Lymphocyte Proliferation as a Means to Assess Cell Cycle Dysregulation in Alzheimer's Disease: Analytical Performance of the LymPro Assay

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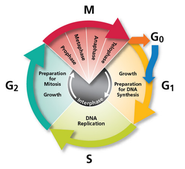
- **Background**: Initial studies by Thomas Arendt *et.al*, at the University of Leipzig identified the mitogenic response of peripheral blood lymphocytes (PLB) as a potential biomarker of Alzheimer's disease (AD) [Stieler, JT, and Arendt, T.; et al. Neuroreport 2001; 12(18):3969-3972]. This finding was confirmed in a second study conducted by Jens Steiler, *et al*, where the authors were able to retrospectively differentiate AD from Other Dementias [(OD), mostly Idiopathic Parkinson's disease] with 95% sensitivity and 90% specificity [Steiler, J et al, 2012. Neurobio Aging 33:234-341].

- **Methods**: Analytical performance of this flow cytometer assay was re-established at a contract GLP laboratory. In collaboration with clinicians, patient blood was sampled and purified for PBMCs. Following the published protocol, a stimulation index was established for CD69 positive expression following mitogenic stimulation. Experiments were conducted on 12 healthy volunteer donor blood specimens to assess analytical performance characteristics of the assay. Pre-analytical and analytical variance was assessed in healthy normal subjects.

- **Results**: Analytical performance data shows excellent reproducibility with coefficients of variation less than 20% for most mitogenic conditions. Additional performance metrics like effect of gating parameters and reagent stability were quantified. The effect of pre-analytical variables such as sample handing conditions were determined as well.

- **Conclusion**: The LymPro test, which measures mitogenic response to peripheral blood lymphocytes, demonstrates suitable analytical performance for use in a fit-for-purpose fashion in the Company's hands

Review of Cell Cycle Dysregulation in Alzheimer's Disease



G1 – gap phase 1
S – synthesis phase
G2 – gap phase 2
M – mitotic phase
G0 – quiescent state

Mature neurons in G0 should not re-enter the cell cycle, but sometimes they do

→ Cell cycle dysregulation

Figure 1

Table 1: Evidence for initiation of the cell cycle in neurons. Alzheimer's Disease (AD) patients show signs of neurons re-entering the cell cycle

Cell Cycle Proteins	Function	AD
Cyclin D	Important in G1 and for G1/S transition	↑
Cyclin E; CDK4; p21	Important in G1 and for G1/S transition	↑
PCNA	Facilitates DNA replication	↑
Cyclin G1	Important for G1/S transition	↑
Cyclin A	Involved in DNA replication and mitosis	↑
Cyclin B	Required for G2/M transition	↑
CDC2/CDK1	Regulates G1 progress and G1/S transition, promotes G2/M transition	↑
Ki67	Cellular proliferation marker present in all active phases (G1, S, G2, M), but not in resting cells (G0)	↑
P16	Inhibits cyclin D, CDK4/6 and G1 progression	↑

Table 2: Neuronal cell cycle re-entry is also apparent in mild cognitive impairment (MCI)

Cell Cycle Proteins	Function	MCI	AD
Cyclin D	Important in G1 and for G1/S transition	↑	↑
Cyclin B	Required for G2/M transition	↑	↑
Cyclin G1	Regulates G1/S transition	↑	↑
CDK2	Regulates G1/S transition	↑	
PCNA	Facilitates DNA replication	↑	↑

Table 3: Evidence for Cell Cycle Dysregulation in Lymphocytes as Surrogate for Neurons

Cell cycle protein / other	Biological role	AD
P53 mutant-like conformation	Regulation of G1/S cell cycle arrest; mutant conformation is functionally inactive	↑
Calmodulin	Regulation of G1/S cell cycle arrest	↑
Cyclin E	Regulation of G1 and for G1/S transition	↑
E_2F-1; CDK2; Rb (retinoblastoma)	Regulation of G1/S transition	↑
CD69	Lymphocyte development and migration	↓
Sensitivity to rapamycin and H_2O_2	Blocks G1/S transition in healthy lymphocytes	↓

Possible links between neuron and lymphocyte cell dysregulation

1. Production of Amyloid β (Aβ Peptides in Alzheimer's disease exceeds the removal capacity of the brain
   → **In brain, Aβ oligomers induce cell cycle re-entry in neurons**
2. Excess Aβ in body fluids activates peripheral lymphocytes and may lead to persistent stimulation of the immune system
   → **May lead to disease-related alteration of lymphocytes**
3. Alteration may include dysregulation of CD69 gene expression regulation
   → **Would affect lymphocyte proliferation and immune response in AD**

Amarantus investigated an improved version of the LymPro assay

- **Eight color antibody cocktail**
- **Becton Dickinson FACSCanto II flow cytometer**

Antibodies and Fluorochromes

Antibody Target	CD3	CD69	CD14	CD4	CD28	CD45	CD19	CD8
Fluorochrome	FITC	PE	PERCP-CY5.5	PE-CY7	APC	APC-H7	V450	V500

Performance Characteristics

Table 4: CVs of CD69 measurements as a percent of lymphocyte class for various stimulation conditions.

