Chapter 25

Semi-Automated Lectin Magnetic Bead Array (LeMBA) for Translational Serum Glycoprotein Biomarker Discovery and Validation

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Abstract

Aberrant protein glycosylation is a characteristic of diverse diseases which has been explored as biomarkers. To support translational serum glycoprotein biomarker discovery and validation, we developed a semi-automated workflow using individual lectin-coupled magnetic beads to conduct lectin pulldowns in a high-throughput format. Lectins are naturally occurring glycoprotein binding proteins widely used in glycobiology. While lectin-affinity isolation has been coupled to mass spectrometry-based proteomics, the lectin magnetic bead array (LeMBA) platform allows technically robust screening and measurement of clinical cohorts. This chapter describes detailed lectin-magnetic bead coupling, serum denaturation, lectin magnetic bead pulldown, and on-bead trypsin digest. The resulting tryptic peptides are analyzed by untargeted or targeted liquid chromatography-mass spectrometry (LC-MS), for biomarker discovery, or qualification/validation, respectively. LeMBA-MS generates quantitative data for glycoforms based on lectin affinity of the glycoprotein coupled with MS measurement of one or more prototypic peptides and has successfully been used to discover and validate novel serum cancer glycoprotein biomarkers. This chapter includes detailed protocols for two different liquid handlers, along with recommendations on quality control measures for clinical biomarker studies.

Key words Glycosylation, Glycoproteomics, Serum biomarkers, Liquid handler, Lectin

1 Introduction

Despite the promise of mass spectrometry-based proteomics in biomarker discovery, few proteomics-discovered markers have progressed from initial discovery by proteomics to clinical mass spectrometry assay. Several factors contribute to the failure in translation of proteomic biomarker research, including resource

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limitations such as poor access to sufficient funding and/or unavailability of high-quality biosamples leading to lack of validation and clinical studies; technical limitations, such as unrecognized biological or analytical variabilities; and use of different biosample type or methodology in discovery and validation phases leading to non-validation of discovered candidates. To overcome these challenges, we sought to develop a high-throughput, cost-effective translational glycoprotein biomarker platform that is reproducible and applicable from discovery to validation and clinical application.

The lectin magnetic bead array-coupled mass spectrometry (LeMBA-MS) method focuses on the glycoprotein subproteome as changes in serum protein glycosylation patterns have been implicated in diverse pathologies from cancer to cardiovascular and neurodegenerative diseases [1–3]. In contrast to sophisticated glycopeptide analyses that aim to identify glycosylation sites and glycan structures [4, 5], LeMBA-MS is a translational biomarker pipeline designed for throughput, technical reproducibility, and translatability. To this end, LeMBA uses naturally occurring glycan-binding proteins of the lectin family for one-step isolation of sub-glycoproteomes and identifies the captured glycoproteins by non-glycosylated peptides only for the purposes of biomarker discovery and validation. Glycopeptides and glycosylation sites of interest can be further investigated in follow-up studies for validated biomarkers.

Lectin affinity chromatography and lectin pulldowns have been widely used in glycobiology, with caveats including specificity and isolation of sub-glycoproteins as each lectin preferentially bind particular glycan structures [1]. In LeMBA, the preferential binding of different lectins is used as a strength to discover differential glycosylated proteins associated with disease, while non-specific binding of non-glycosylated proteins is reduced by the denaturation of serum proteins prior to pulldown to disrupt protein complexes.

The key features of LeMBA are as follows:

1. One step glycoprotein isolation using lectins, with the versatility to use a small or large panel of lectins for initial screening. We have used up to 20 different commercially available lectins with different glycan affinities to broadly profile serum glycoproteins [6]. For diseases with prior literature, a short list of candidate lectins could be generated a priori.
2. Serum/plasma samples are denatured in the first step to disrupt protein complexes while preserving lectin–glycoprotein interactions [6, 7].
3. Spike-in internal standard protein for monitoring sample processing and normalization [6, 8].
4. Compatible with liquid handler systems (recommended) to ensure high precision and reproducible sample preparation across batches [6, 8–10].

5. Pulldown is compatible with diverse downstream analysis methods, including untargeted and targeted mass spectrometry and Western blotting [8, 10].
6. Prior to mass spectrometry analysis, trypsin digestion is performed on-bead with extensive washes but no requirement for peptide cleanup, further reducing technical variability due to non-quantitative sample loss in the cleanup step.
7. Species independent as exemplified with human, mouse, and canine samples [6].

