APO-Easy® Genotyping kit

INSTRUCTION FOR USE

REF FLS-OE-02

For *In Vitro* Diagnostic Use only

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For more information on the device use the QR code

IVD Rx

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List of abbreviations:

SNP: single nucleotide polymorphisms

gDNA: genomic deoxyribonucleic acid

APOE: Apolipoprotein E

AD: Alzheimer's Disease

qPCR: qualitative real-time Polymerase Chain Reaction

IVD: In Vitro Diagnostic

QS5-Dx: QuantStudio 5 Dx Real-Time PCR System

NTC: No Template Control

MUT: Mutant

WT: Wild Type

Het: Heterozygous

RCF: Relative Centrifugal Force

°C: Celsius degree

ng: nanogram

µL: microliter

STD: standard

IFU: Instruction For Use

SDS: Safety Data Sheets

Precautions and warnings:

- The results obtained from this assay must be interpreted within the context of all relevant clinical laboratory findings and are not to be used for any diagnosis.

1. Intended Use

The APO-Easy® Genotyping kit is a qualitative polymerase chain reaction (PCR) test intended for the detection of two single nucleotide polymorphisms (SNPs), rs429358 and rs7412, in the APOE gene using genomic DNA extracted from human EDTA whole blood. The APOE genotype information provided by the test is used with other laboratory and clinical information to aid in the evaluation of the risk of developing late-onset Alzheimer's Disease (AD) in patients presenting with cognitive impairment and/or with predisposing risk factors, who are being evaluated for AD and other causes of cognitive decline.

The APO-Easy® Genotyping kit is not a screening or diagnostic test and does not determine the person's overall risk of developing late-onset AD. It is not intended to replace any clinical and diagnostic examinations.

2. Introduction

Apolipoprotein E (APOE) is a chylomicron apolipoprotein expressed in various organs such as the liver, brain, spleen, kidneys, sex glands, adrenal glands, and macrophages (Marias, 2019). APOE has been shown to be important for various processes such as the metabolism of lipoproteins, fat-soluble vitamins, and glucose/energy as well as signal transduction, metastasis, and angiogenesis. Being important in lipid metabolism, APOE is well-linked to cardiovascular disease as it is required for the normal catabolism of the triglyceride-rich lipoprotein components (Semaev et al., 2022). The importance of APOE in the pathogenesis of neurodegenerative disorders such as frontotemporal dementia, Parkinson's disease, Lewy body dementia, and Alzheimer's disease (AD) has been well documented (Davis et al., 2020; Mishra et al., 2017).

APOE gene has three common alleles (ε2, ε3 and ε4) and six related genotypes (APOE ε3/ε3, APOE ε3/ε2, APOE ε2/ε2, APOE ε3/ε4, APOE ε4/ε4, and APOE ε2/ε4) [Su et al., 2017]. The corresponding isoforms are characterized by amino acids at positions 112 and 158 and determine the affinity for lipoprotein receptors. APOE ε3 is the most common allele believed neither to increase nor decrease the risk of developing AD. APOE ε2 has been shown to have a protective effect against developing AD (Ohm et al., 1999) whereas APOE ε4 reduced the clearance of beta-amyloid (Aβ) that resulted in enhanced Aβ deposition within the neurons in the AD mouse model (Yamazaki et al., 2019). About 25 percent of people carry one copy of APOE ε4, and 2 to 3 percent carry two copies (https://www.nia.nih.gov/health/alzheimers-disease-genetics-fact-sheet). Several studies have evidenced that the APOE ε4 genotype is associated with late onset of familiar and sporadic forms of AD (Corder et al., 1993, Schmechel et al., 1993, Strittmatter et al., 1993, Farrer et al., 1997, Bu G., 2009).

The APO-Easy® Genotyping kit uses qualitative real-time polymerase chain reaction (qPCR) technique for the *in vitro* determination of two APOE single-nucleotide polymorphisms (SNPs) mutation: rs429358 and rs7412. This IVD assay uses fluorescently labelled probes (FAM and VIC, Table 1) allowing allelic discrimination and determination of APOE genotypes.

Table 1. Correspondence between SNP and fluorescently labelled probes.

SNP	VIC	FAM
rs429358	MUT	WT
rs7412	WT	MUT

The Alzheimer's Disease and Related Disorders Therapeutics Work Group, recommends, in the appropriate use recommendations (AUR) for the treatment of AD by monoclonal antibody therapies, that clinicians perform APOE genotyping to better inform patient care decisions, discussions regarding risk, and clinician vigilance concerning amyloid related imaging abnormalities (ARIA). APOE genotyping is recommended to identify patients who are APOE ε4 gene carriers, especially those homozygous for APOE ε4 who are at higher risk for the occurrence of ARIA.

3. Principle of the assay

The APO-Easy® Genotyping kit includes reagents needed to perform qPCR analysis for testing genomic DNA (gDNA) extracted from PAXgene DNA samples to determine APOE genotypes. The assay assesses 2 SNPs (rs429358 and rs7412) in the APOE gene and contains positive control for each SNPs (wild-type, mutant and heterozygous). The genotype is determined based on the mutation status for both mutations analyzed resulting in one of the six genotypes possible (APOE ε3ε3, APOE ε3ε2, APOE ε2ε2, APOE ε3ε4, APOE ε4ε4, and APOE ε2ε4).

For the wildtype allele (ApoE ε3) the amino acids cysteine at position 112 and arginine at position 158 are detected. The mutation ApoE ε4 (SNP rs429358) impacts the amino acid at position 112 resulting in changing thymine to cytosine inducing the translation of arginine instead of cysteine. Whereas the mutation ApoE ε2 (SNP rs7412) impacts the amino acid at position 158 resulting in changing cytosine to thymine inducing the translation of cysteine instead of arginine (Figure 1).

Figure 1. Schematic diagram of human APOE gene and the polymorphisms at 2 single nucleotides resulting in 3 alleles ε2, ε3 and ε4.

