BIOMARKER DIAGNOSIS OF ALZHEIMER'S DISEASE

AD INDEX ASSAY

INTENDED USE AND CLINICAL BASIS

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1. AD INDEX ASSAY

1.1. Intended use

The Intended Use for the AD Index Assay is to confirm a clinical diagnosis of Alzheimer's dementia and discriminate and distinguish Alzheimer's dementia from Non-AD Dementias (such as Parkinson's, Lewy-Body, Huntington's Chorea, etc.).

Requirements for Use

l. Patients tested must first have a clinical diagnosis of dementia (the duration of such dementia to extend from the first year of dementia forward).

2. The intended population will range in age from 55-90 years old.

3. Peripheral skin samples will be collected at the patient's healthcare provider's facility (e.g., primary care physician's office) through a standard 2-3mm skin punch biopsy taken from the bicep/forearm area.

4. Test results will be communicated to the physician in a patient report form.

5. The Assay is for prescription use only.

6. The safety and effectiveness of the AD Biomarkers has not been established for monitoring responses to therapies among patients with a clinical diagnosis of AD.

1.2. Overview of the Developmental and Validation Process

A. Developmental Process and Cohorts. A developmental cohort was established with three clinical diagnostic populations: Alzheimer's Disease (AD), Non-Alzheimer's Disease Demented (Non-ADD), and Age-Matched Controls (AC). With extensive autopsy and genetic confirmation, assay methods were optimized to determine diagnostic cut-offs from Gaussian distributions of the output signals from each assay. Reference intervals between the AD, Non-ADD, and AC were generated to identify an accurate Alzheimer's diagnosis for demented patients. Based on the reference intervals, the cut-offs between AD and AC and AD and Non-ADD were determined for each assay.

B. Validation Process and Cohorts. A validation cohort was then established with three new populations for the same 3 clinical diagnostic cohorts: Alzheimer's Disease (AD), Non-Alzheimer's Disease Demented (Non-ADD), and Age-Matched Controls (AC). The Biomarker Assay was then applied prospectively for each validation cohort. The Gaussian distributions, reference intervals, cut-offs, sensitivity, specificity, and statistical significance were checked with validation cohorts.

C. Developmental/Validation Comparisons. Comparisons were conducted of the results obtained for the developmental and validation cohorts.

1.3. Development Date for the Determination of the Cut-Offs and Reference Intervals Supporting the Intended Use

1.3.1. Introduction

The purpose of this section is to present the study data for the development of the cut-offs and reference intervals for the NeuroDiagnostics AD Index Assay. This section shows how the reference intervals were calculated for each of the three populations: Alzheimer's Disease (AD), Non-Alzheimer's Disease Demented (Non-ADD), and Age-matched Controls (AC), as well as the Cut-Offs between the two pairs of populations AD versus AC, and AD versus Non-ADD.

Clinical diagnosis for AD can be uncertain and inaccurate and can usually only be confirmed in the late stages of progression and with autopsy validation. Within the first 4 years of the onset of AD symptoms, clinical diagnoses of AD have shown to be considerably inaccurate as samples from autopsy-confirmed clinical diagnoses have indicated. AD and cognitive impairment have been found to correlate with inflammatory signals TNF-**α** and IL-1β found in peripheral tissues such as skin fibroblasts. PKC isozymes regulate levels of TNF-**α** and IL-16 and the release of other cytokines and show deficits in AD brains and skin fibroblasts.

1.3.2. Study 1 (Kahn and Alkon, 2006)

1.3.2.1. Methods

Human skin fibroblast cell systems with the diagnoses AD, non-AD dementia (e.g., Huntington's disease and Parkinson's disease, and schizophrenia), and age-matched controls were cultured at 37°C with 5% CO2 to the 90–100% confluence stage in 25-ml cell culture flasks. In addition, the inflammatory agonist bradykinin (BK), a small nano-peptide that induces PKC-mediated phosphorylation of Erk_1 and Erk_2 in fibroblasts, was applied to punch biopsy-obtained human skin fibroblasts. Quantitative imaging of the phosphorylated Erk_1 and Erk_2 bands was then used in a ratio that is mathematically configured into an AD-Index. Basal phosphorylation of $Erk_{1/2}$ (P-Erk) was measured with Western blots from the cell lysates of 90–100% confluent skin fibroblast cells. The following formula was used to create an index that distinguishes between AD and non-AD cases:

$$\text{AD index} = [\text{P-Erk}_1 / \text{P-Erk}_2]^{BK+} - [\text{P-Erk}_1 / \text{P-Erk}_2)^{BK-}$$

1.3.2.2. Results

Results confirmed that the pathophysiology of AD affects other organ systems and not just the brain, and that blood, skin, saliva and extra-brain tissues, all manifest tau and amyloid metabolic pathways.