Using LeMBA as the unifying serum/plasma sample preparation platform from discovery to validation phases, we were able to develop serum biomarkers for esophageal adenocarcinoma [8, 10] as well as canine hemangiosarcoma [9]. The final LeMBA-multiple reaction monitoring mass spectrometry assay has the operational and robustness properties for laboratory-based test for clinical applications, similar to multiplex immunoaffinity mass spectrometry assays [11, 12], with the advantage of a single lectin-affinity isolation in the final assay, rather than multiple antibodies. Alternatively, we reported proof-of-concept translation of a validated biomarker to microfluidic lectin immunoassay [13], while other laboratories have investigated lectin biosensors [14].

This chapter details the four protocols for the LeMBA workflow (Fig. 1), including protocols for two different liquid handlers

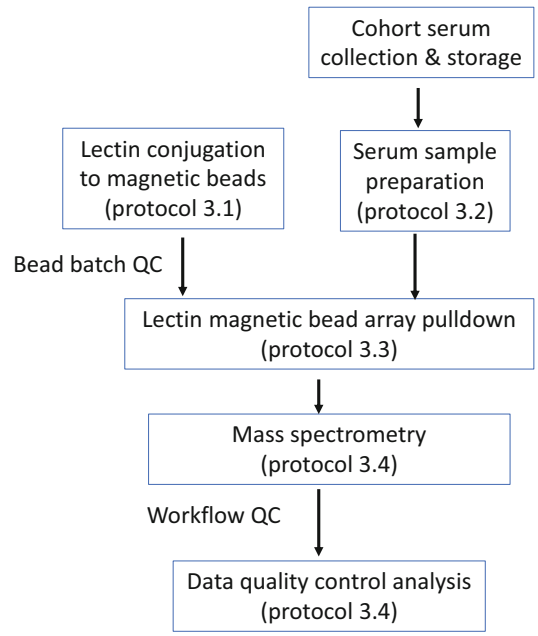

Fig. 1 Workflow for lectin-magnetic bead array (LeMBA) coupled to liquid chromatography-mass spectrometry (LC-MS) for glycoproteomics. Protocols correspond to section numbers in this chapter

used by our teams. The protocol can be scaled down to a manual benchtop workflow using 1.5 mL collection tubes with DynaMag™-2 magnetic rack, or an automatic multichannel pipette with a 96-well ring stand magnet, for trial, small sample batches, or if no liquid handler is available.

2 Materials

All buffers are prepared with Milli-Q water unless otherwise stated.

2.1 Lectin Conjugation to Magnetic Beads

1. Dynabeads™ MyOne™ tosylactivated; 100 mg/mL 10 mL stock (Thermo Fisher, catalogue number 65502). Store beads at 4 °C.
2. Lectins of choice (Vector laboratories). Resuspend lectin powder following manufacturer's instructions to prepare 5 μg/μL stock in lectin coating buffer. Store at −80 °C.
3. DynaMag™-2 magnetic rack (Thermo Fisher, catalogue number 12321D) for washing Dynabeads™ during lectin conjugation.
4. Stock 0.5 M Sodium phosphate buffer = 94.93 mM NaH_2PO_4. $2H_2O$ + 405.07 mM Na_2HPO_4, adjust pH to 7.4. Prepare fresh prior to experiment and store at room temperature.
5. Lectin coating buffer = 0.1 M sodium phosphate buffer + 0.1 mM $CaCl_2$ + 0.1 mM $MnCl_2$.
6. 3 M ammonium sulfate in Milli-Q water. Adjust pH to 7.9. Prepare fresh prior to experiment and store at room temperature.
7. Blocking buffer = 2% glycine solution in 1 M Tris–HCl, pH 7.4. Prepare fresh prior to experiment and store at room temperature.
8. Lectin storage buffer = 20 mM Tris–HCl, pH 7.4 + 150 mM NaCl + 1 mM CaCl2 + 1 mM MnCl2 + 0.5% Triton-X 100 + 1 unit 100× protease inhibitor cocktail (Sigma-Aldrich, catalogue number P8340). Prepare fresh prior to experiment and store at room temperature.

2.2 Serum Preparation

1. Pierce™ BCA protein assay kit (Thermo Fisher, catalogue number 23227).
2. LeMBA serum denaturation buffer = 10 μg/μL serum sample + 20 mM Tris–HCl, pH 7.4 + 1% sodium dodecyl sulfate (SDS) + 5% Triton X-100 + 500 ng chick ovalbumin/ sample. 20 mM dithiothreitol (DTT, Sigma-Aldrich, catalogue number D9779) and 100 mM iodoacetamide (IAA, Sigma-Aldrich, catalogue number I6125) to be freshly prepared and added separately during serum denaturation protocol.

