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MeltMADGE for mutation scanning of specific genes in population studies

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Abstract

MeltMADGE reconfigures the mutation scanning process of denaturing gradient gel electrophoresis (DGGE) so that the independent variable is time rather than space and the dependent (denaturing) variable is temperature rather than concentration of chemical denaturant. Use of a thermal ramp enables use of a homogeneous gel and therefore of high density arrays of wells such as those of microplate array diagonal gel electrophoresis (MADGE). In this configuration, electrophoresis of products on 10-12 96-well meltMADGE gels can be conducted in a 1-2 litre tank in a 1-2hour run, enabling the scanning of a target amplicon in over 1,000 subjects simultaneously. Gels are read by imaging fluorescence of UV-excited ethidium bromide, giving a simple, economical system for identifying rarer sequence variants in target genes which is suitable for large-scale case-control or population studies and other comparable applications. Different amplicons with similar melting characteristics can also be combined into the same run.

INTRODUCTION

Background to mutation detection by melt based methods

The structure of the DNA double helix, first recognised by Watson and Crick in 1953¹, revealed a biological mechanism for its exact duplication through each strand acting as a template for the synthesis of a new strand. From an experimental chemical perspective, one strand can also act as a group to interrogate a test strand to which it might be fully or

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partially complementary. Early studies focused on analysing properties such as the bulk reannealing of total genomic DNA using spectrophotometric techniques and enabled definition of genomic features such as repeated sequence content and complexity². Fisher and Lerman in 1980³ were the first to resolve specific DNA homoduplexes exhibiting a single base pair difference. To do so, they developed the method of denaturing gradient gel electrophoresis (DGGE), which has proven sufficiently reliable that it is still in quite widespread use three decades later. While more complex to set up than more recently emergent techniques not based on duplex melting, such as SSCP⁴ and dHPLC⁵, DGGE has been extensively used in some diagnostic laboratories as a scanning tool to avoid the high costs of direct sequencing of large numbers of amplicons. The inherent predictability of duplex melting⁶, sensitivity of mutation detection in iso-melting domains (see below), and reproducibility of configured assays, have been important features.

DGGE uses a standard vertical polyacrylamide gel electrophoresis format in conjunction with a denaturing chemical gradient incorporated into the gel. As the DNA products are electrophoresed through the gel, they meet an increasingly denaturing environment and consequently will eventually meet a condition where the strands will start to melt. A GC artificial clamp at one end of the duplex prevents complete melting⁷, so the melted but clamped moiety will exhibit the same charge but a much greater length compared with its duplex state, leading to greater retardation in the gel matrix. Strand reannealing post-electrophoresis during staining also enables simple ethidium bromide detection of re-formed DNA duplexes. The bands representing different homoduplexes, which for all transitions will differ by the hydrogen bond representing one base pair (A:T vs G:C), will have been retarded at slightly different concentrations of chemical denaturant and will therefore resolve. In the amplicon for a heterozygote, random strand reannealing at the end of a PCR generates heteroduplexes constituted of the mismatched complementary strands one from each homoduplex—these mismatches have considerably greater destabilising effects and hence heteroduplexes melt rather sooner during the electrophoresis. For scanning for rare sequence variants, most occurrences will be in the heterozygous state ($2pq/q^2 \approx 2/q$ where q is the allele frequency of the specific rare allele and $p=1-q$) and hence in some instances such as in population research, the major emphasis can be placed on heteroduplex detection. DGGE has remained the principal melting approach until recently, even though it contains the implicit inconveniences of requiring vertical polyacrylamide gels which offer low throughput and are labour intensive, of requiring pouring of chemical gradient gels, and of requiring a rig which is supplemented in its capability to achieve melting by being maintained at a constant high temperature (eg 60 °C) relative to ambient temperature.

The other denaturing variable which has been explored is temperature, mainly in the format of spatial temperature gradients. Since duplexes are maintained specifically by hydrogen bonding of base pairs (although base stacking also plays an important secondary role), pH is also crucial to hydrogen bonding although it has never been utilised as the denaturing variable. Temperature in a vertical polyacrylamide gel can be regulated by a heat plate attached to the gel plates and controlled such that the top is cooler than the bottom, giving “TGGE” (temperature gradient gel electrophoresis) with performance characteristics similar with DGGE. This requires a somewhat more specialised apparatus, which may explain the relative paucity of publications using this approach. Laboratories tend therefore to be differentiated according to their hardware specialisation. Much of the development of the TGGE technique can be attributed to Reisner and colleagues⁸. TGGE hardware is commercially available from Biometra, DGGE hardware from Ingeny, BioRad and CBS Scientific, although some laboratories construct their own systems.

