Multiplex Quantitation of pTau-217, Aβ40, Aβ42, NfL, and GFAP in Bio-Hermes-001 Cohort

Introduction

Blood-based biomarkers of neurodegeneration are a cost-effective and minimally invasive tool for Alzheimer's disease (AD) clinical trial patient staging and research.[1] Though AD is a complex disease, changes in specific biomarkers map to disease progression.[2-4] For instance, amyloid aggregation and plaque formation correlates with amyloid beta 40 and 42 (Aβ40 and Aβ42), and the overall amyloid burden and tau pathology can be inferred from phosphorylated tau-217 (pTau-217).[5] Neurofilament light chain (NfL) and glial fibrillary acidic protein (GFAP) are more general indicators of overall neurodegeneration and astrocytic activation.[6-8] Simultaneously measuring these markers in blood provides richer insight into the underlying biology, (Figure 1).

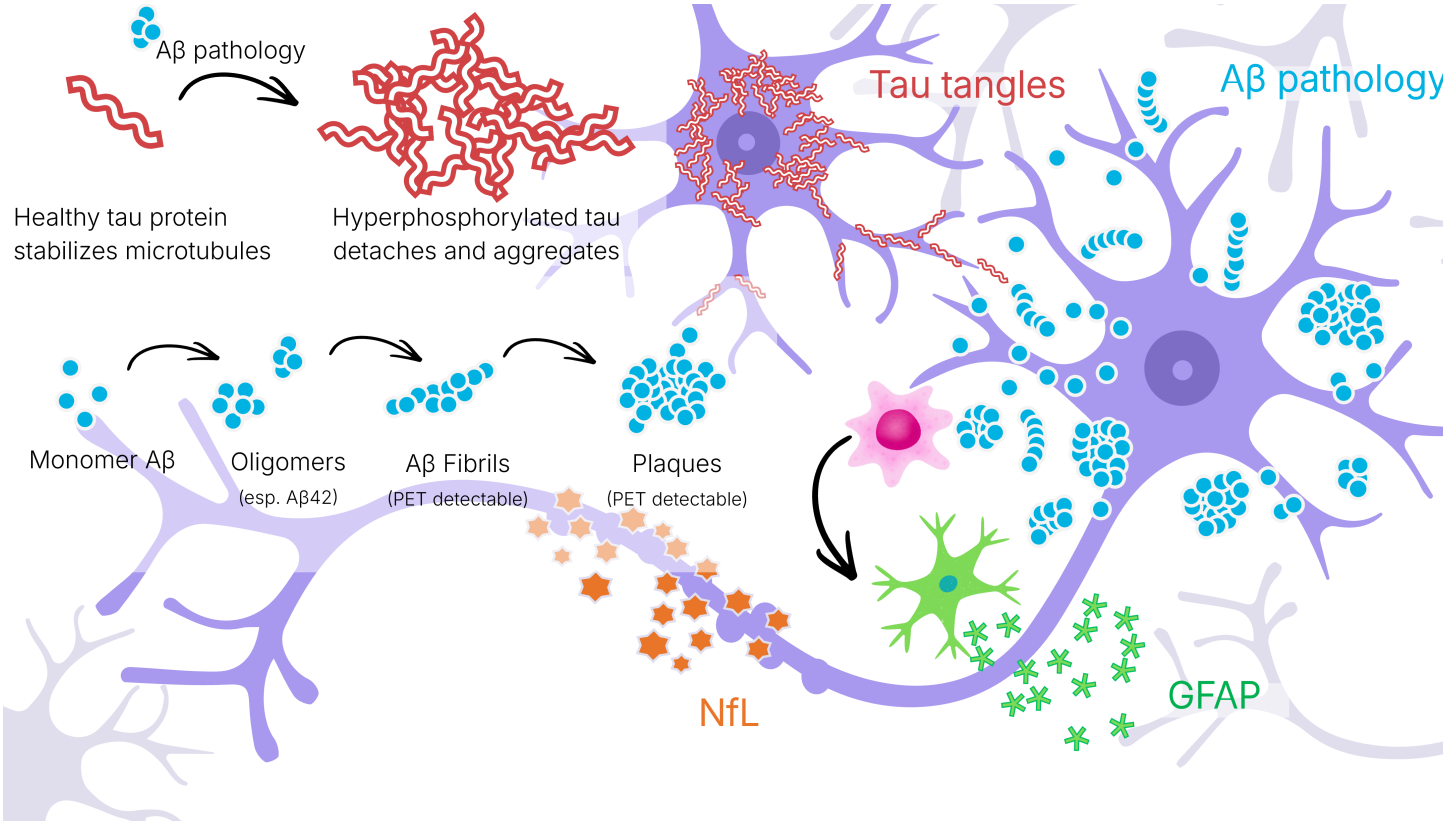

Figure 1. Key biomarkers relevant for AD. Amyloid monomers of various lengths are created from cleavage of the amyloid precursor protein (APP). Amyloid monomers aggregate into oligomers, fibrils, and plaques. Amyloid accumulation may increase phosphorylation on tau proteins. Under normal conditions, tau proteins stabilize microtubules; however, hyperphosphorylated tau detaches and aggregates. In response to neuronal inflammation, microglial cells recruit astrocytes, which in turn release GFAP. Finally, NfL appears as axonal damage advances.