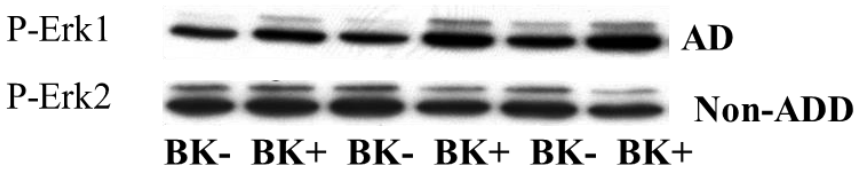

Figure 1: Example of Western Blot Images for Two Cases, Alzheimer's Disease (AD) and Non-Alzheimer's Demented (Non-ADD) Patients (Khan and Alkon, 2006) For BK-treated AD cells (BK+), both the P-Erk1 and P-Erk2 were elevated compared to untreated cells, but not for non-ADD (non-AD dementia) patients.

The AD Index Assay accurately distinguished fibroblasts of AD, normal controls, non-AD dementias, as well as those "mixed" dementia patients suffering from both AD and non-AD etiologies such as Huntington's disease, Lew body disease, and Parkinson's disease.

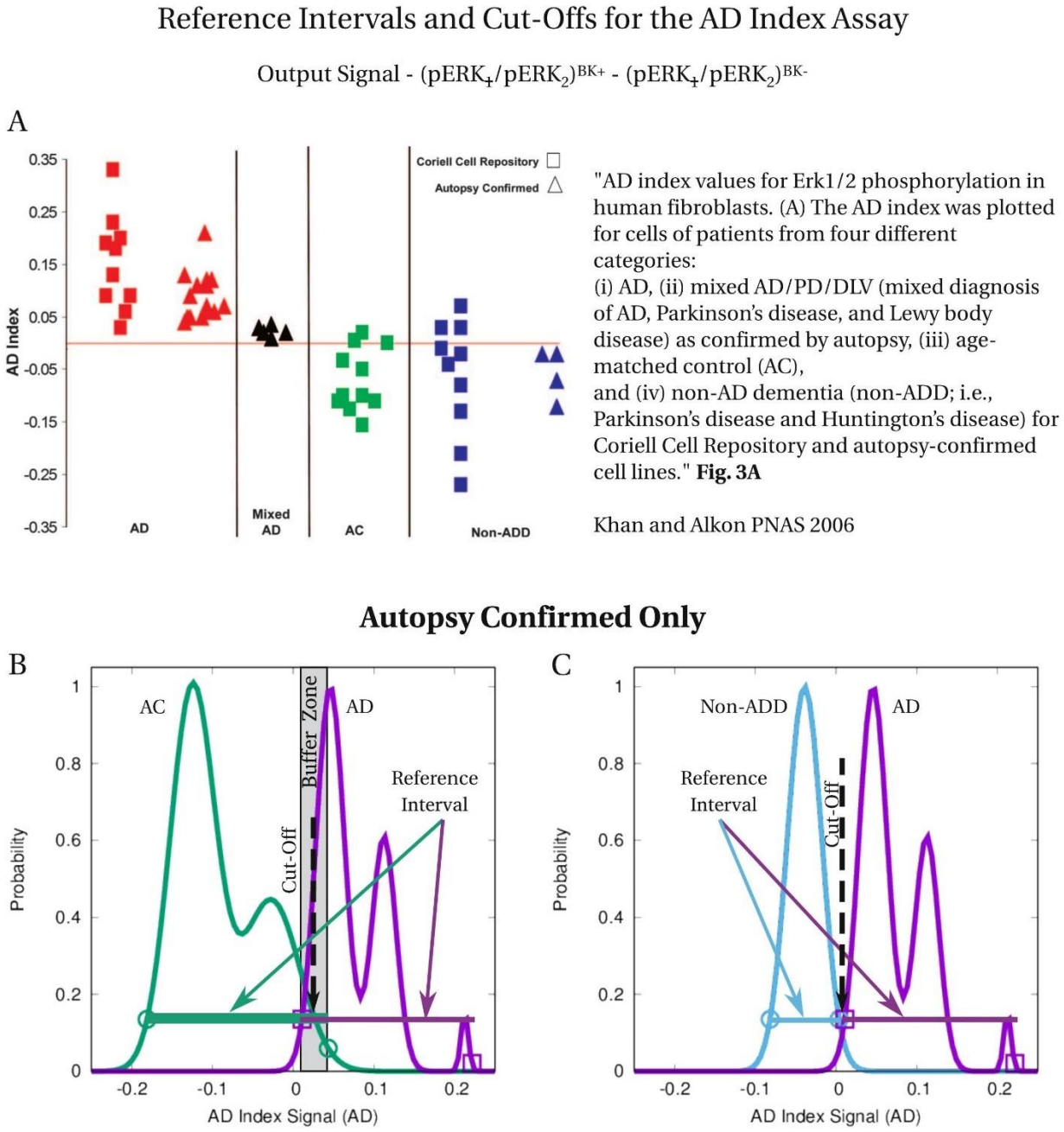

Figure 2: Reference Intervals and Cut-Off determination for AD Index Assay
A. Population data for the AD Index Assay, for AD(red), mixed AD (black), Non-ADD(blue) and AC(green). **B.** AD Index Gaussian distributions for AC(green) and AD populations (purple). The reference intervals for the AC and AD populations are indicated by the horizontal green and purple lines **C.** AD Index Gaussian distributions for Non-ADD (light blue) and AD populations (purple). The reference intervals for the Non-ADD and AD populations are indicated by the horizontal light blue and purple lines. Only the autopsy confirmed cases, which represent the gold-standard, are presented in panels B and C.