Other investigators have sought to capitalise on the advantages of capillary electrophoresis compared with vertical polyacrylamide gel slab electrophoresis^{9, 10}. These potentially offer:

a higher resolution from capillaries (although between-capillary comparisons may be more difficult); greater ease of temperature control of fine capillaries; opportunity to use applied current as a means of temperature control since (unlike gel tracks) each capillary can be thermally independent of adjacent capillaries; and loading, reloading, automation and general throughput advantages similar to those inherent in capillary based sequencing compared with slab gel based sequencing. This has enabled the cycling of temperature to enhance mutant sequence separation and generally does open the possibility to scan for rare mutant copies such as for cancer somatic change or in pools of DNAs¹⁰. Nonetheless, major hardware investments are needed to establish capillary based approaches and these must include an integral system to read the electrophoresis outcomes for example by fluorescence excitation and detectors in the rig. Most of these developments have been concerned with mutation scanning in diagnostic and research contexts where sensitivity not throughput has been a priority issue, either sensitivity to find all germline mutations in potential cases for mutations in particular genes, or sensitivity to identify rare sequence variants in the mutational spectrum for example for rare cellular changes. For the latter, a high fidelity PCR must clearly also be included and even for general DGGE, less background heteroduplex will occur using a high fidelity PCR¹¹.

While total genomic melting was observed and read in liquid phase in early studies, only recently has an assembly of methodology achieved the liquid phase equivalent of the Fisher and Lerman approach for single genes. This has been largely driven by Carl Wittwer^{12, 13}, in conjunction with developments by Idaho Technology Inc. This approach, high resolution melting (HRM), melts an amplicon in liquid phase using a thermal ramp. During the temporal rise of temperature, the amplicon melting is monitored by the change in fluorescence of a dye whose binding (and hence fluorescence) changes when the double strand dissociates. The dye must not “jump” (dissociate from one site and bind at another) and should saturate the sequence such that all positions are interrogated. The other desirable properties of the melting curve are as for DGGE (see below) but no GC clamp is necessary as in DGGE, although a very short clamp may be used to enhance the base stacking at an unstable amplicon end in order to achieve a more perfectly iso-melting domain (see below). The hardware must be able to control temperature both precisely and accurately. This accuracy must extend across all wells of a 96 or 384 well plate where this industry standard format is used. In gel based melt formats such as DGGE, the investigator is simply looking for bands of different mobility - wild type displays a single unsplit band pattern whereas in liquid phase the subtly different melting curves of the four molecular moieties (two heteroduplexes and two homoduplexes) from a heterozygote are superimposed. HRM therefore depends extensively on computational normalisations and comparisons of the temperature-fluorescence traces to detect variant patterns. A disadvantage is the uncertainty (false positive call rate) inherent in some melting profiles (compared with the ease of recognition of band splitting) where the composite melt ‘may’ be different from wild type. A range of diagnostic and research applications and characteristics have recently been presented in a focus issue of *Human Mutation*¹⁴. An advantage of HRM is the ability to retain microplate format throughout the liquid phase procedures of PCR followed by post PCR HRM interrogation. HRM is finding application in diagnostic as well as research laboratories, an indication that it can meet the demands for sensitivity provided that the necessary investments in hardware are made. An important point about HRM is that compared with DGGE and TGGE and other gel based approaches such as SSCP, it permits use of the microplate format as part of the scanning process.

Microplate array diagonal gel electrophoresis (MADGE)

We have focused for research applications on the inherent throughput and accessibility of microplate orientated formats. We developed the microplate array diagonal gel

electrophoresis format. The simplest embodiment is a two piece 'system' (Figure 1) for the preparation of open faced polyacrylamide gels with arrays of wells and tracks (96¹⁵, 192¹⁶, 384 and 768¹⁷ well formats) fully compatible with liquid phase microplates for direct sample transfer using 96 pin passive devices and microplate level sample tracking. 'Simple' non- denaturing open faced MADGE gels can be loaded and used either submerged horizontally¹⁵ or with direct electrode contacts in a 'dry' box¹⁷. These gels are useful for high throughput checking and approximate sizing of PCR amplicons. With attention to specific details, resolution to 1-2% mobility differences is feasible, for example for microsatellite^{18, 19} and minisatellite²⁰ applications, but basic polyacrylamide MADGE gels can readily resolve 5% mobility differences. Agarose MADGE gels are also feasible using gelbond instead of glass backing¹⁵.

The development of MADGE, although it had many other applications, was driven by the need in the mid-1990s for more efficient electrophoretic means to examine PCR products²¹, PCR-RFLP²²⁻²⁴ and allele specific PCR assays of polymorphisms²⁵ in DNA banks for population studies where all other components of the laboratory process (bank operations; and PCR operations) take place in industry standard microplate formats. While there have since emerged many liquid phase microplate compatible hardware platforms for SNP typing such as TaqMan (<https://products.appliedbiosystems.com/ab/en/US/>), Kaspar (<http://www.kbioscience.co.uk/>) and LightTyper (<http://www.roche-applied-science.com/usa/3358950.pdf>), MADGE continues to provide utility for PCR checking, oligo checking, synthetic RNA checking and offers an almost zero entry cost singleplex SNP typing platform for any laboratory with microplate based PCR equipment, a medium-sized agarose gel electrophoresis tank and an ultraviolet illuminator and camera.