4. Instruments and materials

The APO-Easy® Genotyping kit includes consumables to perform the qPCR amplification and fluorogenic detection of APOE mutations using ThermoFisher QS5-Dx (equipment not provided with the kit). The equipment needed for the assay but not provided with the kit and their references are given in Table 2.

Table 2. Reference of the equipment needed to perform APO-Easy® Genotyping kit.

Instrument	Purpose	Manufacturer	Reference no.	Certifications
Nanodrop 2000/2000c	Quantification of gDNA	ThermoFisher	ND-2000	UL/CSA and CE
QS5-Dx	qPCR	ThermoFisher	A31928	FDA 510K (K190302)

For gDNA extraction from whole blood PAXgene DNA tubes, Firalis recommends using the reference given in Table 4 following the manufacturer instruction provided along with the kit.
If another reference is used, Firalis cannot guarantee the results nor performance of the assay.

Samples must be collected in PAXgene® Blood DNA Tube (BD Biosciences, Reference no: 761165) according to the instructions for use provided by the collection tube manufacturer. Whole blood samples are stable under the following conditions:

Table 3. Whole blood specimen stability studies summary at different storage conditions.

Storage Condition	Duration
2-4°C	28 Days
Room Temperature (18–24°C)	14 Days
-20°C	6 Months
-80°C	6 Months

Table 4. Reference of the recommended gDNA extraction kit.
(not provided along with the APO-Easy® Genotyping kit)

Description	Manufacturer	Purpose	Storage	Reference	Quantity
QIAamp DSP DNA Blood Mini kit	Qiagen	gDNA extraction	According to the supplier's recommendation	61104	1kit/50samples

4.1. Reagents and materials provided with the kit

The APO-Easy® Genotyping kit contains ready-to-use master mixes for 32 reactions per SNP, positive controls: Standard A, Standard B, Standard C, Standard D, Standard E, and one vial of no template control (NTC). A total of 32 reactions is possible using the reagents from one kit including 6 reactions for the standards and 2 NTCs for each SNP, and for each analysis (

Table 5).

Table 5. Contents of the APO-Easy® Genotyping kit.

Description	Storage	Reference	Quantity (32 reactions)
TaqMan™ Genotyping **Master Mix 1** rs429358	-15°C to -25°C	FLS-MM1OE- 02	1 vial
TaqMan™ Genotyping **Master Mix 2** rs7412	-15°C to -25°C	FLS-MM2OE- 02	1 vial
No Template Control (NTC)	-15°C to -25°C	FLS-NTC-01	1 vial
Standard A: Mutant (MUT) rs7412	-15°C to -25°C	FLS-OE2S-01	1 vial
Standard B: Wildtype (WT) rs429358 and rs7412	-15°C to -25°C	FLS-OE3S-01	1 vial
Standard C: Mutant (MUT) rs429358	-15°C to -25°C	FLS-OE4S-01	1 vial
Standard D: Heterozygous (Het) rs7412	-15°C to -25°C	FLS-OE23S-01	1 vial
Standard E : Heterozygous (Het) rs429358	-15°C to -25°C	FLS-OE34S-01	1 vial

*Positive controls: Standard A, Standard B, Standard C, Standard D, Standard E. Negative control: NTC

4.2. Miscellaneous materials and equipment needed

- Nuclease-free water
- Use only certified nuclease-free tips and microcentrifuge tubes
- Precision pipettes - Multichannel pipette
- BSL2 cabinet
- Micro centrifuge (17000 Relative Centrifugal Force (RCF))
- Vortex and microcentrifuge
- Real-time PCR plates
- Compact PCR plates spinner

4.3. Shelf-life and in-use stability of the kit

- The kit must be stored at -15°C to -25°C. The kit is stable until its expiration date stated on the box label.
- Once opened, the kit components can be repeatedly thawed and frozen twice without degradation of performance. Exceeding the freeze and thaw cycle recommendation might diminish the kit functionality.
- The reagents may be thawed at room temperature (i.e., +18-24°C, for 60 minutes), on ice for 80 minutes, or in a refrigerator (i.e., a 4-8°C cold room for 16-20 hours).

5. Genomic DNA (gDNA) extraction and quantification

5.1. Extraction

Extraction of gDNA from whole human blood collected into PAXgene Blood DNA tubes using QIAamp DSP DNA Blood Mini kit as given in Table 4 following the user instruction data sheet provided along with the kit.

5.2. Quantification

Quantification of the extracted gDNA is done using Nanodrop. The gDNA input for the APO-Easy® Genotyping test is between 12.5ng and 100ng and a 2µL input volume. If the samples have concentration higher than the recommended concentration, they must be diluted in Nuclease Free water (Molecular grade) according to the input quantity range (12.5ng-100ng).

6. APO-Easy® Genotyping assay procedure

The overview of the APO-Easy® Genotyping assay workflow is presented in Table 6. The required time for the assay is: 2 hours and 30 minutes.

Table 6. Overview of the APO-Easy® Genotyping assay workflow.

Workflow	
Step 1	Real-time genotyping PCR reaction preparation
Step 2	qPCR run
Step 3	Result analysis
Step 4	Interpretation

6.1. Step 1. Real-time genotyping PCR reaction preparation

The kit comprises of two ready-to-use TaqMan™ Genotyping Master Mix to assess APOE SNPs (rs429358 and rs7412), nuclease-free water for the NTC, and standards to mimic wildtype, mutant and heterozygous conditions for each single nucleotide polymorphisms (SNP).

Each master mix is a dualplex assay assessing the SNP of interest:

Master mix 1: composed of 2 probes to assess the SNP rs429358 and allow allelic discrimination for the wildtype nucleotide (T), the mutant nucleotide (C) as well as the heterozygote condition.

Master mix 2: composed of 2 probes to assess the SNP rs7412 and allow allelic discrimination for the wildtype nucleotide (C), the mutant nucleotide (T) as well as the heterozygote condition.

