



# Estimation of the allelic status of apolipoprotein E4 isoforms with fully automated LUMIPULSE® assays

Tatsushi Yuri<sup>1,2†</sup>, Rosina Degrieck<sup>2†</sup>, Dagmara Minczakiewicz<sup>2</sup> , Hideo Sato<sup>3</sup>, Jo Kamada<sup>1</sup>, Takuya Nakazawa<sup>1</sup>, Ina Vandenbroucke<sup>2</sup>, Katsumi Aoyagi<sup>4</sup> , Hisashi Nojima<sup>1†\*</sup> 

<sup>1</sup>Product Development Department, Product Designing 1st section, Fujirebio Inc., Tokyo 107-0052, Japan

<sup>2</sup>Product Development Department, Fujirebio Europe N.V., 9052 Gent, Belgium

<sup>3</sup>Fundamental Research Department, Assay Technology Research Section, Fujirebio Inc., Tokyo 107-0052, Japan

<sup>4</sup>Research and Development Division, Fujirebio Inc., Tokyo 107-0052, Japan

<sup>†</sup>These authors contributed equally to this work.

\* **Correspondence:** Hisashi Nojima, Product Development Department, Product Designing 1st section, Fujirebio Inc., Akasaka Intercity AIR, 1-8-1, Akasaka Minato-ku, Tokyo 107-0052, Japan. [hisashi.nojima@hugp.com](mailto:hisashi.nojima@hugp.com)

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## Abstract

**Aim:** Apolipoprotein E (ApoE) isoforms, especially the ApoE4 isoform, are genetic risk factors for Alzheimer's disease (AD). Moreover, the *APOE*  $\epsilon 4$  haplotype has a dose-dependent association with an increased risk of amyloid-related imaging abnormalities (ARIA) in individuals receiving disease-modifying therapy for AD. Therefore, the importance of *APOE* genotyping or proteotyping has been highlighted. Here, the authors developed fully automated chemiluminescence enzyme-immunoassay kit for ApoE4 and Pan-ApoE, and evaluated their diagnostic concordance with the *APOE* genotyping.

**Methods:** One hundred seventy-eight specimens were analyzed using the Lumipulse® G ApoE4 and Pan-ApoE for the ApoE proteotype and evaluated its diagnostic concordance with the *APOE* genotype.

**Results:** The ApoE4 kit specifically detected the ApoE4 concentration in plasma samples, and the polymorphism could be classified clearly by the ratio of ApoE4 and Pan-ApoE amount in plasma.

**Conclusions:** The combination of Pan-ApoE and ApoE4-specific chemiluminescent enzyme immunoassay (CLEIA) assay is useful for predicting *APOE*  $\epsilon 4$  allele status.

## Keywords

Apolipoprotein E4, Alzheimer's disease, disease modifying therapy, chemiluminescent enzyme immunoassay

## Introduction

Apolipoprotein E (ApoE) is associated with lipid homeostasis by mediating the transport and clearance of lipids between organs via the plasma and interstitial fluids [1]. *APOE* mainly exists in 3 allelic variants,



*APOE*  $\epsilon 2$ ,  $\epsilon 3$ , and  $\epsilon 4$ , which differ in a single amino acid change at position 112 or 158; ApoE2 (Cys112, Cys158), ApoE3 (Cys112, Arg158), and ApoE4 (Arg112, Arg158) [1]. Among them, the *APOE*  $\epsilon 4$  allele has been identified as the leading genetic risk factor for the sporadic form of Alzheimer's disease (AD) [1–3]. Moreover, the presence of one or two copies of the *APOE*  $\epsilon 4$  allele accelerates AD onset by approximately 10–20 years [2, 4]. Furthermore, the *APOE*  $\epsilon 4$  haplotype is a major risk factor for amyloid-related imaging abnormalities (ARIA) in individuals treated with anti-amyloid therapies for AD [5, 6]. Indeed, the combined data from the EMERGE and ENGAGE clinical trials showed that 20.3% of *APOE*  $\epsilon 4$  non-carriers and 43% of *APOE*  $\epsilon 4$  carriers developed amyloid-related imaging abnormalities-edema (ARIA-E) in the high-dose group [5, 7]. In the Clarity AD clinical trial, 5.4% of *APOE*  $\epsilon 4$  non-carriers and 15.8% of *APOE*  $\epsilon 4$  carriers developed ARIA-E [6]. These data indicate that individuals who are *APOE*  $\epsilon 4$  carriers are at greater risk of ARIA occurrence and severe ARIA-related adverse events [8, 9]. Therefore, there is a growing demand to identify the *APOE*  $\epsilon 4$  haplotype.