1.3.2.3. Conclusions

The results, obtained by using both fibroblasts from cultured cell tissue banks and gels obtained from autopsy-confirmed patients, showed high specificity(100%) and sensitivity (100%)that could offer reliable confirmation of the clinical diagnosis of AD vs. other dementias. Furthermore, the separability between Alzheimer's disease (AD) cohort and Non- Alzheimer's Disease Demented Cohort was statistically significant (Fig. 6, P<<0.0001).

1.3.3. Study 2 (Khan and Alkon 2008)

1.3.3.1. Methods

The study included 264 patients, out of which 42 were autopsy confirmed. The autopsy confirmed cases were used for the Reference Intervals and Cut-Off determination. The inflammatory agonist bradykinin, a small nano-peptide, that induces PKC-mediated phosphorylation of Erk1 and Erk2 in fibroblasts, was applied to punch biopsy-obtained human skin fibroblasts. Quantitative imaging of the phosphorylated Erk_1 and Erk_2 bands was then used in a ratio that is mathematically configured into an AD Index Assay.

$$\text{AD Index} = (\text{p-Erk}_1/\text{p-Erk}_2)^{BK+} - (\text{p-Erk}_1/\text{p-Erk}_2)^{BK-}$$

The raw data used for the probability distributions of AD Index Assay where the autopsy confirmed data (**Fig.** 2A). Note that the AC cases are by definition non-demented therefore hyper-validated by the clinical diagnosis.

The output measures for the AD Index Assay were binned into intervals, normalized by the total number of values, and fitted with Gaussian functions. Each of the three patient groups, AC, AD, and Non-ADD, was treated separately. If the distribution of data showed more than one peak, each peak was fitted separately with a Gaussian function and then the global distribution was assembled as a sum. For example, the AC distribution is bimodal while the AD distribution is trimodal.

The **reference intervals** for multi-modal distributions were calculated for each Gaussian function in the distribution, while the final reference interval is from the $(\mu-2\sigma)$ in the leftmost Gaussian to the $(\mu+2\sigma)$ in the rightmost Gaussian. For example, for the AC group the reference interval is from $(\mu-2\sigma)$, in the leftmost Gaussian to the $(\mu+2\sigma)$ in the rightmost Gaussian. The numerical values for the reference interval for the AC group (**Table 1**) are from **-0.181 to 0.043**. For the AD group the same rule, $(\mu-2\sigma)$, in the leftmost Gaussian to the $(\mu+2\sigma)$ in the rightmost Gaussian (squares on the green curve in **Fig. 2B, C**) yielded the following reference interval (**Table 1**) **0.011 to 0.222.** The Non-ADD group yielded the following reference interval (**Table 1**) -**0.083 to 0.003.**

The cut-offs for the AD Index were determined as the middle of the gap between the Non-ADD and AD reference intervals (dashed vertical line in **Fig. 2C**) or the middle of the gray zone (dashed vertical line in **Fig. 2C**). When there is overlap between neighboring reference intervals as for the AC and AD populations (**Fig. 2B**), this overlap was named Gray Area, because in this area cases have a certain probability of being AD and another smaller probability of being AC.

1.3.3.2. Results

Among the 42 autopsy-confirmed cases for which there was also AD Index Assay measurements, the overall accuracy of the AD Index Assay was 98%. Among both the autopsy confirmed and the clinically diagnosed patients. The AD-Index values were inversely correlated with the duration of disease, i.e., the time from the onset of dementia symptoms. Among the autopsy-confirmed cases, the AD Index Assay diagnosis showed remarkably high sensitivity (97%) and specificity (100%) compared to clinical diagnosis (sensitivity: 78% and specificity: 20%). Using autopsy validation, the clinical diagnosis was only accurate at 52% level versus the AD-Biomarker accuracy of 100% for cases with dementia not larger than 4 years of duration. Finally, application of soluble $A\beta_{1-42}$ to the fibroblasts of normal controls induced the abnormal AD Index Assay phenotype, suggesting the pathophysiologic relevance of this AD Index Assay measurement.

The output measure for the AD Index Assay is defined as the subtraction of the percent change of the $pERK_1$ with respect to $pERK_2$ for bradykinin treated (BK+) and untreated (BK-) skin fibroblasts:

$$AD\ Index = \left(\frac{pERK_1 - pERK_2}{pERK_2}\right)^{BK+} - \left(\frac{pERK_1 - pERK_2}{pERK_2}\right)^{BK-}$$

In Table 1, the AD Index Assay Reference Intervals and Cut-Offs are presented. The raw data is provided in Tables 2-4 below.

