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Review article
Pregledni članak

MADGE – MICROPLATE ARRAY DIAGONAL GEL ELECTROPHORESIS

ELEKTROFOREZA U FORMATU MIKROTITARSKE PLOČE

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Summary: Microplate array diagonal gel electrophoresis (MADGE) was invented for molecular genetic epidemiological studies. MADGE is a highly flexible, cost effective, microplate compatible solution to high throughput electrophoresis needs. It enables several thousands to million gel lines per day for direct assay of single-base variation in different capacity laboratories. Variants of the standard 96-well MADGE include: 96-well stretch-MADGE, 192-well MADGE, 384-well MADGE, and 768-well MADGE. Melt-MADGE combines the temporal thermal ramp apparatus to achieve similar throughput for de novo mutation scanning. Basic MADGE principles and procedures, preparation of MADGE gels, electrophoresis, visualization and analysis of these gels, as well as modifications of the basic 96-well MADGE will be discussed in detail. For the first time in our country, this revolutionary polyacrylamid electrophoresis was done in 1998. We shortly review our studies which used MADGE for high throughput genotyping of the apolipoprotein E. MADGE and melt-MADGE will have an important role in the future genetic research of complex diseases and especially in pharmacogenomics.

Keywords: electrophoresis, genetic epidemiology, mutation detection

Kratak sadržaj: »Microplate array diagonal gel electrophoresis« (MADGE) primenjuje se u molekularno genetskim epidemiološkim studijama. MADGE je fleksibilna, jeftina metoda, kompatibilna sa formatom standardnih mikrotitarskih ploča, kojom se može vršiti elektroforeza nekoliko hiljada-miliona uzoraka dnevno kako u malim tako i u velikim laboratorijama. Postoji nekoliko modifikacija standardne MADGE metode sa 96 bunara i to su »stretch« MADGE, kao i MADGE metode u kojima se koristi veći broj bunara (192, 384 i 768). »Melt«-MADGE koristi se za utvrđivanje novih mutacija. U ovom radu biće detaljno prikazani osnovni principi MADGE, priprema gela, elektroforeza, vizuelizacija i analiza tih gelova, kao i modifikacije standardne MADGE metode sa 96 bunara. Ovaj revolucionarni pristup poliakrilamidnoj elektroforezi prvi put je u našoj zemlji primenjen 1998. godine. U radu će ukratko biti prikazani rezultati studija u kojima je za genotipizaciju apolipoproteina E korišćen MADGE. Treba očekivati da će MADGE i »melt«-MADGE imati značajnu ulogu u genetici poligenskih bolesti, posebno u farmakogenomici.

Ključne reči: elektroforeza, genetska epidemiologija, detekcija mutacija

Introduction

Strong evidence from epidemiological and animal studies has implicated genetic influences in the pathogenesis of multifactorial diseases: hypertension, diabetes, ischemic heart disease and cerebrovascular disease. Our knowledge of the three billion base pairs in the human genome could be used to alert patients that they are at risk for certain disease, precisely diagnose disease, and ensure the most effective treatment tailored to patient's genotype is used and develop new treatments at the molecular level. A major challenge for large-scale studies is to compare hundreds of thousands of polymorphisms among numerous individuals. They also depend on user-friendly technologies that can detect polymorphisms rapidly, accurately, cost effectively in both small and large laboratories.

For molecular-genetic epidemiological research, it is important to think about one important step – electrophoresis. It enables ready analysis of size, shape, and charge of molecules. Electrophoresis is also well known and feasible to set up in any laboratory, requiring no expensive hardware. However, gel preparation, long tracks (requiring more cumbersome

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equipment and longer run times), incompatibility with standard microplates, vertical-format polyacrylamide gels (restricting the number of analyses per gel since only one row of samples can be loaded), make electrophoresis an unattractive, but frequently used method in laboratory studies of genetic diversity within populations.

Electrophoresis of DNA is traditionally performed either in an improved-quality agarose capable of higher resolution or a polyacrylamide gel matrix. Polyacrylamide still offers the highest resolution for small fragments such as those from PCR and post-PCR digests. Agarose gels can easily be prepared in an open-faced format (uncovered, with one or more well-forming combs inserted in the molten substrate) to gain the convenience of horizontal electrophoresis. Acrylamide does not polymerize in the presence of air and the usual configuration is vertical between two glass plates.

