A Rapid, Automated Immunoaffinity LC-MS/MS Workflow

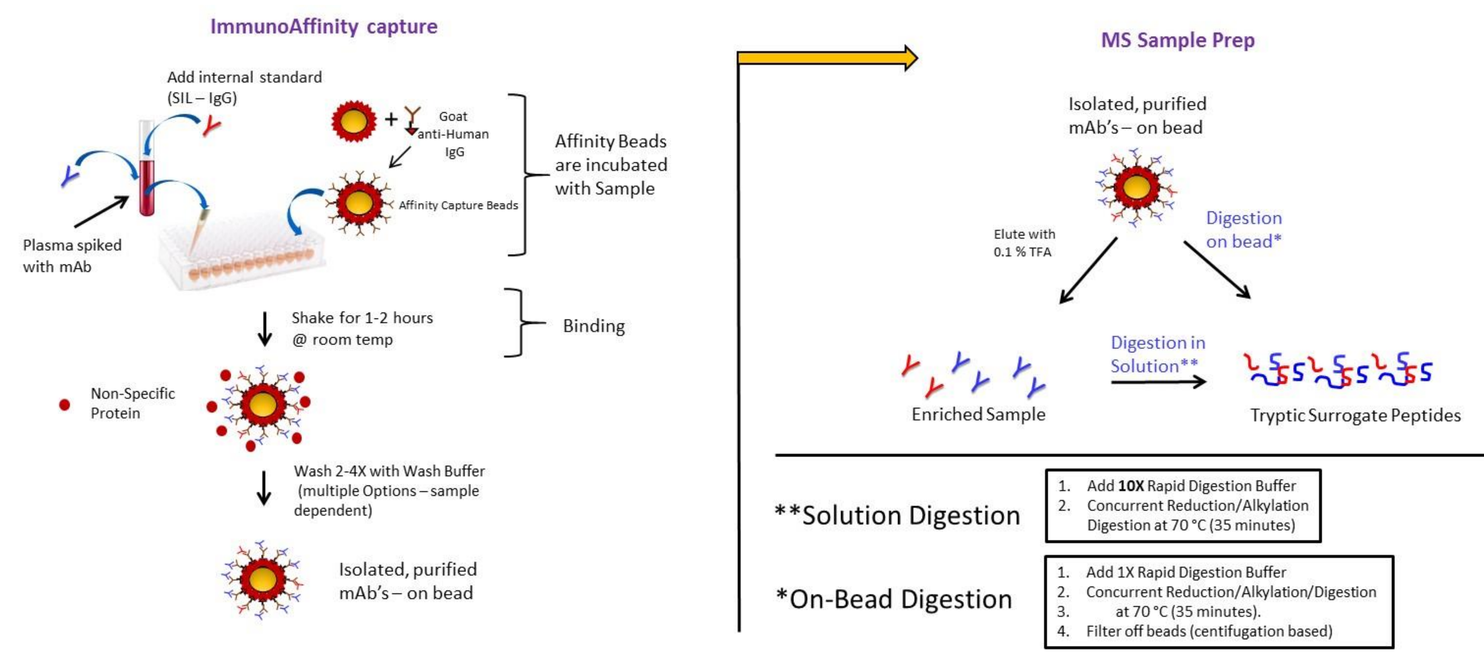
Michael Rosenblatt [1] Gregory Barrett-Wilt[2]; Nidhi Nath[1]; Lyndsey Jager[1]; Marjeta Urh[1]

[1]Promega Corporation, 2800 Woods Hollow Rd, Madison, WI 53711 ; [2]University of Wisconsin, 425 Henry Mall Madison WI 53706

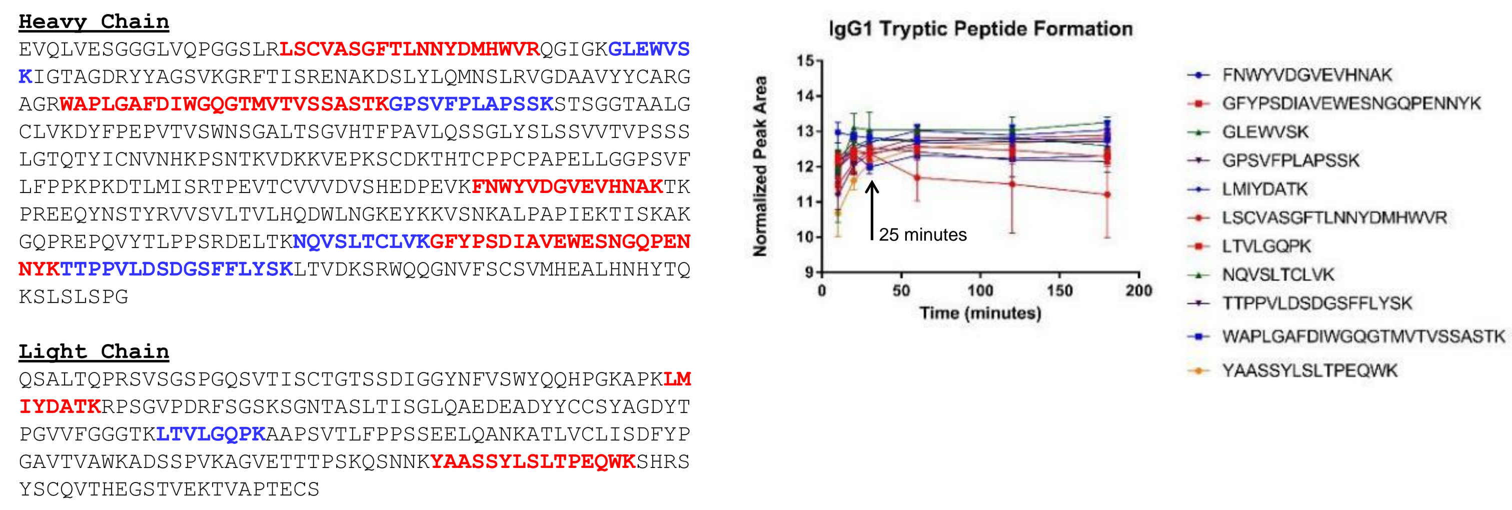
1. Introduction

The analysis of Biotherapeutic antibody metabolism in various animal models is a critical requirement during drug development. As the number of potential antibody targets have increased targets as well as Biosimilar development, there is an immediate need to increase sample throughput. Toward this end, we have developed a streamlined workflow that involves mAb enrichment, using high-capacity magnetic streptavidin particles, followed by proteolysis using a commercially available Rapid Digestion kit. The rapid enrichment/digestion step combined with direct LC-MS/MS analysis (no offline cleanup required) allows for protocols that can be carried out in very short time periods (as short as 2-3 hours). The protocol is very simple, requires routine laboratory equipment, and can be easily automated.