2.3 Lectin Pulldown

1. LeMBA binding buffer = 20 mM Tris–HCl, pH 7.4 + 300 mM NaCl + 1 mM $CaCl_2$ + 1 mM $MnCl_2$ + 1% Triton X-100 + 1 unit 100× protease inhibitor cocktail (Sigma-Aldrich, catalogue number P8340). Prepare fresh prior to experiment and store at room temperature.
2. LeMBA washing buffer = 20 mM Tris–HCl, pH 7.4 + 0.05% SDS + 300 mM NaCl +1 mM $CaCl_2$ + 1 mM $MnCl_2$ + 1% Triton X-100. Prepare fresh prior to experiment and store at room temperature.
3. 96-well deep well plates; 1000 μL/well, polypropylene (Eppendorf, catalogue number 0030501209).
4. Sealing mats for deep well plates (Eppendorf, catalogue number 0030127978).
5. 50 mM ammonium bicarbonate solution in Milli-Q water. Prepare fresh prior to experiment and store at room temperature.
6. Sequencing grade modified trypsin; 100 μg, lyophilized (Promega Australia, catalogue number V5117), stored at −80 °C. Lyophilized trypsin stock is reconstituted in 50 mM ammonium bicarbonate buffer to a working stock concentration of 0.05 μg/μL prior use.
7. Pierce ™ formic acid, mass spectrometry grade (Thermo Fisher, catalogue number 85175).
8. Consumables for Agilent AssayMAP Bravo liquid handler platform.
   - 250 μL tip boxes (Agilent Technologies, catalogue number G8350A).
   - 96-well magnetic-ring stand (Applied Biosystems, P/N AM10050).
   - 96-well U-bottom polypropylene plates; 300 μL/ well (Greiner, catalogue number 650201).
   - 96-well V-bottom polypropylene plates; 300 μL/well (Greiner, catalogue number 651201).
   - 12-well polypropylene reservoir (Agilent reservoir 12 column, part# 201280-100).
   - Adhesive sealing sheets (Thermo Scientific, catalogue number AB-0558).
   - Zone-free sealing film compatible for mass spectrometry injection (Sigma-Aldrich, ZF2721646).
9. Consumables for Integra VIAFLO 96 GripTip and Tecan HydroFlex Microplate washer platforms.
   - 96-Well Microtiter Plate Magnetic Separation Rack (New England Biolabs, catalogue number S1511S).

96-well V-bottom polypropylene plates; 300 μL/well (Greiner, catalogue number 651201).

96-well flat-bottom plates (Greiner, catalogue number 655101).

300 μL Automation GripTips (INTEGRA Biosciences, catalogue number 6433 for Racks, 6432 for Refill).

150 mL Polystyrene Automation Friendly Reagent Reservoir (INTEGRA Biosciences, catalogue number 6318).

96-well plate Sealing Films (Merck Sigma-Aldrich, catalogue number Z721581-100EA).

96-well plate X-Pierce Sealing Films for mass spectrometry injection (InterPath, catalogue number XP-100).

3 Method

3.1 Lectin Conjugation to Tosyl-Activated Dynabeads™ MyOne™

The lectin conjugation protocol is completed over 3 consecutive days. The volumes provided here are for 100 pulldown reactions (i.e., 100 serum samples) for a single lectin and should be scaled to generate a batch sufficient for an entire study. Test the binding activity of the batch prior to analyzing a cohort.

3.1.1 Day 1: Lectin Coupling to Dynabeads™

1. Transfer 500 μL of Dynabeads™ into a 15 mL collection tube and place the tube on an end-to-end rotator to resuspend the Dynabeads™ into a homogenous solution (*see* **Note 1**).
2. Wash the beads three times with 1.5 mL lectin coating buffer. Place the collection tube on the DynaMag™-2 magnetic rack, allowing the magnetic beads to collect at one end. Aspirate the supernatant while being careful not to disturb the pellet and discard wash.
3. Add 500 μL of 3 M ammonium sulfate solution to the washed beads and vortex briefly. Do not discard this solution!
4. Add 500 μL of 5 μg/μL lectin stock to the beads and vortex briefly. Total lectin quantity is 2.5 mg.
5. Incubate the beads and lectin solution at 37 °C for 24 h with gentle rotation on an end-to-end rotator to avoid bead clumping.