Accurate quantitation of these key markers at the pg/mL concentrations present in plasma often require expensive equipment or specialized personnel. Taudia's SPLASH™ (Solid Phase Ligation Assay with Single wasH) immunoassay accurately measures low-abundance biomarkers with a simple benchtop workflow and a standard 96-well qPCR instrument, (Figure 2).

SPLASH is a simplified, highly sensitive proximity-ligation workflow,[9] consisting of three steps: (1) Incubation, (2) Capture and Wash, and (3) Read-out with qPCR.

During incubation, proprietary antibody pairs bind to the target protein in solution to form an immunocomplex. One of the antibodies in the pair is functionalized with biotin, enabling capture by streptavidin-coated magnetic beads.

The magnetic beads with attached immunocomplexes are held in place by a magnetic rack. A single wash step removes unbound antibodies and irrelevant sample matrix material. Signal read-out is accomplished by ligating the unique oligonucleotide sequences connected to each antibody, then amplifying and quantifying each sequence with a unique dye using standard qPCR technology.

SPLASH assays are straightforward to multiplex, allowing the simultaneous quantification of many different protein targets with only 12.5 µL of plasma input. We demonstrate feasibility of a multiplex assay simultaneously quantifying Aβ40, Aβ42, pTau-217, NfL, and GFAP, and show the predictive power of this 5-plex on 101 well-characterized samples from the Bio-Hermes-001 cohort.