Table 1: AD Index Assay – Reference Intervals and Cut-Offs

Pairs					Cut-Off	Buffer Zone
AC versus AD	AC- Gaussian Distribution – Reference Interval		AD - Gaussian Distribution – Trimodal – Reference Interval		0.027	0.011 to 0.043
	μ–2*σ	-0.181	μ–2*σ	0.011		
	μ+2*σ	0.043	μ+2*σ	0.222		
Non-ADD versus AD	Non-ADD Gaussian Distribution – Reference Range		AD - Gaussian Distribution – Trimodal – Reference Range		0.007	0.003 to 0.011
	μ–2*σ	-0.083	μ–2*σ	0.011		
	μ+2*σ	0.003	μ+2*σ	0.222		

Table 2: AD Index Assay-Age Matched Control (AC)

AD Index Assay-Age Matched Control (AC) Hyper-validated samples from Coriell			
Cell **ID**	AD **Index**	Age	Gender
AG09977	-0.155	63	F
AG09878	-0.125	61	F
AG07723	-0.110	58	M
AG07310	-0.110	60	F
AG04560	-0.100	59	M
AG04058	-0.100	53	M
AG08044	-0.050	58	F
AG05271	-0.032	73	M
AG09555	0.000	53	F
AG11363	0.005	73	F
AG11020	0.020	79	F

Table 3: AD Index Assay-Autopsy confirmed Alzheimer's Disease (AD)

AD Index Assay-Autopsy confirmed Alzheimer's Disease (AD) Hyper-validated samples from JHU			
Cell **ID**	AD **Index**	Age	Gender
94	0.04	92	F
45	0.05	82	F
89	0.05	87	M
239	0.05	80	F
321	0.06	83	F
543	0.06	81	M
115	0.07	76	M
121	0.07	89	F
798	0.09	86	F
150	0.11	78	F
68	0.11	83	M
24	0.12	81	M
824	0.12	84	F
110	0.13	81	F
99	0.21	93	F

Table 4: AD Index Assay-Autopsy Confirmed Non-Alzheimer's Disease Demented (Non-ADD)

AD Index Assay-Autopsy confirmed Non-Alzheimer's Disease Demented (Non-ADD) Hyper-validated samples from JHU & Coriell			
Cell **ID**	AD Index	Age	Gender
822	−0.12	79	M
549	−0.02	74	M
569	−0.02	85	M
AG08395	−0.02	85	F

1.3.3.3. Conclusions

These studies showed how the reference intervals for each population Alzheimer's Disease (AD), Non-Alzheimer's Disease Demented (Non-ADD), and Age-Matched Controls (AC) were determined as well as how the Cut-Off's for each of the two pairs of populations, Alzheimer's Disease (AD) versus Non-Alzheimer's Disease Demented (Non-ADD), 0.007 (Table 1), and Alzheimer's Disease (AD) versus Age-Matched Controls (AC), 0.027 (Table 1), were determined.

The results showed no overlap between neighboring Gaussian distribution in the AD-Non-ADD pair therefor there was no gray area, while for the AD-AC pair the gray area stretches from 0.011 to 0043 (Table 1).

1.4. Validation Data for the Cut-Off and Reference Intervals Supporting the Intended Use

1.4.1. Introduction

The purpose of this section is to show how the reference intervals and the cut-offs were validated for the NeuroDiagnostics AD Index Assay.

The assay used three population of patients: Alzheimer's Disease (AD), Non-Alzheimer's Disease Demented (Non-ADD), and Age-matched Controls (AC).

1.4.2. Methods

The raw collected data from the initial studies were used to assess the Gaussian probability distributions for each group of patients, AD, AC, and Non-ADD. From the Gaussian distributions the reference intervals were determined as the 4 Standard Deviations (Sigma), two to the left and two to the right of the average/peak of the distributions. The data has been compiled in accordance with the 4 Sigma standard required by Clinical and Laboratory Standards Institute (CLSI) including 95.45% of values that lie within a two standard deviation band around the mean of the normal distribution. Therefore, the lower (2.5th percentile) and upper (97.5th percentile) limits are used in the Gaussian distribution to determine the reference ranges.

The validation of the reference intervals and cut-offs was performed in the NeuroDiagnostics' Rockville MD Laboratory with some different personnel than the one performing the initial studies. In addition to the banked samples also used in the initial studies, samples from the clinic were used for validation. For the AD index assay the validation samples were entirely new when compared with the samples used for the initial studies.