3.1.2 Day 2: Blocking the Conjugated Beads

1. Post incubation, save 10 μL of the bead solution for quality control analysis by SDS-PAGE (Subheading 3.1.3).
2. Add 500 μL of 2% glycine solution to the conjugated bead solution to block the unreacted tosyl groups on the Dynabeads™.
3. Incubate at 37 °C for 16 h with gentle rotation on an end-to-end rotator.

3.1.3 Day 3: Lectin-Bead Conjugate Storage

1. Post incubation, wash beads three times with 1 mL storage buffer. Place the collection tube on the DynaMag™-2 magnetic rack, allowing the magnetic beads to settle at one end. Aspirate the supernatant and discard.
2. Store the washed beads in 5 mL storage buffer at 4 °C (*see* **Notes 2** and **3**).
3. For each batch, confirm successful conjugation and pulldown efficiency on SDS-PAGE (*see* **Note 4**).

3.2 Serum Sample Preparation

3.2.1 Plate Layout and Controls

1. Serum samples should be randomized across the plate and block-randomized if more than 1 plate is needed, i.e., distribute control and disease samples across plates.
2. Identical aliquots of a "standard" serum should be used as quality control samples on each plate and across batches. For each 96-well plate, four to ten quality control samples are processed in randomly distributed wells alongside the clinical samples.

3.2.2 Serum Protein Denaturation (See **Note 5**)

1. Determine the serum protein concentration using the Pierce™ BCA protein estimation kit, following manufacturer's instructions and calculate starting serum volumes for protein denaturation at a concentration of 10 μg/μL.
2. Serum denaturation is performed on the bench in a 96-well deep well plate. Where possible, a multichannel pipette may be used to add the denaturation reagents. However, for accuracy it is best to manually add the required serum volume to each well.
3. Calculate the total amount of serum required for the number of lectin pulldowns planned. As an example, we have provided the calculations for serum denaturation that will be used for ten pulldown experiments in Table 1. The user should change the

Table 1
Serum denaturation calculations for ten pulldown experiments

Reagent	Stock concentration	Final concentration	Volume to add (μL)
Serum sample	80 μg/μL	800 μg	10
Tris-HCl, mM	1000	20	1.6
SDS, v/v %	20	1	4
Triton, v/v %	20	5	20
DTT, mM	1000	20	1.6
IAA, mM	1000	100	8
MQ water			34.8
		Total volume 80	

required reagent volumes based on their total pulldown experiments (*see* **Note 6**). In the end, 7.5 µL denatured serum is required per lectin pulldown.

4. Prepare the LeMBA denaturation buffer as per Table 1 (without DTT and IAA) and add the required reagent volume (e.g., 60.4 µL for ten pulldowns) to each well of a 96-well deep well plate.
5. Add serum sample to each well at the desired volume. For example, add 10 µL of serum with a concentration of 80 µg/µL to get a final serum concentration of 10 µg/µL in the denaturation buffer.
6. Add the required volume of 20 mM DTT to each sample well. For example, 1.6 µL of 20 mM DTT is added to each well for ten pulldown experiments. Incubate plate at 37 °C for 30 min to reduce disulfide bonds.
7. Allow the plate to cool down to RT and add the required volume of 100 mM IAA to each well. For example, 8 µL of 100 mM IAA is added to each well for ten pulldown experiments. Incubate plate at RT for 30 min in the dark to alkylate free thiol groups.
8. Denaturation is complete.
9. Dilute 15 µL serum solution in 20 times LeMBA binding buffer (example:15 µL denatured serum + 285 µL binding buffer = 300 µL total volume). 100 µL of this diluted buffer will have 50 µg total serum protein (*see* **Note 7**).
10. Aliquot 100 µL diluted denatured serum into a 96-well V-bottom plate; seal and store plate at −80 °C until ready for pulldown protocol (*see* **Note 8**).

3.3 Lectin Magnetic Bead Array (LeMBA) Pulldown

The LeMBA serum pulldown protocol is completed over 2 consecutive days, and all steps are performed on a liquid handler. Here we provide protocols for Agilent AssayMAP Bravo (Subheading 3.3.1), and Integra VIAFLO 96 GripTip system (Subheading 3.3.2) with Tecan HydroFlex Microplate washer. A supplementary video (Video 1) of magnetic bead pulldown on the AssayMAP Bravo is available online.

