Uniform and Reliable Magnetic Beads for Protein Immunocapture Workflows

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Magnetic beads are ubiquitous in the field of genomics but are also a critical component of protein therapeutic analysis [1,2]. Typically utilised in the pharmacokinetic (PK) and pharmacodynamic (PD) laboratories, magnetic beads are used to extract and quantitate proteins from biologic matrices, which is predominantly considered a ligand binding assay (LBA) or immunocapture. The sensitivity, accuracy, and reliability of these quantitation methods are essential during safety and efficacy testing. Enzyme-linked immunosorbent assays (ELISA) are the most established LBA protocol, but magnetic beads are emerging as the tool of choice to streamline the immunocapture process. A common magnetic bead immunocapture approach starts with streptavidin-coated beads that are activated with a biotinylated anti-idiotypic reagent. This strategy allows specific binding to the protein of choice and takes steps toward platforming the protocol.

More modern strategies are geared toward combining LBA with LC-MS/MS which creates a more sensitive process with increased linear dynamic range (LDR) [3,4]. While magnetic beads are a mature technology, advances in the grafting process can lead to increased binding capacity and more dependable data. This article demonstrates a hybrid LBA/LC-MS/MS approach [5-7], utilising immunocapture of large molecule therapeutics with streptavidin-coated magnetic beads, followed by quantitation of signature peptides using LC-MS/MS. This strategy necessitates a consistent and reliable sample preparation procedure that can be platformed to different large molecule therapeutic modalities, which offers a considerable benefit over traditional methods [8,9].

Materials and Methods

Rituximab and insulin aspart (Novolog) were purchased from Myoderm® (Norristown, PA). Trypsin was purchased from Promega® Corporation (Madison, WI). SiLuMab and Dulbecco's Phosphate Buffered Saline (DPBS) were purchased from Sigma-Aldrich® (St Louis, MO). bioZen™ MagBeads, bioZen Peptide XB-C18 LC column, and bioZen Peptide PS-C18 LC column are from Phenomenex® (Torrance, CA). LC-MS/MS methods were performed on an Agilent® 1290 equipped with a SCIEX® 6500+ or a SCIEX X500B QTOF.

Magnetic Bead Activation – Representative Protocol (Figure 1)

A 25 µL aliquot of bioZen MagBeads (20 mg/mL) was washed with 500 µL PBS buffer. Excess liquid was discarded using a magnetic stand (3x). The beads were reconstituted to original volume with PBS. 5 µg of anti-insulin and proinsulin antibodies were added and incubated at room temperature for 1 h with shaking speed of 1200 RPM using a deep well plate thermoshaker. The excess liquid was discarded using a magnetic stand. The beads were washed with 500 µL PBS buffer and the excess liquid was discarded using a magnetic stand (3x). The beads were reconstituted to original volume with PBS.

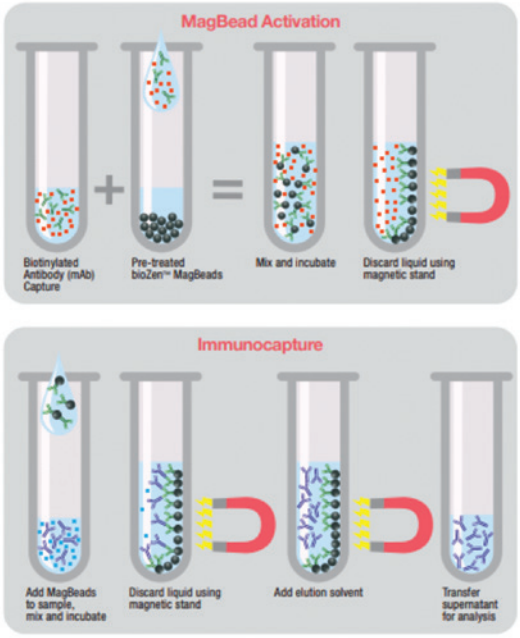

Figure 1: Sample Preparation Procecure Visual.

Immunocapture – Representative Protocol (Figure 1)

250 µL plasma samples were added to a 96-well plate. The activated beads were vortexed to mix thoroughly and 25 µL was added to each well containing plasma. The plate was covered and spun down at 800 RPM for 3 s before incubating at 1200 RPM for 2 h on a thermoshaker. The excess liquid was discarded using a magnetic stand.

