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# High-resolution MADGE

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## 1. Introduction

The previous two articles in this series (Ref. 1, 2) have shown how microplate-array diagonal-gel electrophoresis (MADGE) has facilitated standard genotyping at low cost and high throughput. In this article, we describe how the standard MADGE protocol can be modified to analyse DNA fragments at high resolution, extending MADGE technology to variable number tandem repeat (VNTR) and microsatellite genotyping.

Commercial genetic analysis systems are available that can be used very effectively for VNTR analysis [e.g. ABI Prism™ Hardware with Genescan ™ software (http://www.pebio.com/ab/about/dna/genotyping/GeneScan/)] but such systems are costly. The use of fluorescent detection and analysis using a Fluorimager® (http://www.mdyn.com/products/FluorImager/) offers the potential for more economical analysis (Ref. 3). However, to obtain a high resolution, PCR fragments must be run out on long gels, which are not realistic for high-throughput analysis.

We have now modified the MADGE protocol so that it can be used to resolve products that differ in size by only 2%. The MADGE system can now be used in the analysis of both minisatellite and microsatellite markers, thus extending low-cost, high-throughput genotyping to these analyses.

Some of our VNTR genotyping has been carried out successfully using our conventional MADGE system (Ref. 4), in which tracks follow a 71.6° diagonal between wells, giving a maximum available track length of 24 mm (Ref. 1). However, for microsatellite markers such as tetranucleotide repeats, these tracks are a little too short to resolve the different alleles. The basic MADGE gel former has thus been modified so that the wells are laid out diagonally at an angle of 78.7°, extending the track length to 43.5 mm [stretch MADGE system, MadgeBio (Cat. no. M0031)]. Samples can therefore be run further, considerably improving the resolving power.

By careful attention to detail, it is possible to run relatively uniform high-resolution MADGE gels. However, it is not possible to eliminate intra- and intergel variability completely, and running size markers at the side of the gel will not provide sufficient accuracy to estimate the size of VNTR alleles. To overcome between-track electrophoretic differences, and to guarantee accurate allele calling, it is necessary to include internal sizing markers with every sample. A bracketing pair of molecular weight markers that are fairly similar in size to the amplicon of interest can be used to estimate allele size.

Size-estimation calculations (see below) assume a log–linear relationship between size (in bp) and mobility, and so the calculated allele sizes might not quite be those determined by sequencing. Our aim here is not to provide definitive size estimation but rather to achieve high precision of estimation, in order to allow accurate classification of allele type (correct allele 'binning'). Through focusing on high intertrack precision, we have shown that the correct binning of alleles can be achieved simply and conveniently using short-track electrophoresis.

## 2. Protocol for high-resolution MADGE analysis

### 2.1. PCR

It is essential that the PCR reaction is rigorously optimized (see below) and, ideally, the reaction should standardized as described in Ref. 1. If there is also a restriction enzyme digest step, it is preferable to keep the reaction volume as small as possible.

### 2.2. Gel system

Some degree of experimentation is required to determine which type of gel system will give optimal PCR-product resolution. The standard MADGE format can be used, but stretch MADGE will give better resolution. The most effective range of separation should also be determined by trying different percentages of gel matrix. Table 1 lists the composition of a range of gel mixes we use when

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**Table 1. Composition of different gel systems**

| Gel matrix | Effective matrix concentration (%) | Volume of stock solution (ml) | 10× TAE (ml) | 10× TBE (ml) | $H_2O$ (ml) |
|---|---|---|---|---|---|
| Acrylamide | 3.5 | 5.8 | – | 5.0 | 40.2 |
| | 5.0 | 8.3 | – | 5.0 | 36.7 |
| | 5.0 | 8.3 | 5.0 | – | 36.7 |
| | 7.5 | 12.5 | – | 5.0 | 32.5 |
| | 10.0 | 16.7 | 5.0 | – | 28.3 |
| | 15.0 | 25 | 5.0 | – | 20.0 |
| Duracryl™ | 3.0 | 3.8 | 5.0 | – | 41.2 |
| | 3.5 | 4.4 | 5.0 | – | 40.6 |
| | 5.0 | 6.3 | 5.0 | – | 38.7 |
| | 5.0 | 6.3 | – | 5.0 | 38.7 |
| | 7.5 | 9.4 | 5.0 | – | 35.6 |
| | 7.5 | 9.4 | – | 5.0 | 35.6 |
| | 10.0 | 12.5 | 5.0 | – | 32.5 |
| | 15.0 | 18.8 | 5.0 | – | 26.2 |
| Page-plus | 5.0 | 6.3 | 5.0 | – | 38.7 |

