Supplementary Material

Results

Selection of magnetic beads

We sought a bead system with high antibody coupling efficiency, good functional orientation of antibody for antigen capture, low background binding of serum peptides and proteins, and production of captured samples appropriate for mass spectrometry (i.e. without chemical contamination). Proteins were used to monitor background binding because: i) compared to peptides, proteins show greater propensity to bind nonspecifically and hence enforce stringency on protocols, ii) proteins can be monitored by SDS-PAGE, and iii) residual undigested proteins in biospecimens could interfere with peptide binding. We tested a panel of seven unique Dynal magnetic beads for nonspecific binding to serum proteins, assayed by SDS-PAGE. The results are shown in Figure S1. Tosylactivated beads were associated with the most nonspecific binding. Myone Streptavidin, M280, and epoxy beads showed intermediate levels of nonspecific binding, and M270 and carboxyl beads showed the least nonspecific binding. As expected, Protein G beads capture serum IgG, but otherwise were not associated with significant nonspecific binding.

Note that tosylactivated and epoxy beads are covalently reactive, and hence were expected to bind serum proteins (although in these experiments the temperature and pH were probably not optimal for that coupling). Since the normal use of these beads would be after antibody binding, we also determined non-specific serum protein binding to tosylactivated beads coated with anti IL-8 antibody. The beads coupled with antibody were incubated with serum, washed with buffer containing 0.5 M NaCl, and eluted with 5% acetic acid. The same nonspecific serum proteins were observed in the eluates of the antibody-coated and the uncoated beads (data not shown). According to manufacturer's specifications, the coupling efficiency on this bead surface is 30-50%; hence, the surface of tosylactivated beads is not fully occupied by antibody, leading to non-specific binding of serum protein. (It is possible that the tosylactivated and epoxy bead systems could be useful if conditions were optimized for blocking the remaining reactive sites after antibody coupling.)

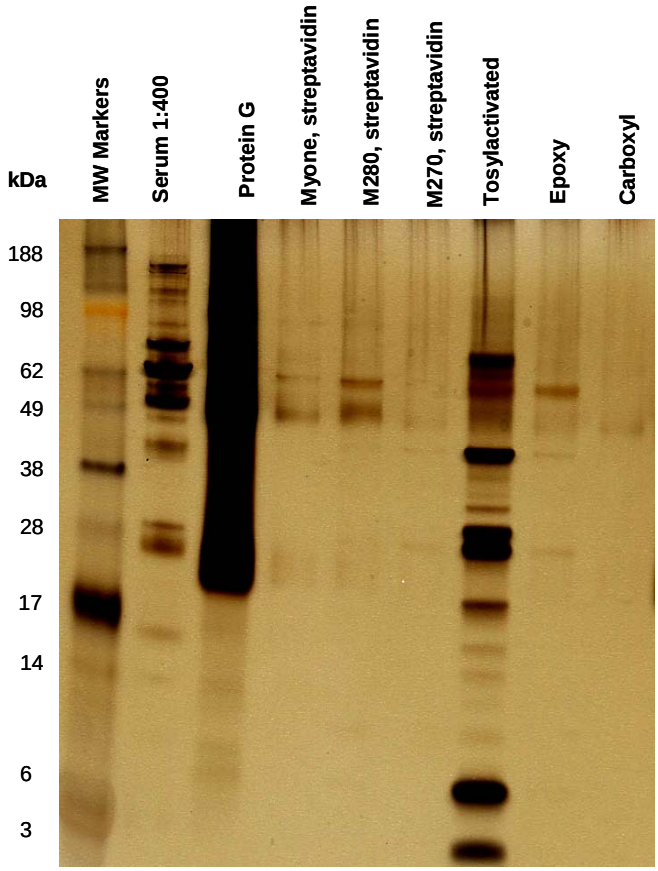

Figure S1. Characterization of non-specific serum protein binding to a panel of magnetic beads. 20 μL each of Dynabeads^{R} Protein G, Myone, M280, M270 streptavidin, tosylactivated, carboxyl, or epoxy beads were incubated with 50 μL of human serum overnight at 4 ^{0}C. The beads were washed with 4 x 1 mL 100 mM Tris, 1 mM EDTA + 0.5 M NaCl, and bound proteins were eluted into 8 μL of 5% acetic acid. The eluates were run on an SDS-PAGE gel, which was subjected to silver staining for determination of the amount of nonspecific binding of serum proteins to beads.