Microplate array diagonal gel electrophoresis

Microplate array diagonal gel electrophoresis (MADGE) was invented by Day in 1994 (1) to enable compatibility between microplate-based liquid phase reactions and polyacrylamide or agarose-gel electrophoretic analysis. This format can be used in optimisation of PCR using gradient thermocyclers. The two-dimensional nature of the electrophoresis system allows the optimal combinations of $MgCl_2$ and temperature to be visualized and compared. MADGE gels are particularly useful in genotyping reactions using restriction fragment linked polymorphism (RFLP) or with amplification-refractory mutation system (ARMS) reaction.

The MADGE gel system is based on a standard 96-well microplate array format with a 9 mm well to well distance and 2 mm cubic wells. The array is set on a diagonal of 71.6° giving a final track length of 26.5 mm (2). MADGE gels can be made using two plain glass plates and plastic 'former' with 96 2-mm cubic »teeth.«

Preparation of MADGE gel

Preparation of MADGE gel consists of:

a) coating of one surface of the plain glass plate (160×100×2 mm) with 1 mL mixture of 99% ethanol, 0.5% glacial acetic acid, and 0.5% v/v γ-methacryloxypropyltrimethoxysilane; it is essential for the polyacrilamide gel to adhere firmly to the glass.

b) preparing the acrylamide gel mix (10×TBE (Tris base, boric acid, 0.5 mol/L EDTA (pH 8.0), deionized water), 30% acrylamide-bisacrylamid (19:1), distilled water, 25% ammonium persulfate and TEMED). Effective separation range (bp) for different acrylamide content is: for 5% 80-500bp, 7.5% 70–400bp, 10% 50–300bp.

c) pouring the gel mix. It could be done in two different ways. The first is to pour the gel mix onto MADGE gel former, and then place the glass plate, silanized side down, onto former. The second is to place the glass plate over the former, leaving a small gap next to one of the broad borders. Hold the plates together with one hand and tip the plates to a slight incline so that the gap is at the top end. Carefully pour the gel mix into the gap so that it runs down in between the two plates and fills the mould up from the bottom. A 100–250 g weight can be placed centrally on the glass to ensure uniform gel thickness. Once the MADGE gel has set (approximately 15 minutes), take a spatula and place under the edge of the glass, where there is a 2 mm gap. The glass plate with firmly attached gel can then be prized off. Gels on glass could be stored wrapped in cling film in buffer at 4 °C for few weeks (3).

Agarose MADGE using hydrophilic plastic backing rather than glass is also feasible but only for examination of larger DNA fragments (over 1 kbp).

Loading and electrophoresis of MADGE gels

The next step is to immerse gel horizontally in the electrophoresis tank or wet the wells with gel buffer and use wicks or direct electrode contacts across the ends of the 'dry' (not submerged) gel. Acrylamide gels are anchored on their glass plate and the bases of the wells are formed by the glass. Well position A1 is placed nearest to the cathode. Samples must be prepared as follows: 5 μL PCR sample to 2 μL loading dye (deionized formamide, 0.5 mol/L EDTA (pH 8.0), xylene cyanol, bromophenol blue, sterile water). This system allows rapid loading with a multi-channel pipette or 96-pin passive replicator under appropriate light source. Typical running conditions would be 10 V cm^{-1} for 30 min in 7.5% polyacrylamide, 1×TBE for a 200-bp PCR product, but conditions can be adapted according to experimental needs (3).

Visualization and analysis of MADGE gels

Gels are usually prestained with ethidium bromide at a concentration of 1 μg/mL by gently agitating gels on a rotary shaker for 30 min. The small-scale gels can be readily read manually and show reasonable resolution, to more than 20 bands per track. Because little UV pass through the glass, it is necessary to place gel-side down on the transilluminator. The use of digital imaging systems (standard CCD camera or a flatbed scanner) and use of suitable software which can take TIFF files enables the

identification of all gel tracks and facilitates analysis. Also, use of dyes such as Vistra Green is more sensitive than ethidium bromide, allowing detection of less than 20 pg (3).