Standard A, B, C, D, and E:

Each standard (STD) is a DNA template designed to mimic the three alleles and the combination of different DNA templates mimicking the heterozygous condition.

Standard A: DNA template of allele APOE ε2
Standard B: DNA template of allele APOE ε3
Standard C: DNA template of allele APOE ε4
Standard D: DNA template with allele APOE ε2 and APOE ε3
Standard E: DNA template with allele APOE ε3 and APOE ε4

Note: Thaw the master mixes and the standards on ice. Mix the reagents thoroughly and spin them down briefly and place them on ice.

1. Dispense 23 µL/well of the detection assay master mix in a 96-well plate (for each sample master corresponding to both SNPs must be added).
2. Add 2 µL of gDNA or the Standards in the corresponding wells and briefly spin down. The total amount of gDNA in a reaction should be 12.5 ng.

- Each individual SNP **requires analysis of WT, MUT, and HET standards** as well as **NTC** (nuclease free Water molecular grade). A model layout plan for the analysis of up to 24 PAXgene DNA samples measured for 2 SNPs in a 96 wells PCR plate is shown in Table 7.
- Note: The APO-Easy® Genotyping kit allows users to perform up to 32 qPCR reactions. Each master mix is sufficient to assay 8 standards (i.e., STD) and up to 24 samples. If a smaller number of samples are to be assessed the kit can be used twice, for example 8 STD plus 8 patients can be assessed once and the second time again 8 STD plus 8 patients can be assessed.

Table 7. Model layout of a 96 well plate for APOE genotyping of 24 samples, 3 controls/SNP in duplicate and 2 NTCs/SNP for APO-Easy® Genotyping kit.

	APOE (rs429358)						APOE (rs7412)					
	Col 1	Col 2	Col 3	Col 4	Col 5	Col 6	Col 7	Col 8	Col 9	Col 10	Col 11	Col 12
A	STD B: WT	Sample #1	Sample #9	Sample #17			STD B: WT	Sample #1	Sample #9	Sample #17		
B	STD B: WT	Sample #2	Sample #10	Sample #18			STD B: WT	Sample #2	Sample #10	Sample #18		
C	STD C: MUT	Sample #3	Sample #11	Sample #19			STD A: MUT	Sample #3	Sample #11	Sample #19		
D	STD C: MUT	Sample #4	Sample #12	Sample #20			STD A: MUT	Sample #4	Sample #12	Sample #20		
E	STD E: HET	Sample #5	Sample #13	Sample #21			STD D: HET	Sample #5	Sample #13	Sample #21		
F	STD E: HET	Sample #6	Sample #14	Sample #22			STD D: HET	Sample #6	Sample #14	Sample #22		
G	NTC	Sample #7	Sample #15	Sample #23			NTC	Sample #7	Sample #15	Sample #23		
H	NTC	Sample #8	Sample #16	Sample #24			NTC	Sample #8	Sample #16	Sample #24		

- The following STD must be used for each SNP assay determination as given below. For rs429358 (Master mix 1), standards B, C and E must be used and to assess rs7412 (Master mix 2), standards B, A and D must be used (Table 8).

Table 8. Standard correspondence for SNP determination of the APO-Easy® Genotyping assay.

APOE (rs429358)	APOE (rs7412)
STD B (WT)	STD B (WT)
STD C (MUT)	STD A (MUT)
STD E (HET)	STD D (HET)

3. Vortex the plate for 5 sec and centrifuge at 500g for 30 seconds.
4. Proceed to start the **run immediately**.

6.2. Step 2. qPCR run

5. Setting the QS5 Dx for APO-Easy® Genotyping assay:

A. On "Properties" page, assign experiment name and select the experiment type as **genotyping** (Figure 2).

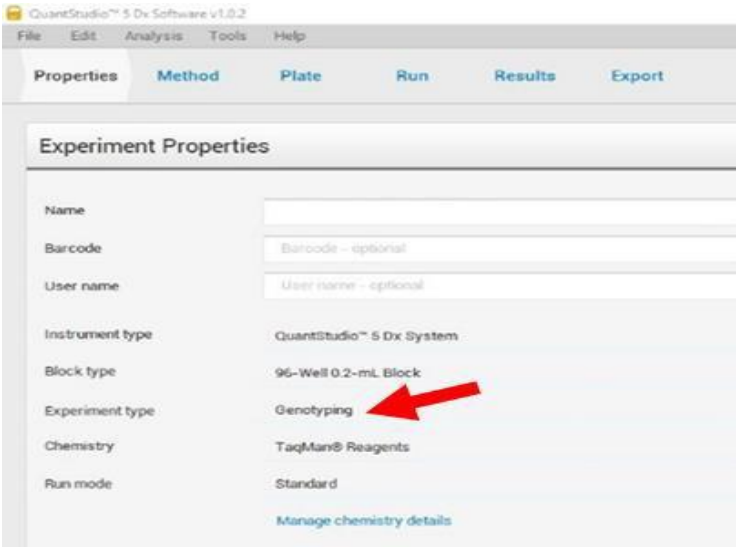

Figure 2. Experiment type selection in the experiment properties on the QS5 software. Make sure to select genotyping (red arrow).