Eluates from each bead type were also subjected to LC-MS analysis to detect chemical contamination (such as polymers) that might interfere with peptide ionization. Dynal Myone streptavidin beads were associated with a high amount of polymer contamination (data not shown), which could not be washed off with methanol, isopropyl alcohol, or acetonitrile. Hence, Myone streptavidin beads were not included in further studies. Identification of the polymers was beyond the scope of our study. Carboxyl and Epoxy beads were also eliminated from further study because they do not work well for antibodies stabilized in 5% trehalose (sugars may interfere with binding of amine groups).

Although Protein G beads capture IgG from native serum (Figure S1), tryptic digests of serum used for peptide antigen capture during SISCAPA gave very little background on these beads since no intact Ig was present in the digest samples (compare lane 1 Figure S1 vs Figure S2). Figure S2 shows the optimization of wash conditions to provide low non-specific serum peptide binding. Antibody capture of target serum peptide was done at 4^{o}C overnight. As shown in Figure S2, very little background binding was observed when trypsin-digested serum was incubated with beads, even with a salt-free wash. This is expected since the bulk of serum protein had been reduced to tryptic peptides. Because of low nonspecific binding, a stringent 0.5 M NaCl wash buffer was chosen for future capture experiments.

Based on their low background binding, lack of chemical contamination, and proper orientation of crosslinked antibodies, we chose Protein G beads for our peptide antigen capture technology.

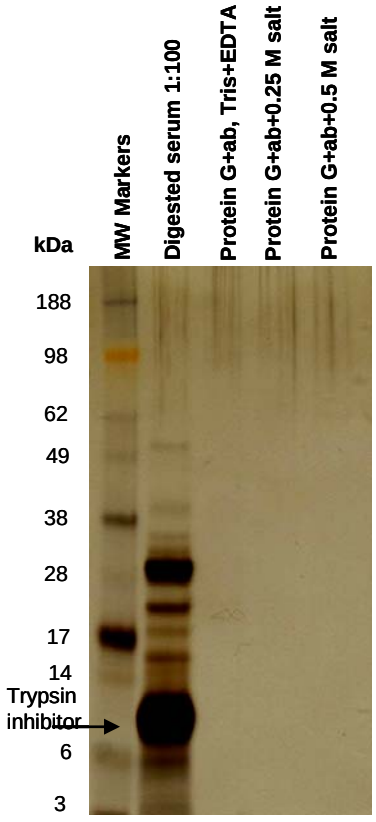

Figure S2. Establishing wash conditions to minimize nonspecific binding of serum peptides (on Protein G beads). Protein G beads were coupled with anti-TNFα antibodies and incubated at 4 ^{0}C overnight with trypsin-digested human serum. The beads were washed four times for 5 minutes at RT with 1 mL of buffer containing 100 mM Tris, 50 mM EDTA and increasing NaCl concentrations. Eluates were analyzed by SDS-PAGE followed by Silverstaining.

Saturation of Protein G bead surfaces with antibody

Due to the high cost of antibody development and because we wanted to minimize prep-to-prep variation, we sought to optimize the coupling efficiencies of antibodies on our chosen bead system. To that end, we sought to determine quantitatively the amount of antibody required to efficiently saturate a given volume of beads.

A series of concentrations of antibody were incubated with a fixed amount of Protein G beads (Figure S3). 600 μg of Protein G beads bound approximately 6 μg of anti-hemopexin polyclonal antibody (Figure S3), within the range of the manufacturer's specifications (Protein G: 8.3 μg human IgG/mg beads). This corresponds to about 1 amol rabbit anti-hemopexin antibody molecules per Protein G bead.

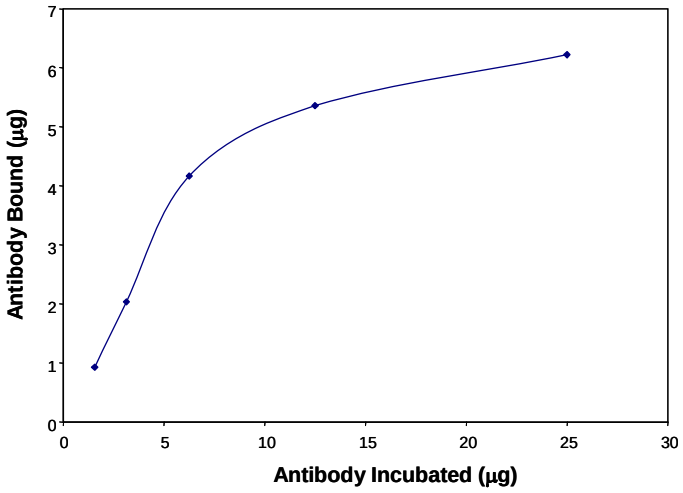

Figure S3. Antibody binding capacity of Protein G beads. 20 μl (600 μg) of Protein G beads were incubated with an increasing amount of anti-hemopexin polyclonal antibody. An estimate of the amount of antibody bound to Protein G beads was determined by comparing protein concentrations (measured by Bradford assay) of the supernatants (containing unbound antibody) before and after incubation of beads with antibody.