2. A Flexible and Rapid IA LC-MS/MS Workflow

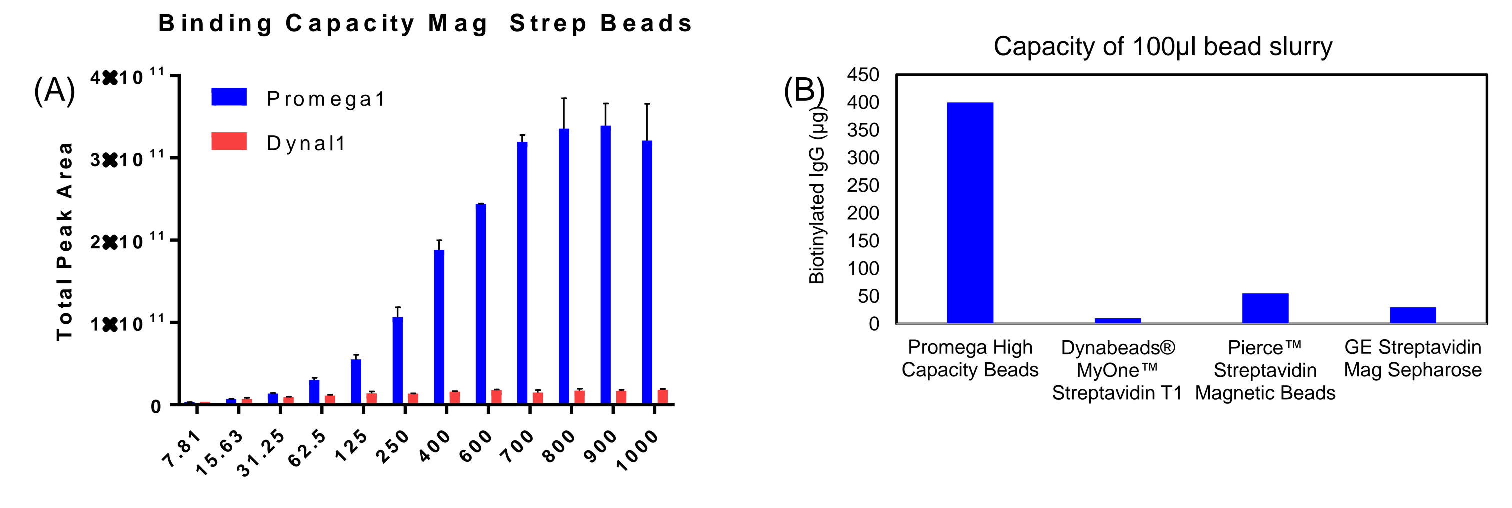


3. Time Course of Digestion



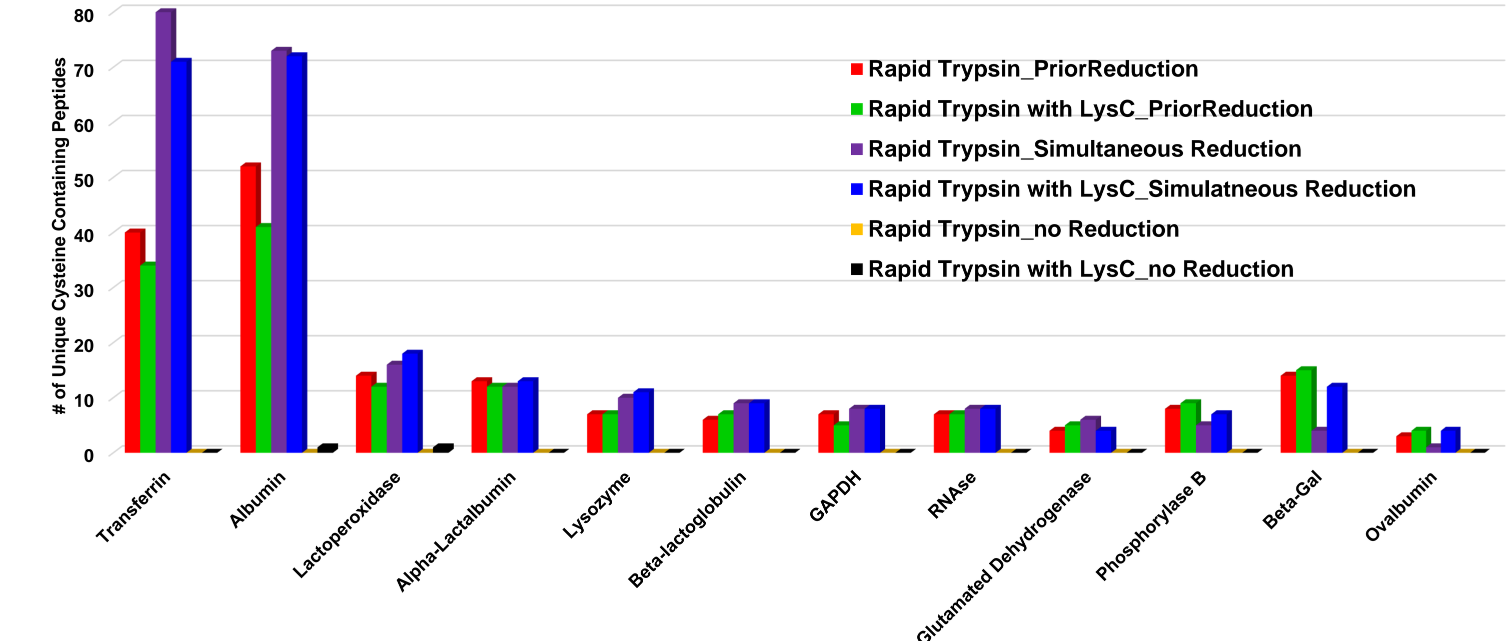
IgG1 (Sigma; SILu™lite) at 0.1 mg/mL was prepared in rapid digestion buffer and then aliquoted into multiple tubes and digested at 70 C.. At the appropriate time point, the sample was digested using the Rapid Digestion protocol. Rhe samples were then removed from the heat block, allowed to cool on ice, and then quenched with 5 microliters of neat formic acid. Peak areas for multiple signature peptides appear to saturate within 25 minutes.