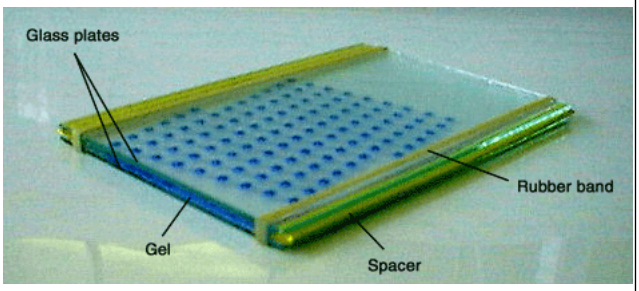


**Fig. 1.** High-resolution microplate-array diagonal-gel electrophoresis (MADGE) gel. Sandwiching the MADGE gel between two glass plates and sealing the sides of the gel with a rubber spacer ensures that the gel runs evenly.

optimizing effective range of separation. Standard acrylamide can be used but we have found that a Duracryl™–TAE combination consistently gives the best resolution.

Gels are prepared as described in Ref. 1. When the gel has set, soak it for 1 h in 100 ml of 5 mM KCl in 1× TAE buffer, changing the salt buffer once.

### 2.3. *Loading the MADGE gel*

Each loaded sample is composed of 1 $\mu$l MADGE dye (Ref. 1), 1 $\mu$l 4× TAE buffer, 1 $\mu$l of an appropriate dilution of the reference markers and 1 $\mu$l PCR reaction.

1. For one 96-well MADGE plate, prepare sufficient loading mix for all of the samples (100 $\mu$l MADGE dye, 100 $\mu$l 4× TAE buffer, 100 $\mu$l of the diluted reference markers). Split the mix between eight wells of a 96-well plate.

**Fig. 2.** MADGE gel electrophoresis rack. In our set-up, the microplate-array diagonal-gel electrophoresis gel plate sandwiches are horizontally stacked in a rack so that up to 12 gels can be placed in the electrophoresis tank for each run.

2. Use an eight-channel pipette to transfer 3 $\mu$l of the mix into each well of a second 96-well plate, one row at a time. Transfer 1 $\mu$l of the PCR product from the reaction plate to the loading plate, maintaining equivalent array number.

**Fig. 3.** Temperature-controlled MADGE electrophoresis tank (INGENY phorU system). The loaded rack is carefully lowered into the MADGE electrophoresis tank. Air bubbles should be removed from both ends of the gel-plate sandwiches by slightly tilting the rack. The leads from the lid of the tank are connected to the anode and cathode on the rack. Once the cover is shut, the power can be turned on.

3. Transfer the 4 $\mu$l of PCR mix, one row at a time, from the loading plate to the MADGE gel, maintaining the array order. In between rows, rinse the tips out in fresh 1× TAE buffer and blot the rinsed tips with paper tissue.
4. After loading the gel, slide a glass cover [part of stretch MADGE kit or separately from MadgeBio (Cat. no. MA0012)] carefully onto the gel. It is important to make sure that there are no air bubbles between the gel and the glass cover plate.
5. Seal up both sides of the gel–plate sandwich with seal spacers, leaving the anode and cathode ends unsealed. Secure the plates with two longitudinally placed rubber bands (Fig. 1).
6. In our set-up, several loaded gels can be stacked in the tank (Fig. 2).
7. Lower the gel sandwich rack into the electrophoresis tank (Fig. 3). The tank contains 1× TAE buffer pre-equilibrated for temperature. Electrophoresis voltage, temperature and time will need to be determined empirically; we have used 150–200 V, 30–40°C and 90–120 min.

### *2.4. Gel staining and band visualization*

For high-resolution analysis, we routinely use the stain Vistra Green®, which gives a tenfold sensitivity increase over ethidium bromide. The dye is diluted 1: 10 000 in 1× TAE buffer just before use.

1. After electrophoresis, detach the glass cover plate and stain the gel in 100 ml of stain by gentle rocking for 30 min.