In the study of *APOE* polymorphism, both genotyping and proteotyping methods have been used [10]. Among the numerous available genotyping techniques, including gene-based analyses such as polymerase chain reaction (PCR) plus sequencing, and real-time PCR [11, 12], HhaI restriction fragment length polymorphism (RFLP) analysis of a PCR-amplified domain of the *APOE* gene appears to be the conventionally adapted assay [13]. On the other hand, the characterization of the different ApoE proteotypes is carried out by isoelectric focusing and immunoblotting (IEF-IB), enzyme-linked immunosorbent assay (ELISA), mass spectrometric immunoassays (MSIAs), or biochip arrays [14–17]. Among them, IEF-IB is a commonly used assay method based on charge differences and separation of common isoforms [14, 18], but the main drawback of IEF-IB is that posttranslational modifications such as physiological sialylation/desialylation, diabetes-induced glycation or oxidative modification such as imine adduct formation can alter the charge, leading to the disagreement between genotype and proteotype [13, 14, 19]. In addition to the above, these genotyping and proteotyping assays pose several limitations; they can be time-consuming, technically complex, require additional sample processing, lack high-throughput capacity, or lack of fully automation. These technical disadvantages might be one of the reasons to hamper the implementation of *APOE*  $\epsilon 4$  analysis in routine clinical practice.

In this study, we have developed fully automated chemiluminescent enzyme immunoassay (CLEIA) kit for ApoE4 and Pan-ApoE on the LUMIPULSE<sup>®</sup> platform using monoclonal antibodies recognizing ApoE proteins, including the ApoE4-specific antibody [15]. Here, we evaluated its diagnostic concordance with *APOE* genotyping and showed that the CLEIA ApoE4 and Pan-ApoE assays robustly distinguish between *APOE*  $\epsilon 4$  carrier status in plasma.

## Materials and methods

### Participants

The study included 178 Japanese volunteers, who were from our company and provided informed consent prior to enrollment. After the blood samples were collected into BD Vacutainer<sup>®</sup> K<sub>2</sub>EDTA tubes (Catalog number: 365900; BD Biosciences, NJ, USA), all tubes were gently inverted 8–10 times to avoid micro-clotting, and centrifuged at room temperature within 30 min (1,500× *g* for 10 min). The plasma layer was aliquoted into 2.0 mL polypropylene tubes (Catalog number: 72.694.600; Sarstedt, Numbrecht, Germany), followed by freezing in liquid nitrogen. Samples were kept at –80°C until use. Since the *APOE* gene is hereditary, collected samples were completely anonymized so that biographic and medical information about these participants was not collected. Study procedures were compliant with the principles of the Declaration of Helsinki. The ethics committee approved the study at H.U. Group Holdings, Inc. in the 2022 fiscal year (22-012-01).

### ApoE proteotyping

ApoE4 and Pan-ApoE were sequentially analyzed using the Lumipulse<sup>®</sup> G ApoE4 and Pan-ApoE test (Fujirebio Europe N.V., Gent, Belgium). The LUMIPULSE<sup>®</sup> systems, e.g., LUMIPULSE<sup>®</sup> G1200 or G600II, are instruments using chemiluminescence quantitative sandwich immunoassay principle, with an assay time of

30 min per test. Samples were tested according to the instructions for use. The measurements were carried out on the LUMIPULSE® G1200 automated immunoassay analyzer with a single calibration run. Each assay required the following sample volume: 20 µL for ApoE4, and 20 µL for Pan-ApoE. For each analyte, one assay lot was used to measure all the samples.

