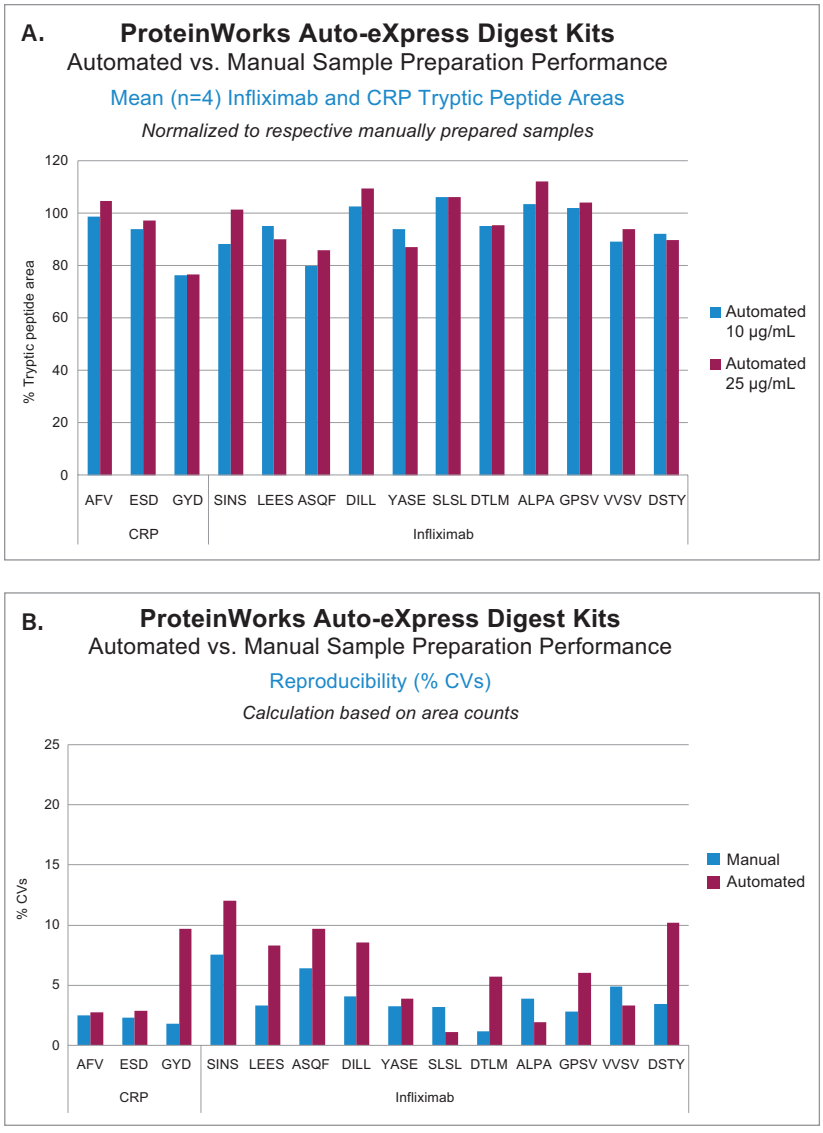

Figure 2. Comparable automated (STAR) vs. manual sample digestion performance using the ProteinWorks Auto-eXpress Digest Kits and LC-MS/MS analysis of signature tryptic peptides from CRP and infliximab. Panel A: Comparison of mean (N=4) area counts and Panel B: 10 µg/mL protein digest reproducibility (%CV) comparison.

	Peptide	Curve range (µg/mL)	Weighting	Linear fit (r^2)	Mean % accuracy
CRP	AFV	1.0–100	1/x	0.997	100.60
	ESD	1.0–100	1/x	0.996	100.60
	GYS	1.0–100	1/x	0.996	100.90
Infliximab*	SINS	0.75–250	$1/x^2$	0.994	101.000
	LEES	1.0–250	1/x	0.995	99.00
	ASQF	1.0–250	$1/x^2$	0.992	98.10
	DILL	1.0–250	1/x	0.994	100.20

Table 1. Linear and accurate quantification for CRP and infliximab. Representative standard curves for signature peptides used to quantify CRP (A) and infliximab* (B) in plasma which were digested using ProteinWorks Auto-eXpress Digest Kits and performed on the STAR.*Subsequent peptide level clean-up of the digested infliximab samples was performed on the STAR with the ProteinWorks µElution SPE Clean-Up Kits.*