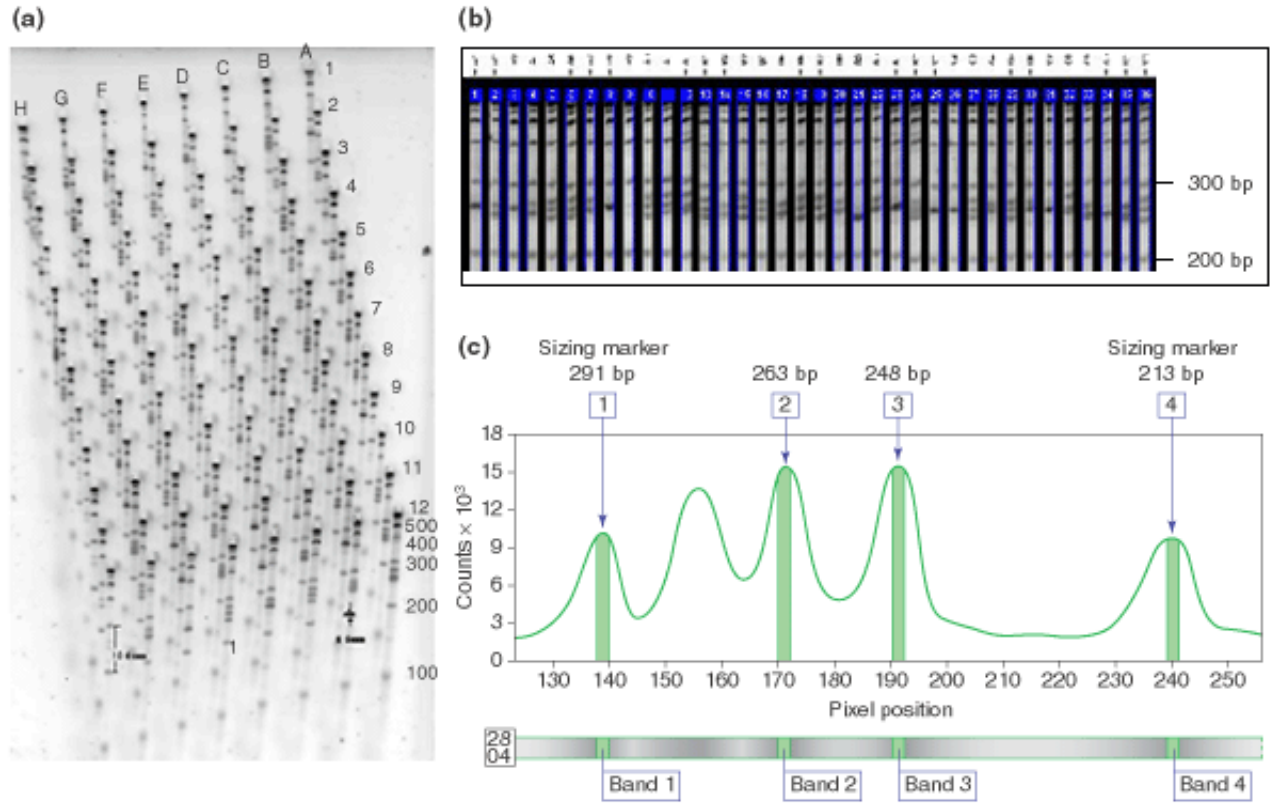


**Fig. 4.** (a) Original 96-well stretch microplate-array diagonal-gel electrophoresis (MADGE) image. In stretch MADGE, the wells are laid out diagonally at a larger angle than in the conventional MADGE gel, extending the track length. (b) Phoretix™ software single-row presentation of the 96 MADGE lanes. This layout illustrates the power of MADGE to resolve the different genotypes clearly. (c) Peak intensity profile produced by the Phoretix software. Bands are indicated with arrows; algorithms are then used to assign sizes to the unknown bands. This example shows NPHS II N8 with TH tetra markers and size markers of 213 bp and 291 bp.

2. Destain the gel for 5 min in deionized water.
3. View the wet gel using the Fluorimager® 595 Fluorescent Scanning system.

### 2.5. *Computer analysis of the MADGE gel digital image*

Image analysis software (Phoretix™) is available to allow direct analysis of MADGE gels (http://www.phoretix.com/1d.htm).

