THE LymPro® TEST: A FIT FOR PURPOSE VALIDATION OF A FLOW CYTOMETRIC ASSAY TO ASSESS LYMPHOCYTE PROLIFERATION IN PERIPHERAL BLOOD LYMPHOCYTES IN ALZHEIMER'S DISEASE.

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ABSTRACT

Initial studies by Arendt *et.al*, at the University of Leipzig identified differential mitogenic response of peripheral blood lymphocytes as a potential biomarker of Alzheimer's disease (AD). The LymPro® test is a flow cytometric assay that measures the response of peripheral blood lymphocytes to mitogenic stimulation. In this study we report on a fit-for-purpose validation of the LymPro test conducted by a contract clinical laboratory.

INTRODUCTION

The LymPro test is a blood assay, that has shown differential mitogenic activation in lymphocytes from AD subjects compared to cognitively intact controls (1, 2), and is intended to be used as a biomarker for detection of AD. The assay is based on the cell cycle dysregulation hypothesis for AD (3), which states that neurons in AD have inappropriately re-entered the cell cycle with downstream events such as tau hyperphosphorylation and increased liability for neuronal cell death. This cell cycle dysregulation (CCD) is likely one of the earliest key pathologies in AD (4). CCD has systemic manifestations and has been measured in white blood cells by several groups. Dr. Thomas Arendt et al, at Leipzig University developed the specific technique (1, 2) we use here.

Amarantus Diagnostics (AMDx) will offer this test to clients, including pharma, biotech, and academic institutions, wishing to identify with accuracy individuals with Alzheimer's disease. In preparation for this offering, AMDx is working with ICON Central Laboratories (ICON) to implement and validate the LymPro test at ICON's clinical laboratory in Long Island, New York, USA. Here we present the results of that validation.

METHODS

Whole blood samples were drawn from normal, healthy donors in Becton Dickinson (BD) 10 mL cell preparation tubes (CPT) containing sodium heparin. To mimic the shipping time of clinical samples, the whole blood samples were stored at ambient temperature for 24 hours before any processing.

Peripheral blood mononuclear cells (PBMC) were isolated from the CPT tube and incubated unstimulated or stimulated with either PWM or PHA for 4 hours or 20 hours. Post incubation, the samples were frozen in cell freezing medium, FBS with 10% DMSO, and were kept at -80º C for at least 24 hours. The cells were thawed, washed, viability determined, and white blood cells (WBC) enumerated on an ADVIA hematology analyzer and then labeled with monoclonal antibody-fluorophore conjugates.

Samples were acquired on FACSCanto II Flow Cytometers (BD Biosciences) using FACSDiva Software (version 6.1.3).

Instrument quality control measures were performed using the automated Cytometer Setup and Tracking program (CS&T, BD Biosciences) that is integral to FACSDiva software. The QC measures laser power, time delay, and multiple components of each detector, including determining system voltage based on target fluorescent values.

BD CompBeads™ were labeled with monoclonal antibodies in single color tubes with the study-specific fluorochromes to set compensation. Assay spectral compensation was experiment-, label-, and preparatory method-specific.

Samples were analyzed using FCS Express (Ver 3.00.0808, DeNovo Software Clinical Edition). The assay panel included an unlabeled sample for background fluorescence measurement. Cell types were identified and reported according to defined phenotypes.

Intra and inter-assay precision measurements were obtained using samples from five normal healthy donors; intra assay results were determined by analyzing the same sample three times. Inter assay results used three stimulated samples per donor.

Operator to operator and instrument to instrument comparisons were conducted with two operators and two instruments, respectively. For the operator comparison, two operators each independently prepared and analyzed samples from five normal, healthy donors on one instrument. For the instrument comparison, one operator prepared and analyzed five samples on two instruments.

Data were acquired for the following parameters: event counts, median fluorescent intensity (MFI) and frequency (%) for CD28 and CD69 cell surface markers on CD3, CD4, CD8, CD14, CD19 and CD45 cell types.

RESULTS

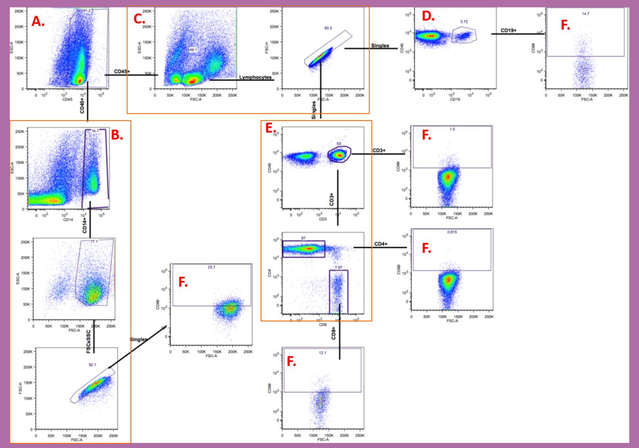

Figure 1: Representative gating strategy for LymPro assay. (A) Lymphocytes were identified by expression of the pan-lymphocyte marker CD45. (B) CD14 was used to identify monocyte population. (C) Lymphocytes were identified by scatter parameters. (D) B-lymphocytes were identified by CD19 expression (E) T-Lymphocytes were identified by CD3 expression, and further subset by expression of CD4 and CD8. (F) In each case, CD69+ (or CD28+, not shown) positive cells were identified. Gates were placed based upon appropriate controls. Compensation was performed using automatic compensation in the FACSDIVA software.