B. On "Method" page, set up the total volume of PCR reaction, **25 µL** of reaction mix.

C. On "Plate sheet," go in advanced Setup, to set up fluorescence, click on "(+) Add button" to obtain **two SNP assays** (SNP Assay 1 & SNP Assay 2). Click on OK button to proceed. Then for each SNP Assay, click on the Action button and select Edit to fill the name as following (Figure 3):

NOTE: For **SNP Assay 1: rs429358** (Master mix 1). Make sure to have **Allele 1** set with the reporter **VIC (MUT)** and **Allele 2** set with the reporter **FAM (WT)**

For **SNP Assay 2: rs7412** (Master mix 2). Make sure to have **Allele 1** set with the reporter **VIC (WT)** and **Allele 2** set with the reporter **FAM (MUT)**

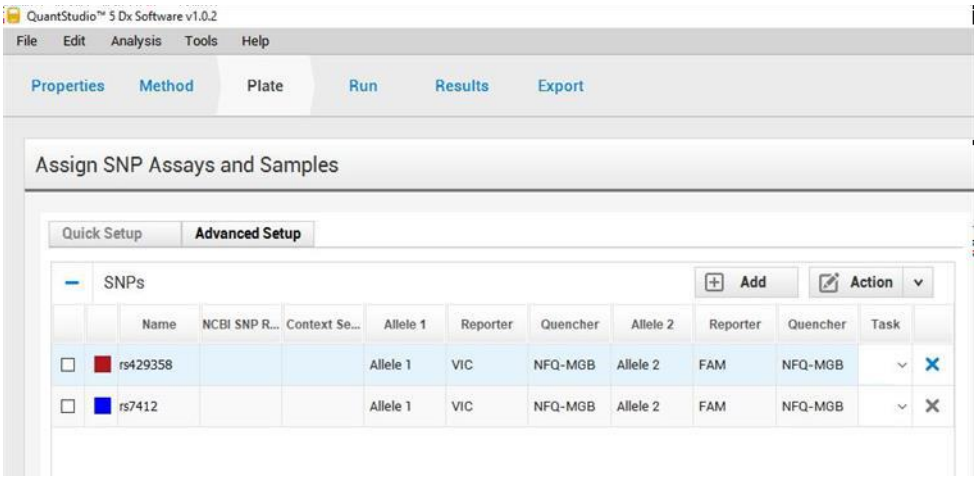

Figure 3. SNP setup for the APO-Easy® Genotyping kit on the QS5 software.

6. To define Standards for rs429358 (Master mix 1), select column 1 in the plate design and select SNP Assay1 (rs429358). Then, select A1 and B1 ("STD B (WT)") and assign 2/2 in the task bar. Select C1 and D1 ("STD C (MUT)") and assign 1/1 in the task bar. Select E1 and F1 ("STD E (HET)") and assign 1/2 in the task bar. Select G1 and H1 and assign N in the task bar "NTC".

7. To define Standards for rs7412 (Master mix 2), select column 7 in the plate design and select SNP Assay2 (rs7412). Then, select A7 and B7 ("STD B (WT)") and assign 1/1 in the task bar. Select C7 and D7 ("STD A (MUT)") and assign 2/2 in the task bar. Select E7 and F7 ("STD D(HET)") and assign 1/2 in the task bar. Select G7 and H7 and assign N in the task bar "NTC".

8. To define Samples for rs429358 (Master mix 1), select columns 2 to 4 in the plate design and select SNP Assay1 (rs429358).

9. To define Samples for rs7412 (Master mix 2), select columns 8 to 10 in the plate design and select SNP Assay2 (rs7412).

10. Once the plate ready and the set up done, go to "Run" sheet and the start the run.

11. The qPCR run program will be automatically select by the QS5-Dx and the program specification should be as indicated in Table 9:

Table 9. qPCR run program in the QS5 device.

Program	Temperature °C	Time	Cycles
Activation	95	10 min	
Denaturation	95	15 sec	40
Extension	60	1 min	

6.3. Step 3. Result analysis

Before the sample result analysis, verify the signal obtained for each standard (STD) with the respective detection assay in allelic discrimination mode. The qPCR run results can be found on the results page of the QS5 Dx software (Figure 4). In this page select Allelic Discrimination (red arrow). The analysis validity should be evaluated for each mutation separately. The selection of rs429358 or rs7412 can be done by clicking on the icon highlighted by the green arrow.

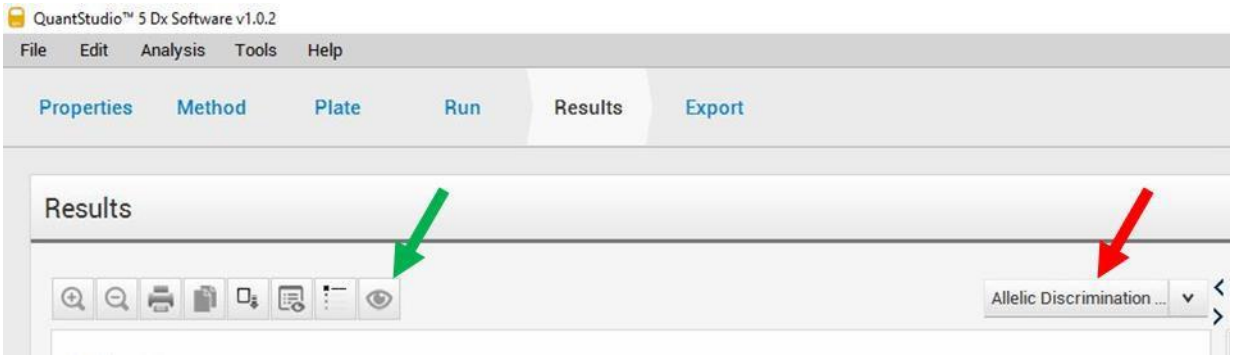

Figure 4. qPCR run results page on the QS5 software. The red arrow indicates the type of result to select (Allelic Discrimination) and the green arrow indicates the icon to select the SNPs.

For the STD and NTC, analysis can proceed when the STD signals correspond to the following example. Make sure your standards have a similar pattern as shown in Figure 5 and Figure 6. For rs429358 the wild-type (WT) will have the signal on the top left region of the graph in the y-axis (allele 2) corresponding to the homozygous allele2/allele2 (e3). The mutant (MUT) standard will have a signal close to the x-axis (allele 1) at bottom right area of the graph corresponding to the allele1/allele1 (e4). The heterozygous standard will be in between MUT and WT corresponding to the heterozygous allele1/allele2 (e3/e4) as shown in Figure 5. NTC will not generate any signal and will be found at the bottom left of the graph.