1. Digitize the image (Fig. 4a), import the image into the Phoretix analysis software and create lanes as described in Ref. 1.
2. Select 'Detect Bands' from the 'Analysis' menu. This generates an image of all the 96 lanes in a single row (Fig. 4b). The software can analyse all the bands in every track, presenting them as a profile of peak intensities with bands being indicated by arrow marks (Fig. 4c). The distance from the well migrated by each detected band is displayed. Manual selection (mouse button click on peak) can be used to remove irrelevant marker bands if a commercial sizing ladder is being run in the lane or if there are any background noise peaks. The remaining measurements are then the distances run by the two markers and the distance(s) run by the unknown band(s).
3. The data can be then be transferred to a Microsoft Excel® spreadsheet by selecting 'Save current Window to Excel' from Edit in the menu bar. Macros can then be set up to calculate the sizes of the bands in base pairs using Eq. (1), where $U$ is the size of the unknown allele in bp, $x$ is the molecular weight of the smaller marker, $y$ is the molecular weight of the larger marker, $d_x$ is the distance between the $x$ and $y$ bands, and $d_U$ is the distance between the unknown allele and the larger marker. Macros can also be used to calculate allele or genotype frequency, and for homoduplex analysis.

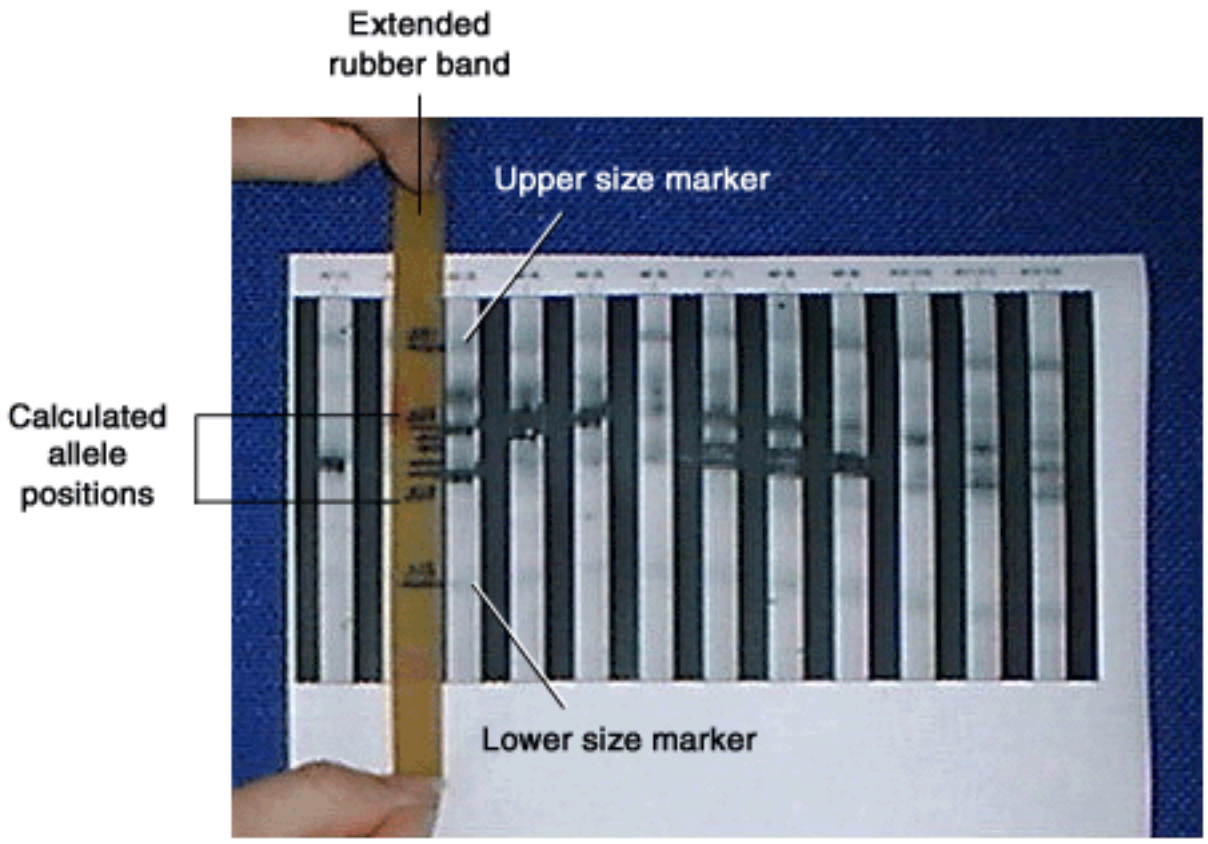


**Fig. 5.** 'Rubber-band' size estimation. An appropriately calibrated rubber band can be used very successfully to calculate the sizes of unknown alleles.