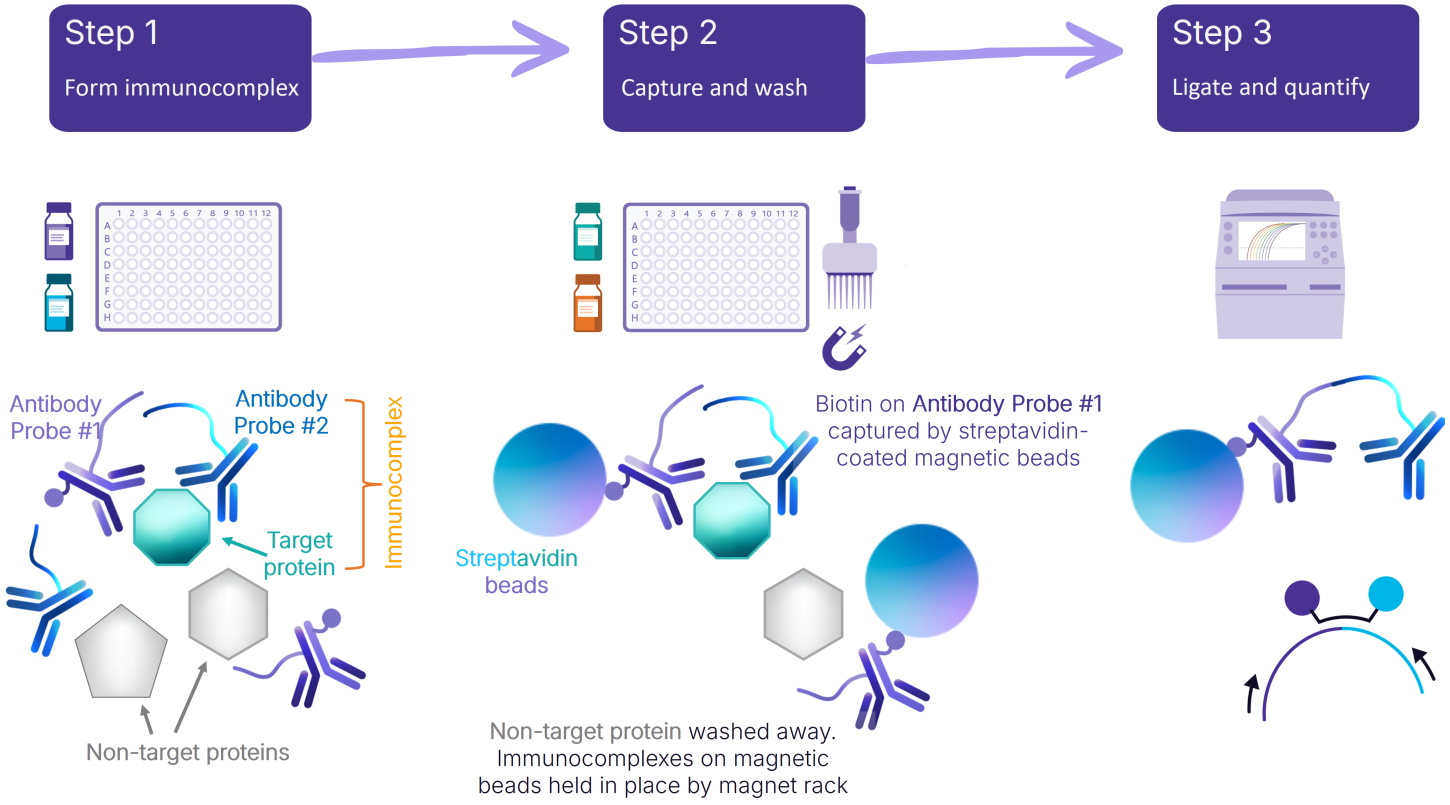

Figure 2. The SPLASH benchtop workflow quantifies low-abundance biomarkers with proprietary antibody pairs and minimal user touchpoints. In step 1, the user adds the SPLASH antibody pairs to the sample. An immunocomplex is formed during a 2-hour, room temperature incubation. The immunocomplexes are captured on streptavidin-coated magnetic beads in step 2, then non-target proteins are washed away. DNA ligation is possible on proximal antibodies, and then the resulting sequence is amplified and quantified on a standard qPCR instrument in step 3.

Results

Human plasma samples were sourced from the Bio-Hermes-001 study (ClinicalTrials.gov ID: NCT04733989) organized by the Global Alzheimer's Platform Foundation (GAP). The cohort was composed of clinically normal (CN, n=36), mild cognitive impairment (MCI, n=33), and probable AD (n=34). There were no significant differences in sex, Positron Emission Tomography (PET) positivity, or APOE4 status across the cohort. The probable AD group was slightly older than the other cohorts, consistent with the known increase in AD risk with age (Table 1).

Variable	Healthy (n=34)	MCI (n=33)	Probable AD (n=34)	Total (n=101)	P-value
Sex					**0.206**
Female	23 (67.6%)	20 (60.6%)	16 (47.1%)	59 (58.4%)	
Male	11 (32.4%)	13 (39.4%)	18 (52.9%)	42 (41.6%)	
Age					**0.005***
Mean (SD)	69.9 (6.8)	73.2 (6.1)	74.8 (5.6)	72.6 (6.5)	
Range	60 - 85	63 - 83	61 - 85	60 - 85	
PET positivity					**0.990**
Positive	17 (50.0%)	17 (51.5%)	17 (50.0%)	51 (50.5%)	
Negative	17 (50.0%)	16 (48.5%)	17 (50.0%)	50 (49.5%)	
APOE4 positivity					**0.407**
Positive	12 (35.3%)	17 (51.5%)	15 (44.1%)	44 (43.6%)	
Negative	22 (64.7%)	16 (48.5%)	19 (55.9.0%)	57 (56.4%)	

Table 1. Cohort demographics by diagnostic grouping. P-values computed via chi-square test for categorical variables sex PET positivity, and APOE4, and one-way ANOVA for continuous variable age. * $p < 0.05$.