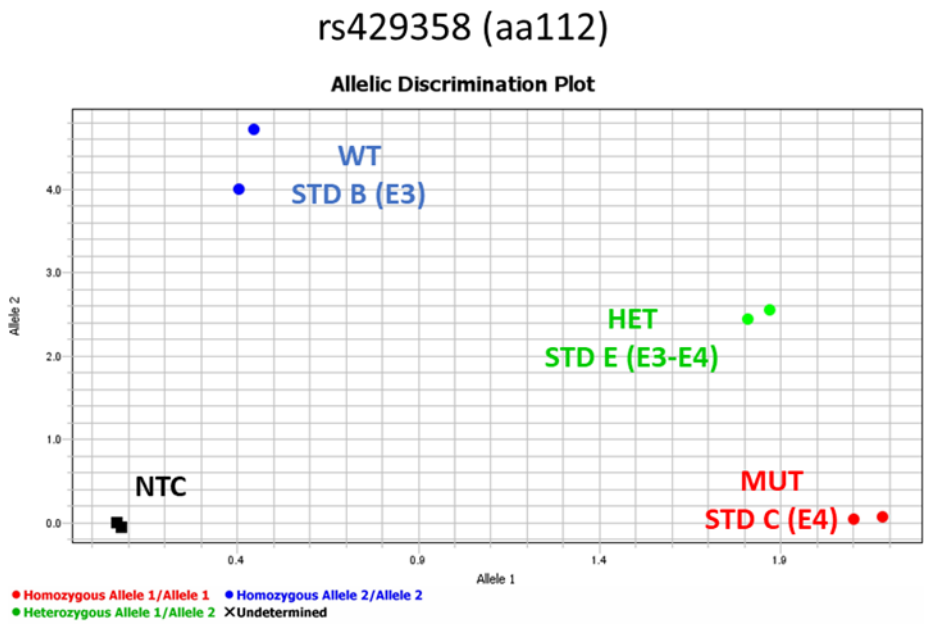

Figure 5. Pattern of the signals of the standards for SNP rs429358 (Master mix 1) after qPCR analysis.

For rs7412 the wild type (WT) will have the signal on the bottom right close to x-axis region of the graph corresponding to the homozygous allele1/allele1 (e3). The mutant (MUT) standard will have a signal to the top left area of the y-axis of the graph corresponding to the allele2/allele2 (e2). The heterozygous standard will be in between MUT and WT corresponding to the heterozygous allele1/allele2 (e2/e3) as shown in Figure 6. NTC will not generate any signal and will be found at the bottom left of the graph.

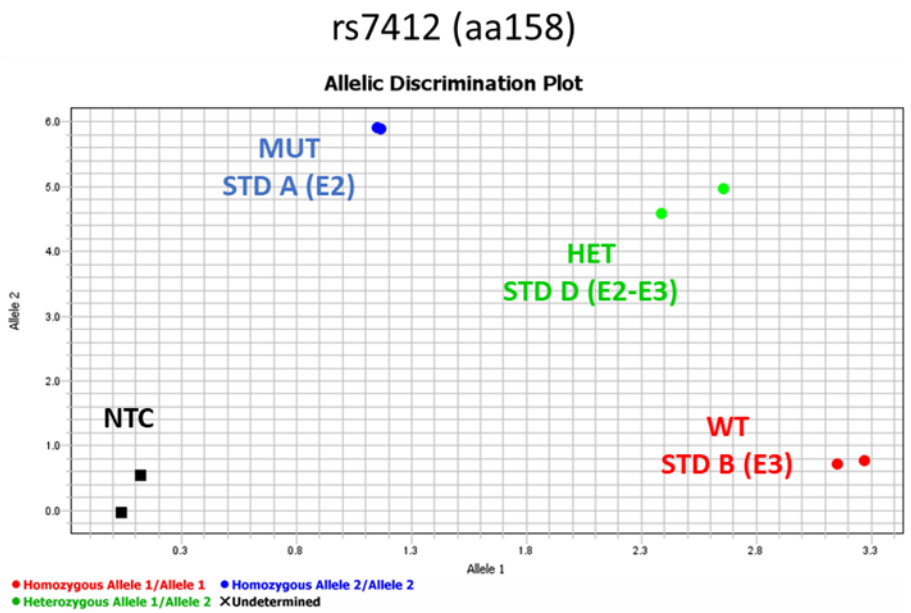

Figure 6. Pattern of the signals of the standards for SNP rs7412 (Master mix 2) after qPCR analysis.

6.4. Step 4. Interpretation

Once the controls are validated, the sample signals are interpreted by clicking on the icon highlighted by the red arrow. For each SNP, patient status is available in the column "Call" (green arrow; Figure 7).

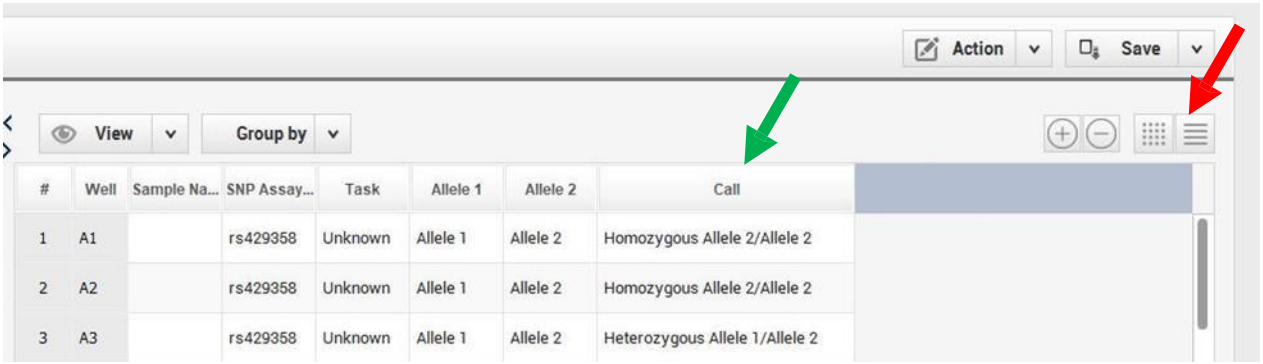

Figure 7. Interpretation of the results on the QS5 software.
The red arrow indicates the icon which interprets the signal, and the green arrow indicates the patient status for each SNP determined by the software after the results analysis.

