

Development of Two Multiplex Immunoassays and Preliminary Application in Determining Protein Depletion for Seppro IgY14/SuperMix Depletion Columns

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Abstract

Depletion of Highly Abundant Protein (HAP) and Moderately Abundant Protein (MAP) from plasma or serum samples can be achieved through Sigma Seppro Tandem IgY14/ SuperMix columns. A range of 77–129 MAPs has been identified from the bound fraction of Seppro SuperMix resin by LC/MS-MS method. However, the efficiency and lot-to-lot consistency of MAP depletion using SuperMix resin has not been determined and requires a robust method to monitor. By taking advantage of the Luminex technology, we have developed two multiplex immunoassays to monitor protein targets of depletion or retention. The MAP-10PLEX assay can simultaneously quantify 10 MAPs (Ceruloplasmin, C4, Plasminogen, IgD, C1q, Antithrombin III, Hemopexin, α -Antichymotrypsin, CRP and Prealbumin) from plasma or serum samples depleted utilizing IgY14/Supermix columns. The LAP4PLEX assay, which is used to track loss of low-abundant proteins (LAP) during depletion, is able to simultaneously quantify Adiponectin, soluble L-selectin, soluble ICAM-1 and TIMP-1. The preliminary assay data on Seppro IgY-SuperMix depleted samples has suggested the two assays to be accurate, fast and cost-saving methods to monitor depletion consistency from lot-to-lot of the SuperMix resin in the R & D, Operations and QC departments.

Introduction

An avian IgY based immunodepletion technology is used for Sigma Sepro depletion products. The Human IgY14 Columns can remove 14 highly-abundant proteins (HAP) and the unique Human SuperMix System is to remove moderately-abundant proteins (MAP). The combination of IgY14/Supermix columns can deplete approximately 96–99% of total protein mass from human serum or plasma (Figure 3). While the depletion of 14 HAPs has been well established, the further removal of MAPs is less well characterized. Previously, a range of 77–129 MAPs has been identified from the bound fraction of Seppro SuperMix resin by LC/MS-MS. However, the degree of specific depletion of MAPs as well as the non-specific impact on low-abundant proteins (LAP) is still unclear. This also makes tracking the lot-to-lot depletion consistency of SuperMix products a challenge. Regular ELISA methods are time-consuming and commercial ELISA kits are costly. Therefore, finding a fast and economical alternative analysis has appeared to be important.

Luminex xMAP technology is a widely used multiplex platform to quantify multiple analytes from biological fluid samples. It combines the accuracy of ELISA and multiplicity of antibody arrays through protein-antibody interaction on the surface of microspheres and is characterized by speed and minimal consumption of samples (Figure 2). In order to evaluate Seppro SuperMix resins in the R & D department and to monitor the lot-to-lot consistency of materials produced by Operations in the QC department, we developed two Luminex immunoassays to simultaneously quantify 10 representative MAPs (Ceruloplasmin, C4, Plasminogen, IgD, C1q, Antithrombin III, Hemopexin, α -Antichymotrypsin, CRP and Prealbumin) and 4 representative LAPs (Adiponectin, soluble L-selectin, soluble ICAM-1 and TIMP-1) from depleted plasma via Seppro SuperMix columns.

Materials and Methods

1. Instrument: Luminex® 100 (Figure 2, Luminex Corp., Austin, Texas, USA)
2. Vacuum pump, 96-well microtiter filter plates and filtration manifold (Millipore Corp)
3. Capture antibodies were immobilized on Luminex® beads (Luminex Corp., Austin, Texas, USA), detection antibodies were biotinylated before use, streptavidin-phycoerythrin conjugate (Sigma E4011)
4. Data were calculated using STATLIA® software with a 5-parameter logistic curve-fitting method (Brendan Scientific, CA)
5. Depletion procedure: 50 or 100 μ L human plasma (Sigma P9523) was loaded to Seppro IgY14-LC5/SuperMix-LC2 columns with tandem connection (Figure 3). Depletion was run in Waters 2695 HPLC System (Alliance). After collection of flowthrough from combined columns (SuperMix depleted sample), bound proteins were eluted from IgY14-LC5 and SuperMix LC2 column separately.
6. Multiplex assay protocol: 50 μ L standards or samples are mixed with 25 μ L antibody conjugated bead mixture in 96-well filter plate. Shake 2 hr at RT or overnight at 4 °C and wash plate twice. Add 50 μ L/well detection mixture, shake 1hr at RT and wash plate once. Add 50 μ L/well SAPE, shake 30 min at RT and wash plate twice. Add 100 μ L/well sheath fluid and shake 2–5 min at RT. Read plate on Luminex Instrumentation.