4. High Binding Capacity of Magnetic Streptavidin Beads



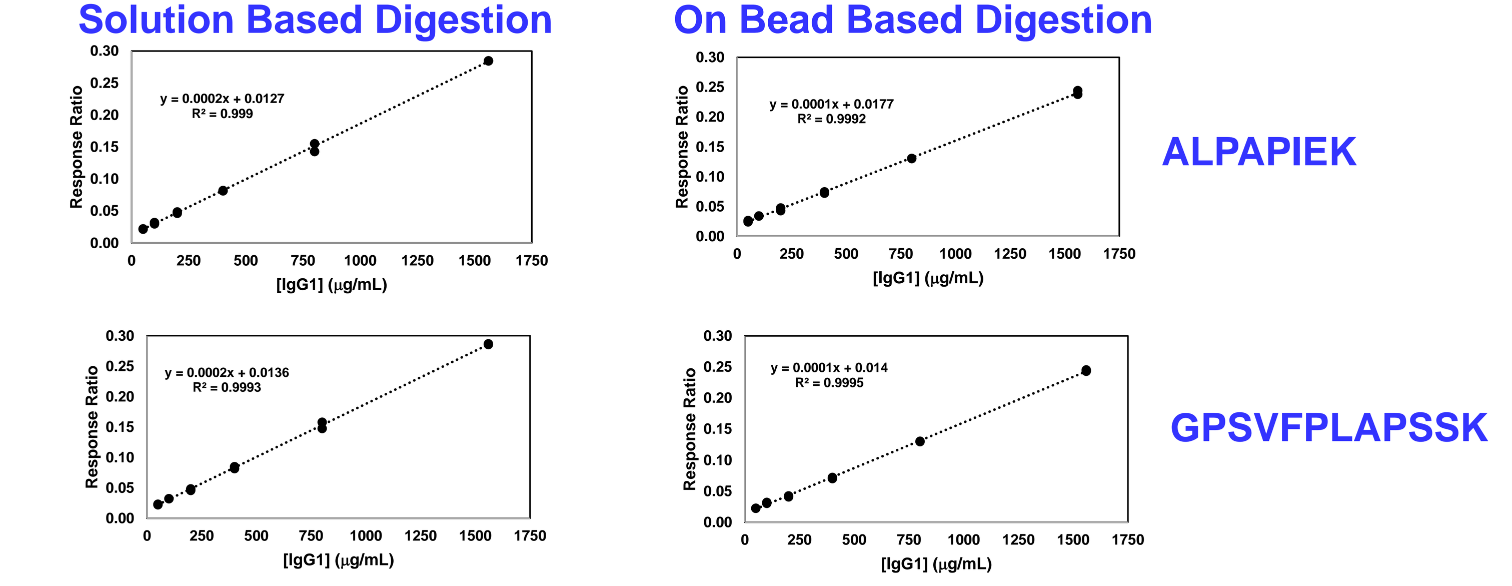
(A) IgG1 at the described concentrations in a Rat Plasma matrix (4 µg/mL IgG1 SIL-IS) was isolated using either 25 µLs (20 % slurry) of Promega High Capacity Magne® Streptavidin beads or 1 mg (100 µLs) of Dynal® beads, which is the suggested manufacturer protocol. Peak areas for the Promega beads are substantially larger than Dynal ®, which translates to a wider assay dynamic range window. (B) Binding capacities of 100 µL bead slurries are compared, confirming the superior capacity of the Promega beads. Mass Spec and protein assay both confirm a binding of at least 15 mg of IgG per 25 µL of slurry.