$$U = \frac{x}{\left[\frac{x}{y}\right]^{\frac{d_x - d_U}{d_x}}} \qquad (0.1)$$

### 2.6. *'Rubber band' size estimation*

Should computerized allele calling not be an option, allele size can be estimated by use of a simple calibrated rubber band placed on a printed track image (Fig. 5). Allele size controls the velocity of migration in a log–linear manner. As the distance travelled by an allele band of a given size is proportional to its velocity and the stretching of the rubber band is proportional to the force applied to it, the distance stretched by the rubber band at a given point is a valid representation of the allele size.

Calibration points are fixed by marking the two markers $x$ and $y$ from the gel image on the unstretched band and measuring the distance between them ($d_x$ in Eq. (1)). Then, for each allele of $U$ bp, $d_U$ can be calculated from the formula. The position of the alleles between the $x$ and $y$ reference points can be marked on the rubber band. Placing the band against the printed image of each track then allows direct reading of allele sizes. The band can be stretched if necessary to accommodate any intertrack variation in migration, so that the reference $x$ and $y$ bands can be accurately positioned over the image. Several calibrated rubber bands can be prepared to accommodate a range of standard marker migration distances.

### 2.7. *Classification of allele type*

In high-throughput genotyping, it is very difficult to ensure complete PCR standardization and to eliminate gel-to-gel variability. Thus, in common with other genotyping methods, high-resolution MADGE generates a range of DNA fragments. When many individuals are being investigated, the sized alleles do not fall into discrete groups according to genetic predisposition. Instead, data points tend to cluster in allele groupings, with variability around each allele.

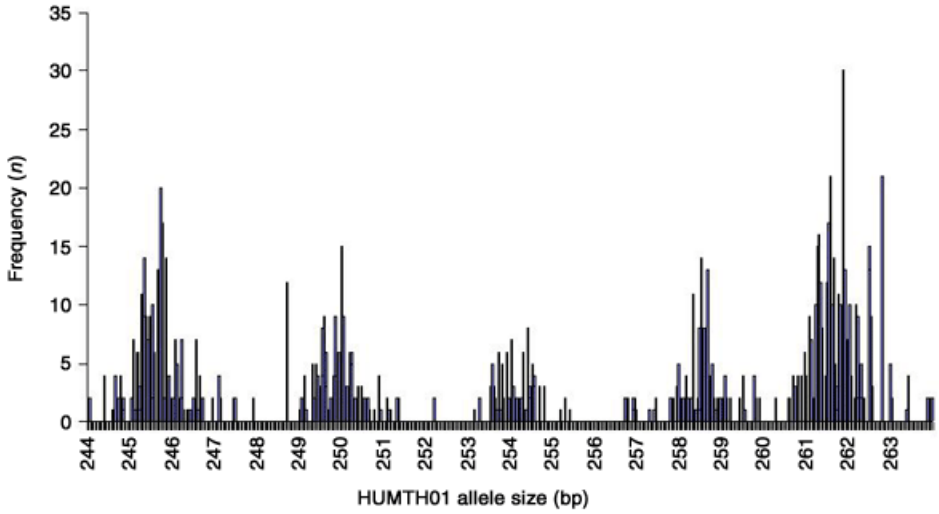


**Fig. 6.** 'Binning' of allele type. High-resolution microplate-array diagonal-gel electrophoresis (MADGE) usually allows the clear assignment of bands to the closest expected allele size.