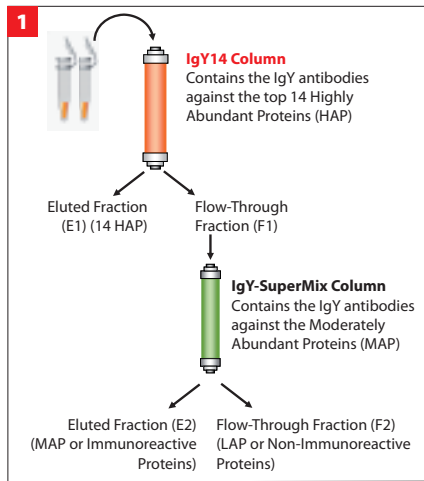


Figure 1: Seppro SuperMix Depletion System

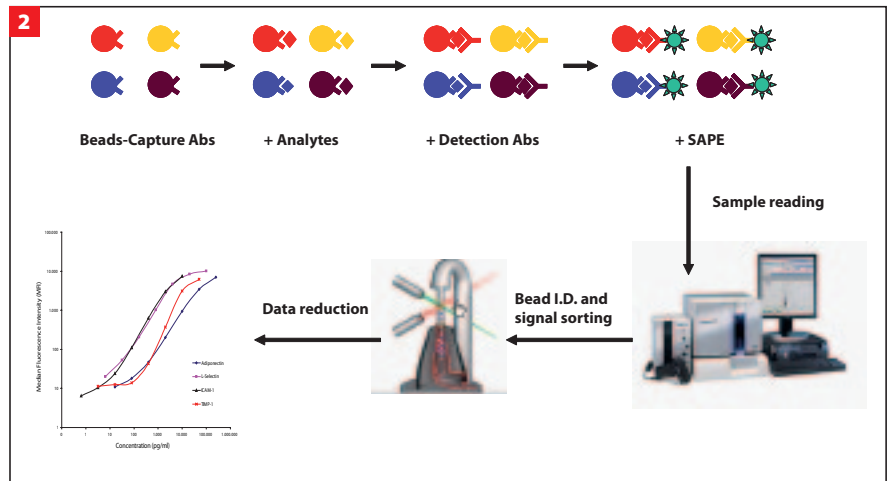


Figure 2: Principle of Luminex Multiplexing Assay

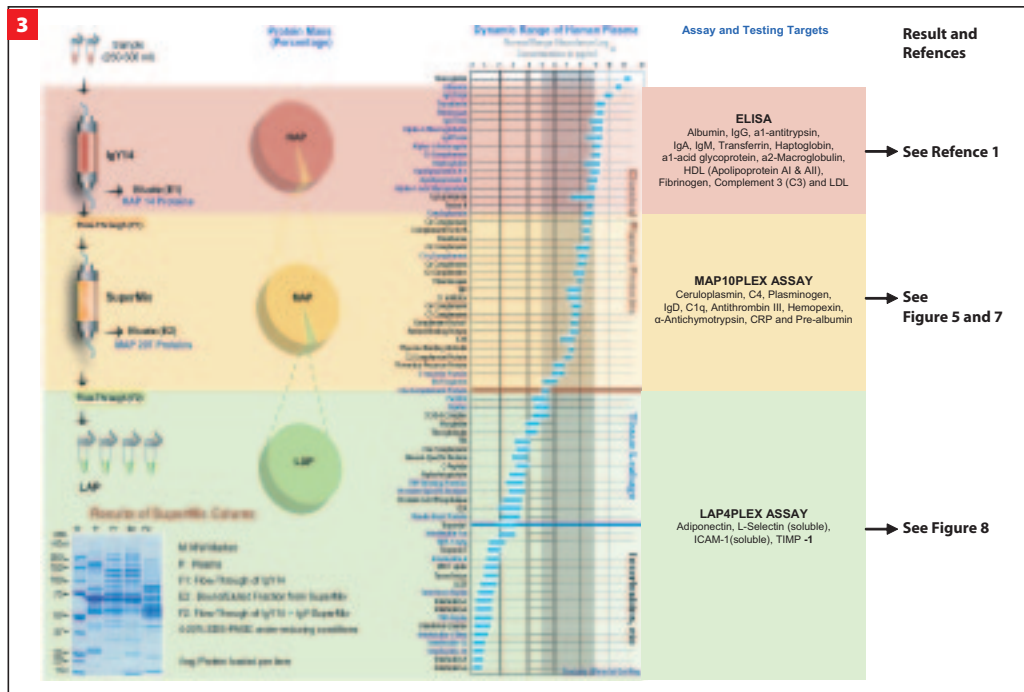


Figure 3: Flowchart of Seppro SuperMix Depletion Process and the Targets to Test

The top14 HAPs (plasma level ranges in 0.5–50 mg/ml) compose 95% of total plasma proteins. The 10 targets to test for SuperMix depleted plasma cover a wide range concentration (2.3 µg/ml for CRP, 0.5 mg/ml for Ceruloplasmin) of MAPs. The 4 LAP targets in normal human plasma ranges in 0.2–10 µg/ml.

Results

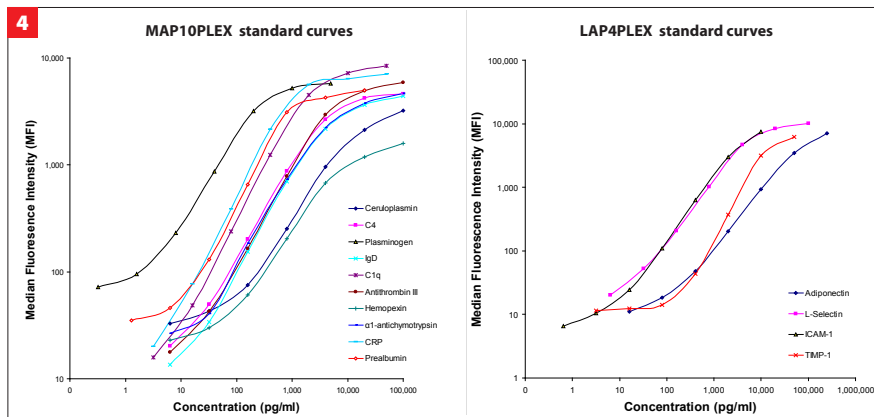


Figure 4: MAP10PLEX and LAP4PLEX assays are performed with protocol described above and standard curves are plotted for each assay.

MAP10PLEX

Final diution of plasma (folds)	C4	C4	Plasminogen	IgD	C1q	Antithrombin III	Hemo- pexin	α 1-antichymo- trypsin	CRP
512000	103.73	74.21	153.77	112.98	125.17	127.80	104.88	115.24	86.50
256000	105.03	69.44	137.89	99.57	11.08	109.75	97.32	99.54	102.85
128000	105.01	68.59	120.09	114.09	103.57	101.52	104.10	93.74	110.09
64000 (start)	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00