1.4.3. Results

The raw data are presented in **Tables 5 to 7.**

Table 5. AD Index Assay – Age Matched Controls Validation Samples

#	ID	AD Index	Age	Gender
1	1	-0.030	22	M
2	4	-0.010	23	M
3	19	-0.020	33	M
4	25	-0.011	39	M
5	27	-0.020	32	M
6	29	-0.004	21	M
7	31	-0.009	43	F
8	32	-0.011	23	M
9	36	-0.006	46	M
10	37	-0.005	65	F
11	38	-0.010	68	M
12	39	-0.017	65	F
13	50	-0.007	61	M
14	75	0.004	38	F

#	ID	AD Index	Age	Gender
15	77	0.000	18	M
16	78	0.003	45	F
17	80	-0.003	45	M
18	AG12998	-0.060	65	M
19	AG04461	-0.090	66	M
20	AG08044	-0.040	58	F
21	AG04058	-0.050	53	M
22	AG05271	0.020	73	M
23	AG04560	0.000	59	M
24	AG07723	-0.110	58	M
25	AG09555	0.000	53	F
26	AG11363	0.010	74	F
27	AG07310	-0.100	60	F
28	AG09977	-0.150	63	F
29	AG09878	0.010	61	F
30	AG11020	0.020	79	F

Table 6. AD Index Assay – Alzheimer's Disease Validation Samples

#	Sample ID	AD Index	Age	Gender
1	40	0.011	59	M
2	42	0.015	78	M
3	43	0.024	43	M
4	AG04401	0.18	53	F
5	AG08259	0.11	90	M
6	AG05809	0.06	63	F
7	AG10788	0.13	87	NA
8	AG06848	0.11	56	F
9	AG05810	0.03	79	F
10	AG06848	0.06	56	F
11	AG04402	0.19	47	M
12	AG07377	0.05	60	M
13	AG06263	0.11	67	F
14	AG08527	0.008	61	M
15	GM00364	0.131	53	M
16	AG05770	0.021	70	M
17	AG08170	0.009	56	M
18	AG08243	0.055	72	M
19	AG00364	0.203	53	M
20	AG14149	0.054	80	NA

#	Sample ID	AD Index	Age	Gender
21	AG11369	0.022	50	F
22	AG06264	0.065	62	F
23	AG08541	0.016	59	F
24	AG11415	-0.024	55	M
25	AG06265	0.029	61	M
26	AG07376	0.023	60	M
27	AG06262	0.017	66	M
28	AG07375	-0.006	71	M
29	AG06205	0.007	67	M
30	AG06840	0.01	56	M
31	AG04159	0.003	52	F
32	AG07374	0.219	73	M
33	AG11368	0.0459	77	M

Table 7. AD Index Assay – Non-Alzheimer's Disease Demented Validation Samples

#	ID	AD Index	Age	Gender
1	GM05031	-0.21	60	M
2	GM04198	0.03	63	F
3	GM04232	-0.04	70	M
4	GM05030	-0.13	56	M
5	GM04226	0.07	74	M
6	AG08395	-0.02	85	F
7	GM06274	-0.08	56	F
8	GM02165	-0.27	57	M
9	GM02038	0.03	22	M
10	GM02167	-0.02	59	F