MeltMADGE: temporal thermal ramp MADGE, an overview

The complex array of tracks in a MADGE gel precludes implementation of any spatial gradient of chemical denaturant or temperature in which tracks could all be subjected to equivalent conditions. However, by using a temporal thermal ramp, a DGGE like process can be achieved²⁶. Thus, in effect, meltMADGE applies the same principle as DGGE but instead of using a chemical denaturing gradient in space to interrogate the sample, uses a thermal ramp in time as the independent variable, migration distance of band(s) being the dependent variable. This has enabled population-based mutation scanning of specific genes such as *LDLR*, *BRCA1*²⁶ and *MC4R*²⁷. In overview, the meltMADGE process contains the following typical steps. Firstly, amplicons for mutation scanning are designed in a way similar to those for DGGE. Most if not all amplicons suitable for DGGE or TGGE should also be suitable for meltMADGE. Secondly, predicted amplicon mobility in meltMADGE is tested in trial runs, including a PCR generated artificial positive control if no natural mutant is available. Thirdly, analytical runs are conducted in which 10-12 meltMADGE gels each loaded with the amplicon from up to 96 different study participants per gel, are processed in a 1-2 hour run in a 1-2 litre tank. These gels are then stained with ethidium bromide, imaged under ultraviolet illumination and scored by eye or by MADGE image analysis software (e.g. Phoretix) for variant band patterns. Where only a single band is expected for wild type and mutants are rare, scoring by eye to identify templates meritorious of direct sequencing, is straightforward.

Apparatus for meltMADGE

MeltMADGE is differentiated from DGGE and TGGE in several aspects. Firstly, small horizontal MADGE gels are used. Secondly, the denaturing temperature change takes place during time during the time-course of the run - there is spatial homogeneity of temperature and denaturant within each gel in the tank. Thirdly, compared with a single slab gel DGGE, the tank and gels present a high cross-sectional conductive area hence taking quite a high

current, for example one amp - however, the heating effects of this current need to be neutralised by the temperature control imposed by the system designed to deliver the controlled thermal ramp. Any apparatus must be designed to accommodate these requirements. Each gel is anchored on standard 2mm glass and covered with an identical glass plate following sample loading and prior to electrophoresis. Glass is an efficient conductor and distributor of heat and therefore in addition to acting as both the support for MADGE gel formation and effectively the shelf on which each gel sits, it also participates usefully in the thermal control. A fully functional system can therefore be established with the following components

1. a simple rack to carry the glass backed gels and leave space for buffer circulation between each gel.
2. a cuboid electrophoresis tank with four Perspex walls and base; with a housing at one end in which an impellor is mounted. The principal role of the impellor is to ensure thermal homogeneity in the tank. However, this circulation of the electrophoresis buffer also reduces buffer exhaustion. The housing also contains inlet and outlet for silicon tubing connected to a glass serpent mounted in the base of the tank. This serpent carries a high flow of temperature controlled water supplied from a standard programmable temperature controlled recirculating water bath - this sets the thermal ramp for the run, although in term of quantities of heat, mainly acting to counteract amperometric heating. Lastly, the ends of the tank are lined with zig-zag platinum electrodes attached to connectors in the lid of the tank.
3. a recirculating temperature programmable water bath (at least covering the range from ambient temperature, e.g. 20°C, to 80°C) which can deliver a thermal ramp, e.g. linear 5°C over 1-2 hours
4. an electrophoresis powerpack capable of an amperage up to 1.5Amps such as the type typically used for Western blotting.
5. an accurate, precise, calibrated thermometer used to check the correspondence between the temperature ramp in the electrophoresis tank and that intended for the course of the run.

Overall, this design is simple and economical. Components 3-5 are widely commercially available if not already available in the laboratory. Components 1-2 are straightforward for a basic workshop to construct.

Schematics, descriptions and photographs sufficient to build meltMADGE apparatus, are given under the Procedure section and as Supplementary figures 1-17.

Applications of meltMADGE

The principal utility of meltMADGE is in the large number of gel tracks and gels which can be processed simultaneously in one tank. This readily lends the technique to large-scale population and case studies, for which it was originally developed^{26, 27}. In this configuration, every gel track on every gel may represent the same target genomic region, amplified from many different subjects. There is also scope to include different amplicons on the same gel, where the necessary thermal ramps for interrogation are identical or very similar. For example many exons of a specific gene may have very similar melting characteristics, although first exons near the promoter region may exhibit higher %G+C. Since different concentrations of urea and formamide can be incorporated into each gel, and since each 1M urea increment corresponds with approximately a 2.5°C decrease in melting temperature for any given fragment, wider combinations of different amplicons can also be incorporated into a single run in one tank. Equally, DNA templates may derive from any

species or cells, for example to screen target genes in shotgun mutagenesis experiments. This defines the applicability of the technique.

Concerning emergent technologies, genomewide association typing of SNPs has not ablated, rather it has enhanced the need for focused single SNP typing technologies for replication and follow up studies. Analogously, mutation scanning methodologies such as meltMADGE which focus on single targets with a high level of sample parallelism, are likely to experience a similar demand as next generation sequencing identifies genes meritorious of further focused scanning.