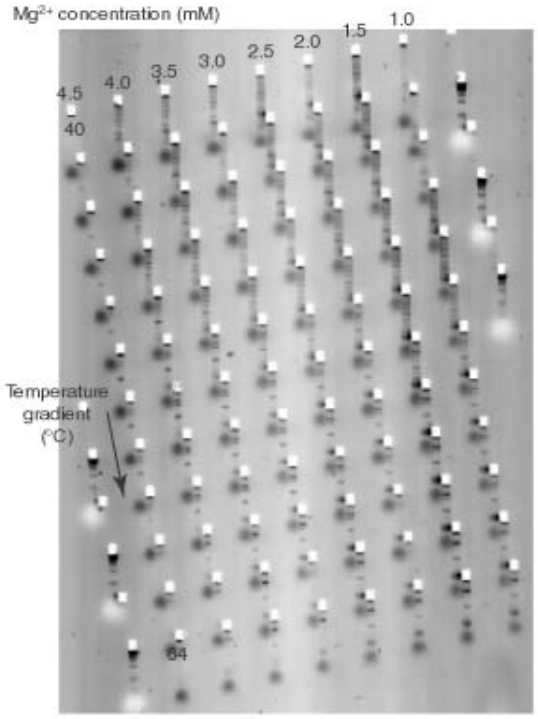


**Fig. 7.** PCR optimization for microplate-array diagonal-gel electrophoresis (MADGE). In this example, a tetraprimer PCR reaction with three products is simultaneously optimized with respect to $Mg^{2+}$ concentration and a range of annealing temperatures using a Tetrad gradient block (MJ Research).

With very few exceptions, computed allele sizes fall within two standard deviations of the ascribed value (Fig. 6). Bands do not normally fall mid-way between the respective allele groups and can be assigned to the closest expected allele size. At the moment, this assessment must be done manually but it is hoped that software will in the future allow fully automated peak identification.

## 3. Factors important for high resolution of similarly sized DNA fragments

Successful use of MADGE for high-resolution discrimination of DNA fragments is dictated by a number of factors. It is sensible to bear the following points in mind when applying the MADGE set up to a new assay.

### *3.1. PCR reaction optimization*

MADGE gels have relatively short tracks, with bands bunched together rather more than in a conventional long gel. It is very important to optimize PCR reactions so that nonspecific amplification is reduced to an absolute minimum and there are no bands contributing to the track background. Many thermal cyclers come with a temperature gradient block so that thorough PCR optimization can be accomplished with relative ease (Fig. 7).

### *3.2. PCR product loading*

It is important to load an appropriate amount of PCR product onto the MADGE gel. Enough must be loaded to visualize reaction products, but the bands run sharper and straighter with less product. Loading too little sample causes

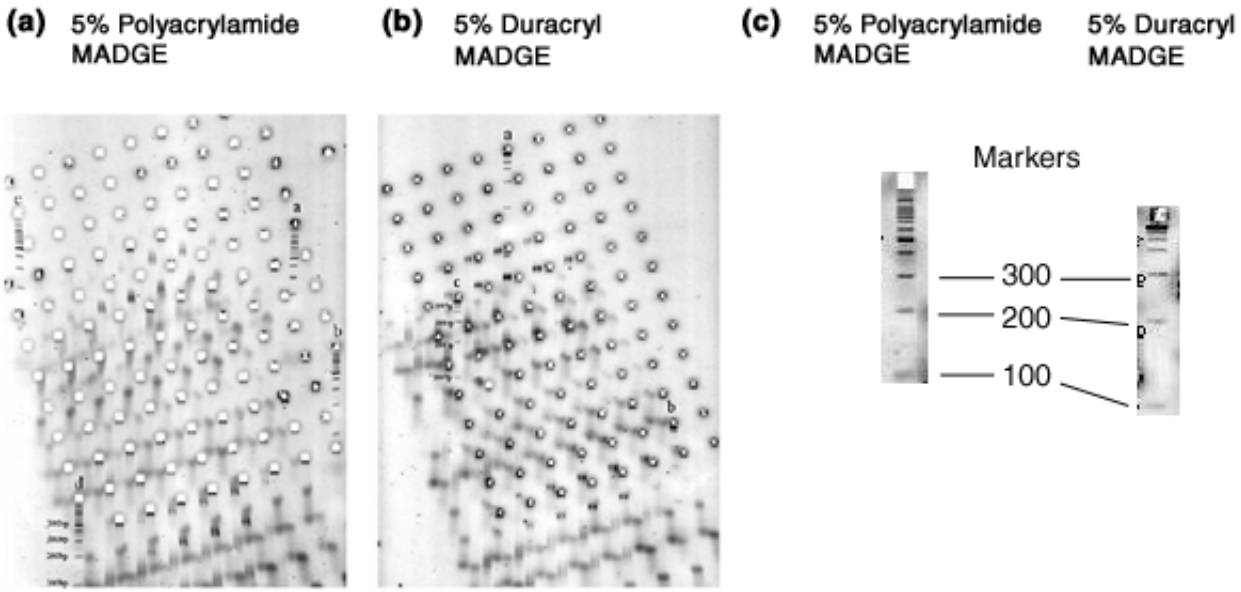


**Fig. 8.** Resolution of DNA ladder markers with (a) polyacrylamide and (b) Duracryl™. 100 bp DNA ladder markers have been loaded in opposite corners and electrophoresed on 5.0% polyacrylamide microplate-array diagonal-gel electrophoresis (MADGE) gel or a 5% Duracryl MADGE gel. (c) The superior resolving power of Duracryl for 100–300 bp fragments.

the tracks and bands to narrow. For us, 4 $\mu$l of loading mix gave the highest quality banding. Testing a range of different sample buffers showed that the conventional MADGE loading dye (Ref. 1) gave the sharpest bands.

