Abstract #2268

A Rapid Method for Identification of *APOE* Alleles ε2, ε3, and ε4 via Electrochemiluminescence

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❶ Abstract

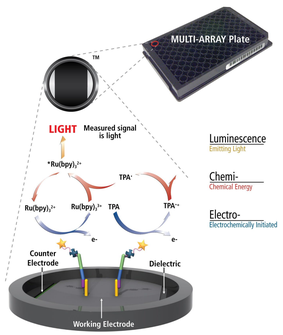
Apolipoprotein E, a lipoprotein encoded by human gene *APOE*, is the principal cholesterol carrier in the brain. Three alleles (ε2, ε3, and ε4) have been identified as risk factors for Alzheimer's disease and are differentiated by single nucleotide polymorphisms (SNPs) at two sites in the gene. A multiplex assay was developed to quickly distinguish between the three alleles as an alternative to sequencing, qPCR, or traditional oligonucleotide ligation assay (OLA) methods.

A single amplicon that spans both *APOE* mutation sites was generated using a fast PCR protocol (runtime ≈50 minutes). For each target, two probes that differ only at the polymorphic base were tagged with capture sequences specific for spots on an N-PLEX plate and paired with a probe that contained a 5' phosphate for ligation and a 3' biotin for detection. These probes were combined for the OLA (initial denaturation and 30 cycles of ligation: total ≈1.5 hours) or a single ligation (initial denaturation and static temperature 15 minutes). The product was transferred to 96-well, 10-spot N-PLEX plates for hybridization of the ligated probes to their corresponding plate-bound oligonucleotides. SULFO-TAG labeled streptavidin was then bound to biotin on ligated probes, and plates were read on an MSD instrument for electrochemiluminescence detection (total optimized assay time ≈3.5 hours).

The OLA and single-ligation methods were able to clearly discriminate single-base changes at both SNP sites in 79 extracted blood samples. Allele frequencies were 6.3% (ε2), 77.5% (ε3), and 16.3% (ε4), similar to previously reported U.S. data. Additionally, the ε4/ε4 genotype, shown to be a significant biomarker related to the development of late-onset Alzheimer's disease, was 1.4%. The genotyping results were confirmed by Sanger sequencing. This optimized protocol shortened total assay time from ≈5.5 hours to ≈3.5 hours, while still allowing for clear base discrimination at both SNP sites.

❷ Methods

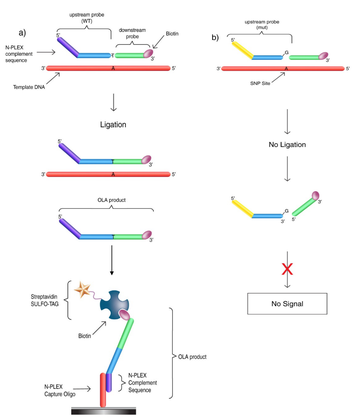
MSD Technology:
MSD's electrochemiluminescence detection technology uses SULFO-TAG™ labels that emit light upon electrochemical stimulation initiated at the electrode surfaces of MULTI-ARRAY® and MULTI-SPOT® microplates.



Electrochemiluminescence Technology

- Minimal non-specific background and strong responses to analyte yield high signal-to-background ratios.
- The stimulation mechanism (electricity) is decoupled from the response (light signal), minimizing matrix interference.
- Only labels bound near the electrode surface are excited, enabling non-washed assays.
- Labels are stable, non-radioactive, and directly conjugated to biological molecules.
- Emission at ~620 nm eliminates problems with color quenching.
- Multiple rounds of label excitation and emission enhance light levels and improve sensitivity.
- Carbon electrode surface has 10X greater binding capacity than polystyrene wells.
- Surface coatings can be customized.

SNP Detection with OLA/Single Ligation and N-PLEX:
The oligonucleotide ligation assay (OLA) and single ligation used probes that are specific for DNA sequences upstream and downstream of the SNP of interest, with only exact matches at the SNP site allowing for the ligation of the two probes. The ligated probes were hybridized to spot-specific capture oligos on the N-PLEX® plates to allow for detection.