5. Simultaneous Reduction/Akylation/Digestion



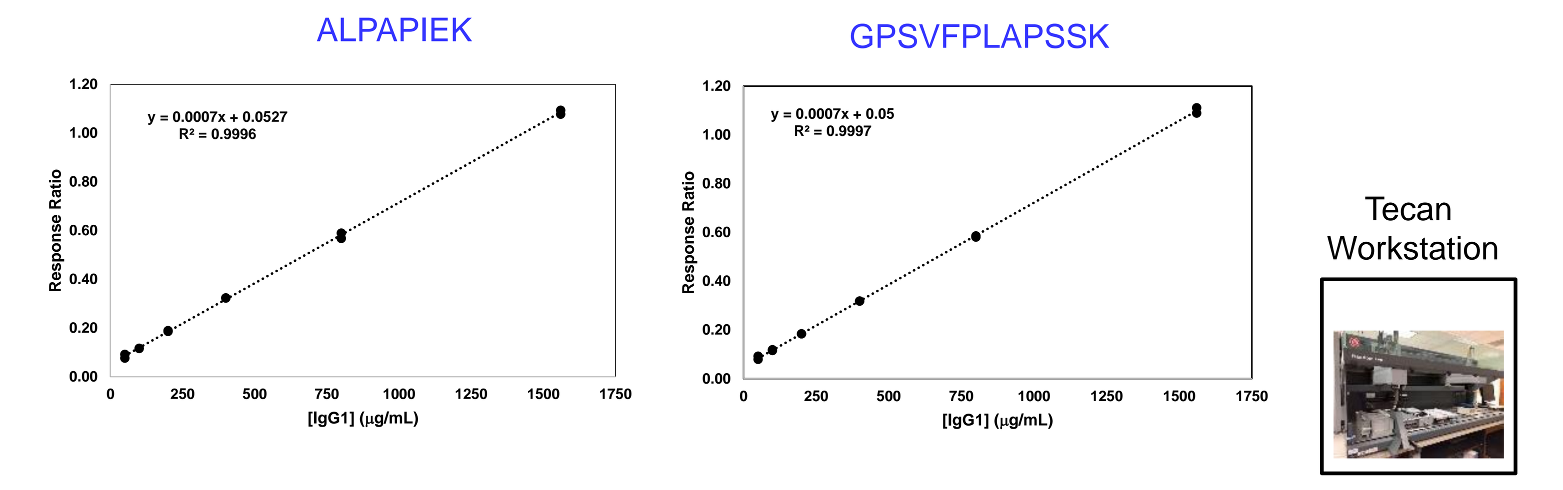
A panel of disulfide-rich proteins were incubated with 1 mM TCEP/2.5 mM Iodoacetamide/0.5 micrograms of Trypsin for 1 hour at 70 °C. In all cases, similar or more unique cysteine-containing peptides produced in a simultaneous Red/Alk protocol versus those in which a prior reduction/alkylation step was performed.