Evaluation of Trypsin Digestion Protocol

Complete and reproducible proteolysis is very important for the SISCAPA-MS approach to be accurate because the approach relies on peptide quantification to infer the concentrations of the original protein(s). We sought a digestion protocol that would yield complete proteolysis, be applicable to serum and plasma, and

minimize sample handling steps (e.g. desalting). To this end, we developed the protocol described in the manuscript using an organic-aqueous solvent system. Organic solvents (such as methanol) can unfold and solubilize proteins, acting as a denaturant, while retaining the activity of trypsin. Following digestion, methanol can be removed by evaporation, thus minimizing sample-handling and maximizing sensitivity and reproducibility. To evaluate the efficiency of the trypsin digestion, we compared the products of the methanol-based protocol with those of a popular urea-based protocol. Figure S4 shows the SDS-PAGE of the digestion products at four timepoints following addition of trypsin. The absence of proteins at high mass (>25 kDa) indicates trypsin is efficient. The predominant band at ~24 kDa corresponds to trypsin. Predominant bands around 10 kDa may be autodigestion or degradation products of trypsin.

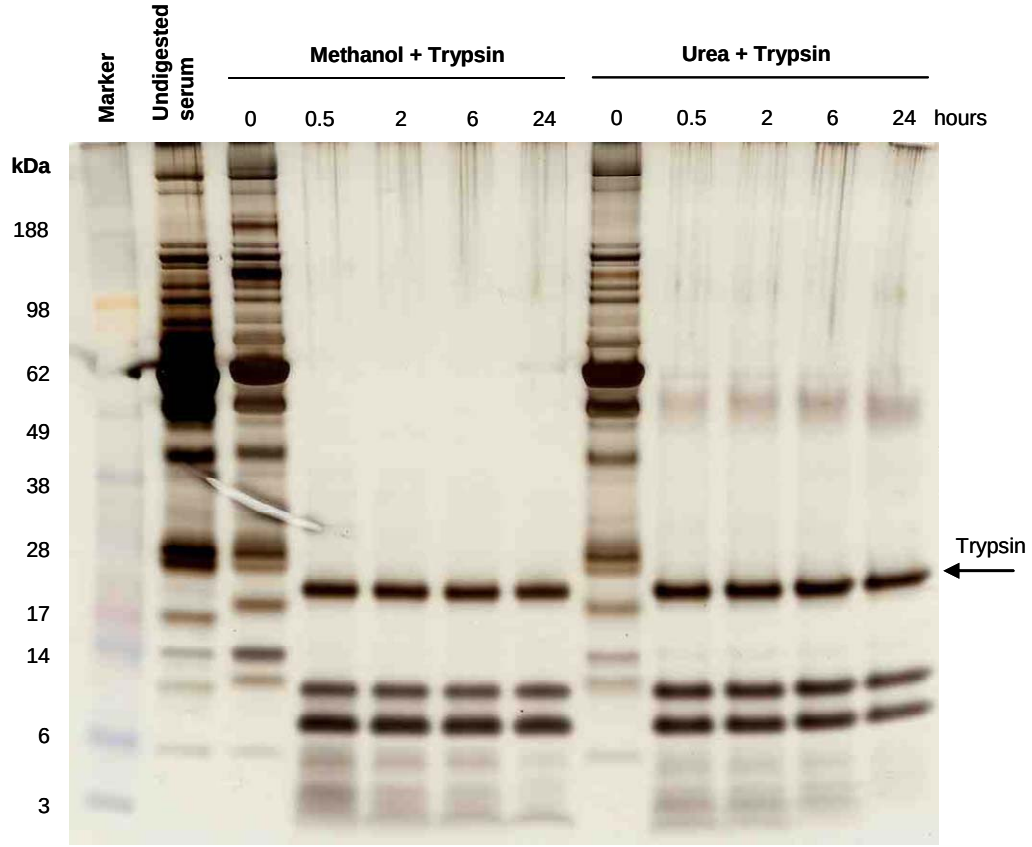

Figure S4. Evaluation of Trypsin Digestion. Serum was diluted to 6 mg/mL and digested according to the protocol found in the Experimental Section. For the urea digestion, 8M urea was used for denaturation and was diluted to 1M following reduction and alkylation of proteins. Aliquots were removed from the digestion at 0.5, 2, 6, and 24 hours and were subjected to analysis by SDS-PAGE with silver staining. The lane marked "Undigested serum" is 6 μg non-denatured serum.