3.3.1 AssayMAP Bravo System Start-Up

1. Switch on the AssayMAP Bravo including the pumps and the connected computer.
2. Access the "Protein Sample Prep Workbench" software. This opens the user interface dashboard of the AssayMAP Bravo.
3. From the dashboard, access the "Utility" option and select the "start-up/shut down protocol" option to check the movement and alignment of the pipettor head and functioning of the tip washing station. Ensure that water is flowing from each channel

of the tip wash station; otherwise, it will introduce error in the protocol. Air pockets within the chimney can interfere with water flow, which can be removed by inserting a pipette tip into the chimney.

4. Once the start-up protocol is complete, the AssayMAP Bravo is ready to use.

AssayMAP Bravo Deck Layout

1. AssayMAP Bravo has nine deck positions of which seven can be customized as per user requirements.
2. We customized the deck layout for our protocol as per Table 2.

Serum Glycoprotein Capture with Lectin-Conjugated Beads

1. Prior to experiment, place the lectin conjugated Dynabeads™ on a roller at 4 °C overnight to ensure homogenous bead suspension (*see* **Note 9**).
2. Place the 96-well magnetic holder on position 7 of the Bravo platform.
3. Pre-chill deck position 4 to 4 °C.
4. Thaw the 96-well V-bottom plate aliquoted with denatured serum samples and place it at position 4 of the Bravo platform.
5. Place a fresh 250 µL Agilent tip box on position 3 of the Bravo platform.

Table 2
Deck positions of the AssayMAP Bravo

Deck position	Function	Comment
1	Tip washing station	Pre-fixed
2	Tip loading station	Pre-fixed
3	Tip box	Bravo was programmed to move the tips from position 3 to position 2
4	Serum plate	Temperature-controlled spot where the serum plate was positioned
5	Buffer plate	96-well deep well plates for wash buffers were positioned on this spot
6	Waste plate	12-well Seahorse plate for waste collection was positioned on this spot
7	Magnet holder	U-bottom plates with beads were positioned on this spot
8	Plate holder	Flexible spot programmed for bead washing steps
9	Plate holder with shaker	Flexible spot programmed for bead washing steps

6. In a fresh 96-well U bottom plate, manually pipette 50 µL of the beads to each well. Repeatedly rotate the bead stock during this procedure to ensure equal bead distribution across wells and to avoid bead clumping at the bottom on the tube (*see* **Note 9**).
7. Place the bead plate on the magnetic holder and wait for 1 min to allow beads to collect at the bottom of the well. The beads will deposit as a ring along the edge of the well bottom.
8. Using Bravo, wash the beads thrice with 100 µL LeMBA wash buffer. Discard wash (*see* **Note 10** and Video 1).
9. Transfer 100 µL of the prepared, diluted serum to the beads.
10. Incubate the beads with the serum at 4 °C for 1 h on a plate shaker at a gentle speed and allow for the pulldown of serum glycoproteins. This step is not performed on the Bravo as the plate shaker (deck position 9) cannot be temperature controlled.
11. Place the bead plate on the magnetic holder on position 7 and remove supernatant (*see* **Note 10**).
12. Wash the beads thrice with 100 µL LeMBA wash buffer. Discard the washings.
13. Transfer the washed beads into a fresh U-bottom 96-well plate (*see* **Note 11**).

On-Bead Trypsin Digestion

1. Replace the wash buffer plate with a deep well plate containing 50 mM ammonium bicarbonate solution.
2. Wash the beads seven times with 100 µL of 50 mM ammonium bicarbonate and transfer the beads to a new plate after every third wash. Discard the washings (*see* **Note 12**).
3. Post wash steps, perform on-bead digestion. Resuspend 100 µg lyophilized trypsin stock in 50 mM ammonium bicarbonate to a concentration of 0.05 µg/µL. Add 20 µL of the prepared trypsin solution to each well to achieve a final concentration of 1 µg trypsin/well. Seal the plate with a plastic seal and incubate at 37 °C overnight with gentle shaking (*see* **Note 13**).

Collect Digested Peptides

1. Dispense 10 µL of 1% formic acid to a fresh V-bottom 96-well plate, which will receive the digested peptides.
2. Place the plate on the magnetic holder and transfer the supernatant containing the peptides to the prepared plate with 1% formic acid.
3. Wash the beads with 50 µL of 50 mM ammonium bicarbonate solution and pool the washes with the collected digested peptides. Repeat to collect any remaining peptides.

4. Check the pooled peptide mixture to ensure no beads are present, as the introduction of beads into the LC system is detrimental to the columns and will lead to potential system blockage.
5. Dry down the collected peptides in a vacuum concentrator, seal the plate, and store the dried peptides at −80 °C until mass spectrometry injection.
6. After protocol is completed, perform AssayMAP Bravo system shutdown.