6. Solution versus On Bead



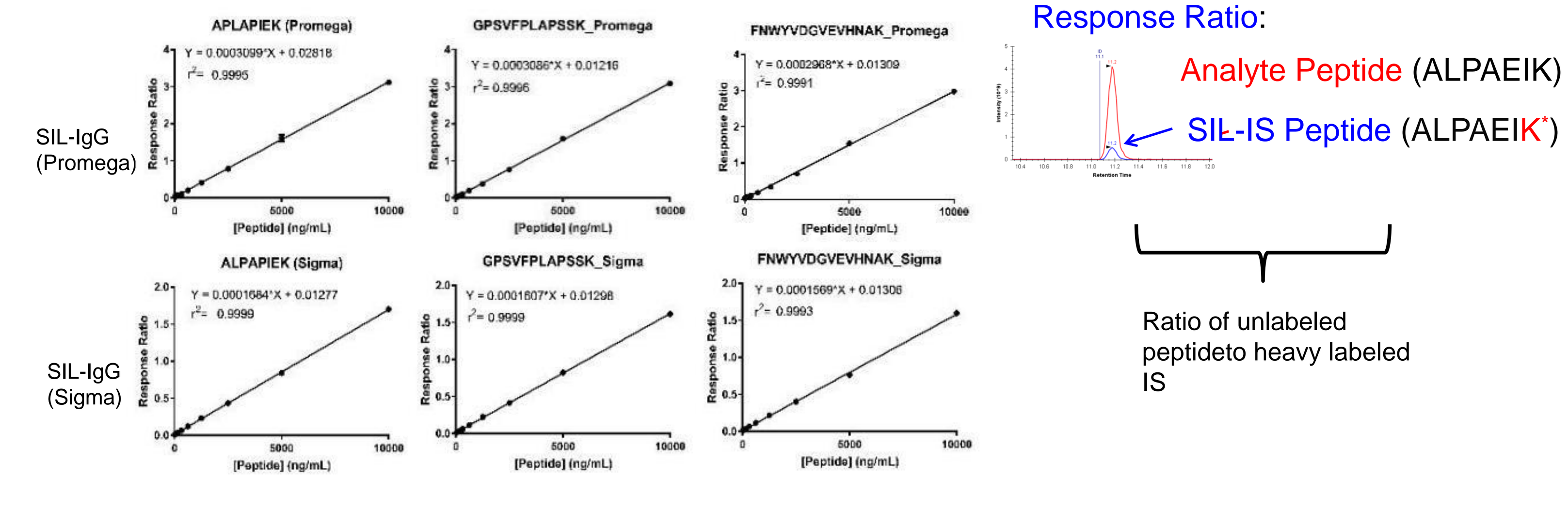
Two experimental protocols were compared. The first protocol involved elution with 0.1 % TFA, addition of a 10X Rapid Digestion Buffer, and digestion for 30 minutes at 70 °C. The second protocol, eliminated the elution step and involved a direct, on-bead digestion using a 1X digestion buffer. Both protocols gave comparable results, although the on-bead approach required less manipulation and therefore shorter processing times. In addition the variance of IS peak areas for the solution digestion was 19.46 % whereas that of the "on-bead" digestion was 13.04 %.

7. Automation of Assay



We have developed an automated protocol. The results of the automated protocol, which involved the enrichment and all liquid handling steps on a Tecan workstation (only the digestion at 70 °C was done offline using Eppendorf thermo-mixer) gave results that match well against the manual protocol. This data shows the feasibility of automating the process.

8. A Universal SIL-IgG Internal Standard



A Universal Stable-Isotopically labeled IgG1 (IS) was used as an internal standard. A calibration curve from 10 mg/mL – 0.05 mg/mL was produced with the IS held at a constant concentration of 4 mg/ mL. As seen above, the linearity between the Promega IS and the Sigma is virtually identical for multiple signature peptides.

9. Conclusions

- A rapid and simple protocol for quantification of mAbs from animal matrices has been demonstrated.
- The entire protocol takes less than ½ day.
- High capacity magnetic beads facilitate wider assay dynamic range.
- The protocol is very sensitive, down to at least 50 ng/mL.
- Reduction/alkylation/denaturation (not required) can be performed concurrent with the digestion.
- The protocol can be automated.

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