The ionic strength of the product buffer mix is different to that of the gel and this difference can cause smearing within the tracks and 'skiing' of the sample to either side of the track, and can also cause bands to distort to dumbbell shapes (Ref. 5). Such electrophoresis artefacts compromise resolution and might lead to the miscalling of genotypes.

Should these artefacts be encountered, they can usually be eliminated by a closer matching of the ionic strength of the sample buffer and electrophoresis buffer. Delaying the start of electrophoresis to allow ionic diffusion and to reduce the ionic imbalance between sample and gel also aids high resolution. With a standard PCR reaction, we have found that a combination of PCR product, MADGE loading dye and 10× TAE in the ratio 5:1.5:1.5 (vol:vol:vol) along with a 5 min delay to the start of electrophoresis gives considerable improvements in band quality (Ref.5).

### 3.3. *Temperature control*

Band patterns can be distorted within MADGE gels through uneven heating during electrophoresis. This amperometric heating effect can be prevented by running at a low voltage or using direct thermal control. We use electrophoresis tanks that can be thermostatically controlled [INGENY phorU-system (http://www.ingeny.com/); Fig. 3]. Sandwiching the 2 mm MADGE gel between 2 mm glass plates ensures even distribution of heat, eliminating amperometric heating effects at the centre of each gel.

### 3.4. *Anode–cathode equalization*

While running any gel system, the local ionic environments around the electrodes change as an consequence of electrophoresis. The different electrolyte compositions at the anode and cathode can result in variability in the way samples run at either end of the gel, primarily affecting the speed at which samples run. This can lead to problems in correctly estimating the size of fragments. Local anode and cathode effects can be equalized by rinsing the MADGE gel with 5 mM KCl before loading the sample. This rinsing in KCl coincidentally contributes to enhanced resolving capability by bringing the ionic strength of the gel closer to that of the sample (see above).

### 3.5. *Gel matrix*

As with all polyacrylamide gel electrophoresis systems, the resolving power of MADGE gels can be optimized by changing the acrylamide concentration (Ref. 1). We have also found that using the polymer-reinforced polyacrylamide gel matrix Duracryl is particularly valuable in resolving