AssayMAP Bravo System Shutdown

1. Ensure all consumables are removed from the Bravo workstation.
2. Navigate to the "Utility" option on the Bravo dashboard and select the "start-up/shut down protocol."
3. Select the "shut down" protocol. Input the desired number of syringe washes. Recommended three wash rounds.
4. Once completed, AssayMAP Bravo is ready for shutdown.

3.3.2 Overview of the Integra VIAFLO 96 GripTip and Tecan HydroFlex Microplate Washer

1. The Integra VIAFLO96 Griptip has 96 channel heads for transferring of beads and liquid. Programming this takes place in the software @INTEGRA Biosciences AG. Version VIALINK 5.4.0.
2. The Tecan HydroFlex Microplate washer dispenses and aspirates liquid into and out of samples in a flat bottom 96-well microplate. Programs for alternate aspiration and dispensing of solutions into the wells are set up in the HydroControl™ software.
3. The two solutions, LeMBA washing buffer and 50 mM ammonium bicarbonate, are prepared in at least 500 mL in glass bottles for connection with the HydroFlex Microplate washer.

Programming of the Tecan HydroFlex Microplate Washer

1. Two programs are required for the Tecan HydroFlex Microplate washer using the HydroControl™ software:
2. (Program #1) Dispense 100 μL of LeMBA washing buffer into each well, with shaking at 1100 rpm for 15 s, and then leave the plate on the magnet inside the HydroFlex for 3 min. This is followed by aspiration of the washing buffer from all wells to waste. This is repeated twice so that the beads are washed three times.
3. (Program #2) Start with prompt to leave plate on magnet inside the HydroFlex for 3 min before aspirating liquid from each well to waste and then dispense 100 μL liquid, followed by prompt to shake the plate at 1100 rpm for 15 s. This is repeated twice so that the beads are washed three times.

Programming of the Integra VIAFLO 96 GripTip

1. Three programs are set up in the VIAFLO 96 GripTip:
2. (Program #1) Aspirate beads in liquid from flat-bottom 96-well plate and dispense into another flat bottom plate.
3. (Program #2) Transfer the supernatant containing digested peptides from a flat-bottom plate (on magnet) to a V-bottom plate for subsequent drying and resuspension for mass spectrometry analysis.
4. (Program #3) Transfer 50 µL of 50 mM ammonium bicarbonate into the microplate wells.

Procedures to Work with the Tecan HydroFlex Microplate Washer and the Integra VIAFLO 96 GripTip Systems for LeMBA

1. In a fresh 96-well flat-bottom plate, add 50 µL of the beads/well.
2. Prepare LeMBA Washing buffer. Connect the solution to Tecan HydroFlex Microplate Washer and prime the line accordingly.
3. Run Program #1 from Subheading "Programming of the Tecan HydroFlex Microplate washer". Insert the plate into the Microplate washer and wash the beads three times with 100 µL LeMBA washing buffer (*see* **Note 11**).
4. Manually add 100 µL of the diluted serum (from Subheading 3.2) to the microplate wells with washed beads using a multichannel pipette.
5. Incubate the beads with the serum at 4 °C for 1 h on a plate shaker at a gentle speed and allow for the pulldown of serum glycoproteins. Ensure the bead plate is secure in the shaker.
6. Run Program #2 from Subheading "Programming of the Tecan HydroFlex Microplate washer" on the HydroFlex washer. Insert the plate and wash the beads three times with 100 µL LeMBA washing buffer.
7. Transfer the beads to a new plate using the VIAFLO 96 Program #1 from Subheading "Programming of the Integra VIAFLO 96 GripTip" (*see* **Note 12**).
8. Prepare fresh 50 mM ammonium bicarbonate and replace the bottle containing the LeMBA Washing buffer with the 50 mM ammonium bicarbonate on the Tecan HydroFlex Microplate washer. Prime the line accordingly.
9. Run Program (#2) on the HydroFlex washer from Subheading "Programming of the Tecan HydroFlex Microplate washer". Insert the plate and wash the beads three times with 100 µL 50 mM ammonium bicarbonate (*see* **Note 12**).
10. Transfer the beads to a new plate using the VIAFLO 96 Program #1 from Subheading "Programming of the Integra VIAFLO 96 GripTip".
11. Repeat the above two steps to wash the beads six times in total.