SNP Detection

- Each well in an N-PLEX 96-well plate has 10 unique capture oligos attached to the surface of the plate, allowing for the detection of up to 5 SNPs per well.
- Three probes were needed per target: a biotinylated (downstream) probe and two (upstream) probes that recognized either polymorphic base and contain a sequence complementary to a specific capture oligo.
- Probe characteristics: 13-18 nucleotides in length; probe ligation temperature between 64-67°C for a given pair.
- DNA ligase was used to join upstream and downstream probes that aligned correctly on a given DNA sample. Fragments of unmodified template complements were added to prevent bridging of nonligated probes.
- OLA/single-ligation products (from synthetic oligos or PCR products from DNA samples) were hybridized to the appropriate capture oligo on the N-PLEX plate, bound by SULFO-TAG labeled streptavidin, and analyzed using an MSD® instrument.
- Synthetic oligos were obtained from IDT.
- DNA was extracted from HL-60 cells using the PureGene Cell and Tissue Kit.
- Blood samples were obtained from BioIVT, and DNA was extracted using the MagMAX DNA Multi-Sample Ultra 2.0 Kit.
- PCR was conducted with site-specific primers using the MyTaq HS Mix or Platinum II *Taq* Hot Start DNA Polymerase (fast PCR mix), according to manufacturer recommendations.

❸ Selected SNPs

SNP	Base	Allele	Chromosome
rs7412	C	Major	19
	T	Minor	
rs429358	T	Major	
	C	Minor	

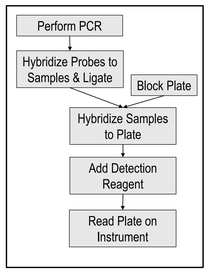
Allele	rs7412	rs429358	Population Allele Frequencies (n=10,623)[1]
ε2	T	T	6.7-10.0%
ε3	C	T	75.3-82.8%
ε4	C	C	7.5-15.6%

Genotype	rs7412	rs429358	Population Genotype Frequencies (n=10,623)[1]	Comment[2]
ε2/ε2	T/T	T/T	0.6%	Lowest risk for AD development
ε2/ε3	C/T	T/T	12.5%	
ε2/ε4	C/T	C/T	2.3%	
ε3/ε3	C/C	T/T	60.7%	Most common genotype
ε3/ε4	C/C	C/T	22.1%	>3x increased risk for Alzheimer's
ε4/ε4	C/C	C/C	1.9%	~12x higher risk for late-onset Alzheimer's

Two SNPs, rs7412 and rs429358, in the *APOE* gene shown to be genetic risk factors of Alzheimer's disease (AD) were chosen for the study.

❹ Results – Assay Design and Optimization

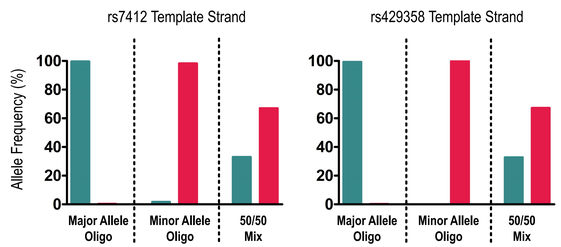
Assay Workflow



	Standard Protocol	Optimized Protocol
PCR	Standard: ≈1 h 45 min.	Fast: ≈50 min.
Probe Hybridization & Ligation	OLA: 1 h 35 min.	Single Ligation: ≈20 min.
Plate Blocking	30 min.	15 min.
Plate Hybridization	1 h	30 min.
Sample Detection	30 min.	15 min.
Plate Reading	≈2 min.	
Total Time	≈5 h 30 min	≈3 h 30 min

Optimized Protocol

1. Perform fast PCR on extracted samples with site-specific primers and DNA polymerase (2 min at 95°C and 35 cycles of 15 sec at 95°C, 15 sec at 60°C, and 15 sec at 72°C).
2. Perform single ligation on PCR product with multiplexed probe sets and DNA ligase (2 min at 95°C and 15 min at 65°C). Block N-PLEX plate for 15 min at 37°C.
3. Wash plate and add ligation product in hybridization buffer (50 µL per well) to plate. Incubate for 30 min at 37°C.
4. Wash plate and add detection solution (50 µL per well). Incubate 15 min at room temperature.
5. Wash plate and add read buffer (150 µL per well). Analyze with MSD instrument.