**APOE genotyping with PCR-RFLP or sequence**

The *APOE* genotyping with PCR-RFLP method was previously reported [20]. Briefly, the DNA was purified from peripheral whole blood, and the extracted DNA was used as the template for PCR. The PCR products were purified using QIAquick PCR Purification Kit (QIAGEN Inc., CA, USA) and the purified PCR products were digested with 10 units HhaI (New England BioLabs Inc., MA, USA) at 37°C for 2 h. For the restricted patterns, 20 µL of the digested fragments was separated by electrophoresis on a 15% polyacrylamide gel using SuperSeptTMDNA (FUJIFILM Wako Pure Chemical Corporation, Japan) at 30 mA for 1 h. After electrophoresis, the gel was stained with ethidium bromide, and the polymorphic patterns were analyzed. Furthermore, the PCR fragments were sequenced by ABI PRISM® 310 Genetic Analyzer (PE Applied Biosystems, CT, USA) following the manufacturer’s instructions.

**Statistical analyses**

All statistical analyses were performed in version 4.2.2 of R (R Foundation for Statistical Computing, Vienna, Austria). Group comparisons for continuous variables were performed using the Wilcoxon rank-sum test with Bonferroni correction. Cross-table analyses were performed by  $\chi^2$  test. The significance level of the statistical analyses was set at  $P < 0.05$ .

**Results**

We conducted both PCR-RFLP and PCR plus sequencing, and confirmed that the genotype results from both methods are consistent. The distribution of *APOE* genotypes among the subjects in this study was summarized in Table 1; 78.7% (140/178) of subjects were *APOE*  $\epsilon 4$  non-carriers ( $\epsilon 2/\epsilon 3$  or  $\epsilon 3/\epsilon 3$ ), and 21.3% (38/178) had at least one *APOE*  $\epsilon 4$  allele ( $\epsilon 2/\epsilon 4$ ,  $\epsilon 3/\epsilon 4$ , and  $\epsilon 4/\epsilon 4$ ).

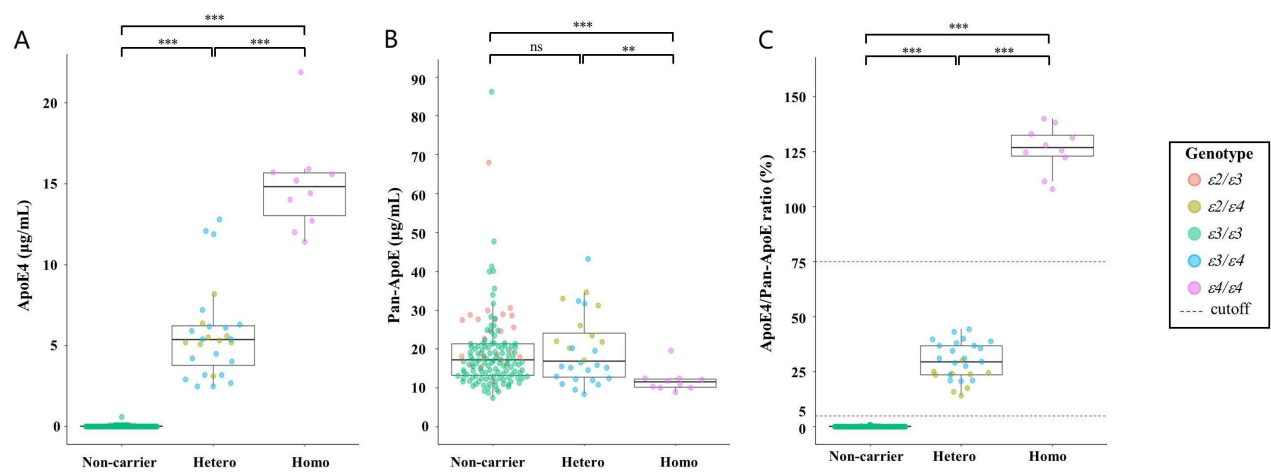
**Table 1.** *APOE* genotype distributions in this study

Genotype	<i>n</i>	Frequency
$\epsilon 2/\epsilon 2$	0	0.00%
$\epsilon 2/\epsilon 3$	24	13.48%
$\epsilon 3/\epsilon 3$	116	65.17%
$\epsilon 2/\epsilon 4$	9	5.06%
$\epsilon 3/\epsilon 4$	19	10.67%
$\epsilon 4/\epsilon 4$	10	5.62%
Total	178	-