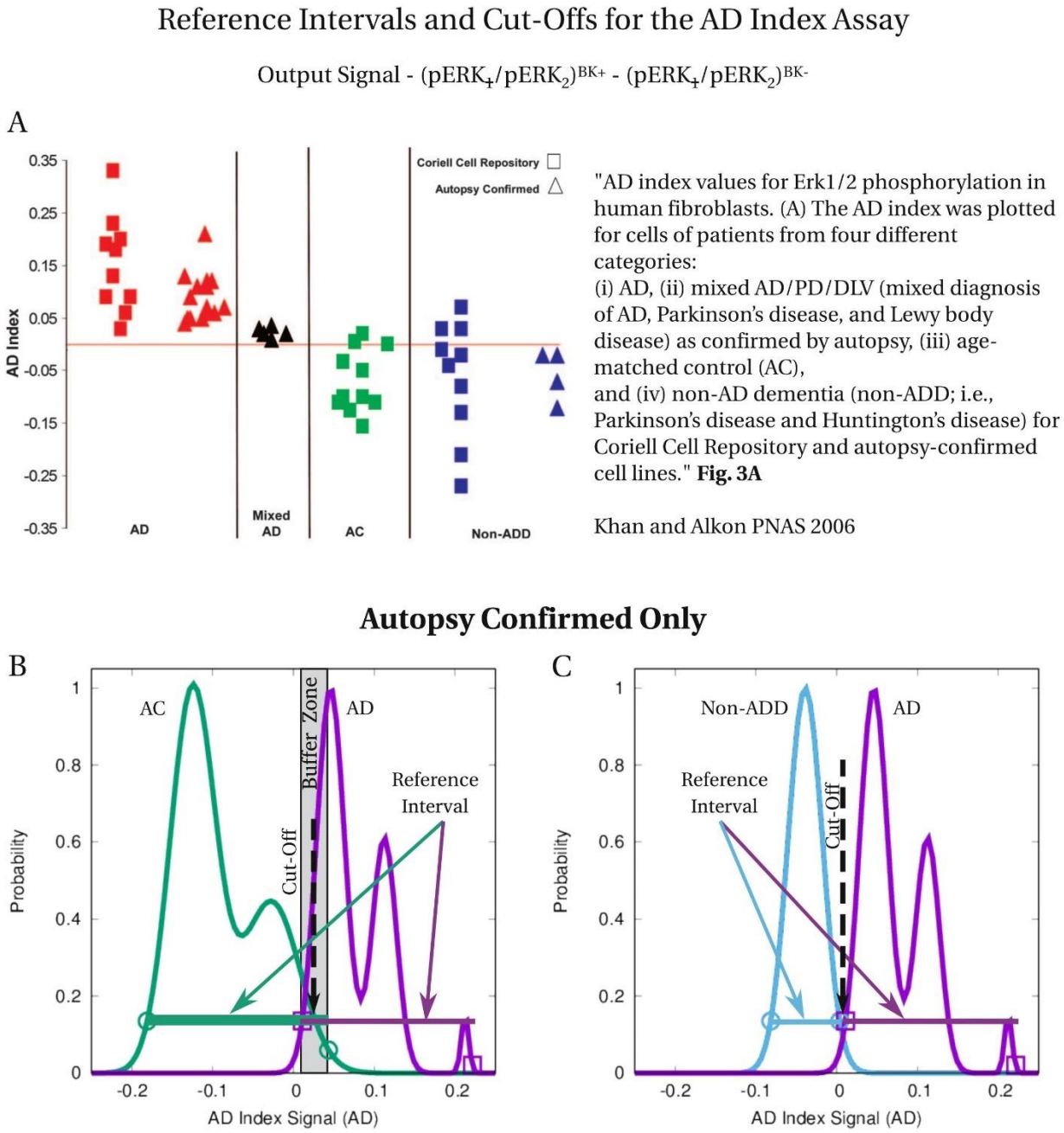

Figure 4: Validation of the Reference Intervals and Cut-Off for AD Index Assay. A. Population data for the AD Index Assay, for AD (red), mixed AD (black), Non-ADD (blue) and AC (green). **B.** AD Index Gaussian distributions for Non-ADD (light blue) and AD populations (purple). The reference intervals for the Non-ADD and AD populations are indicated by the horizontal light blue and purple lines **C.** AD samples used for validation are represented on the

Gaussian function with squares while the triangles represent the Non-ADD samples.

