Development of Two Multiplexed Immunoassays for Study of Nine Blood-Based Biomarkers of Alzheimer's Disease

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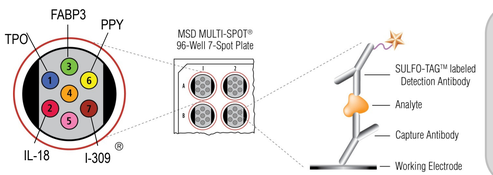
1 Abstract

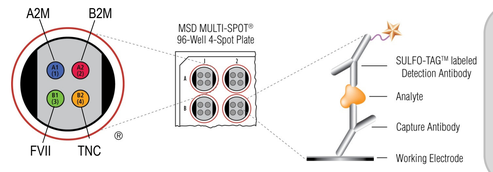
Objective: To develop multiplexed immunoassays that measure candidate biomarkers for Alzheimer's disease (AD) in blood.
Background: Blood-based biomarkers provide a cost-effective means to screen for AD at the population level. Circulating alpha-2-macroglobulin (A2M), beta-2-microglobulin (B2M), factor VII (FVII), tenascin C (TNC), pancreatic polypeptide Y (PPY), interleukin-18 (IL-18), fatty acid binding protein 3 (FABP3), thrombopoietin (TPO), and I-309 are proteins that have been identified as candidate AD biomarkers. We describe 2 multiplexed immunoassay panels that measure these biomarkers in human plasma and serum.
Design/Methods: Multiplex panels were developed according to fit-for-purpose assay development and validation principles. The panels were tested for precision, sensitivity, specificity, and matrix tolerance. Protein levels were measured in EDTA plasma and serum samples from patients with and without AD.
Results: The panels were assembled based on biomarker abundance in human plasma and serum. A low abundance panel (2-fold dilution) included TPO, FABP3, PPY, IL-18, and I-309, and a high abundance panel (4,000-fold dilution) included A2M, B2M, FVII, and TNC. All biomarkers were detectable in plasma and serum at their respective dilutions with minimal levels of non-specific binding. For both panels, dilutional linearity and spike recovery were within 80–120% of the expected values with the exception of PPY. Differences in analyte levels between AD and non-AD samples were protein-dependent.
Conclusions: These multiplex panels provide reproducible results across multiple blood-based matrices. They support efforts to study blood-based biomarkers of AD and may accommodate inclusion of additional novel AD biomarkers.

2 Methods



Panel 1 Protocol

1. Add 150 µL Blocker A. Incubate for 1 hour at RT.
2. Wash with PBS-T. Add 50 µL of calibrator or 2-fold diluted sample. Incubate for 2 hours at RT.
3. Wash with PBS-T. Add 25 µL of detection antibody. Incubate for 1 hour at RT.
4. Wash with PBS-T. Add 150 µL of 2X Read Buffer T. Read on SECTOR® Imager.



Panel 2 Protocol

1. Add 150 µL Blocker A. Incubate for 1 hour at RT.
2. Wash with PBS-T. Add 50 µL of calibrator or 4,000-fold diluted sample. Incubate for 2 hours at RT.
3. Wash with PBS-T. Add 25 µL of detection antibody. Incubate for 1 hour at RT.
4. Wash with PBS-T. Add 150 µL of 2X Read Buffer T. Read on SECTOR Imager.

Two multiplexed immunoassay panels were developed and optimized for electrochemiluminescence detection. Analytes were stratified into 2 panels based on common dilution factors that allowed us to detect all analytes in each panel (2-fold dilution for Panel 1 and 4000-fold dilution for Panel 2). Analyte specificity was assessed by measuring samples spiked with blended calibrators in assays using individual detection antibodies. Dilutional linearity and spike recovery tests were conducted to assess matrix interference. Matched EDTA plasma and serum samples from patients diagnosed with AD and from patients without AD were evaluated using both panels.