12. Run Program #2 from Subheading "Programming of the Tecan HydroFlex Microplate washer" on the HydroFlex washer using 50 mM ammonium bicarbonate for the seventh wash. Press STOP and remove the flat-bottom microplate after the first round of aspiration and dispensing (rather than the three washes of the program).
13. After the seventh wash, add 1 μg (10 μL of 0.1 μg/μL) of sequencing grade Trypsin in water to the beads using a multichannel pipette. Seal plate and incubate at 37 °C overnight with gentle shaking (*see* **Note 13**).
14. Place the microplate onto a magnetic plate. Using the VIAFLO 96 Program #2 from Subheading "Programming of the Integra VIAFLO 96 GripTip", transfer the supernatant containing the digested peptides into a fresh V-bottom 96-well plate.
15. Improve peptide recovery by rinsing the beads twice with 50 μL of 50 mM ammonium bicarbonate using Program #3 from Subheading "Programming of the Integra VIAFLO 96 GripTip" on the VIAFLO 96. Add the rinses to the V-bottom 96-well plate containing the supernatant digested peptides.
16. To inactivate the trypsin, add 10 μL of 2% formic acid to the peptide solution using a multichannel pipette.
17. Dry down the peptides in a vacuum concentrator, seal the plate, and store the dried peptides at −80 °C until ready for mass spectrometry analysis.

3.4 Mass Spectrometry

1. Resuspend the dried peptides in 25 μL 0.1% v/v formic acid (*see* **Note 14**).
2. Spin the plate at 1000 g for 3 min to remove air bubbles and to collect all the sample droplets in the well bottom.
3. Seal the plate with zone-free sealing film that is compatible with mass spectrometry injection.
4. Mass spectrometry can be performed using any data-dependent acquisition for biomarker discovery, or multiple reaction monitoring using a custom method for biomarker validation. Example protocols can be found in our publications [8, 10]. Alternatively, data-independent acquisition may be used.

3.5 Data Quality Control Analysis

1. Reproducibility is measured by calculating the coefficient of variation (CV) of peptides from the spiked in chicken ovalbumin protein, with CV <20% accepted (*see* **Note 15**).
2. Quality control is assessed at two levels: the experimental reproducibility is monitored by running a "standard" serum sample multiple times on and across plates/batches, while

individual sample quality control is monitored by pulldown of the spiked-in chicken ovalbumin protein in each clinical sample. If experimental QC is >30% CV, then the method should be examined for any potential sources of variability. If specific samples report >30% CV, the samples should be repeated.

3. The chicken ovalbumin peptides may be used for normalization prior to statistical analyses (*see* **Note 16**).

4 Notes

1. We selected 1 μm diameter tosylactivated MyOne™ Dynabeads™, as the small diameter of the beads offers more surface area for protein capture. Epoxy Dynabeads also performed well in initial evaluation, but tosylactivated had the highest conjugation efficiency against three tested lectins (Loo 2010). We have also tested the pulldown efficiency of MagReSyn® carboxyl and amine magnetic beads as they are a cost-effective alternative to the MyOne™ Dynabeads™. However, the activation protocol for the amine beads requires the use of highly toxic chemicals, such as sodium cyanoborohydride, which is neither user-friendly nor compatible with automated liquid handling platforms. Additionally, we experienced bead clumping issues with the MagReSyn® beads, despite following manufacturer's guidelines, and observed a low pulldown efficiency when compared to the tosylactivated MyOne™ Dynabeads™.
2. Storage buffer is added at a volume such that the conjugated beads have a final concentration of 100 μL original bead suspension/1 mL storage buffer. We use 50 μL of this dilution of conjugated beads per pulldown in tube or microplate format. Users may titrate the required bead volume to lower levels that allows minimal usage of the bead stock without compromising on pulldown efficiency.
3. Conjugated beads retain binding activity for at least 6 months, and possibly longer. However, for best practices it is advised to prepare new conjugated bead stocks after 6 months.
4. To confirm successful lectin conjugation, place 10 μL of the conjugated bead aliquot on the magnetic rack. Allow beads to settle at the bottom of the tube, transfer supernatant to a separate tube, and add 30 μL of 4× Laemmli buffer to both the tubes, i.e., tube with beads and tube with the supernatant. Heat both tubes at 95 °C for 5 min to denature the proteins and load 20 μL of the denatured sample on an SDS-PAGE gel. Beads with successful lectin conjugation will show a band corresponding to the molecular weight of the conjugated lectin, while the band should be mostly absent in the supernatant.