Accessibility of meltMADGE: cost considerations

Large scale genomic and genetic research is rapidly being transformed by next generation sequencing. Complete human genome resequencing is already possible at under \$50,000 per genome, with a five-year goal of the \$1,000 genome²⁸. The thousand genomes project (www.1000genomes.org) is expected to deliver 1,000 complete genome sequences in a similar timeframe. By target capture, for example all exons, complete exome resequencing can be achieved considerably more readily²⁹. Plans are also under way for a 10,000 genomes (species) project in a 10 year timeframe. At the opposite extreme, diagnostic laboratories must at any point in time focus attention specifically on one or a few genes diagnostically relevant to a small number of recent clinical consultations in their catchment. Many research laboratories have specific target genes and sample collections on which they focus, which fall somewhere between these two extremes in terms of throughput requirements. Furthermore, specialist and costly hardware requirements limit research for many groups worldwide. In contrast, MADGE and meltMADGE costs both for hardware and reagents, are considerably less than those for PCR (approximately one seventh of PCR costs, mainly for acrylamide and electrophoresis reagents), which opens immediate access to these methodologies for any laboratory which has been able to afford PCR. This defines the accessibility of the technique. Since the reagent costs are very low for meltMADGE (water, urea, acrylamide, simple buffer, ethidium bromide), the main consideration is staff time, i.e. overall PCR-meltMADGE costs are low. We estimate that it takes approximately twice as long for a technician to complete the meltMADGE set up and analysis, as to set up the PCR reactions. Staff time is therefore the major cost to consider. Batch processing of 20-40 96 well gels per day is possible per technician. At a technician cost of £100 per day, the main cost is about 2.5-5 UK pence per amplicon analysed. Since most time consuming steps must still be undertaken for single gels, only a modest time saving would be made, i.e. cost per sample for 96 PCR products still likely to be 10-20 pence. Thus the technique is most cost efficient in batch mode. Diagnostic and research laboratories continue to use a variety of mutation scanning approaches in order to circumvent the costs of conventional direct sequencing when focusing on specific target genes. This applies in developed as well as developing nations. With increasing numbers of genes attracting interest for follow up; and rapidly increasing numbers of banks of DNAs for analysis (research, diagnostic, case, population), scanning methods can be expected to continue to play an important role.

Experimental design

Outline—Setting up meltMADGE involves amplicon design including also positive control design; trial runs and imaging to confirm design and melting conditions; and large scale analytical runs and imaging.

Amplicon design—Target sequences in the template DNA are chosen according to the study design. Mutation scanning of exons, splice sites, and other functional regions of genes or of other genomic elements, is a frequent choice. Target sequence extraction will often be from online genomic sequence databases such as those maintained by NCBI (<http://>

www.ncbi.nlm.nih.gov/) or EBI (<http://www.ebi.ac.uk/>). Amplicons in the size range 150 to 300 base pairs are suitable for meltMADGE, although somewhat larger or smaller amplicons may also suffice. Sequence regions, for example exons, are first examined for their melting characteristics. A number of computer programs to predict DNA melting have been developed, *Melt87* for DOS from Lerman's group³⁰ having been widely utilised for DGGE planning for some years. *Melt94* from the same laboratory offers a graphical output (<http://web.mit.edu/osp/www/melt.html>). Other developments have also incorporated more user-friendly graphical and menu driven interfaces. We have implemented *Melt87* and extended its algebra substantially in a Visual Basic package which offers a graphical interface and annotation of a range of sequence features (Tixis - available from E. Spanakis). Alternative commercial applications are available from MedProbe (Oslo, Norway) (<http://www.medprobe.com/xx/melt.html>) or Ingeny (<http://www.ingeny.com/>). The chemical model and algorithm for melting was first developed by Poland⁶. This incorporates empirical data for the melting characteristics of any base pair in any of its 16 possible adjacent base pairs contexts, i.e. giving a model which takes account both of hydrogen bonding and of the equally important base stacking interactions. Fixman and Freire provided a more computationally efficient approach³¹. Web implementation of the Poland algorithm³² is also available at <http://www.biophys.uni-duesseldorf.de/local/POLAND/poland.html>. Addition of a GC rich clamp, introduced in the 5' end of one PCR primer at the less stable end of the target sequence, tends to flatten the melting profile further. In general, profiles where there is a single domain fluctuating within a predicted melting range of about 3 degrees, seems to perform reasonably, whereas if there are two domains with distinctly different melting ranges, they may be better examined in separate amplicons, since the melting of the lower melting domain would limit the gel resolution then possible for the second domain if it were incorporated in the same amplicon. PCR primer design locating primers at the ends of the approximate target region is undertaken in a parallel process, in conjunction with electronic PCR check of the relevant genome that a unique amplification should be achieved. We frequently clamp both ends, the higher melting end with a much smaller clamp (5–10 GC) to obtain more perfectly flat melting domains.

If no heterozygous template is available, an additional PCR primer (at the end without the GC clamp) should also be synthesised which is a few (e.g. 10) bases longer and contains a template mismatch (base transition from the 'wildtype' primer) at position -4 or so. Reannealing amplicon made with the 'wildtype' primer and the 'artificial mutant' primer will generate heteroduplexes in addition to the differing homoduplexes, thus giving a positive control for the trials of thermal ramp conditions.