Sample concentrations were determined using an in-run calibration standard curve, which converts the Cq (quantification cycle) from the qPCR instrument into absolute protein concentration. All five proteins achieved R^2 values greater than 0.999 across a wide dynamic range (Figure 3). The average plasma precision (coefficient of variation (%CV)) on apparently normal human plasma samples was less than 5% (4 replicates, 3 runs) (Figure 4).

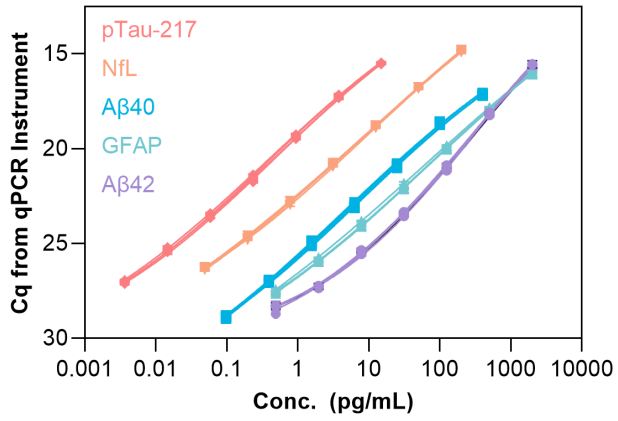

Figure 3. Four repeats of standard curves (7 point, 4-fold serial dilution, 2 replicates) for the five targets. Standard curve Cq values are highly similar between the four runs.

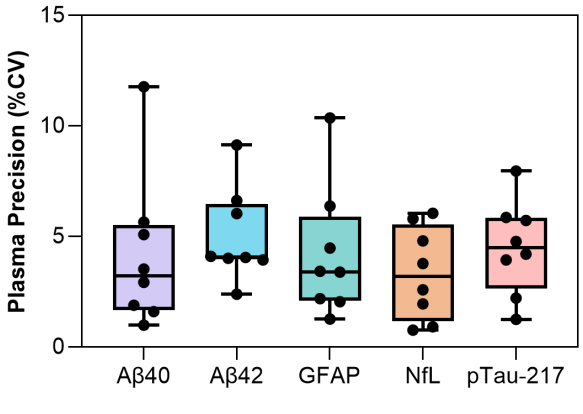

Figure 4. Plasma precision on apparently normal, human plasma samples. Average precision (%CV) of 4.2% (Aβ40), 5.0% (Aβ42), 4.2% (GFAP), 3.3% (NfL), and 4.5% (pTau-217).

Bio-Hermes-001 samples (n=101) were quantified with the AD 5-plex assay. All targets were clearly distinguishable from the functional Lower Limit of Quantitation (fLLOQ) (Figure 5a). Next, the individual plasma protein quantification values were used to predict amyloid PET positivity (Figure 5b). pTau-217 alone was reasonably predictive of PET positivity (AUC=0.839 [0.753 - 0.912]), but adding the other four biomarkers markedly improved performance (AUC=0.919 [0.860 - 0.970]).