To test the pulldown efficiency of each batch of conjugated beads, we suggest conducting pulldown on a control serum sample (Subheadings 3.2 and 3.3) and visualize the pulldown, the supernatant, and the starting serum sample on SDS-PAGE. Follow the steps as detailed in Subheading 3.1.3 for preparing the lectin pulldown and supernatant samples for SDS-PAGE. For the control serum sample, we suggest using 10 μL denatured serum (diluted 20 times in binding buffer), adding 30 μL of 4× Laemmli buffer to it and loading 30 μL on SDS-PAGE. In parallel, load 25 μL each of the pulldown serum sample and the supernatant on the gel for analyzing pulldown efficiency.

5. Denaturation disrupts protein complexes in the serum sample, to reduce the co-isolation of binding proteins.
6. The serum denaturation is conducted in a large batch to avoid small volume pipetting and minimize batch effects in denatured serum preparations. For discovery, we screened each serum sample against 7–20 different lectins and then shortlisted 3–4 lectins with the highest number of candidates for the validation cohort.
7. Denatured serum is diluted to reduce the concentration of detergents, DTT, and IAA and avoid interference in the pulldown protocol.
8. When screening against multiple lectins, use one microplate per lectin. Aliquot and freeze prepared serum samples to multiple microplates as stated at the end of Subheading 3.2. For example, pre-aliquot denatured serum to ten individual microplates when performing ten pulldown experiments and freeze plates at −80 °C until use. This will reduce the number of freeze-thaw cycles for the stock denatured serum.
9. The beads have a tendency of settling at the bottom of the tube, and thus it is essential to ensure a homogenous bead suspension while adding beads to the pulldown plate. This can be achieved by vortexing for 2–3 min, or continual mixing on a roller at 4 °C for 2–3 days prior to use.
10. It is advised to set the Bravo aspirate/dispense setting on a gentle speed to allow for proper bead washing and to minimize bead loss in the wash flow-through. It is advised that the user should first test the AssayMAP Bravo mixing speeds using spare conjugated beads in LeMBA buffer to determine optimum bead washing and bead transfer speeds. Supernatant can be collected after every major bead wash step to check for pulldown efficiency by comparing bound proteins versus unbound by separating on a SDS-PAGE (*see* **Note 4**).
11. After every transfer step, check the original plate to ensure no residual beads are left in the wells. If beads are present, repeat the wash and transfer **steps** to achieve complete bead transfer to the new plate.

12. Multiple washes and plate changes are done to remove trace Triton X-100 levels remaining in the wells, which will interfere with mass spectrometry. Although pulldown can be performed without Triton X-100 in the pulldown buffer, the pulldown efficiency is lowered due to incomplete dissociation of protein complexes. After developing the described washes and plate change protocol, we have had no contamination issues. It is possible to check for contamination in the final mass spec-ready sample using Fourier transform infrared (FTIR) spectroscopy by comparing spectra to Triton X-100 diluted in solvent.

    Beads will tend to clump up in ammonium bicarbonate due to the properties of the bound lectins. We have used two different mitigation strategies. One option is to use a magnet to disperse the beads by manually scrubbing the bottom of the microplate before dispensing. Alternatively, we programmed the Bravo to perform three to four rounds of plate shaking on deck position 9 to ensure beads are uniformly resuspended in the ammonium bicarbonate solution and then to resuspend and dispense beads at a slow speed (without changing the speed for buffer dispensing steps). It is possible to configure the Bravo shaker to perform direction-based shaking, i.e., north-south, east-west directions, to achieve complete bead resuspension. We advise users to test the shaker settings to best suit their workflow.

13. Always seal the plate with a plastic seal, prior to overnight digestion to avoid buffer evaporation and drying of the beads. The plastic seal can be further fixed in place by taping down the edges of the plate seal with masking tape, to avoid the seal from detaching off the plate surface during the incubation period.

14. The protein recovery will vary depending on the lectin used for pulldown. For each lectin, the mass spectrometry loading should be trialed by testing the injection volume range (e.g., from 1 to 10 μL) of a control pulldown before running a cohort. As an alternate, users may choose to perform a Micro BCA™ assay on the digested peptides to calculate the peptide concentrations and decide the injection volume and amount based on their LC system requirements.

15. When setting up the technique in a new laboratory, three to five intraday quality control samples over 3–5 days should be processed and analyzed to establish the robustness of the technique setup. Intraday and inter-day samples should be $<20\%$ CV.

16. Normalization is not necessary if CV is $<10\%$.

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