Amplimers are designed according to the same principles as for DGGE, CDGE or TGGE. To avoid complete dissociation of the strands, and thus to maximize the electrophoretic retardation achieved by partial melting, the variable sequence needs to be 'clamped' with a small GC-rich thermostable domain. Clamps can be added by PCR using a GC-rich extension in one of the primers. The following extension has been repeatedly tested and can be used as a universal clamp at either end:

5'-CGCGGCGGAGCGAGGCCCGCGGGCCCGCCCGCGCCCC-3'

Locations of primers will likely be substantially dominated by their necessary locations to focus on specific target sequences such as exons. However, precise position may be adjusted and one of numerous primer design programs can be used to ensure that self-priming, primer dimer formation and hairpin formation are all avoided. Additionally, electronic PCR should be used to check that no related sequence in the DNA template would be amplified. Many useful programs are listed at <http://molbiol-tools.ca/PCR.htm>. Typical primers would be matched for annealing temperature (ignoring the GC-clamp) which would be in the range 50–65°C in 150mM NaCl. Primers require no 5' nor 3' labels nor other modifications.

Typical example primer pairs are 5'-CGCCCGCCGCGCCCGCGCCCGTCCCGCCGCCCCGCCCCGTCGGCCTCAGTGGGTCTTTC-3' (sense) and 5'-ACTCCCCAGGACTCAGATAGGC-3' (antisense) representing human *LDLR* exon 3; and 5'-CGCCCGCCGCGCCCGCGCCCGTCCCGCCGCCCCGCCCCGTCCTCCACCAAGCCTCTTTCT CTC-3' (sense) and 5'-CCACCACTGCTGCCTGTAAG-3' (antisense) representing human *LDLR* exon 8. See below for examples of similar primers to generate artificial positive controls for the meltMADGE runs.

MeltMADGE thermal ramp design

The T_m of a homoduplex amplicon predicted from any implementation of the Poland algorithm, provides the crucial indicator for the choice of thermal ramp. Using constant temperature denaturing electrophoresis to estimate the temperatures of maximal rate of change of mobility, we showed for 35 *BRCA1* amplicons, a very good correlation between predicted T_m and observed T_m (Figure 2).

Heteroduplexes will melt, at least partially, at a couple of degrees lower temperature than the homoduplexes. In general, we observe that for single base mutations, the mismatch pairs will usually have quite different destabilising effects for the two heteroduplexes. Most mutations are transitions (interchange of A and G on one strand; and of C and T on the other). Heteroduplexes for all transition mutations therefore involve A:C and G:T mispairs. The G:T mispair is much more stable than the A:C mispair, so these heteroduplexes will therefore usually melt at quite different temperatures from each other. By contrast, the homoduplexes, which differ by just one hydrogen bond, will melt at a fraction of a degree (e.g. 0.3°C) difference. By contrast, the heteroduplexes formed by a small (minimally 1 base pair) deletion or insertion tend to co-run, as do their cognate homoduplexes. A thermal ramp starting about 4 degrees below the T_m and rising to one degree above the T_m is, in our experience, likely to prove quite suitable for analytical runs. During the set up phase, this ramp, and two equivalent ones starting respectively 2°C lower and 5°C higher, for identical voltage and run times, should prove sufficient to confirm the approximate melting transition and mobility transition (sudden marked decrease) of the wild type amplicon and to confirm that the heteroduplexes of an artificially constructed positive control (see below) display expected resolution, which should usually be optimal for the expected ramp. Complex ramps are possible, for example a biphasic linear ramp with a steep phase to resolve heteroduplexes and a shallow phase to resolve homoduplexes. However, in practice, the exact optimal inflection point is difficult to determine and for most applications a simple linear ramp suffices. A short stabilising period of a few minutes at the base temperature to start the ramp, is necessary after the ambient temperature gels have been loaded into the tank. We have also experimented with declining ramps (reannealing ramps) but possibly due to the random events of reannealing as opposed to the sudden change of denaturation, bands in this approach were not as sharp. The thermal ramp electrophoresis starts directly at a temperature point a short interval below that for heteroduplex melting, so much of the time of the electrophoretic run will be spent resolving heteroduplexes and homoduplexes, utilising much of the resolving length available in the short track length format of MADGE gels.

MATERIALS

REAGENTS

CRITICAL—General reagents are from Sigma-Aldrich unless otherwise stated. We are not aware of any special requirements where there might be a preference for one supplier's product.

PCR Reagents:

- Sequence specific PCR primers (MWG Biotech, <http://www.eurofinsdna.com>). 16pmol of each primer is typically required per 10µl PCR.
- CRITICAL meltMADGE PCR primers often require the addition of a GC-clamp to stabilise amplicon melting, *in silico* melt profile analysis is recommended (see experimental design)
- Tris (pH 8.3) (USB, Ohio, USA cat. no. US75825),
- KCl (BDH, Poole, Dorset),
- gelatine (Amersham Life Science, Buckinghamshire, UK),
- MgCl₂,
- dATP, dCTP, TTP and dGTP mix (Promega, cat. no. U1240)
- standard Taq DNA polymerase (GibcoBRL Life Technologies, Paisley, UK) plus
- DMSO (Sigma-Aldrich, cat. no. D5879) some GC-rich sequences may require the addition of DMSO (e.g. 0.5% v/v) to the PCR master mix.
- template DNA (if human genomic DNA, 5 or 10ng required per 10µl PCR)

Gel Reagents:

- Sticky silane (0.5% v/v γ - methacryloxypropyltrimethoxy silane and 0.5% v/v glacial acetic acid in ethanol)
- 30% (w/v) acrylamide-bisacrylamide (19:1) (AccuGel, National Diagnostics, Fisher Scientific, cat. no. ELR-138-020T), CAUTION Liquid acrylamide is potentially neurotoxic and skin or other contact must be avoided by use of gloves and clean laboratory practice.
- 10x Tris-acetate-EDTA (TAE) (BDH, Leuven, Belgium), Stock 10XTAE can be stored for 1-2weeks (probably longer).
- urea (Fisher Scientific, cat. no. U/0500/63),
- ammonium persulphate (APS) (Sigma-Aldrich, cat. no. A9164),
- NNN'N'-tetramethylethylenediamine (TEMED) (Sigma-Aldrich, cat. no. T9281). ethidium bromide (Sigma-Aldrich, cat. no. E1510 – a 10mg/ml stock) Store the stock at 4°C protected from light by aluminium foil wrapping. CAUTION Ethidium bromide is a potential mutagen and skin or other contact must be avoided by use of gloves and clean laboratory practice.
- Electrophoresis tank running buffer (see reagent setup).

Equipment:

- Standard PCR microplates or tubes (E.g. ABgene 96xwell PCR plates, Thermo Scientific)
- Pipettes & tips
- Adhesive PCR film (Thermo Scientific, cat. no. AB-0558)
- PCR thermocycler (MJ Research DNA Engine™ Tetrad series thermal cyclers, Genetic Research Instrumentation Ltd, Rayne, UK)

- External temperature monitor (E.g. Platinum resistivity thermometer, M-Squared, UK calibrated to national standards) Tracker 120 (Data Track Instruments, New Milton, UK); calibrated by Universal Calibration Laboratories (Romsey, UK)]
- Programmable temperature controlled recirculating water bath (Thermo Haake, Newton USA & Multi-TEMP II, Pharmacia LKB, UK).
- Power supply, 200V/2Amp output (BioRad Laboratories Inc., CA, USA)
- Purpose built meltMADGE tank (see experimental design). Optional – a purpose built box for multiple simultaneous meltMADGE gel pouring.
- MADGE gel formers (NBS Biologicals Ltd, Huntingdon, UK, MadgeBio catalogue number M0011)
- Glass plates (cut from standard float glass - 110mm × 170mm × 2mm)
- Stationery rubber bands
- Flexible silicon rubber tube, 1mm internal diameter
- 96-split-pin replicator (V&P Scientific, San Diego, CA, USA)

EQUIPMENT SETUP

MeltMADGE apparatus—Prototype apparatus was purpose built as follows. Tanks are (internal dimensions) 23cm long (anode to cathode), 11cm wide and 15cm high (Figure 3a) and contain two platinum electrodes, one zigzag down each end of the tank; a motorised propeller stirrer; a glass serpent (Figure 3a) and a removable gel rack. An overview engineering drawing of the tank is shown in Figure 3b. Full details, exploded views and further measurements, electrical specification are given in a set of supplementary drawings and plan, as are further representative photographs views (Eighteen supplementary files). The electrical contents of the box housing the impellor motor, are also given in supplementary material (Supplementary Table 1 - Parts and Components list; and Figures 12 and 13). The electrodes (specified below and in the Supplementary figures 2,3, and 14) are connected through the cover of the tank to a commercial 200V, 2A power supply (Figure 3c), spatial thermal homogeneity is achieved by vigorous stirring. The glass serpent is connected to a programmable heating-cooling circulator (Figure 3c), and a digital thermometer (Figure 3c) is used to monitor the temperature.

The glass serpent is handmade with 2cm vertical inlet and outlet adjacent in the centre of one end, the horizontal serpent having its inlet leading directly into the first of seven fingers each containing one turn of the glass tubing with gaps of about 0.4cm between each parallel of glass tube. Following the last side of the last finger, the tube turns through a right angle back along beside the fingers and at a right angle toward the inlet before forming the outlet. Fingers are approximately 8.5cm from 'outer' turn to 'inner' turn, and the serpent occupies approximately a 17cm by 9.5cm rectangle. The serpent was stabilised against flexion damage by a small glass bridge connecting the bases of inlet and outlet arms. Silicon rubber tubing (5mm) was used to connect the serpent ends to the external connectors housed in the lid from which connections to the recirculating water bath are made. The exact dimensions of this serpent are not crucial but by occupying most of the base of the tank and combined with action of the impellor, efficient homogeneous heat transfer can take place between the buffer compartment and the water in the serpent delivered from a temperature controlled programmable water bath. Note that other integrated designs would be possible, with temperature control integral to the purpose built apparatus, but the presented design capitalises on the use of equipment already available in many laboratories, therefore limiting new construction to the meltMADGE tank itself.