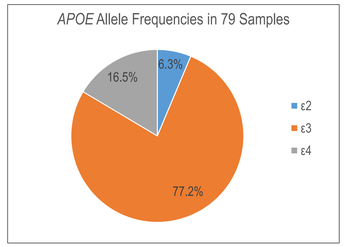


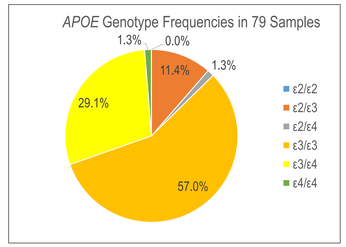
Synthetic oligo templates show specificity for the appropriate allele using the optimized conditions of no ligation cycling and a ligation temperature of 65°C. **Both SNP assays fit within our allele frequency guidelines for calling SNPs: Homozygous ≥80%, Heterozygous =30-70%, Not Present ≤20%.**

❺ Results – Sample Testing

	Allele Frequency % (Major / Minor)				
Sample	rs7412	rs429358	Sample	rs7412	rs429358
1	99.9 / 0.1	88.7 / 11.3	41	99.3 / 0.7	98.7 / 1.3
2	43.9 / 56.1	99.0 / 1.0	42	99.7 / 0.3	98.9 / 1.1
3	99.7 / 0.3	35.8 / 64.2	43	99.6 / 0.4	98.9 / 1.1
4	99.8 / 0.2	98.6 / 1.4	44	44.6 / 55.4	98.9 / 1.1
5	43.7 / 57.3	98.7 / 1.3	45	99.7 / 0.3	98.9 / 1.1
6	44.6 / 55.4	98.9 / 1.1	46	99.4 / 0.6	98.6 / 1.4
7	99.8 / 0.2	37.5 / 62.5	47	99.7 / 0.3	31.2 / 68.8
8	99.6 / 0.4	41.9 / 58.1	48	99.7 / 0.3	31.9 / 61.1
9	99.7 / 0.3	32.4 / 67.6	49	99.7 / 0.3	98.9 / 1.1
10	99.6 / 0.4	98.9 / 1.1	50	99.7 / 0.3	32.0 / 68.0
11	99.8 / 0.2	33.0 / 67.0	51	99.5 / 0.5	98.9 / 1.1
12	99.6 / 0.4	99.0 / 1.0	52	44.2 / 55.8	99.0 / 1.0
13	42.1 / 57.9	37.7 / 62.3	53	99.7 / 0.3	99.0 / 1.0
14	99.8 / 0.2	98.8 / 1.2	54	99.7 / 0.3	99.0 / 1.0
15	99.7 / 0.3	32.8 / 67.2	55	99.7 / 0.3	99.0 / 1.0
16	99.7 / 0.3	99.0 / 1.0	56	99.6 / 0.4	99.0 / 1.0
17	99.8 / 0.2	99.1 / 0.9	57	98.5 / 1.5	32.9 / 67.1
18	99.8 / 0.2	98.8 / 1.2	58	99.8 / 0.2	99.0 / 1.0
19	99.8 / 0.2	98.8 / 1.2	59	99.7 / 0.3	99.0 / 1.0
20	99.6 / 0.4	98.8 / 1.2	60	99.7 / 0.3	33.3 / 66.7
21	99.6 / 0.4	98.8 / 1.2	61	99.7 / 0.3	99.0 / 1.0
22	99.7 / 0.3	98.8 / 1.2	62	44.1 / 55.9	98.9 / 1.1
23	99.5 / 0.5	35.7 / 64.3	63	99.7 / 0.3	99.0 / 1.0
24	99.8 / 0.2	99.0 / 1.0	64	99.3 / 0.7	98.5 / 1.5
25	99.6 / 0.4	34.2 / 65.8	65	99.7 / 0.3	98.9 / 1.1
26	43.5 / 56.5	98.5 / 1.5	66	99.8 / 0.2	98.9 / 1.1
27	99.6 / 0.4	99.0 / 1.0	67	99.7 / 0.3	98.9 / 1.1
28	99.7 / 0.3	98.8 / 1.2	68	87.5 / 12.5	82.9 / 17.1
29	99.7 / 0.3	32.3 / 67.7	69	99.5 / 0.5	98.8 / 1.2
30	99.8 / 0.2	98.9 / 1.1	70	99.7 / 0.3	32.3 / 67.7
31	99.8 / 0.2	98.9 / 1.1	71	99.6 / 0.4	32.3 / 67.7
32	99.7 / 0.3	34.1 / 65.9	72	99.6 / 0.4	98.8 / 1.2
33	99.7 / 0.3	0.1 / 99.9	73	99.7 / 0.3	98.9 / 1.1
34	99.6 / 0.4	34.4 / 65.6	74	99.7 / 0.3	99.0 / 1.0
35	99.7 / 0.3	33.3 / 66.7	75	99.6 / 0.4	31.5 / 68.5
36	99.7 / 0.3	32.7 / 67.3	76	55.5 / 44.5	99.2 / 0.8
37	99.8 / 0.2	98.9 / 1.1	77	99.0 / 1.0	99.2 / 0.8
38	99.7 / 0.3	36.7 / 63.3	78	99.6 / 0.4	99.2 / 0.8
39	99.6 / 0.4	98.9 / 1.1	79	99.5 / 0.5	38.3 / 61.7
40	43.3 / 56.7	98.5 / 1.5	HL-60	99.9 / 0.1	99.1 / 0.9