	Peptide	QC concentration (µg/mL)	Mean calculated concentration (µg/mL)	Mean % accuracy	% CV	Replicates
CRP	AFV	3.00	2.97	98.89	12.76	3 of 3
		20.00	21.27	106.33	5.95	3 of 3
		90.00	89.27	99.19	5.22	3 of 3
	ESD	3.00	2.87	95.56	7.26	3 of 3
		20.00	21.80	109.00	2.10	3 of 3
		90.00	92.03	102.26	2.65	3 of 3
	GYS	3.00	3.10	103.33	8.53	3 of 3
		20.00	21.70	108.50	1.30	2 of 3
		90.00	88.30	98.11	8.48	3 of 3
Infliximab*	SINS	4.00	4.17	104.43	3.67	3 of 3
		40.00	44.60	111.50	7.67	3 of 3
		200.00	188.90	94.47	7.00	3 of 3
	LEES	4.00	3.83	95.23	10.86	3 of 3
		40.00	42.10	105.25	7.39	2 of 3
		200.00	183.67	91.87	2.29	3 of 3
	ASQF	4.00	3.95	99.05	1.79	2 of 3
		40.00	39.63	99.00	5.58	3 of 3
		200.00	179.70	89.87	5.98	3 of 3
	DILL	4.00	4.25	106.25	4.99	2 of 3
		40.00	39.07	97.67	10.26	3 of 3
		200.00	198.83	99.42	8.67	3 of 3

Table 2. QC sample statistics for tryptic peptides used to quantify CRP and infliximab in plasma. Sample digestion and SPE extraction, using the ProteinWorks Auto-eXpress Digest and µElution SPE Clean-Up Kits, was performed on the STAR. *Subsequent peptide level clean-up of the digested infliximab samples was performed on the STAR with the ProteinWorks µElution SPE Clean-Up Kits.*

SUPPLEMENTAL INFORMATION

LC gradient

Time (min)	Flow rate (mL/min)	%A	%B	Curve
0.0	0.3	98	2	6
1.0	0.3	98	2	6
6.5	0.3	60	40	6
7.0	0.3	10	90	6
8.0	0.3	10	90	6
8.5	0.3	98	2	6
10.0	0.3	98	2	6

MRM conditions for CRP and infliximab

	Peptide	Parent (*m/z*)	Daughter (*m/z*)	Cone (V)	Collision (V)
CRP	AFVFPK	354.7076	244.1656	35	9
	ESDTSYVSLK	564.7746	347.2289	35	17
	GYSIFSYATK	568.7848	716.3614	35	11
Infliximab	SINSATHYAESVK	469.5686	603.7911	35	13
	LEESGGGLVQPGGSMK	773.3825	576.281	35	24
	ASQFVGSSIHWYQQR	598.6288	631.3073	35	17
	DILLTQSPAILSVSPGER	632.6861	545.2678	35	16
	YASESMSGIPSR	642.7981	359.2037	35	19
	DTLMISR*	418.2207	506.2755	35	13
	ALPAPIEK*	419.7553	327.6947	35	10
	GPSVFPLAPSSK*	593.827	418.2296	35	25
	VVSVLTVLHQDWLNGK*	603.3403	805.4385	35	16
	DSTYSLSSTLTLSK*	751.8829	836.4724	35	23

**Indicates generic signature peptides*

LC CONDITIONS: ACQUITY CLASSIC

- Column temp.: 55 °C
- Injection vol.: 10 µL
- Loop size: 20 µL
- Column type: ACQUITY UPLC HSS T3, 100Å, 1.8 µm, 2.1 mm x 50 mm (p/n 186003538)

MS CONDITIONS: XEVO TQ-S

- Capillary: 3 kV
- Cone: 30 V
- Source offset: 50 V
- Source temp.: 150 °C
- Desolvation temp.: 600 °C
- Cone gas flow: 150 L/Hr
- Desolvation gas flow: 1000 L/Hr
- Collision gas flow: 0.17 mL/min
- Nebulizer gas flow: 7 Bar

SUMMARY

Accurate and robust protein quantification in plasma was achieved using a generic, kit-based approach (with simple step-wise protocols and standardized, pre-measured reagents) and fully automated sample digestion and subsequent peptide purification workflows.

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