For rs429358, Homozygous Allele 2 / Allele 2 corresponds to WT, Homozygous Allele 1 / Allele 1 corresponds to MUT and Heterozygous Allele 1 / Allele 2 corresponds to HET.

For rs7412, Homozygous Allele 1 / Allele 1 corresponds to WT, Homozygous Allele 2 / Allele 2 corresponds to MUT and Heterozygous Allele 1 / Allele 2 corresponds to HET.

Once assigned the status for each SNP, the genotype of the sample can be determined using the Table 10 given below.

Table 10. Interpretation of genotype from SNPs rs429358 and rs7412 results.

rs429358	rs7412	Genotype
WT	MUT	e2/e2
WT	HET	e2/e3
WT	WT	e3/e3
HET	HET	e2/e4
MUT	WT	e4/e4
HET	WT	e3/e4

The estimated risk of developing Alzheimer's Disease (i.e., AD) is associated with each genotype, as supported by the clinical information provided in section 9 below and as described in Table 11.

Table 11. The estimated risk of developing Alzheimer's Disease in association with each APOE genotype that may be identified using the APO-Easy® Genotyping kit.

APOE Genotype	Likelihood Ratio	AD Risk Category
ε2/ε2	0.33	Decreased Risk
ε2/ε3	0.39	
ε3/ε3	0.58	Average Risk
ε2/ε4	1.24	Increased Risk
ε3/ε4	1.90	
ε4/ε4	6.679	

7. Troubleshooting

- Assessment of the assay results should be performed after the positive and negative controls have been examined and determined to be valid and acceptable. If the controls are not valid, the Sample results cannot be interpreted.
- If assay failure is encountered:
  - Check the expiration dates of the individual reagents and ensure that all the reagents have been stored as indicated on the product label. If the storage conditions were not strictly followed throughout, the results are not reliable, and the kit cannot be used anymore.
  - Ensure that the calibrations of the pipettes used are up to date.
  - Ensure that the cycler settings are as per the IFU.

Possible reasons and corrective actions for some of the findings during the assay are given in

Table 12 below.

Table 12. APO-Easy® Genotyping kit troubleshooting recommendations.

Findings	Possible reasons and corrective actions
No calls in all channels for a sample	Possibility: problems with the quality of gDNA or interference substances **Recommendation**: extract again the sample and redo the qPCR.
No signal in one or two channels	Possibility: if for only one sample then redo the qPCR; if for all the samples, then check the expiration date of the kit and storage condition.
Undetermined genotype for one SNP	Possibility: the gDNA input was not sufficient. **Recommendation**: Redo the test. During the same assay, for the same sample, use 2µL of sample's gDNA with 23µL of master mix and 4µL of sample's gDNA with 21µL of master mix.
After retest, the genotype is still undetermined	Possibility: presence of an interfering substance in the sample. **Recommendation**: - The addition of K2EDTA in the PAXgene DNA tube might interfere with the sample's genotyping, please check if EDTA were added in the tube or if the volume of blood sample was properly collected according to the manufacturer's instructions for use. - Triglycerides in the blood should not exceed the physiological concentration (normal blood range concentration: 0,5 - 1,5 g/L). If an excess of triglycerides is present in the blood sample, it might interfere with the correct genotyping of the patient. - Albumin should not exceed the physiological concentration (normal blood range concentration: 35 - 50 g/L). If the presence of albumin in the patient's blood sample exceed this concentration, it might interfere with the genotyping results. If the problem persist, contact the technical assistance.
Controls have no signal	Confirm that the storage conditions and instructions given were followed. A pipetting problem might have occurred. **Recommendation**: Redo the test. If you still do not have a signal, contact the technical assistance.
Signal in NTC	Possibility: the reaction mix were most probably contaminated with a template. **Recommendation:** Redo the qPCR. If the problem persists, do not use the kit further and contact the technical assistance.

8. Analytical Validation

Accuracy: the APO-Easy® Genotyping kit is 100% accurate (95%CI: 96.8%-100%) in detecting APOE genotypes when compared to a reference method (Table 13 i.e., Sanger bidirectional sequencing).

Table 13. Comparison of the APO-Easy® Genotyping test with bidirectional Sanger sequencing.

APOE genotype	APO-Easy® Genotyping kit	Sanger sequencing	Correct genotype calls	Accuracy	95%CI
ε2/ε2	10	10	10	100%	(72.3%-100%)
ε2/ε3	21	21	21	100%	(84.5%-100%)
ε2/ε4	17	17	17	100%	(81.6%-100%)
ε3/ε3	25	25	25	100%	(86.7%-100%)
ε3/ε4	23	23	23	100%	(85.7%-100%)
ε4/ε4	21	21	21	100%	(84.5%-100%)
Total	117	117	117	100%	(96.8%-100%)

Limit of Detection: the lowest genomic DNA level at which ≥95% of correct calls are obtained by QuantStudio 5 Dx Real-Time PCR System is 12.5 ng of genomic DNA for each APOE genotype.

Precision: Within-lab precision, lot-to-lot precision, and site-to-site reproducibility of the APO-Easy® Genotyping kit were evaluated with a panel of genomic DNA samples representing all six *APOE* genotypes. The APO-Easy® Genotyping kit is reproducible with 100% agreement results for each *APOE* genotype when testing different lots or when used by different operators. The results of the site-to-site reproducibility, which followed the recommendations contained in CLSI EP05-A3: *Evaluation of Precision of Quantitative Measurement Procedures; Approved Guideline-Third Edition*, are presented in Table 14.

Table 14. Site-to-site reproducibility results.