Washing and Elution – Representative Protocol

200 µL of 0.5 % CHAPS in PBS buffer was added to the beads and the solution was mixed then centrifuged at 800 RPM for 3 s. The liquid was then discarded using a magnetic stand. The beads were washed with 200 µL PBS, mixed and shaken for 10 m at 1200 RPM using a deep well plate thermoshaker. The resultant mixture was centrifuged at 800 RPM for 3 s and the excess liquid was discarded using a magnetic stand for 2 m. 70 µL methanol/water/acetic acid (50:48:2) was added, mixed, and shaken at 1200 RPM for 10 min using a deep well plate thermoshaker. The mixture was centrifuged at 800 RPM for 3 s then placed on a magnetic stand for 10 m. The supernatant was transferred to a different 96-well plate and 45 µL water was added and

mixed. The plate was plated on a magnetic stand for 10 m then the supernatant was transferred to an injection plate.

Trypsin Digestion Protocol

For samples requiring a trypsin digestion, the supernatant of the wash/elution step was placed into a 96-well plate. The eluted samples were diluted with ammonium bicarbonate, ensuring pH >7.0. Samples were then heat denatured to 95°C. After cooling to <50°C, trypsin was added, and the mixture was shaken at 300 RPM for 1 h at 50°C. After centrifugation, the sample was used for LC-MS/MS analysis.

Results and Discussion

The uniformity of the bioZen MagBeads was assessed by scanning electron microscopy (SEM). The particle size of the streptavidin-coated beads was 1 µm and displayed excellent uniformity (Figure 2). Along with a patented streptavidin coating process, the consistent particle size lends to more efficient binding, thus lower background.

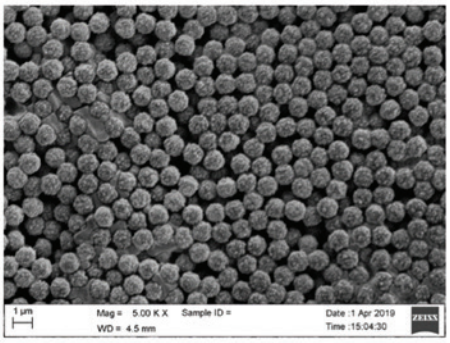

Figure 2: Uniform Particle Size of Magnetic Beads Determined by SEM.

To assess consistent immunocapture, two different lots of the activated magnetic beads were evaluated (Figure 3). With correlation coefficients greater than 0.99 for both lots, a robust LBA protocol was confirmed. This lot-to-lot reproducibility assessment is important to ensure consistency in biotinylated capture antibody, which could lead to potential variation in the linear dynamic range of the assay.

Mag Bead	Correlation Coefficient
bioZen MagBeads Lot 1	0.9914
bioZen MagBeads Lot 2	0.9941

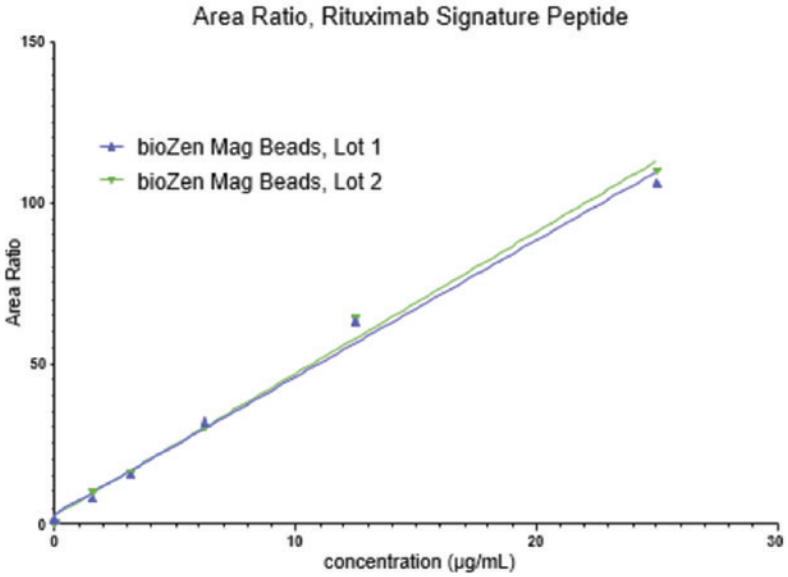

Figure 3: Correlation Coefficient Assessment of Multiple Bead Lots.

Insulin analogues are growing in the biotherapeutic industry [10] and thus, the bioanalytical immunocapture workflow is significant. The experiment commenced with a calibration curve to ensure good linearity with the external standard (Figure 4). A correlation coefficient of 0.99814 was determined for a LDR from 50-10,000 pg/mL. Proceeding to analyse insulin, we assessed samples at 50 pg/mL and 500 pg/mL to qualify the binding capacity of the magnetic beads (Figures 5 and 6, respectively).