Figure 3b is a schematic view with one long side of the tank detached. For further schematics, see Supplementary Figures 1-5. The long sides are mirror images, except that one side has a fine groove cut in its top edge to lead a platinum wire from the lid contact to the electrode plate. One electrode plate is shown partly raised from the tank: the other is not shown but the side wall inset anchoring it can be noted in the top left aspect of the tank. It is constituted of a rear plate with a fine groove in which the platinum electrode is set, threaded at turns through the plate, with each horizontal segment exposed to the tank via a slot (eight in total) in the front plate. The ends of the tank are simple slabs. The base is a simple slab. The secondary base is a simple slab bracing the walls and electrode plates.. Each side wall has two sequential recesses at each end, the first to accommodate the edges of the end walls, the second to accommodate the electrode plates. The lid section which bears the impellor, connectors between glass serpent (not shown in base of tank) and recirculating water bath, and holes for temperature probes, is shown raised. The other part of the cover (not shown) slots over the two electrode posts to which electrophoresis power pack cables are connected during operation. Dimensions - base $262 \times 132 \times 5$ mm, inner base $222 \times 112 \times 4$ mm. Side walls $262 \times 160 \times 10$ mm recesses 12×5 mm to accommodate end walls, 10×2 mm to accommodate electrode plates. Ends $160 \times 122 \times 12$ mm. Electrode assemblies: 'grooved' plate $114 \times 158 \times 4$ mm, with zig zag groove for electrode spanning 100mm for each horizontal, dropping 15mm at each turn to next horizontal, four turns in total, each turn threaded through the plate and with a vertical drop from the top entry point of 37mm located 7mm from the side. Groove of 2mm to accommodate the run of platinum wire; 'window' plate with eight 'windows' each opposite a platinum wire horizontal when the two plates are opposed. Windows each 100×8 mm, 15mm between top edge of one window and top edge of next, first window top edge being 35mm below top edge of plate. Lid section bearing impellor housing $90 \times 130 \times 10$ mm but recessed at 3 edges to slot down into sides and end of tank – lid secured by one large thumbscrew to end; and containing two 10mm holes for temperature probes and two bidirectional silicon tubing plastic connectors. The remaining lid similarly designed, giving complete tank closure and with two holes to locate over the two electrode contact pins. The impellor motor (see supplementary Table 1) is anchored and controlled as shown in supplementary figures 7-13 and 17, and is connected to a shaft of long plastic (Acetal or Nylon or Polyethylene) rod that is turned into a tapered shaft. The fins at the end of the rod were machined out of a solid plastic block (see Supplementary figures 10).

Gel casting—this only takes a few seconds. For a single gel, a MADGE gel former and a glass plate with the same dimensions are required (Figure 4a). Wipe one surface of the glass plate with sticky silane. Hold the glass with the silanized surface against the gel former. Initiate polymerization of the gel (see Regaent setup; gel mixture) and immediately pour the gel into the gap between the former and the glass (Figure 4a). Gels are left to set for at least 40 min, before prising open-faced gels (anchored on one glass plate) from formers. For pouring large batches of gels, a purpose built pouring box can be used (Figure 4).

REAGENT SETUP

Gel mixture (50 ml)—

10ml of 30% (w/v) acrylamide-bisacrylamide (19:1), 5ml of 10xTAE buffer, 6M (18 g) urea and 35ml of warmed (30-40°C) dH₂O to dissolve the urea. After cooling to room temperature, 100μl of 20% (w/v) APS and 100μl of TEMED are added to initiate polymerisation – gels should be poured immediately. For batch gel pouring, volumes are scaled up accordingly. **CRITICAL: NOTE** This mix may be modified depending on the properties of the amplicon: 5–6% (w/v) acrylamide-bisacrylamide (depending on the size of the amplimer – a 445bp amplimer requires 5% (w/v) acrylamide but shorter amplimers resolve at either concentration); 4–8 M

urea (depending on the melting point of the sequence assayed) CAUTION Liquid acrylamide is potentially neurotoxic and skin or other contact must be avoided by use of gloves and clean laboratory practice.

Ethidium bromide solution (for gel staining)—10 μ l of 10mg/ml ethidium bromide in 100ml of 1XTAE buffer. Store the stock at 4°C protected from light by aluminium foil wrapping. CAUTION Ethidium bromide is a potential mutagen and skin or other contact must be avoided by use of gloves and clean laboratory practice.

Electrophoresis tank running buffer—1 $\times$ TAE, 2mM APS. Stock 10XTAE can be stored for 1-2weeks (probably longer) but mixes containing urea, APS or other reagents should be made daily.

PROCEDURE

Primer design and synthesis for meltMADGE

1. Identify the target DNA sequence from a sequence database, including slightly more sequence (eg 25 bases at each end) than might be in the final amplicon
2. Copy the sequence (which must read 5' to 3' left to right, top to bottom) into a melt analysis program running on a local PC or online (eg online at <http://www.biophys.uniduesseldorf.de/local/POLAND/poland.html>). For large scale planning a local program rather than a web tool may prove more efficient. Primer location is important for capturing information about functionally important sequence regions. Usually, a reasonably flat melting profile exists over a region such as an exon, but two amplicons might best be chosen if the region has domains of distinctly different (e.g. >3C) melting temperatures. The long GC clamp should be located at the lower melting end of the amplicon which will help render the overall melting profile to be more level.
3. Select the arrangement with the flattest melting profile
4. Using the chosen region, use a primer design program (links at <http://molbiol-tools.ca/PCR.htm>) to locate suitable positions for PCR primers near the ends of the sequence. Check that there is no primer dimer or hairpin formation risk. Run an ePCR against the relevant template DNA (assuming there exists a complete genome sequence) to ensure that a unique amplification will result.
5. Examine the melt profile of the sequence with or without a GC clamp on either end.
6. Order the primers from an oligonucleotide primer supplier such as MWG-Biotech (<http://www.mwgdna.com>). When developing new assays for population scanning to identify unknown mutations, also order a primer with a one base chemical mutation at position -4 from 3' end (for example 5'-ACTCCCCAGGACTCAGACAGGC-3' for human LDLR exon 3, see above; and 5'-CCACCACTGCTGCCTGCAAG-3' for human *LDLR* exon 8). This will be used to generate artificial positive controls and test sample heteroduplexes (see Box 1 and Figure 5).