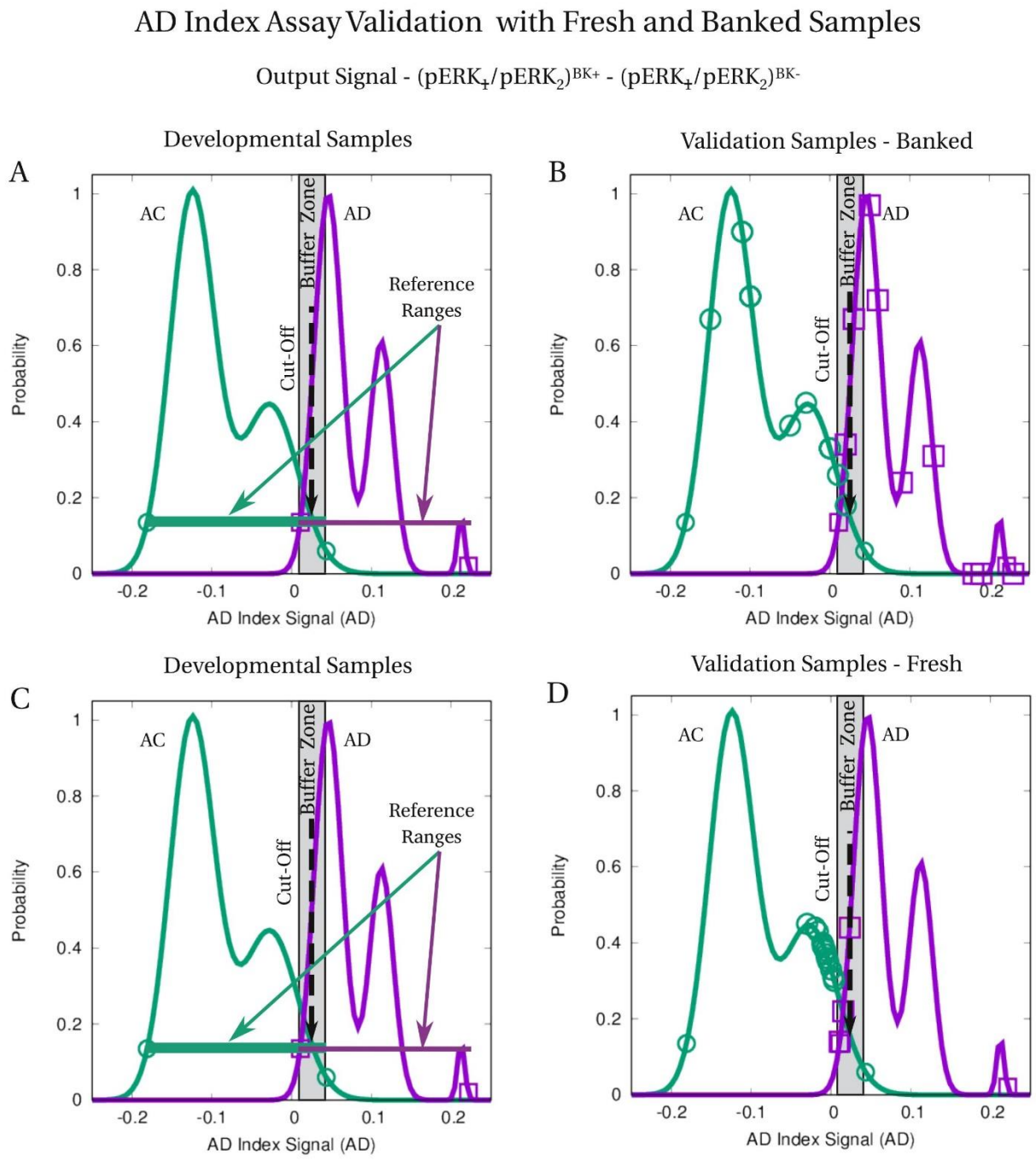

Figure 5: Validation of the Reference Intervals and Cut-Off for AD Index Assay. **A and C.** AD Index Gaussian distributions for AC (green) and AD populations (purple). The reference intervals for the AC and AD populations are indicated by the horizontal green and purple lines **B.** Banked samples are represented here. AD samples used for validation are represented on the Gaussian function with squares while the circle represent the AC samples. **D**. Fresh samples from the clinic are represented here. AD samples used for validation are represented on the

Gaussian function with squares while the circle represent the AC samples. Several of the AC and AD samples are in the gray zone. This error can be eliminated by requesting agreement in diagnosing a patient for the two out of the three assays.