3 Assay Specificity

Individual assays are specific to the targeted analytes. Signal and calculated cross-reactivity are presented below.

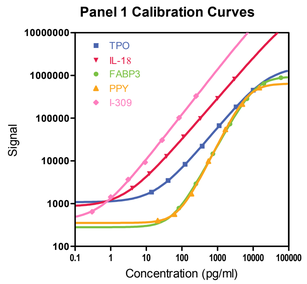
	Panel 1: Analyte Specificity				
Detection Antibody	Assay Signal (Capture Ab)				
	TPO	IL-18	FABP3	I-309	PPY
TPO	**59 199**	34	29	41	42
IL-18	88	**251 714**	72	56	43
FABP3	152	129	**436 984**	155	94
I-309	7	23	36	**9749**	25
PPY	1	23	22	48	**86 429**

	Panel 2: Analyte Specificity			
Detection Antibody	Assay Signal (Capture Ab)			
	A2M	B2M	FVII	TNC
A2M	**21 584**	3	24	13
B2M	23	**36 782**	71	14
FVII	275	140	**710 079**	251
TNC	19	3	367	**7310**

Detection antibody	Panel 1: % Cross-reactivity				
	TPO	IL-18	FABP3	I-309	PPY
TPO	-	0.01	0.01	0.42	0.05
IL-18	0.15	-	0.02	0.57	0.05
FABP3	0.26	0.05	-	1.58	0.11
I-309	0.01	0.01	0.01	-	0.03
PPY	0.001	0.01	0.01	0.49	-

Detection antibody	Panel 2: % Cross-reactivity			
	A2M	B2M	FVII	TNC
A2M	-	0.01	0.001	0.18
B2M	0.1	-	0.01	0.17
FVII	1.27	0.38	-	3.43
TNC	0.09	0.01	0.06	-

4 Panel 1: Dynamic Range and Sensitivity



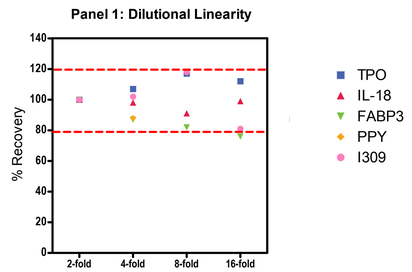
Calibration curves for each assay in Panel 1: Intra-plate precision between triplicate measurements was typically <10%.

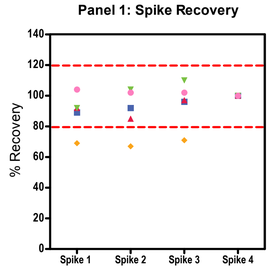
Analyte	Panel 1: Sensitivity Average LLOD (pg/mL)
TPO	17.4
IL-18	4.94
FABP3	103
I-309	0.431
PPY	118

The lower limit of detection (LLOD) is a calculated concentration corresponding to a signal 2.5 standard deviations above the background (zero calibrator). Average LLODs across 3 lots are shown.

5 Panel 1: Matrix Tolerance: Linearity and Spike Recovery

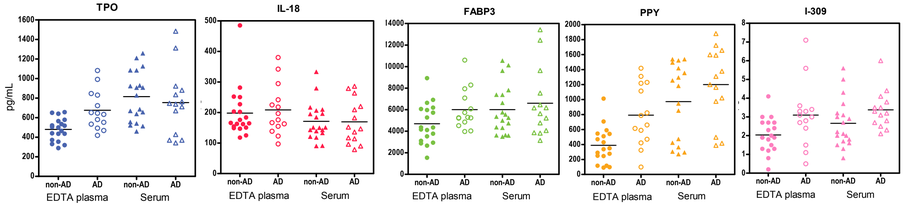
Left: Linearity was assessed by serially diluting EDTA plasma (n=5 samples). PPY calibrator was spiked into samples prior to testing due to low endogenous levels. The average percent recoveries at each dilution factor are shown. Linearity was within 20% of the expected concentrations for all but 1 sample; PPY was not detectable at 8- and 16-fold dilutions.
Right: Spike recovery was assessed by diluting EDTA plasma samples 2-fold and spiking with calibrators at 4 levels throughout the range of the assays. Recoveries were within 15% of the expected concentrations with the exception of PPY which was slightly under-recovered. The low spike for PPY was not detectable. Dashed red lines in both figures represent boundaries of ±20% recovery.