-: not applicable

All 178 samples were analyzed using the Lumipulse® G ApoE4 and Pan-ApoE. The concentration levels ranged from 0.0 µg/mL to 21.9 µg/mL for ApoE4 (Figure 1A), and from 7.5 µg/mL to 86.2 µg/mL for Pan-ApoE (Figure 1B). There was the trend that the ApoE4 concentration mostly dose-dependently increased with the copy number of *APOE*  $\epsilon 4$  allele, allowing for the statistically significant ( $P < 0.001$ ) distinction among these groups (Figure 1A). Although some samples in the *APOE*  $\epsilon 4$  heterozygous group had higher ApoE4 concentrations which are comparable to the concentrations of the *APOE*  $\epsilon 4$  homozygotes, the two groups were significantly different ( $P < 0.001$ ) (Figure 1A). On the other hand, Pan-ApoE concentrations in *APOE*  $\epsilon 4$  homozygous group was significantly lower than those in heterozygous and null group ( $P < 0.01$  and  $P < 0.001$ , respectively) (Figure 1B), but there was no statistical difference in Pan-ApoE concentrations between heterozygous and null group. Furthermore, the ApoE4/Pan-ApoE ratio could clearly classify the *APOE*  $\epsilon 4$  carrier status (Figure 1C and Table 2). The range of the ApoE4/Pan-ApoE ratio in this study was between 0.0% and 140% (Figure 1C). Then, we preliminary set the cutoff values at 5% and 75% in the

ApoE4/Pan-ApoE ratio (Figure 1C), and cross-table analyses were performed by  $\chi^2$  test, resulting in a significant difference (Table 2).



**Figure 1.** Distribution of biomarkers. Beeswarm boxplots of the assays ApoE4 (A), Pan-ApoE (B), and ApoE4/Pan-ApoE (C) by *APOE*  $\epsilon 4$  carrier status. Boxplots display the median values with the interquartile range (lower and upper hinge) and  $\pm 1.5$ -fold the interquartile range from the first and third quartile (lower and upper whiskers). Data were analyzed using Wilcoxon signed-rank tests with Bonferroni correction. \*\*  $P < 0.01$ ; \*\*\*  $P < 0.001$ .  $\epsilon 2$ : *APOE*  $\epsilon 2$ ;  $\epsilon 3$ : *APOE*  $\epsilon 3$ ;  $\epsilon 4$ : *APOE*  $\epsilon 4$ ; ns: not significant

**Table 2.** Cross tabulation analysis of *APOE*  $\epsilon 4$  genotype versus proteotype

Category	-/-	$\epsilon 4$ /-	$\epsilon 4/\epsilon 4$	Total
-/-	140	0	0	140
$\epsilon 4$ /-	0	28	0	28
$\epsilon 4/\epsilon 4$	0	0	10	10
Total	140	28	10	178

The horizontal cells are genotype, and the vertical cells are proteotype.  $\chi^2 = 356$ ;  $\text{df} = 4$ ;  $P < 0.0001$

## Discussion

In our study, we established ApoE4 and Pan-ApoE assays on the LUMIPULSE<sup>®</sup> platform, and demonstrated their performances by setting cutoff values for the ApoE4/Pan-ApoE ratio to optimize its concordance with the *APOE* genotype. Consistent with previous reports [15], we found that the ratio could completely match the *APOE*  $\epsilon 4$  allele status.

It has been reported that the levels of plasma ApoE decrease in a perfect parametric fashion across the six *APOE* genotypes ( $\epsilon 2/\epsilon 2 > \epsilon 2/\epsilon 3 > \epsilon 2/\epsilon 4 > \epsilon 3/\epsilon 3 > \epsilon 3/\epsilon 4 > \epsilon 4/\epsilon 4$ ) [21]. In this regard, our results showed a similar trend (Figure 1B), although the *APOE*  $\epsilon 2$  homozygotes were not obtained in this study because it is a rare variant [22]. On the other hand, the frequency of the *APOE*  $\epsilon 4$  homozygotes or heterozygotes in this study was comparable to the previous reports [3, 23]. Further study with a larger sample size could address these points.