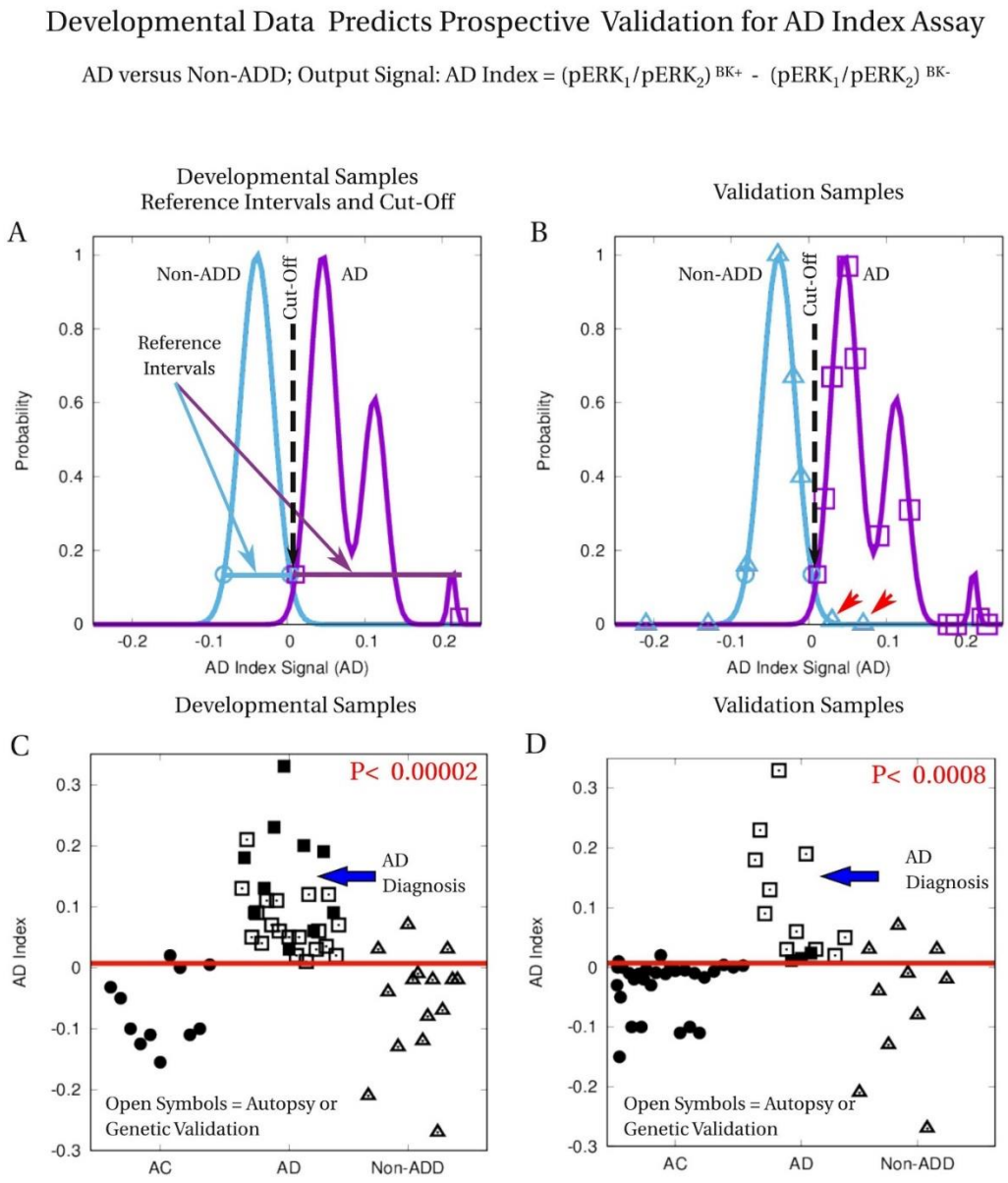

Figure 6: Developmental data Predicts Prospective Validation for AD Index assay. A. Reference intervals and cut-off resulted from the Gaussian distributions of developmental data. **B** Validation samples are represented on the Gaussian functions resulted from the developmental data. None of the samples from the clinic go over the pre-determined cut-off. **C.** Raw data for developmental samples. Separability between AD and Non-ADD cohorts is statistically significant (P<0.00002; two-tailed, unequal variance) **D.** Raw data

for validation samples. Separability between AD and Non-ADD cohorts is statistically significant (P<0.0008; two-tailed, unequal variance).

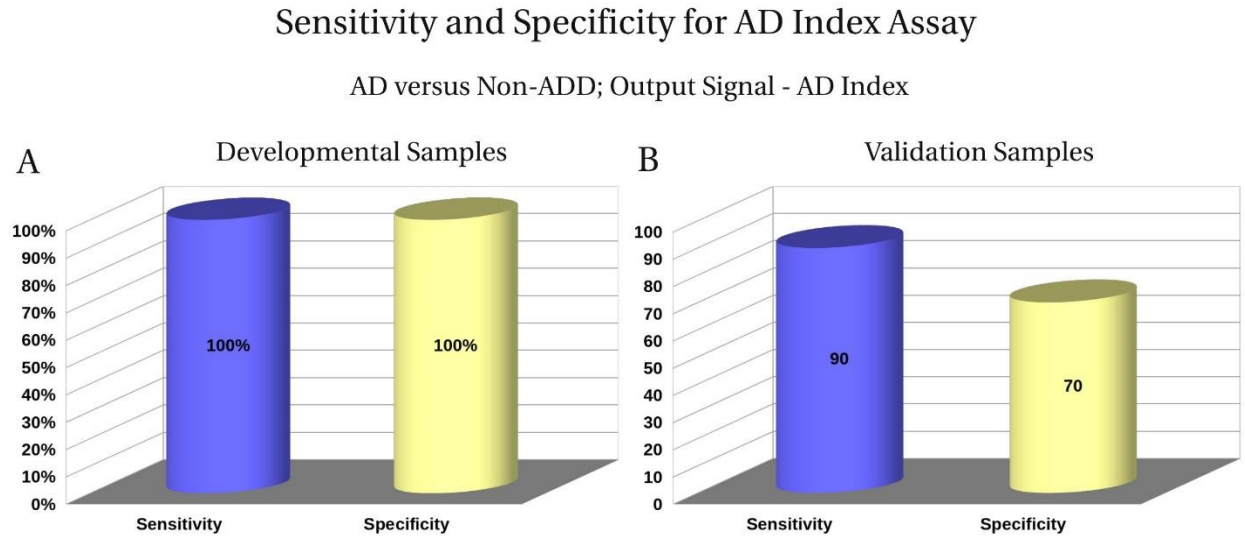

Figure 7: AD Index Sensitivity and Specificity

1.4.4. Conclusions

This report shows how the reference intervals for each of the three populations, Alzheimer's Disease (AD), Non-Alzheimer's Disease Demented (Non-ADD), and Age-Matched Controls (AC) were determined and validated by using Gaussian distributions of the output signals for the AD Index Assay. Furthermore, this study shows how the Cut-Offs between the two pairs AD-AC and AD-Non-ADD were determined by using the middle of the gap between the reference intervals for the adjacent population at the 4σlevel, and prospectively validated.

The validation data confirmed the non-overlapping distributions found initially (Khan and Alkon 2006, and 2008) between the AD and Non-ADD pair of population (P<<0.0001). The gray area between the AC and AD was also confirmed. The high sensitivity and specificity initially found for this assay (greater than 95%) was confirmed by the validation data. Furthermore, the cut-offs of 0.027 and 0.007 from the developmental samples were confirmed**.**

2. REFERENCES

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2. Khan T, Alkon D. Early diagnostic accuracy and pathophysiologic relevance of an autopsy-confirmed Alzheimer's disease peripheral biomarker. Neurobiology of Aging. 2010;31:889–900 (available online 2008).

3. Khan T, Alkon D. Peripheral Biomarkers of Alzheimer's Disease. Journal of Alzheimer's Disease. 2015b; 44:729–44.