AVG	104.59	70.74	137.25	108.88	113.27	113.02	102.10	102.84	99.81

LAP4PLEX

Table 1: Assay Accuracy – Dilution Linearity for Plasma Samples

Dilution linearity is commonly used to evaluate assay accuracy that is one of the most important requirements for immunoassays. A serial dilution of a pooled plasma was tested with MAP10PLEX and LAP4PLEX assay. The numbers in the table represent percentage of concentrations at each dilution point against start point. Both assays perform well. For normal human plasma samples, dilutions in the range from 5×10^4 to 5×10^5 for MAP10PLEX assay or 250 to 2000 for LAP4PLEX assay are suggested.

Final diution of plasma (folds)	Adipo- nectin	L-Selectin	ICAM-1	TIMP-1
2000	140.59	98.95	107.32	87.11
1000	127.67	96.97	118.63	96.45
500	114.96	93.20	108.45	99.48
250 (start)	100.00	100.00	100.00	100.00
AVG	127.74	96.37	111.47	94.35

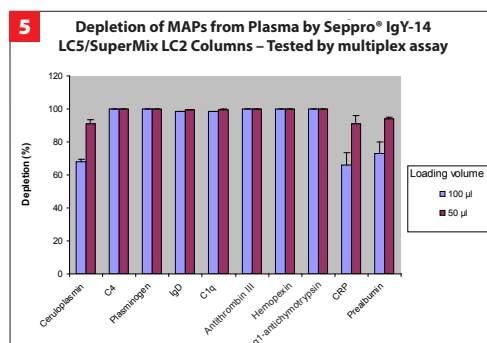


Figure 5: Depletion Efficiency of Seppro SuperMix Columns Tested by Luminex MAP10PLEX Assay

Depletion efficiency of 10 representative MAPs is related to loading volume of plasma samples. > 90% of all 10 MAPs can be depleted from 50µL human plasma.