APOE Genotype	No. of Samples	No. of Replicates	No. of Correct Calls	% of Correct Calls
ε2/ε2	1	75	75	100%
ε2/ε3	2	150	150	100%
ε2/ε4	2	150	150	100%
ε3/ε3	2	150	150	100%
ε3/ε4	2	150	150	100%
ε4/ε4	2	150	150	100%
Total	**11**	**825**	**825**	**100%** **95% CI: (99.5-100%)**

Interferences: The effect of endogenous or exogenous substances that might interfere with the downstream application of the APO-Easy® Genotyping kit was evaluated using a panel of genomic DNA samples representing all six *APOE* genotypes in accordance with the CLSI guideline CLSI EP07-Ed3: *Interference Testing in Clinical Chemistry*. The potential interferants and corresponding solvents were added directly into each EDTA whole blood sample prior to extracting the genomic DNA. The APO-Easy® Genotyping kit may tolerate up to 20% volume/volume ethanol, 200 mg/L of bilirubin (unconjugated), 100 g/L of hemoglobin, 25 g/L of Albumin, 18.2 g/L of triglycerides, or 20mg/mL of

K_2EDTA without interference with the genotyping results.

Cross-reactivity: An In Silico DNA sequence analysis of the APO-Easy® Genotyping kit primers and probes was conducted to determine potential cross-reactivity. The analysis confirmed sequence specificity and the absence of cross reactivity.

9. Clinical Performance

Late-onset Alzheimer's disease is characterized by onset of the disease in people that are 65 years of age or older. Many factors have been suggested to be associated with a decreased or increased risk of developing late-onset Alzheimer's disease, including age, genetic variants, family history of dementia, and lifestyle. One of the well-known genetic determinants that has been strongly associated with decreased or increased risk of developing Alzheimer's disease across various ethnicities in peer-reviewed scientific literature is the *APOE* allele, including the *APOE ε2* and *APOE ε4* allele (Mayeux *et al.*, 1998). The clinical performance of the APO-Easy® Genotyping kit and the association of identified *APOE* alleles with the risk of late-onset Alzheimer's disease is supported by data from the Alzheimer's Disease Neuroimaging Initiative (ADNI), (https://adni.loni.usc.edu/data-samples/adni-data/; Mueller *et al.*, 2005), in addition to peer-reviewed scientific literature (Farrer *et al.,* 1997; Neu *et al.,* 2017).

The presence of two copies of the *APOE* ε4 allele (i.e., *APOE ε4/ε4* homozygotes) was associated with a 6.67 (95% CI: 6.03–7.39)-fold increased risk of developing AD for individuals between 55 and 85 years of age, when compared to the average risk of AD for all *APOE* genotypes. Similarly, individuals between 55 and 85 years of age carrying one *APOE* ε4 allele demonstrated an elevated risk, with *APOE ε3/ε4* heterozygotes showing a 1.90 (95% CI: 1.84-1.96)-fold increase and ε2/ε4 heterozygotes showing a 1.24-fold (95% CI: 1.09–1.96) increase relative to the average risk of AD for all *APOE* genotypes (Table 15).

Table 15. Likelihood ratios for different genotypes (ε2/ε2, ε2/ε3, ε3/ε3, ε2/ε4, ε3/ε4, ε4/ε4).

AD Risk Category	APOE Genotype	AD	Non-AD	Likelihood Ratio	95%CI*
Decreased Risk	ε2/ε2	31	111	0.33	(0.22-0.48)
	ε2/ε3	732	2211	0.39	(0.36-0.42)
Average Risk	ε3/ε3	5889	11841	0.58	(0.57-0.59)
Increased Risk	ε2/ε4	439	413	1.24	(1.09-1.41)
	ε3/ε4	7121	4362	1.90	(1.84-1.96)
	ε4/ε4	2400	419	6.68	(6.03-7.39)
	Total	16612	19357		

To ensure demographic and ethnic diversity in these findings, data from the Alzheimer's Disease Neuroimaging Initiative (ADNI), (Mayeux et al., 1998), were utilized alongside key publications identified through a comprehensive literature review (Farrer *et al.,* 1997; Neu *et al.,* 2017). Results from this analysis are presented in Table 16.

Table 16. Likelihood Ratios Stratified by Race/Ethnicity.

Caucasian					
AD Risk Category	**APOE**	**AD**	**Non-AD**	**Likelihood Ratio**	**95% CI***
Decreased Risk	ε2/ε2	10	53	0.26	(0.13–0.51)
	ε2/ε3	250	904	0.38	(0.33–0.44)
Average Risk	ε3/ε3	1916	4437	0.60	(0.57–0.62)
Increased Risk	ε2/ε4	137	179	1.06	(0.85–1.32)
	ε3/ε4	2177	1604	1.88	(1.78–1.98)
	ε4/ε4	790	135	8.10	(6.78–9.69)
	Total	5280	7312		
Asian					
AD Risk Category	**APOE**	**AD**	**Non-AD**	**Likelihood Ratio**	**95% CI***
Decreased Risk	ε2/ε2	1	8	0.75	(0.12-4.56)
	ε2/ε3	13	143	0.54	(0.31–0.93)
Average Risk	ε3/ε3	167	1522	0.66	(0.58–0.73)
Increased Risk	ε2/ε4	3	16	1.12	(0.35–3.56)
	ε3/ε4	124	312	2.37	(1.99–2.81)
	ε4/ε4	30	17	10.54	(5.92–18.72)
	Total	338	2018		

African Americans					
AD Risk Category	**APOE**	**AD**	**Non-AD**	**Likelihood Ratio**	**95% CI***
Decreased Risk	ε2/ε2	4	3	2.16	(0.54-8.57)
	ε2/ε3	23	65	0.57	(0.37–0.89)
Average Risk	ε3/ε3	86	186	0.75	(0.61–0.91)
Increased Risk	ε2/ε4	5	12	0.68	(0.25–1.81)
	ε3/ε4	94	115	1.33	(1.06–1.65)
	ε4/ε4	31	13	3.87	(2.09–7.18)
	Total	243	394		
Hispanic					
AD Risk Category	**APOE**	**AD**	**Non-AD**	**Likelihood Ratio**	**95% CI***
Decreased Risk	ε2/ε2	1	1	1.26	(0.08–20.01)
	ε2/ε3	25	36	0.87	(0.54–1.41)
Average Risk	ε3/ε3	142	215	0.83	(0.72–0.95)
Increased Risk	ε2/ε4	6	2	3.77	(0.88–16.27)
	ε3/ε4	83	69	1.51	(1.15–1.99)
	ε4/ε4	7	9	0.98	(0.38–2.50)
	Total	264	332		