The validation generated over 1800 individual data points. Clinical studies have indicated that CD4+CD69+, CD19+CD69+ and CD45+CD69+ markers are the most informative. Data for these markers are presented in Table 1 and a summary of all results is shown in Table 2 below.

Reportable Result	Intra Assay %CV	Inter Assay %CV	Op to Op %Δ	Inst to Inst %Δ
PWM-4H CD45+CD69+ %	1.83	3.24	19.87	10.27
PWM-4H CD45+CD69+ MFI	2.08	1.95	6.84	4.9
PWM-4H CD19+CD69+ %	2.21	2.62	13.57	10.6
PWM-4H CD19+CD69+ MFI	3.62	3.28	10.41	4.22
PWM-4H CD4+CD69+ %	3.41	3.03	24.27	8.82
PWM-4H CD4+CD69+ MFI	3.21	1.98	8.03	9.05
PWM-20H CD45+CD69+ %	1.02	7.18	12.92	4.46
PWM-20H CD45+CD69+ MFI	1.02	10.04	16.78	2.86
PWM-20H CD19+CD69+ %	1.05	3.81	7.92	3.14
PWM-20H CD19+CD69+ MFI	1.48	13.13	20.55	7.21
PWM-20H CD4+CD69+ %	1.66	8.01	18.62	5.83
PWM-20H CD4+CD69+ MFI	2.51	6.65	16.88	3.98
PHA-4H CD45+CD69+ %	4.22	2.86	9.85	17.74
PHA-4H CD45+CD69+ MFI	2.95	3.81	18.46	14.14
PHA-4H CD19+CD69+ %	7.22	9.29	19.14	11.32
PHA-4H CD19+CD69+ MFI	9.41	8.42	15.4	14.96
PHA-4H CD4+CD69+ %	3.94	6.21	16.15	8.33
PHA-4H CD4+CD69+ MFI	10.59	11.94	17.19	15.3

Table 1, Representative Precision Data

Op = Operator; Inst = Instrument; MFI = Median Fluorescent Intensity

Parameter	Acceptance Criteria	Range of Results	Percent Passing	Number Passing
Intra-Assay Precision				
Frequency of CD28+/CD69+	≤ 10%	0.12 to 37.3%	88.8%	32 of 36
MFI of CD28+/CD69+	≤ 30%	1.02 to 33.2%	97.2%	35 of 36
Inter-Assay Precision				
Frequency of CD28+/CD69+	≤ 10%	0.10 to 27.9%	97.2%	35 of 36
MFI of CD28+/CD69+	≤ 30%	1.75 to 20.7%	100%	36 of 36
Operator to Operator Diff.				
Frequency of CD28+/CD69+	≤ 20%	0.07 to 39.4%	83.3%	30 of 36
MFI of CD28+/CD69+	≤ 30%	2.87 to 58.4%	91.7%	33 of 36
Inst to Inst Difference				
Frequency of CD28+/CD69+	≤ 20%	0.35 to 64.2%	83.3%	30 of 36
MFI of CD28+/CD69+	≤ 30%	1.92 to 95.8%	91.7%	33 of 36

Table 2, Summary of Results

DISCUSSION

Flow cytometry is a powerful tool for the rapid characterization of cell subtypes from clinical samples. In the clinical setting, this tool is used in routine diagnosis of a wide variety of diseases and the effects of treatment on those diseases. Building on the work of Arendt et al., the LymPro assay has been developed to aid in the diagnosis of Alzheimer's disease based on the response of peripheral blood mononuclear cells to mitogenic stimulation.

Using the LymPro test, the effect of these stimulations on the expression of CD69, a marker of early activation on lymphocytes, and CD28, an activation marker found on T-lymphocytes, can be measured on the major lymphocyte subsets including: B-lymphocytes, T-lymphocytes (both CD4+ and CD8+ sub-sets), as well as CD14+ monocytes. In total, this panel allows for sub-setting of 7 major populations.

As described by Maecker (5), the largest source of variability in flow cytometry is due to the operator effect in the analysis of the data. As reported in that paper, the mean CV of analysis is over often 20%, but is reduced when the analysis is performed in a central facility. The LymPro test exhibits good agreement between operators with the majority of the differences below 20%. In addition, to ensure consistent analysis, the test will be performed in a central facility.

CONCLUSIONS

The LymPro test was validated in a fit-for-purpose manner as recommended by the US FDA (6). The most relevant and informative markers demonstrated excellent intra- and inter-assay precision as well as good agreement between analysts and instruments. The results of this study validate the analytical performance of the LymPro Test for use in clinical trials.

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