Variants of the standard 96-well MADGE

Later, the basic MADGE system was adopted to analyze PCR reactions set up as higher density arrays, 192-well MADGE (*Figure 1 B*), 384-well MADGE (*Figure 1 C*) and 768-well MADGE (*Figure 1 D*).

The 192-well MADGE system is designed to allow two arrays of 96 samples to be run on the same gel. This is used for genotyping by ARMS, with one reaction for each allele. The same angle of electrophoresis as 96-well MADGE is used (71.6°), the same well size 2 mm × 2 mm × 2 mm, but track lengths are 11 mm. For computer analysis of 192-well MADGE gels, the two allele-specific tracks are treated as one line (4).

The 384-well gel can therefore be used to handle four 96-well arrays of samples, or two arrays for an ARMS assay. The 384-well MADGE system is used in conjunction with 384-well PCR for very high throughput genotyping. 384-well gels are loaded with a 96-pin passive replicator. The angle 78.7° and the well size 1.5 mm × 1.5 mm × 1.5 mm are used, allowing four arrays of 96 samples to be run in tracks that are 1 mm wide by 11 mm long. The gel is prestained by soaking for 15–20 min in 1×TBE with 1 μg/mL

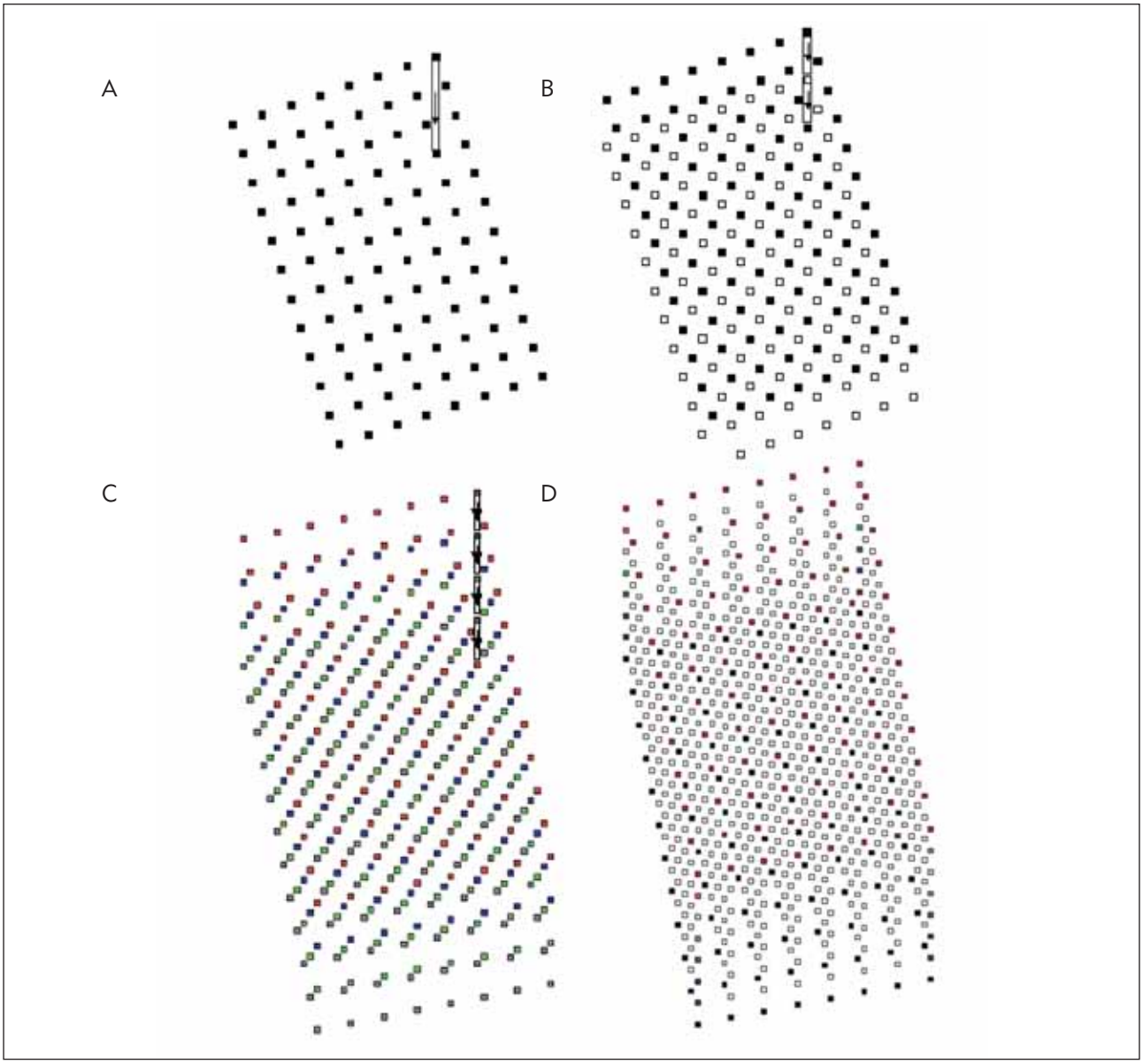

Figure 1 Scheme of a MADGE gel image. A. 96-well MADGE, B. 192-well MADGE, C. 384-well MADGE (10), D. 768-well MADGE (6).

ethidium bromide. Sample buffer (5 μL) is added to each well and gel is placed on a colored grid to aid sample loading (2 μL sample form PCR plate) using a 96-slot pin replicator. Four arrays are loaded on one gel. MADGE-384 gels are loaded dry and voltage applied directly across the gel for 8 to 10 minutes at 150 V. MADGE-384 gels have to be analyzed using digital imaging systems (5).

Day et al. (6) reconfigured the MADGE format and associated procedures and made a 768 well-MADGE. A design was adopted which uses an 11.3° angle between the 12 well rows of each intercalated 96 well component array and the direction of electrophoresis. Track width in this 768 well format is 1.5 mm corresponding with width of the wells, gel thickness is 1.5 mm and track length is 4.24 mm. Using this method it is possible to run 1 500 000 gel tracks per day with minimal hardware investment and cost reduction for gel-based approaches to PCR checking and SNP genotyping.