10. Precautions

The risks of using the APO-Easy® Genotyping kit are minimal if test is used as intended. Users can further minimize risk by following the recommendations below:

Samples Contamination: contamination of samples with extraneous DNA or possible interferents can lead to inaccurate results. The use of clean workspaces, disposable gloves, and dedicated equipment for sample preparation will mitigate this residual risk.

Cross-contamination: improper handling of reagents or samples could result in cross-contamination between samples, leading to erroneous results. Maintain separate work areas for sample preparation, DNA extraction, and PCR setup, and work in a clean environment when handling pipettes and other equipment to prevent cross-contamination.

Results Readout: The interpretation of the APO-Easy® Genotyping assay must be done by highly trained personnel to minimize any risk of incorrect results determination.

Inadequate Training: users with insufficient training or experience may be more prone to errors in assay setup or interpretation. We recommend the use of the APO-Easy® test only to highly qualified personnel.

11. Limitations of the Procedure

- Not an automated method and not an over the counter (self-test) device.
- This assay is designed to detect the APOE genotype from human PAXgene DNA tubes, within the assay detection range.
- Results obtained from this assay must be interpreted within the context of all relevant clinical laboratory findings and are not to be used alone for any diagnosis.
- Contamination of genomic materials from external sources must be avoided by careful handling of the samples, kit reagents, and a clean working environment.
- Operators must avoid microbial contaminants during the procedures and should not use the kit components if evidence of microbial growth is observed.
- This kit is intended to be used with the QS5-Dx Real-Time PCR System (ThermoFisher).
- Strict compliance with the recommendations given in the Assay Kit IFU is essential to obtain optimal results.
- Attention must be paid to expiration dates and storage conditions for each box in the kit. Do not use it if expired or if incorrectly stored.
- Do not mix or substitute reagents with those from other lots or sources.
- Use only certified nuclease-free tips and microcentrifuge tubes.
- Use Nuclease-free water stored in clean containers.
- Any variation in the process described in the IFU can cause variations in the result.
- APO-Easy® Genotyping is not intended to predict or detect response to therapy, or to help select the optimal therapy for patients.

12. Chemical safety guidelines

To minimize the hazards of chemicals:

- Read and understand the Safety Data Sheets (SDSs) provided by the chemical manufacturer before you store, handle, or work with any chemicals or hazardous materials.
- Avoid direct contact with chemicals.
- Wear appropriate personal protective equipment when handling chemicals such as safety glasses, gloves, and protective clothing.
- Avoid the inhalation of chemicals. Do not smoke nor leave the chemical containers open. Use only with adequate ventilation or a fume hood.
- For additional safety guidelines, consult the SDS.
- Check regularly for chemical leaks or spills. If a leak or spill occurs, follow the manufacturer's cleanup procedures as recommended in the SDS.
- Comply with all local, state/provincial, or national laws and regulations related to chemical storage, handling, and disposal.

13. Technical hints

- For technical assistance related to DNA extraction refer to the datasheet provided with the recommended QIAamp DSP DNA blood mini-Kit (ref: 61104) by Qiagen.
- Avoid any contamination among samples and reagents. For this purpose, change tips at each step. Bacterial or fungal contamination in any reagents may cause erroneous results.
- Dispose of consumable materials and unused contents in appropriate containers.
- The procedure is only suitable for use with whole blood from PAXgene DNA tubes.

14. Liability

- This kit is only intended for the *in vitro* determination of APOE genotype in human whole blood from PAXgene DNA tubes.
- This kit is only intended for use by qualified personnel.
- Firalis shall not be responsible for any damage or loss due to using the kit in any way other than as expressly stated in these instructions.
- Firalis is not responsible for any patent infringements that might result from the use or derivation of this product.

Technical assistance

For technical assistance, call Firalis SA Technical Services at +33-389 911 320 or visit the Firalis SA website at http://www.firalis.com or contact "contact@firalis.com".

Serious incident report notice

A serious incident is the cause of any malfunction or deterioration in the characteristics or performance of a device, and can be classed in at least one of the following consequences:

- The death of a patient, user or other person.
- A temporary or permanent serious deterioration in a patient's, user's or other person's state of health.
- A serious public health threat.

In case of doubt, users should always report.

If you conclude that a reportable incident is involved, please contact the manufacturer at clinical@firalis.com. You can also find the contact information of the competent authority in your country.

When reporting a serious incident please compile the following information:

- Trade name of the device (TM for Trade Mark, © for Copyright).
- Name and address of the manufacturer.
- Lot number.
- Serial number.
- UDI (Unique Device Identification) Code.
- An accurate and concise description of the serious incident and the serious or possibly serious consequences for the patient, user or a third party.

The device concerned should not be disposed of but should be made available to the manufacturer for further analysis to determine the causes of the incident. You can add your return shipment request when contacting the manufacturer for reporting the incident.

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Symbols used in the IFU and labels of the kit

IVD	*In vitro* diagnostic device	NON STERILE	Non-sterile medical device
REF	Catalogue number		Do not use if package is damaged
LOT	Batch code		Warning
	Temperature limitations	Σ	Contains sufficient for < n > tests
	Use by date		Toxic or very toxic
	Date of manufacture		Consult operating instructions
	Manufacturer		Do not reuse
	Biological risk	Rx	Prescription use only

Thank you for choosing Firalis

For any further information, MSDS or

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contact@firalis.com