Major
Heterozygous
Minor





Genomic DNA was extracted from HL-60 cells and 79 human whole blood samples and amplified with site-specific primers via PCR. The optimized protocol was used to run a single ligation and fast N-PLEX on the two target SNPs in multiplex.

The *APOE* allele and genotype frequencies in the sample set are similar to those reported from population studies in 11 countries. The majority of the samples (45/79) have the ε3/ε3 genotype. There is one sample with ε4/ε4 (major alleles for rs7412 and minor alleles for rs429358). None of the 79 samples has the rarest (ε2/ε2) genotype. All samples fit in our allele frequency guidelines for SNP calling.

❻ Results – Genotype Confirmation via Sanger Sequencing

SNP	Allele	Number of Samples	Percentage of Samples	Matches Sequencing?
rs7412	Major	69	87.3	✓
	Minor	0	0.0	✓
	Heterozygous	10	12.7	✓
rs429358	Major	54	68.4	✓
	Minor	1	1.3	✓
	Heterozygous	24	30.4	✓

APOE PCR reactions for all 79 samples were loaded and run on 2% agarose gels. The desired fragment was excised and purified using the QIAquick Gel Extraction Kit. The purified PCR products, along with the forward primer, were used for Sanger sequencing.

There was 100% concordance in genotyping results between N-PLEX and Sanger sequencing for *APOE* rs7412 and rs429358.

❼ Conclusions

This report highlights a novel method to quickly identify *APOE* genotypes. The assay improves sample throughput, making this a viable alternative to sequencing and other methods. Lastly, this method can be easily adapted to target SNPs or insertions/deletions that are identified as risk factors for other diseases.

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Citation

1. McKay GJ, et, al. Variations in apolipoprotein E frequency with age in a pooled analysis of a large group of older people. Am J Epidemiol. 2011 Jun 15;173(12):1357-64. doi: 10.1093/aje/kwr015. Epub 2011 Apr 15. PMID: 21498624; PMCID: PMC3145394.
2. Corder EH, Saunders AM, Strittmatter WJ, et al. Gene dose of apolipoprotein E type 4 allele and the risk of Alzheimer's disease in late onset families. *Science*. 1993;261(5123):921-923. doi:10.1126/science.8346443.

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