Excellent recovery of each sample was observed even at 50 pg/mL, although some inherent background noise was also observed at this concentration.

Hybrid LBA/LC-MS/MS

Rituximab is a classic example of a commercial therapeutic monoclonal antibody in the biopharmaceutical industry. Utilising the magnetic bead LBA protocol in tandem with the trypsin digestion protocol described above, six rituximab signature peptides were analysed on a bioZen Peptide XB-C18 LC column (Figure 7). Good chromatographic separation of the peptides was observed, and each peptide demonstrated appropriate ionisation for the LC-MS/MS analysis. Notably, the integrity

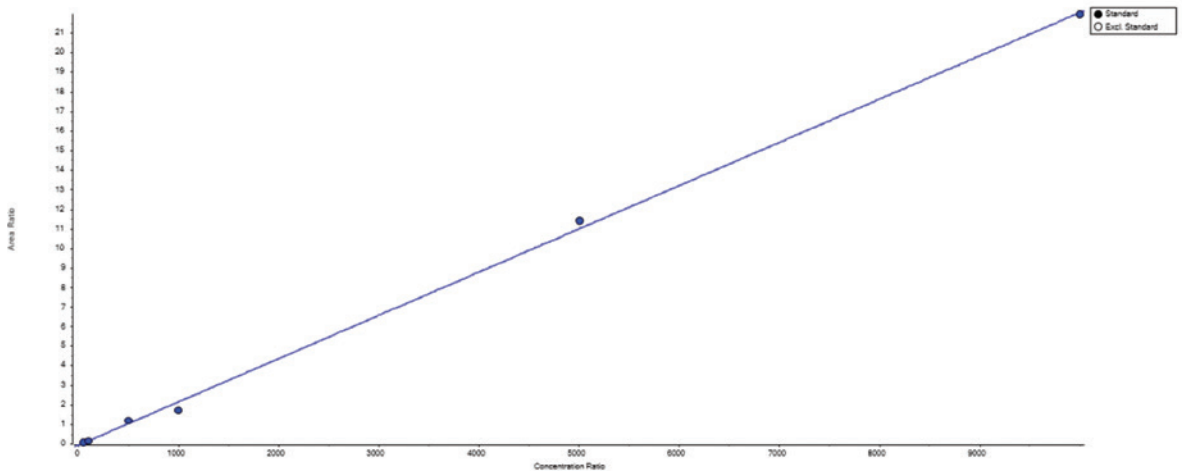

Figure 4: Calibration Curve from 50 pg/mL-10,000 pg/mL.

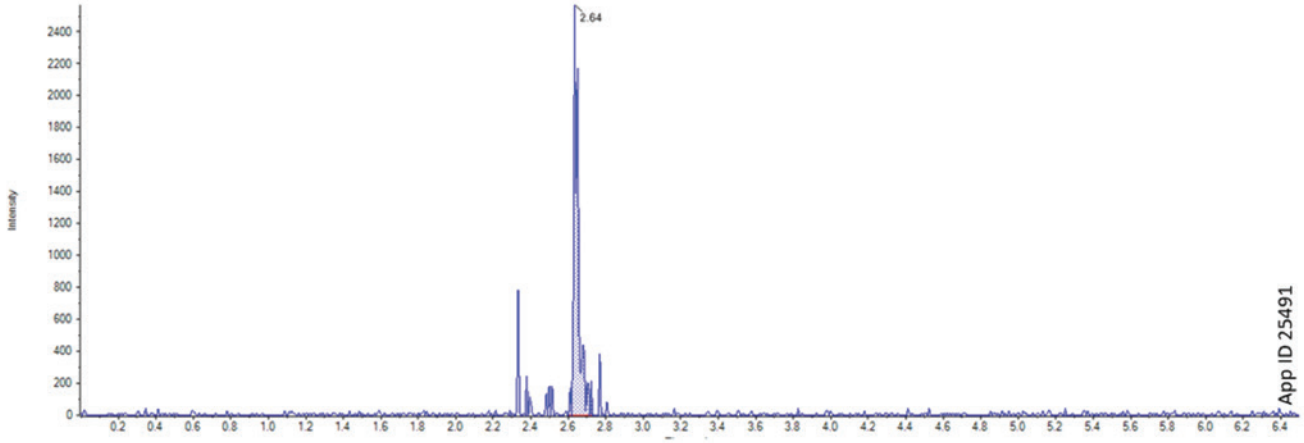

Figure 5: 50 pg/mL Extracted Insulin Aspart Standard Using bioZen MagBeads.

