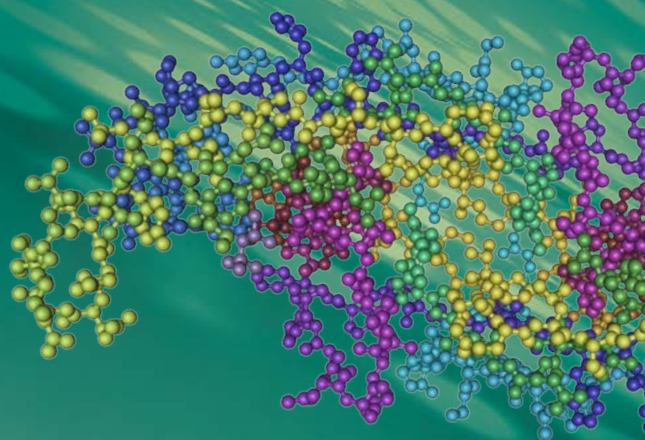


A Universal, Optimized SPE Protocol for Clean-up of Tryptic Peptides in Protein Bioanalysis

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GOAL

To demonstrate the performance of an optimized method for tryptic peptide clean-up in protein bioanalysis.

BACKGROUND

During a typical protein bioanalytical workflow, it is common to either directly digest an unpurified plasma/serum sample or to perform affinity purification followed by digestion. An affinity step may be included to isolate a class of proteins (for example, the use of Protein A/G which isolates an IgG fraction) or a specific target from the plasma/serum sample. Depending on the degree of protein-level clean-up, which ranges from none (direct plasma digestion) to significant (specific affinity capture of a target) the concentration of resultant peptides can be extremely high. In small molecule SPE the majority of proteins (undigested, of course) pass through the extraction device on the load step. In contrast, in a protein bioanalysis workflow, 10's of thousands of digested peptides from as high as 50–60 mg/mL starting protein concentration, are all now capable of binding to the SPE sorbent bed. This creates a significant capacity challenge. A possible solution that might occur to a “small molecule” scientist would be to use a larger sorbent bed size, for example 10, 30 or even 60 mg sorbent beds. However, these larger bed sizes require larger volumes to elute, which must then be dried down and reconstituted. The same practices, when applied in large molecule quantification, could result

As of 2013, there were 338 new monoclonal antibody drugs in development,¹ representing the largest class of biologics in drug development pipelines. Recently, LC-MS has been gaining ground as the technique of choice over ligand binding assays (LBAs) for the support of biologic drug development programs. An important component of these LC-MS workflows is the post-digest purification of tryptic peptides. While SPE is commonly used in small molecule sample preparation, the same methods and guidelines do not apply to peptide extraction. Knowledge of peptide handling concerns as well as the ability to efficiently manipulate wash and elution conditions for tryptic peptides have become critical capabilities in bioanalytical labs. Historically, these labs have been dominated by work on small molecule therapies with far simpler and more straightforward sample prep and analysis methods. The expertise in peptide and protein bioanalysis, if it exists, tends to be a rare and valuable skill, held by only a few individuals.

in significant peptide/protein losses due to adsorption during evaporation. Therefore, it is critical to avoid the dry-down step commonly associated with small molecule SPE methods.

In addition to the requirement for clear capacity guidelines for loading protein digests, a method tailored to targeted elution of tryptic peptides is also needed. A broadly applicable protocol which effectively removes excess digest reagents, digestion buffer, phospholipids, and other plasma/serum components could not only increase sensitivity by reducing matrix effects, but would also improve system robustness.