Both genotyping and proteotyping methods have been used for *APOE* polymorphism analysis [10]. However, it has been reported that the ApoE proteotype by the IEF-IB method can be susceptible to protein modification and degradation, resulting in discordant results of the proteotype-genotype correlation [13, 14, 19]. In this point, while the immunoassay examined in this study, ELISA [15], MSAs [16], or biochip arrays [17] might show robustness toward both post-translational modification and protein degradation, the Lumipulse<sup>®</sup> G ApoE4 and Pan-ApoE assays could be better than the other proteotyping assays in terms of improved accuracy and reproducibility, elimination of technical skill influence, and reduced labor and costs.

Recently, it has been reported that the *APOE*  $\epsilon 4$  carrier or non-carrier could be classified with automated immunoassay for ApoE4 [24, 25]. However, it might be difficult to separate homozygotes and

heterozygotes completely by ApoE4 assay alone because peripheral blood ApoE levels might be affected by individual differences or even diets [26]. Indeed, some ApoE4 concentrations in the *APOE*  $\epsilon$ 4 heterozygous group were similar to those of the *APOE*  $\epsilon$ 4 homozygotes (Figure 1A). By contrast, the Lumipulse® G ApoE4/Pan-ApoE ratio could distinctly separate *APOE*  $\epsilon$ 4 status among null, heterozygote, and homozygote because Pan-ApoE could serve as an internal control to minimize potential variability of plasma ApoE4 concentrations. Furthermore, LUMIPULSE® G1200 can perform 120 tests/h, suggesting that this system is suitable for high-throughput screening. Additionally, the LUMIPULSE® platform has a product portfolio for AD-related biomarkers, allowing sequential measurement of several biomarkers [27–29].

The limitations of this study are the lack of various analyses using specimen information, the limited study population, and the lack of a validation cohort. Since the samples were collected from our internal volunteers, the genetic information related to AD needs to be protected from leakage, which affected the availability of the biomedical information about the study participants. In addition, the samples are collected from our internal volunteers aged between 20s and 50s, consequently suggesting no serious illnesses that might affect the modification of the ApoE protein. Therefore, further investigations using patient specimens with underlying diseases such as diabetes mellitus, would be necessary in future. Besides, the samples in this study were composed of Japanese volunteers, and it would be necessary to compare the results with other racial groups. Furthermore, the cutoff values which were used in this study, could not be validated in an independent secondary cohort, suggesting that further studies are required to address these points.

In conclusion, this study successfully demonstrated that the combination of ApoE4 and Pan-ApoE CLEIA assays was useful for predicting *APOE*  $\epsilon$ 4 allele status. It has been reported that *APOE*  $\epsilon$ 4 genotype is strongly associated with ARIA and exhibits its allele copy-number dependent effects [5–8]. Indeed, it is recommended to perform a test for *APOE*  $\epsilon$ 4 before initiating anti-amyloid treatments [7, 8]. Therefore, the Lumipulse® G ApoE4 and Pan-ApoE assays could contribute to risk discussions and safety considerations of disease-modifying therapies for AD.

## Abbreviations

AD: Alzheimer's disease

ApoE: apolipoprotein E

ARIA: amyloid-related imaging abnormalities

CLEIA: chemiluminescent enzyme immunoassay

IEF-IB: isoelectric focusing and immunoblotting

PCR: polymerase chain reaction

RFLP: restriction fragment length polymorphism

## Declarations

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### Author contributions

TY: Resources, Data curation, Investigation, Methodology, Writing—review & editing. RD: Resources, Data curation, Methodology, Writing—review & editing. DM: Resources, Methodology, Writing—review & editing. HS: Resources, Data curation, Investigation, Writing—review & editing. JK and TN: Resources, Investigation, Methodology. IV and KA: Supervision, Writing—review & editing. HN: Data curation, Formal analysis, Supervision, Investigation, Visualization, Writing—original draft, Project administration, Writing—review & editing.



## Conflicts of interest

TY, HS, JK, TN, and HN are employees of Fujirebio Inc. TY, RD, DM, and IV are employees of Fujirebio Europe N.V. KA is a board member of Fujirebio Inc.

## Ethical approval

Study procedures were compliant with the principles of the Declaration of Helsinki. The ethics committee approved the study at H.U. Group Holdings, Inc. in the 2022 fiscal year (22-012-01).

## Consent to participate

Informed consent to participate in the study was obtained from participants.

## Consent to publication

Not applicable.

## Availability of data and materials

Not applicable.

## Funding

Not applicable.

## Copyright

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