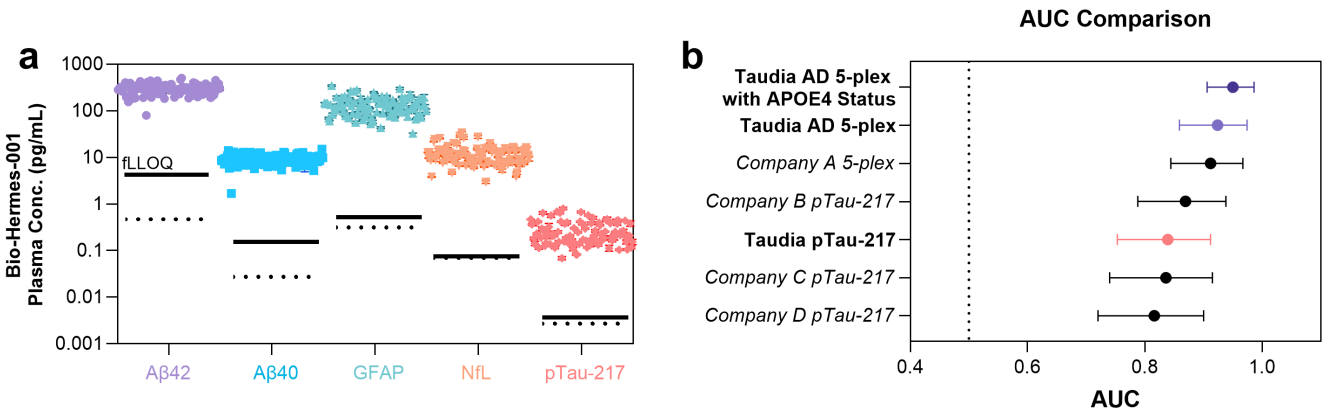

Figure 5. (a) Protein quantitation on 101 plasma samples, showing individual variation and signals well-above the fLLOQ (solid lines) and functional lower limit of detection (fLLOD, dashed lines). **(b)** Comparison of AUC (area under the curve) between different assays. The Taudia pTau-217 performance matches the industry standard. Adding more markers (5-plex) improved performance. If the APOE4 status is used in the model, the predictive power is further enhanced (AUC= 0.9416 [0.8992 - 0.9840])

Discussion

We have demonstrated feasibility of a benchtop workflow to simultaneously and precisely measure five key protein targets and predict PET positivity. Both the pTau-217 marker individually and the AD 5-plex assay can predict PET positivity, with AUC power in-line with the industry leaders.

The predictive power a given assay will vary, depending on the patient poplation; however, adding more markers related to the biological progression of AD seems to improve discrimination.

Multiplexed AD biomarker detection in plasma is a cost-effective option to screen patients prior to expensive PET imaging scans. The predictive power of a given assay will vary, depending on the patient population.

Materials and Methods

Cohort acquisition

Plasma samples were sourced from the BioHermes-001 study (ClinicalTrials.gov identifier: NCT04733989), organized by the Global Alzheimer's Platform Foundation (GAP). All participants provided written informed consent prior to sample collection and study protocols were administered by approved clinical trial sites. All data access and experimental methods complied with institutional review board (IRB) standards. The samples were thawed and aliquoted upon receipt and stored at -80ºC until analysis.

SPLASH Methods

Plasma samples and standards were run in duplicate following the standard SPLASH protocol. Lyophilized protein standards were reconstituted, then serial diluted

4-fold to create a 7-point standard curve. The plasma samples were diluted 1:4, then loaded into individual wells in a 96-well qPCR plate. Standards and samples were incubated with the antibody probes for two hours at room temperature, creating immunocomplexes with the target protein. Streptavidin-coated magnetic beads were added to capture the immunocomplexes (10-minute incubation). The samples were then washed by placing the plate on a magnetic rack and decanting the supernatant, then washing 2x with 200 µL of wash buffer. A custom qPCR master mix containing ligase and qPCR probes was then added to the magnetic beads with captured immunocomplex and placed directly in a benchtop qPCR instrument (QuantStudio 7 Pro 96-well 0.2 mL, Thermo Fisher Scientific, Waltham, MA, USA) for ligation, amplification, and signal read-out. The protocol for signal amplification consisted of a 30 °C ligation step (15 minutes), followed by inactivation at 95 °C (3 minutes), 3 cycles of PCR (95°C for 30 seconds, 60°C for 1 minute), and finally 37 cycles of PCR (95°C for 15 seconds, 60°C for 30 seconds). Data was collected in the FAM (GFAP), VIC (Aβ42), TAMRA (Aβ40), ROX (NfL), and CY5 (pTau-217) channels.

Data Analysis

Cq (quantification cycle) values from the qPCR curves were obtained from the instrument manufacturer's analysis software. The calibration standard curves were fit with four-parameter logistic (4PL) regression, then used to convert plasma Cq values to concentrations in pg/mL. The ROC curves were fit with logistic regression, using the PET status as a dependent variable and the five protein targets as input parameters.

References

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