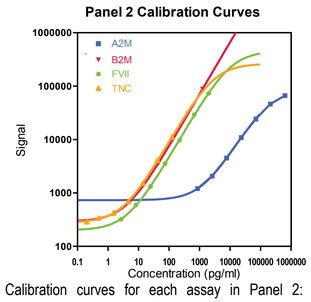


6 Panel 1: Sample Quantification

Proteins were detectable in all AD and non-AD EDTA plasma and serum samples. Protein concentrations of some analytes were modified in AD samples as compared to non-AD samples based on a comparison of median measurements. Analyte concentrations are reported as pg/mL.



7 Panel 2: Dynamic Range and Sensitivity



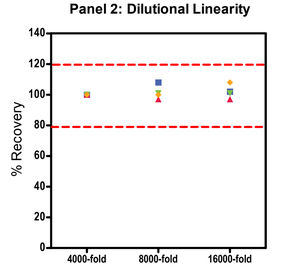
Calibration curves for each assay in Panel 2: Intra-plate precision between triplicate measurements was typically <10%.

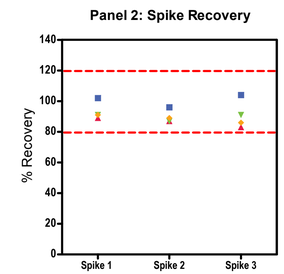
Analyte	Panel 2: Sensitivity Average LLOD (pg/mL)
A2M	933
B2M	2.04
FVII	3.88
TNC	0.460

Average LLODs across 2 lots are shown.

8 Panel 2: Matrix Tolerance: Linearity and Spike Recovery

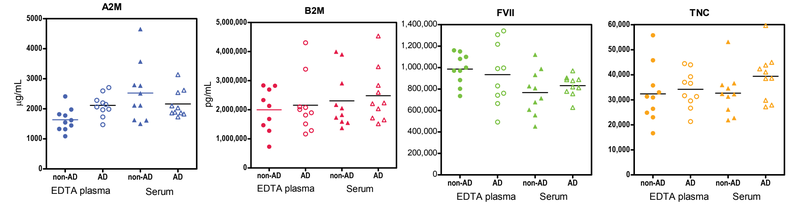
Left: Linearity was assessed by serially diluting EDTA plasma (n=3 samples). The average percent recoveries at each dilution factor are shown. Linearity was within 10% of the expected concentrations for all samples.
Right: Spike recovery was assessed by diluting EDTA plasma samples 4,000-fold and spiking with calibrators at 3 levels throughout the range of the assays. The average percent recovery at each spike level is shown. Recoveries were within 20% of the expected concentrations. Dashed red lines in both figures represent boundaries of ±20% recovery.





9 Panel 2: Sample Quantification

Proteins were detectable in all AD and non-AD EDTA plasma and serum samples. Protein concentrations of some analytes were modified in AD samples as compared to non-AD samples based on a comparison of median measurements in EDTA plasma samples. A2M concentrations are presented as µg/mL; other analytes are presented as pg/mL



10 Conclusion

Two multiplexed immunoassay panels were developed and tested for suitability to measure candidate biomarkers of AD in blood. The panels were shown to have reproducible performance demonstrated by precision of calibration curves, and analytes were quantitated in multiple blood-derived matrices. This study suggests that biomarker levels may differ between EDTA plasma and serum; this should be further explored with a larger sample set. These panels will support ongoing efforts to study blood-based biomarkers of AD and may be further expanded to accommodate additional novel AD biomarkers.

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