Chen et al. (7) modified the standard MADGE protocol and made a high resolution MADGE (stretch-MADGE) for accurate and precise analysis of DNA fragments. Stretch-MADGE can be used to resolve products that differ in size 2%, and can be used in the analysis of minisatellite and microsatellite markers. This system uses a 78.7° angle of rotation with narrower 1.5 mm square wells to extend the track length to 43.5 mm. Duraacryl™ in combination with TAE was used for preparing the gel. Each loaded sample is composed of 1 μL MADGE dye, 1 μL 4× TAE buffer, 1 μL of an appropriate dilution of the reference markers and 1 μL PCR reaction. After loading the gel, glass cover slides carefully onto the gel and it is put in the temperature-controlled MADGE electrophoresis tank in 1×TAE buffer under empirically determined conditions. The gel stains with Vistra Green R®, which gives a tenfold sensitivity increase over ethidium bromide. The dye is diluted 1: 10 000 in 1× TAE buffer just before use. Using the scanning system and Microsoft Excell®, it is possible to calculate the size of the bands in bp. It is necessary to include internal sizing markers with every sample.

Temporal thermal ramp electrophoresis MADGE (Melt-MADGE)

Day et al. (8, 9) described temporal-thermal-ramp electrophoresis MADGE (melt MADGE), in which the analysis of duplex melting provides a method of de novo mutation scanning. Using a purpose-built temporal-thermal-ramp-electrophoresis apparatus for programmable melting display (PMD) (melt-MADGE), a DGGE-like analysis was undertaken in MADGE format. A single band indicates no base variation, whereas band splitting during electrophoresis (resolving up to four bands–two homoduplexes and two heteroduplexes) indicates heterozygosity; several different patterns representing different mutations are evident in the gel. The run time is 1 h and ten MADGE gels can be analyzed simultaneously in one 2 L tank. In the absence of partial melting, each PCR product appears as the same sized single band on MADGE.

MADGE in population-scale analysis for gene polymorphisms: the apolipoprotein E gene as an example

The apolipoprotein (apo) *E* gene is one of the most widely studied genes in vascular and neurodegenerative diseases. Its protein product, apoE, is a glycoprotein with 3 common isoforms, E2, E3, and E4, encoded by the alleles *E2*, *E3*, and *E4*, giving rise to 6 genotypes, with the genotype E3/3 occurring in about one half to two thirds of people in most populations. The molecular basis of apoE polymorphism is cysteine-arginine interchange. The protein plays a major role in lipid transport and metabolism and is also significantly expressed in brain. Individuals with an *E4* allele are at higher risk for ischemic heart disease, ischemic stroke, Alzheimer's disease and other neurodegenerative disorders, and E2/2 homozygotes for the type III hyperlipoproteinemia (12–16).

We performed apoE genotyping by restriction isotyping of the amplified apoE gene sequence and MADGE analysis in different patient groups.

The frist study included a random subset of 622 healthy subjects (422 men and 200 women, mean age 39.7±13.5 years), of different professions, social and family history. The other study included 96 elderly Serb (mean age 80 years): 32 patients with late-onset Alzheimer's disease (AD), 32 patients with vascular dementia (VD) and 32 age- and gender-matched controls. The third study included 72 patients with ischemic stroke (mean age 41.7±13.1 years) and age and BMI-matched controls.

Informed consent was obtained from each participant in these studies; all procedures were in accordance with the Helsinki Declaration of 1975, revised in 1983. Fasting peripheral blood samples were collected in sodium citrate-containing tubes.

DNA preparation

DNA was extracted by Triton X-100 lysis, proteinase K digestion, and phenol/chloroform extraction (17). When the procedure could not be carried out within 2–4 days after blood collection, the blood was frozen at –20 °C and DNA was extracted within the following 4–8 weeks. The concentration of isolated DNA was determined by a spectrophotometer (LKB-Pharmacia, Uppsala, Sweden).

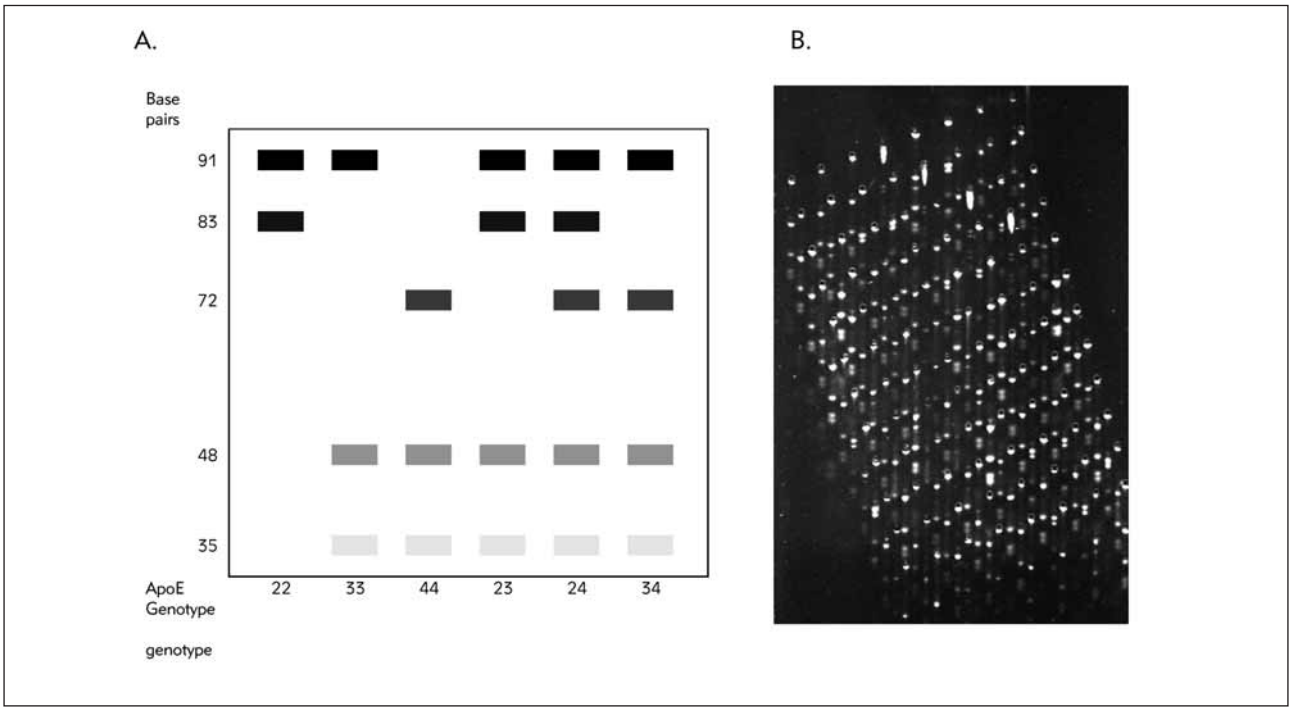

Figure 2 ApoE genotyping by using HhaI digests in combination with MADGE. A. Schematic identifyng six tracks, each representing one of the six possible genotypes. B. View of full 96 well MADGE gel of a set of HhaI from PCR.

DNA amplification and restriction isotyping

A 244 bp sequence of the apoE gene including the two polymorphic sites was amplified by PCR in a Hybaid Omnigene thermocycler (Teddington, UK) using the oligonucleotide primer pair F4 and F6 described by Emi et al. (18). The reaction mixture contained the following reagents: 0.125 mmol/L each of dNTP, 100 mL/L dimethylsulfoxide, 20 pmol of each primer, 1.5 mmol/L $MgCl_2$, 0.5–1 mg DNA, Nonidet P40 in a final volume of 50 mL. Each reaction mixture was heated at 94 °C for 2 min and subjected to 35 cycles of amplification (94 °C for 40 s, 62 °C for 30 s, and 72 °C for 1 min) (19). ApoE alleles were identified by restriction fragment analysis. Ten units of *Hha*I were added to PCR products and incubated at 37 °C at least 3h.

MADGE imaging and analysis

After digestion, 3 μL of each sample was added to 1.5 μL of formamide dye mix (formamide, 0.5 mol/L EDTA, and 0.25 g/L each of bromphenol blue and xylene cyanole), and 4.5 mL of each mix was loaded into the wells of a 12 g/L MADGE gel. Gel was prestained in a solution of 10 mL ethidium bromide in 100 mL of 1xTris-borate-EDTA, pH 8.3 for 10 min in an electrophoresis tank. A DNA ladder was also loaded into one well of the gel. Electrophoresis was performed at 10 V/cm at room temperature for 45 min, and then we observed the gel with the use of an ultraviolet transilluminator and acquired a digital image of the gel with a CCD camera and frame grabber (GS8000 Documentation System, UVP Inc, USA). ApoE genotypes were determined by comparison with a combination of fragment sizes described elsewhere.

ApoE allele and genotype frequencies in the Serbian population

Figure 2 shows apoE genotyping MADGE of *Hha* I digests of apoE gene PCR products.

Using an RFLP/MADGE method for the determination of apoE allele and genotype frequencies in the healthy Serbian population and different patient groups we obtained the following results:

The relative frequencies of the common apoE genotypes in healthy Serbian subjects were: E2/2 3.8, E2/3 18.8, E2/4 3.4, E3/3 55.5, E3/4 17.5, and E4/4 1.0. The most frequent allele was E3 (0.736 relative frequency), the next common E2 (0.149 relative frequency), followed by E4 (0.115 relative frequency). ApoE allele frequencies in the Serbian population studied were within Hardy-Weinberg equilibrium ($\chi_5^2 = 11.5$).

Among patients who had AD, there was a statistically significant excess of the E4 allele, compared to non-demented subjects ($\chi^2 = 4.48$, $P=0.034$). No association between the distribution of apoE2 allele and dementia status could be detected in this study ($P<0.01$) when the relative E2 frequ-

encies of the AD cases were compared with the frequency of non-demented persons. There was no statistically significant difference in the apoE allele frequencies between patients with VD and controls ($P>0.05$).

Compared with persons without the E4 allele, carriers of an E4 allele had 2.8 (95% CI 1.61–4.91) times higher risk of incident stroke.

Conclusion

MADGE has an important role in molecular-genetic epidemiological studies. As an electrophoretic equivalent of the microtiter plate, this short-track electrophoresis enables a throughput of up to several thousands to million gel lines per day for the direct assay of single base variation, rapid interpretation of results and good resolution of bands and also very important fact-cost reduction. It is also robust and convenient for handling because gels are attached to the glass and palm sized; gels are directly stackable for storage and electrophoresis and the gels can be reused. MADGE and its modifications are suitable for both, small and large laboratories. We believe that this method will have an important role in the future genetic research of complex diseases and pharmacogenomics as a practical and economically viable option.

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