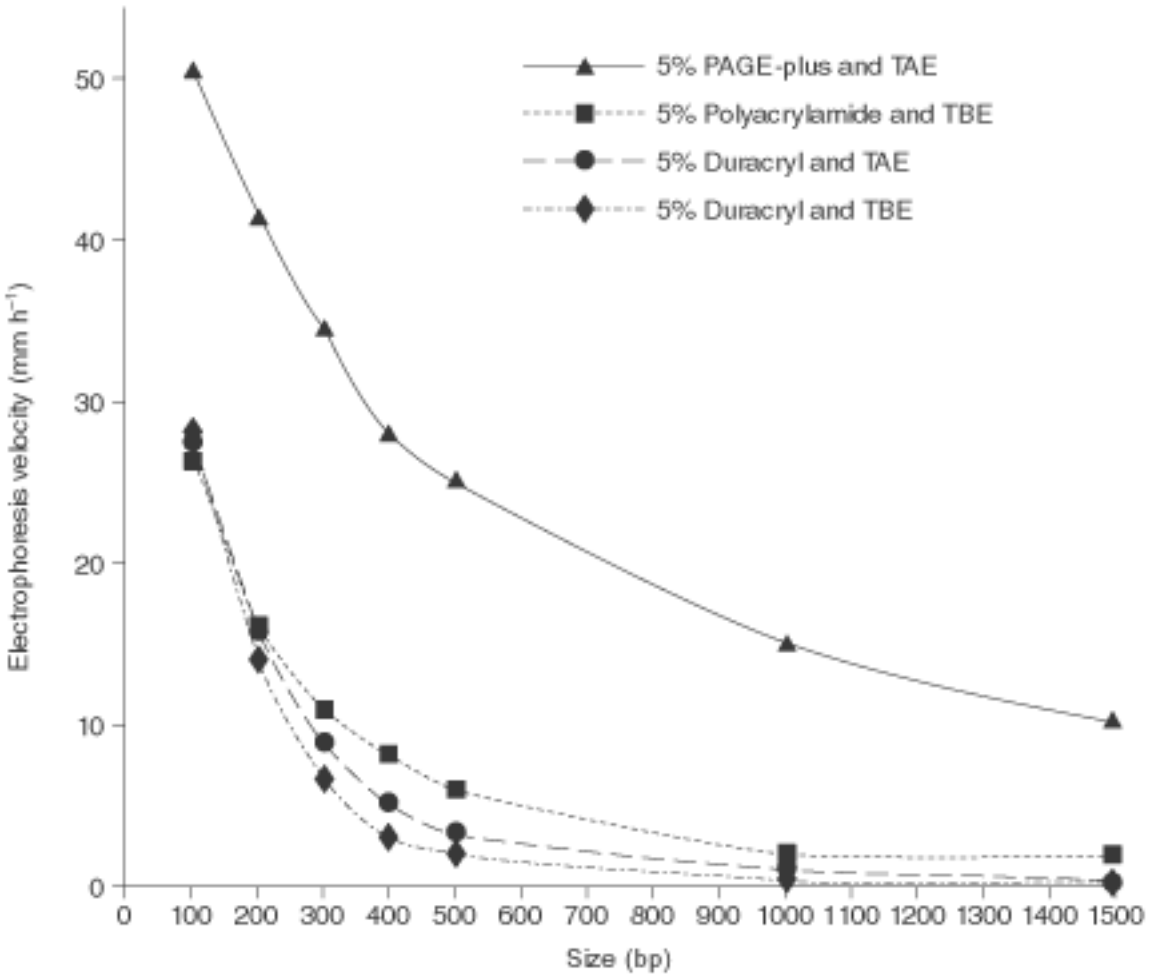


**Fig. 9.** Mobility of 100 bp DNA markers in different gel matrices. DNA markers were run out on a microplate-array diagonal-gel electrophoresis (MADGE) H-PAGE gel (MadgeBio, Cat. no. M0021) to illustrate varying electrophoresis velocities with different gel matrices. H-PAGE is PAGE using gels that are the same size and thickness as MADGE gels but with the wells in a conventional linear arrangement; the track length is 2.4 mm.

similarly sized DNA fragments. Duracryl is marketed as a high-tensile-strength acrylamide, originally formulated to prevent tearing of large gels. However, over the size range 200–300 bp, Duracryl gives significantly better resolution than conventional gels with the same acrylamide concentration (Fig. 8), giving resolving power much closer to that of a denaturing gel system (Fig. 9. A Duracryl–TAE combination also appears to give sharper bands than Duracryl–TBE (X-H. Chen, unpublished).

### *3.6. Molecular weight markers*

In spite of paying attention to all of the above issues, it is not possible to eliminate between-track electrophoretic differences completely. To guarantee accurate allele calling, it is necessary to include internal sizing markers with every sample. Absolute sizing is not the aim here and so setting up a complex sizing curve (as with the five or more markers used by the Genescan software) is unnecessary.

A bracketing pair of molecular weight markers that are fairly similar in size to the amplicon of interest can be used to calculate the intervening unknown allele (see above). These could be components of a commercially available digest ladder marker, and we have used the ΦX 174 DNA *Hae*III digest ladder (New England BioLabs, Cat. no. 302-65) for insulin VNTR analysis (Ref. 4). The ladder can be diluted 1:500 and so can be used cost effectively. Alternatively, 'in house' PCR amplicons of appropriate size can be engineered. Here, any allele size can be accom-

modated and the markers can be generated extremely cheaply.

This peer-reviewed article can be cited as: Chen, X-H. *et al.* (2001) High-resolution MADGE. *Technical Tips Online* T02156.

## References


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### Products Used

**Genescan:** Genescan from PE Applied Biosystems
**FluorImager:** FluorImager from Molecular Dynamics Inc
**Page-Plus:** Page-Plus from Amresco Inc
**MADGE kit:** MADGE kit from Madgebio
**Vistra Green:** Vistra Green from Amersham Pharmacia Biotech
**FluorImager:** FluorImager from Molecular Dynamics Inc
**Genescan:** Genescan from PE Applied Biosystems