Analyte	Stimulation	Median % CV	Range of %CV	Upper 95% CI Limit (% CV)	% of observations with > 20% CV
Total CD69⁺ (as a % of CD45⁺ lymphs)	Unstim	7.5	0.6-15.9	10.9	0.0
Total CD69⁺ (as a % of CD3⁺)	Unstim	8.2	0.0-29.9	20.3	22.2
Total CD69⁺ (as a % of CD3⁺4⁺)	Unstim	27.9	0.0-56.8	39.6	77.8
Total CD69⁺ (as a % of CD3⁺8⁺)	Unstim	10.3	5.0-20.6	16.6	11.1
Total CD69⁺ (as a % of CD19⁺)	Unstim	5.3	0.3-8.9	7.2	0.0
Total CD69⁺ (as a % of CD14⁺)	Unstim	18.8	4.4-81.3	44.3	44.4
Total CD69⁺ (as a % of CD45⁺ lymphs)	PWM	5.8	0.3-9.9	7.9	0.0
Total CD69⁺ (as a % of CD3⁺)	PWM	5.4	0.2-11.6	9.2	0.0
Total CD69⁺ (as a % of CD3⁺4⁺)	PWM	4.7	0.2-11.8	8.9	0.0
Total CD69⁺ (as a % of CD3⁺8⁺)	PWM	8.0	0.5-43.4	20.8	11.1
Total CD69⁺ (as a % of CD19⁺)	PWM	4.9	0.3-6.9	6.0	0.0
Total CD69⁺ (as a % of CD14⁺)	PWM	11.0	2.7-33.8	19.7	11.1
Total CD69⁺ (as a % of CD45⁺ lymphs)	PHA	2.3	0.4-4.9	3.5	0.0
Total CD69⁺ (as a % of CD3⁺)	PHA	2.2	0.4-5.1	3.5	0.0
Total CD69⁺ (as a % of CD3⁺4⁺)	PHA	3.0	0.8-4.6	3.6	0.0
Total CD69⁺ (as a % of CD3⁺8⁺)	PHA	2.1	1.0-4.7	3.5	0.0
Total CD69⁺ (as a % of CD19⁺)	PHA	2.3	0.4-5.2	3.3	0.0
Total CD69⁺ (as a % of CD14⁺)	PHA	9.0	2.4-20.1	12.9	11.1

PWM: Pokeweed mitogen; PHA: Phytohemagglutinin

Table 5: Summary of ANOVA for difference between fresh and 24 hr harvesting of PBMCs

Analyte	ANOVA p-value for CV difference between Fresh versus 24 hr harvest	ANOVA p-value for Mean difference between Fresh versus 24 hr harvest
Total CD69⁺ (as a % of CD45⁺ lymphs)	0.7049	0.5690
Total CD69⁺ (as a % of CD3⁺)	0.7687	0.5196
Total CD69⁺ (as a % of CD3⁺4⁺)	0.1547	0.9577
Total CD69⁺ (as a % of CD3⁺8⁺)	0.3196	0.4715
Total CD69⁺ (as a % of CD19⁺)	0.0221	0.2779
Total CD69⁺ (as a % of CD14⁺)	0.7180	0.2939

Table 6: General linear mixed models (GLMs) for CV

Analyte	p value, Donor	p value Stimulation	p value Fresh vs. 24 hr harvest
Total CD69⁺ (as a % of CD45⁺ lymphs)	0.8354	***0.0395***	Nested within Donor
Total CD69⁺ (as a % of CD3⁺)	0.6579	***0.0207***	Nested within Donor
Total CD69⁺ (as a % of CD3⁺4⁺)	0.5704	***<0.0001***	Nested within Donor
Total CD69⁺ (as a % of CD3⁺8⁺)	0.2032	***0.0334***	Nested within Donor
Total CD69⁺ (as a % of CD19⁺)	***0.0278***	***0.0156***	Nested within Donor
Total CD69⁺ (as a % of CD14⁺)	0.2043	***0.0210***	Nested within Donor

Table 7: GLMs for Mean value

Analyte	p value, Donor	p value Stimulation	p value Fresh vs. 24 hr harvest
Total CD69⁺ (as a % of CD45⁺ lymphs)	0.0566	***<0.0001***	Nested within Donor
Total CD69⁺ (as a % of CD3⁺)	***0.0075***	***<0.0001***	Nested within Donor
Total CD69⁺ (as a % of CD3⁺4⁺)	0.0701	***<0.0001***	Nested within Donor
Total CD69⁺ (as a % of CD3⁺8⁺)	0.0815	***<0.0001***	Nested within Donor
Total CD69⁺ (as a % of CD19⁺)	***0.0065***	***<0.0001***	Nested within Donor
Total CD69⁺ (as a % of CD14⁺)	***0.0002***	***<0.0001***	Nested within Donor

GLMs for CV and Mean value treating Donor and Stimulation condition as random effects, and Harvest point (Fresh or 24 hr) as a nested effect within Donor.

Summary

The Total CD69+ (as a % of CD45+ Lymphocytes) displays:
- Consistently low CVs in general and across stimulation conditions
- No effect of PBMC harvest point (Fresh or 24 hr)
- No inter-individual effect on CV in GLM models
- No inter-individual effect on Mean value in GLM models (which may indicate a tight distribution in the healthy control population)

The Total CD69+ (as a % of CD19+) also displays consistently low CVs as well as lack of significant difference on CV by stimulation condition, but shows an effect of harvest point and an inter-individual effect on CV.

Conclusions

The LymPro test, which measures mitogenic response to peripheral blood lymphocytes, demonstrates acceptable pre-analytical and analytical variation in this study. The test will transition into multiple clinical studies for differentiation of individuals at risk for Alzheimer's Disease from other dementias.

Key References
1. Stieler, JT, and Arendt, T.; et al. Neuroreport 2001; 12(18):3969-39722.
2. Stieler, J et al, 2012. Neurobio Aging 33:234-341

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For References: