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SNP Genotyping by Combination of 192-Well MADGE, ARMS and Computerized Gel Image Analysis

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ABSTRACT

A new modification of the microplate array diagonal gel electrophoresis (MADGE) system accommodates the dual amplification refractory mutation system (ARMSTM) products of 96 samples on one 192-well gel. Simultaneous electrophoresis of a number of horizontal ARMS-MADGE gels achieves high throughput. Gels are imaged digitally, here using the FluorImager[®] 595 fluorescent scanning system. Customised software by PhoretixTM enables rapid computerized calling of band patterns in ARMS-MADGE arrays, in which the two wells receiving a pair of allele-specific assays for a single template are juxtaposed to form one virtual track, with genotype data exported directly into Microsoft[®] Excel[®] for statistical analysis. An ARMS assay of the A/T base change at the -23/HphI RFLP in the insulin gene promoter, which initiates from 2.5 ng template DNA, was used here to demonstrate this improved general approach for population SNP analyses.

INTRODUCTION

Single-nucleotide polymorphisms (SNPs) are the most frequent category of sequence variation in the genome (1). In ultra high-throughput SNP genotyping, extreme miniaturization permits high densities of analysis, but it is likely that capital investments in sophisticated equipment will be necessary. By contrast, in medium-throughput applications, more easily distributed approaches suited to laboratories with specific projects focused on single genes and regions of interest, with minimal capital expense and running costs, will also be attractive.

For many SNPs, RFLP analysis is not an option because there is no suitable restriction enzyme recognition sequence or, as in the case of the *INS* -23/*HphI* RFLP (3), the cost of enzyme is very high. We have devised a dual-reaction amplification refractory mutation system (ARMSTM) (7) assay to genotype the A/T base change at the *INS* -23/*HphI* site.

In this paper, we describe a new modification of the microplate array diagonal gel electrophoresis (MADGE) (2) system that accommodates the dual ARMS products of 96 samples on one 192-well gel. In the new design, a second 8 × 12 array consists of wells positioned half way down each 24.5-mm track of the first array. Hence, the two ARMS products undergo electrophoresis in pairs of vertically aligned tracks, each with half the track length available in the standard gel (Figure 1). We have collaborated with Phoretix International (Newcastle upon Tyne, UK) in the design of a software package for the analysis of electrophoresis bands in lanes arrayed in the double MADGE 8 × 12 format. Clicking on selected lanes allows automatic designation of genotype and export of data into a Microsoft[®] Excel[®] spreadsheet for data analysis. Taken together these innovations represent a general approach to SNP typing that requires only microplate PCR, a simple gel system, a standard digital image system and gel image analysis software. This methodology supersedes 96-well MADGE, PCR-RFLP analysis and manual data calling; it is permissive of several thousand SNP calls (several SNPs in thou-

sands of subjects from large DNA banks) per technician per day.

MATERIALS AND METHODS

Design of Illustrative Dual-Reaction ARMS Assays

The *INS* -23/*HphI* polymorphic site is situated between nucleotides 4460 and 4464 on GenBank[®] sequence L15440 (3). The rare allele (non-cutting) sequence TCTCC is present in GenBank; the transition T to A creates the common allele (cutting) sequence TCACC. Primers with destabilizing mismatches were designed to selectively amplify the alleles containing either A or T. The ARMS primers were designed according to Little (5) and Kwok et al. (4).

The allele-specific forward primers (priming off the antisense strand) were respectively 5'-CTTCAGCCTGCCTC-AGCCCTGCCTGTGA-3' and 5'-CTTCAGCCTGCCTCAGCCCTGCCTGT-*TT*-3' where the italicized base is the destabilizing mismatch with target, and the base in bold type matches the specific allele. The common reverse primer (priming off the sense strand) was 5'-GGCGGGTCTTGGGTGTGTAGAAGAAGCC-3', giving an ARMS product of 216 bp. An internal PCR co-amplification control was used, as suggested by Little (5). Primers were forward AATF: 5'CCCCACCTTCCCCTCTCTCCAGGCAAATGGG-3' and reverse AATR: 5'-GGGCCTCAGTCCCAACATGGCTAAGAGGGTG-3', representing a 360-bp region in the human α_1 -antitrypsin (AAT) gene.

Primer concentrations of 8 pmol each ARMS and control primer were used in each 10- μ L reaction, which contained 1.2 mM MgCl₂, 50 mM KCl, 10 mM Tris, pH 8.3, 0.01% gelatin, 200 μ M each dNTP and 0.2 U *Taq* DNA polymerase (Life Technologies, Paisley, UK). Approximately 2.5 ng DNA were dried into 96-well Omniplates (Hybaid, Teddington, UK) before adding the PCR mixture. The PCR cycling conditions must be capable of amplification of both ARMS and control reactions. PCR amplification (1 cycle at 94°C for 6 min; 35 cycles at 94°C for 1 min, 60°C for 1 min, 72°C for 1

Protocol

1. Gel image acquisition: An image of the ARMS-MADGE gel is digitized by FluorImager scanning using a photomultiplier tube (PMT) voltage setting of 800 V with a 514-nm excitation filter and a 610 RG long-pass emission filter. The image is saved as a 16-bit tagged image file format (TIFF) file with a ".gel" file-name extension. [Digital images from a standard charge-coupled device (CCD) camera are also suitable for this application.]
2. Import into analysis software: The gel image is imported into Phoretix 1D Advanced image analysis software (Phoretix International), running on Microsoft Windows® NT, Microsoft Windows 95 or 98 by selecting the New Experiment option, then selecting the file name of the gel.
3. Analysis of MADGE gels: Analysis is supported within the software by selecting the MADGE gel option from the Analysis menu. For the purposes of ARMS-MADGE, the two allele-specific lanes for each sample are treated as one, resulting in 96 double (concatenated) lanes per 192-well gel.
4. Lane creation: There are three steps to creating lanes:
 - (1) Locating wells: From the Analysis menu, select the Create Lanes option. Set columns at 12, rows at 8 and lane width at 10. Use the mouse to drag the 8 × 12 grid from the center of well A1(i) to the center of well H12(ii) (where "i" represents the first 96-well array) (Figure 1). The grid can be repositioned if necessary by dragging the corners with the mouse.
 - (2) Lane selection: Select the lane tool (second button on the Lane Creation toolbar), then use the mouse to drag a rectangle from the top of lane A1(i) to the bottom of lane A1(ii) (where "ii" represents the second 96-well array). The software will then create lanes for the other 95 wells on the grid (Figure 2). Individual lanes can be adjusted using tools on the Lane Creation toolbar.
 - (3) Lane naming: Select the Automatic Naming option, then click on the Naming Options button. This allows the naming scheme set-up for the array, using letters or numbers in ascending or descending order for both columns and rows. These settings can be stored by selecting the Save Template option from the File menu. Clicking the Accept button completes the operation.
5. Allele calling: select the Matching option on the Analysis menu. This generates an image of the 96 double lanes in a single row (Figure 3). Select the MADGE Lane Classification option from the Matching toolbar and in the left-hand window assign allele calls to the numbers on the numeric keypad (Figure 3). Then, select the first lane with the mouse. Allele calling is now performed by pressing the button on the numeric keypad that corresponds to the genotype in the highlighted double lane.
6. Data export: Select the Match table button from the toolbar at the bottom and then click on the MADGE tab. The resulting table can be exported by selecting Copy Current Window option from the Edit menu, then pasting into a spreadsheet. A Copy Current Window to Excel option is also available for direct export.

min and 72°C for 10 min) was in 96-well Omniplates.

Genotyping by 192-Well ARMS-MADGE

A standard MADGE gel (2) is an open-faced polyacrylamide gel with well spacing that preserves the 8 × 12 array and pitch of the industry-standard microplate, in tracks following a 71.6° diagonal between wells, giving a maximum available track length of 24.5 mm.

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Figure 1. ARMS-MADGE arrays: locating wells for lane creation. The two 96-well arrays are set on a diagonal of 71.6° to the perpendicular. As electrophoresis proceeds from top to bottom of the gel, tracks extend on the 24.5-mm diagonal between four columns and two rows of wells, each track with a maximum track length availability of 12.25 mm. The 8×12 grid is positioned with lines intersecting over the first array of 96 wells carrying the common A allele-specific ARMS products from each sample before selecting the lanes. Vertically below each intersection, in the center of each grid square in the next row and at a distance 12.25 mm away, is the well carrying the corresponding rare T allele-specific ARMS product. These form the second array of 96 wells in the ARMS-MADGE format. The direction of electrophoresis is vertical from top to bottom of the gel, with maximum track lengths of 12.25 mm available to each well.

Figure 2. ARMS-MADGE arrays: lane selection for analysis of two allele-specific ARMS products. Lanes of equal width to the wells are drawn vertically from the intersection of grid lines over the first array of 96 wells carrying the A allele-specific ARMS products. The gridlines disappear after creation of the lanes. The well carrying the corresponding T allele-specific product is located 12.25 mm down each lane, and the lane is made to terminate at the maximum track length available just before the next well on the grid diagonal. Each well carrying A allele-specific product is flagged with the PCR microarray coordinate of the sample.

In the ARMS-MADGE modification, a second 8×12 array consists of wells positioned half-way down each 24.5-mm track. The two ARMS products undergo electrophoresis in pairs of vertically aligned tracks, each with half the track length available in the standard gel (Figure 1). A track length of 12.25 mm is sufficient to distinguish the 360-bp control product and the presence or absence of allele-specific 216-bp products on a 5% polyacrylamide gel (Severn Biotech, Kidderminster, UK). The gels were prestained with ethidium bro-

mid (10 mg/mL stock solution diluted 1:10000 in TBE buffer) (6) for 10 min. A volume of 5 μ L PCR product was mixed with 2 μ L formamide dye mixture (6), and 5 μ L were then loaded onto the gel by a multichannel pipet. A set of 96 A allele-specific samples was loaded in the first array, followed by the corresponding set of 96 T allele-specific samples in the second array of wells. Electrophoresis was in TBE buffer at 150 V for 25 min.

Gels were examined using a Fluor-Imager[®] 595 fluorescent scanning sys-

tem (Amersham Pharmacia Biotech, Piscataway, NJ, USA). Excess buffer was removed before scanning. The images were viewed using ImageQuant[™] fragment analysis software (Amersham Pharmacia Biotech). Normal sensitivity scan mode and 100- μ m resolution scan settings were used in these experiments.

RESULTS AND DISCUSSION

The digitized image of an ARMS-MADGE gel containing two sets of 96

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samples is shown in Figure 1. The arrangement of the two 96-well arrays carrying the allele-specific products is explained in the legend. Accurate positioning of the 8×12 grid over the first array of 96 wells carrying the common A allele-specific ARMS products defines the selection of the lanes for analysis. When lanes of equal width to the wells are drawn vertically from the first array of wells carrying the A allele-specific product, the well carrying the corresponding T allele-specific product is located 12.25 mm below, with each lane terminating just before the next well on the grid diagonal. This well carries an A allele-specific product and is sited four columns and one row away in the microplate array (e.g., A1 and B4 at opposite ends of the A1 track) (Figure 2). Figure 3 shows six tracks with numbering that corresponds to the microplate array coordinates flagged in Figure 2, selected from the

Automatic Naming option. The Matching option on the analysis menu generates an image of 96 double lanes in a single row, the A allele-specific tracks above and the T allele-specific tracks below. The wells carrying T allele-specific products appear half-way (12.25 mm) down each lane. Genotype is determined by the presence or absence of an allele-specific 216-bp band in all tracks in which the control 360-bp product has amplified successfully. Genotype AA has a band in the A allele-specific track but not in the T allele-specific track, while genotype AT has bands in both tracks and genotype TT has a band only in the T-specific track. As described in Materials and Methods, allele calling for subsequent statistical analysis is numeric, so that in Figure 3, the common A allele is designated 1 and the rare T allele is 2.

This ARMS procedure reliably genotypes the A/T transition at the *INS*

-23/*HphI* site, verified by comparison with results using a method based on PCR and *HphI* digestion, for which it provides a less costly alternative. Simple liquid phase analyses can economically assay many samples simultaneously and are convenient to set up. In MADGE (2), the open-faced horizontal polyacrylamide gel provides a matrix with a high density of wells for sample loading and analysis by electrophoresis in short track lengths. The 8×12 configuration of wells in the standard microplate is preserved, and gels are of sufficient size to enable personal handling and visualization. A number of gels can be set up simultaneously, for example, separate tanks with submerged gels or semi-dry gels with wicks, or multiple gels loaded and stacked in a rack for submersion in a single tank. We have stacked up to six 192-well gels in a 1.2-L electrophoresis tank, handling nearly 1200 ARMS re-

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actions in a 25-min electrophoresis run. One technician can therefore generate electrophoretic data for 600 genotypes per hour with due allowance made for gel loading and set-up time. The rate at which genotypes can be determined is limited by the PCR capacity and the time taken for image analysis, rather than electrophoresis.

MADGE (2) was invented to serve a need in human molecular genetic epidemiological studies for higher-throughput, microplate-compatible electrophoresis. For the examination of genetic influences on common complex diseases, it is often necessary to examine DNA banks of hundreds or thousands of families or individuals by association using SNPs. The development of a format of 192-well MADGE, accommodating separate allele-specific reactions, in combination with newly developed software for gel image analysis, is suitable

ed to detailed examination of candidate gene regions (multiple SNPs, perhaps 10–1000) within a single project. By continuing to use assays based on electrophoresis, a variety of well-established analytical molecular genetic methodologies such as ARMS retain their utility. This contrasts with higher-density set-ups such as microscope slide-based arrays and chips that demand sophisticated equipment.

Although a FluorImager was used here, a standard CCD camera would be sufficient for this MADGE application, so there is minimal capital expense. The approach is thus amenable to “distributability” and to centralized scale-up.

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Combining Enhanced Metabolic Labeling with Immunoblotting to Detect Interactions of Endogenous Cellular Proteins

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ABSTRACT

Metabolic labeling, immunoblotting and two-dimensional isoelectric focusing/SDS-PAGE are powerful techniques for characterizing endogenously expressed cellular proteins and their interactions. We achieved improved resolution and sensitivity for the detection of metabolically labeled proteins separated on two-dimensional gels by electroblotting the proteins onto polyvinylidene difluoride or nitrocellulose membranes and detecting the ³⁵S signal on a bio-image analyzer. We obtained independent detection of specific proteins from the same blot by subsequent rehydration of the membrane and immunoblot analysis. The combination of these enhanced detection techniques with immunoprecipitation and two-dimensional electrophoresis on precast minigels provides a simple, sensitive method for detecting interactions between endo-

Figure 3. ARMS-MADGE arrays: allele calling. The tracks numbered 54 to 59 correspond to microarray coordinates C7, B7, A7, G8, F8 and E8, flagged in Figure 2. The image of 96 double lanes in a single row is generated from the arrayed lanes selected in Figure 2. The A allele-specific tracks are above and the T allele-specific tracks are below. Wells carrying the T allele-specific products appear half way (12.25 mm) down each lane. Genotypes are designated by the presence or absence of an allele-specific band, provided that the slower migrating control PCR band is also present. Allele calling is numeric, the common A allele is 1 and the rare T allele is 2. PCR failure in the T allele-specific ARMS reaction prevents a genotype call in track 59.