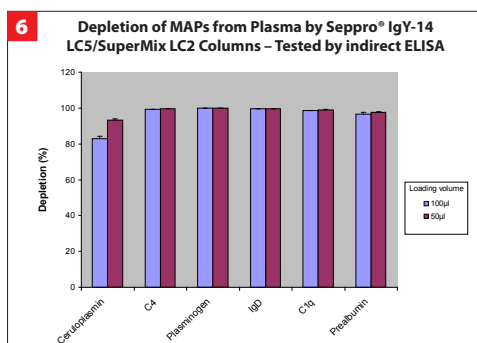


Figure 6: Depletion Efficiency of Seppro SuperMix Columns Tested by Indirect ELISA (for 6 targets only)

The same set of SuperMix depleted samples were tested by indirect ELISA and displayed a comparable result as Luminex MAP10PLEX assay.

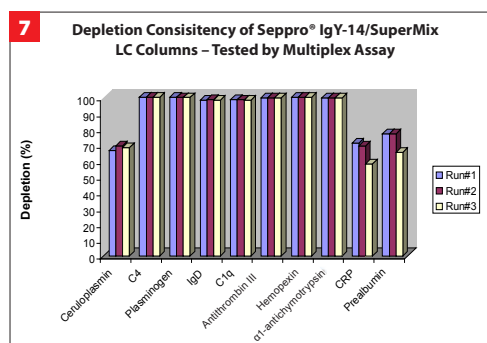


Figure 7: Run-to-Run Depletion Consistency of SuperMix Columns

Three depletion runs were performed with 100 µL plasma utilizing Seppro IgY14 LC5/SuperMix LC2 columns and the depletion efficiency was determined by Luminex MAP10PLEX assay. Depletion of all 10 targets is consistent (CV% < 15%)

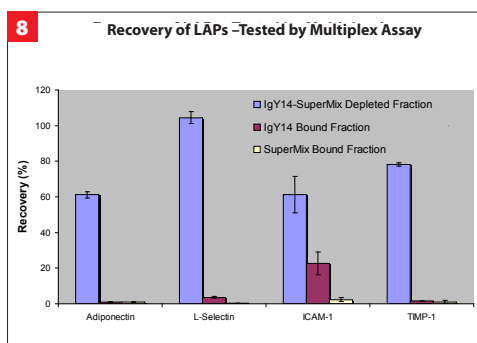


Figure 8: Impact of SuperMix Depletion Process on LAPs

Four representative LAPs were tested by Luminex LAP4PLEX assay to monitor the loss of LAPs during SuperMix depletion process. Depending on different biological properties of the individual target, approximately 1-40% loss has been observed from SuperMix depleted plasma (Note: some proteins in bound fraction are unstable).

Conclusions

1. The MAP10PLEX and LAP4PLEX assays are able to simultaneously measure 10 MAPs or 4 LAPs from human plasma or serum samples within 4 hours
2. These two multiplex assays can be used to replace regular ELISA methods to determine MAP depletion and LAP retention from Seppro IgY-14/SuperMix depleted samples
3. The preliminary test indicated that depletion of 10MAPs from human plasma through the tandem IgY14/SuperMix columns were reproducible and is consistent with Dr. Smith's result based on spectral count data (Ref. 3)
4. In order to remove > 90% of MAPs from plasma, columns with equal volume of IgY14 and SuperMix resins (IgY14/ SuperMix ratio = 1 : 1) are suggest to be combined

Summary

The newly developed MAP10PLEX and LAP4PLEX assays using Luminex technology have been proved to be both time and cost saving solutions to monitor the robustness of protein depletions from lot to lot and sample to sample.

References

- 1) Seppro® Depletion Product Info: <https://www.sigmaldrich.com>
- 2) Technical Bulletin, May 2001, Luminex Corp. <http://www.luminexcorp.com>
- 3) Qian WJ et. Al., Mol Cell Proteomics. 2008 Oct; 7(10):1963-73