THE SOLUTION

A total of 17 signature peptides (a combination of both generic and unique) were used in the development and verification of an SPE protocol, as well as an optimal device format, which specifically targets tryptic peptide clean-up during protein bioanalysis experiments. The result of this research is the ProteinWorks™ μ Elution SPE Clean-up Kit (p/n [186008304](#)). Key attributes of the kit include: the ability to concentrate digests without evaporation, orthogonal retention mechanism for maximum specificity, and a protocol designed expressly for efficient purification of tryptic peptides. Oasis® MCX was chosen as the ideal sorbent due to its strong ion exchange binding to the positively charged termini produced through cleavage at the basic residues arginine and lysine during trypsin digestion. In addition, very polar peptides are more efficiently trapped by ion exchange on the Oasis MCX plate than they would be on a traditional reversed-phase only sorbent. The μ Elution 96-well device format is characterized by elution in as little as 25 μ L, which enables significant concentration of extracts without evaporation. The entire 96-well plate can be processed in under 20 minutes using either vacuum or positive pressure, and is amenable to automation.

Performance of the SPE Kit was evaluated using a broad range of chemically diverse representative peptides. Key peptide characteristics are summarized in Table 1.

Through extensive testing and optimization, recovery was maximized for all peptides and sample loading guidelines were created. The comprehensive guidelines, which are related to starting protein content, address both direct plasma/serum digest samples and extracts resulting from affinity purification of the target protein or class of proteins. Recovery of the various peptides using the optimized protocol in the SPE Kit is summarized in Figure 1. The average peptide recovery for the antibody drugs is 104%. Peptides from the generic murine mAb IS average 84% recovery.

Table 1. Relevant characteristics of signature peptides tested during the development and verification of the ProteinWorks μ Elution SPE Clean-up Kit

Protein Name	Peptide	MW	pI	# of Residues	HPLC Index
Herceptin	FTISADTSK	969	6.2	9	26.5
Humira	APYTFGQGTK	1069	9.1	10	39.3
Herceptin	DTYIHWVR	1089	7.4	8	31.1
Generic Human IgG	GPSVFLAPSSK	1186	9.4	12	54.7
Murine	MNSLQDDETAK	1223	4.1	11	21.5
Murine	VNSAAFPAPIEK	1243	6.4	12	47.4
Avastin	STAYLQMNSLR	1283	9.2	11	48.4
Avastin	FTFSLDTSK	1285	7.3	11	40.5
Generic Human IgG	STSGGTAALGC[+57.0]LVK	1322	9.4	14	50.8
Humira	NLSYLQMNSLR	1339	9.2	11	54.6
Remicade	SINSATHYAESVK	1407	7.4	13	7.1
Humira	NYLAWYQKPGK	1496	9.7	12	52.2
Generic Human IgG	DSTYSLSTLTLSK	1503	6.2	14	47.8
Generic Human IgG	VVSVLTVLHQDWLNGK	1808	7.4	16	77.7
Murine	SVSELPIMHQDWLNGK	1854	5.5	16	52.5
Remicade	DILLTQSPAILSVSPGER	1869	4.3	18	74.2
Murine	NTQPIMDTDGSYFVYSK	1966	4.1	17	35.1

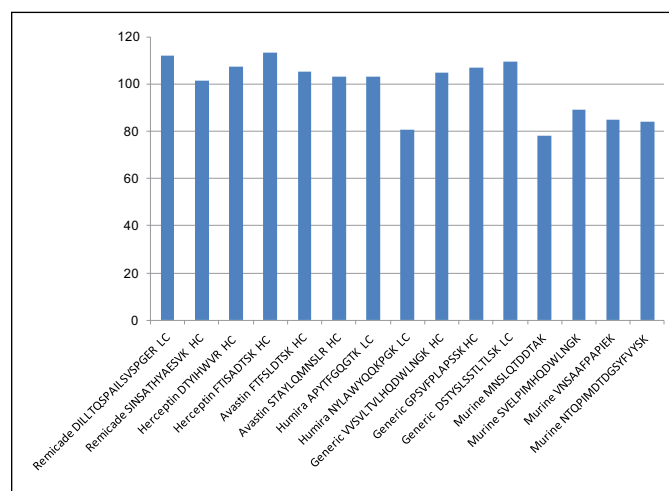


Figure 1. MCX μ Elution SPE recovery of unique and generic signature peptides from infliximab, bevacizumab, adalimumab, trastuzumab, and a generic murine mAb internal standard.