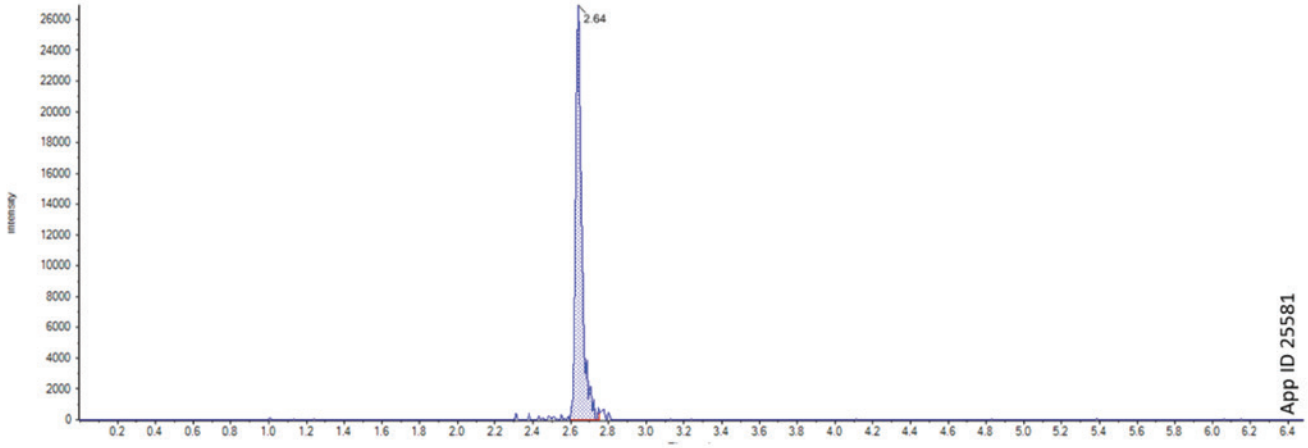

Figure 6: 500 pg/mL Extracted Insulin Aspart Standard Using bioZen MagBeads.

of the signature peptides is excellent which confirms a robust sample preparation protocol.

An important feature of this protocol is the similarity of the immunocapture procedure of rituximab compared to insulin. While the proteins are very different in structure, the immunocapture procedure had minimal modifications to achieve similar results. This fact suggests the broad applicability of the magnetic bead protocol and the ability to adopt it as a platform method.

Conclusion

As the complexity of therapeutic modalities increases, the bioanalytical field must adapt. Robust immunocapture and hybrid LBA/LC-MS/MS protocols using bioZen MagBeads are demonstrated. Because hybrid LBA/LC-MSMS utilises MS as the detection, specificity of the assay can be improved while sensitivity can be similar to traditional ELISA methods.

By utilising streptavidin-coated magnetic beads, the method can be platformed in such a way that only the capture antibody or biotinylated target can be modified. This concept is demonstrated by the workflow being used for both a monoclonal antibody, as well as for a peptide therapeutic. Finally, this workflow not only extends to different therapeutic modalities, but it also applies to other large molecule bioanalytical workflows including intact quantitation or biotransformation, which also could implement a similar sample preparation strategy.

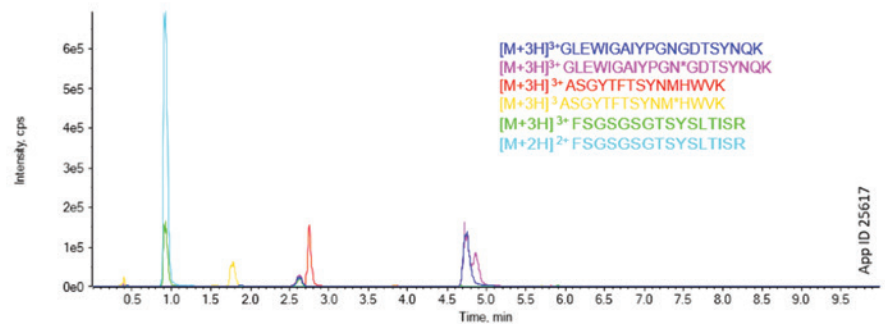

Figure 7: XIC of the Signature Peptides of Rituximab on a bioZen Peptide XB-C18 column.

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