It is recommended that the total protein load in the digested sample on the Oasis MCX μ Elution plate not exceed 1.25 mg. In addition, for best performance, the minimum SPE loading volume range is 15–25 μ L. The sample loading guidelines, summarized in Table 2, were designed to produce the highest sensitivity by identifying the maximum digest volume which should be loaded.

Table 2. Recommended maximum digest loading volumes for the ProteinWorks μ Elution SPE Clean-up Kit

Oasis MCX 96-well μ Elution Plate Recommended Maximum Digest Loading Volumes		
Starting Plasma/Serum Volume (μ L) [*]	SPE Loading Volume (μ L)	
	Direct Digest ^{**}	Post-Generic Affinity Digestion [†]
15	110-200	Total Digest Supernatant
25	70-140	Total Digest Supernatant
35	50-100	Total Digest Supernatant
50	35-70	170-200
70	25-50	120-200

^{*} Starting volume of plasma added for protein digestion or affinity purification using the ProteinWorks eXpress Digest Kit and Protocols, with a final digestion volume of 200 μ L.

^{**} Based on a total protein content of 75 mg/mL in whole plasma/serum.

[†] Based on a total protein content 15 mg/mL post-generic affinity purified plasma (Protein A/G) and assuming all Affinity captured sample is used for digestion.

Plasma was spiked with infliximab, trastuzumab, adalimumab, bevacizumab, and a common murine mAb IS and then digested. The resultant tryptic peptides were isolated from digest reagents (which are washed off during loading and wash steps), phospholipids (which remain stuck to the SPE plate after peptide elution), and concentrated using the ProteinWorks SPE Clean-up Kit. Chromatographic purity of the signature peptides extracted from the plasma digest sample is demonstrated in Figure 2.

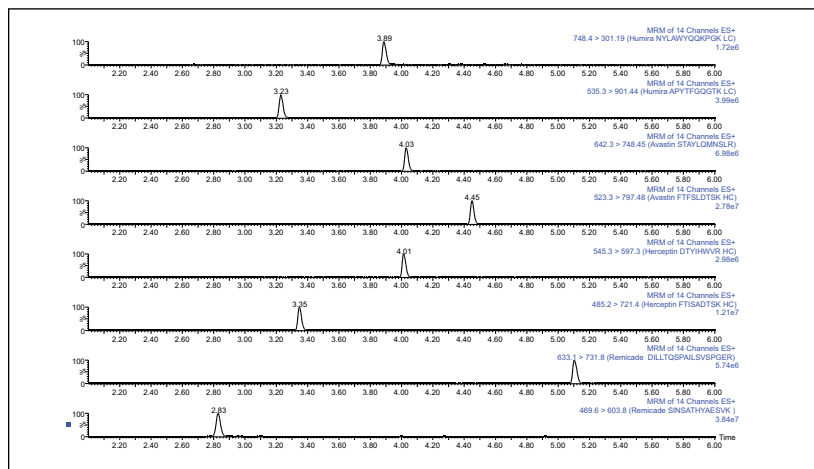


Figure 2. LC-MS chromatograms for representative unique signature peptides from a plasma digest containing adalimumab, bevacizumab, trastuzumab, and infliximab.

SUMMARY

The ProteinWorks μ Elution SPE Clean-up Kit was successfully used to isolate peptides resulting from plasma digests of bevacizumab, trastuzumab, adalimumab, and infliximab as well as a common murine mAb internal standard. The simple 96-well μ Elution format enabled clean-up and concentration of digest samples in <20 minutes, without the need for evaporation and reconstitution, preserving low levels of precious tryptic peptides. Furthermore, the kit takes advantage of the orthogonal nature of mixed-mode SPE (binding tryptic peptides by ion-exchange) to provide the degree of specificity required for low-level protein quantification studies. Finally, the specific sample loading guidelines and an optimized kit-based approach allow novice users to quickly, easily, and effectively clean-up protein digests. This strategy generates data with the best possible reproducibility and highest peptide recovery for challenging protein bioanalysis studies.

References

1. Medicines in Development: Biologics, PhRMA, phrma.org report 2013.

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