PCR amplification of DNA for meltMADGE

7. Pre-load 5 or 10ng template DNA into each well (eg in 1 μ l water then air dried within days before use) of a PCR microplate. If scanning for unknown mutations for the first time, proceed as described in Box 1. Otherwise, continue as described below. It

would be expected that many test templates will show a wildtype pattern but if a sequence verified wildtype control is available, this should also be used as a control template **CRITICAL STEP:** If carrying out the experiment for the first time, it is recommended to first optimise the temperature and the Mg^{2+} concentration for the PCR as described in Box 2.

8. Prepare a PCR mastermix as tabulated below. Note that some %G+C-rich sequences might require the addition of DMSO (e.g. 0.5% v/v) to obtain good amplification. Note that separate reactions using different primers will be needed for generating an artificial positive control (generated using a wildtype template), compared with the standard reaction for test templates.

9. Add 10 μ l PCR mastermix to the DNA in each well of the microplate from step 7. Run the reaction using the cycling conditions tabulated below, which are likely to suffice for most reactions. However, for sequences with melting points well above 72°C, annealing and extension can be combined into a single step at 72°C for 1 min. Also, a final denaturation step of 96°C for 30 sec followed by immediate cooling to 55°C for 5 minutes then to room temperature (20-22°C), may increase heteroduplex yield. If samples have not been snap frozen prior to storage, this final step should be carried out immediately prior to electrophoresis

Component	Amount per 10 μ l reaction	Final
Taq DNA polymerase	0.15U	0.15Units/10 μ l
100 mM Tris (pH 8.3)	1 μ l	10 mM
500mM KCl	1 μ l	50 mM
gelatine		0.01% w/v
15mM $MgCl_2$	1 μ l or 2 μ l	1.5mM or 3 mM
2mM mixed stock dNTP's (for each of N = A,C, G and T)		200 μ M
Each primer Stocks at 8pmoles/ μ l	1ul	8 picomoles/10 μ l
dH ₂ O	6 μ l or 5 μ l	
TOTAL	10 μ l	

CRITICAL STEP—the reaction should be in plateau phase not log phase, because in a log phase reaction, if all duplexes are being formed by new strand synthesis rather than random reannealing of existent strands, then no heteroduplexes will have formed.

PAUSE POINT—If amplifications are to be stored, they should be placed on ice until frozen to -20°C or lower. If storage is at 4°C, then it is wise to repeat the heteroduplex generation step immediately before electrophoresis, because heteroduplexes, particularly unstable ones, will tend to rearrange in solution to homoduplexes over time.

Step number	Denature	Anneal	Extend
1	96°C for 2 min	--	--
2-31	96°C, 30 sec	55°C, 30 sec	72°C, 30 sec
32	--	--	72°C, 2 min

meltMADGE

10. Set up the meltMADGE apparatus as described in the Equipment setup section.
11. Prepare and pour the meltMADGE gel as described in the Reagent and Equipment setup sections.
12. Load approximately 2 μ l of each PCR product from microplates in step 9 by passive transfer from plate to gel using a 96-slot pin replicator (Figure 4c).
13. Cover each gel by sliding a clean (untreated) glass plate over it. To do this, drop 2-3ml of buffer (or water) onto the edges of the gel in order to facilitate sliding of the cover and to eliminate formation of air bubbles in wells.

CRITICAL STEP—A 96-split-pin replicator (V&P Scientific, San Diego, CA, USA) loads 2 μ l at a time, which is sufficient for most PCR reactions. Larger volumes can be loaded with repeated transfers or with a pressure-driven replicator [e.g. simultaneous plate-loading and transfer tool (SplatT) (Intelligent Bio-Instruments, Cambridge, MA, USA)]. If the volume of the samples is less than 8 μ l, complete with water; a few drops placed on the surface of the gel will fill up the wells during covering. Any air bubbles trapped in under-loaded wells will severely affect band definition in the corresponding tracks. However, never lift the cover glass to release trapped air as this will remove all samples from their wells and mix them up. Loading more than 8 μ l might cause visible well-to-well contamination but, when all PCR products have approximately equal concentrations, such contamination does not impair interpretation because contaminant bands are much fainter than real ones.

14. Seal the long edge of the gel with silicon rubber tubing stretched and inserted between the glass plates in order to prevent electrophoretic edge artefacts (Figure 4d). Secure the assembly with two stationery rubber bands (Figure 4d).
15. Electrophorese for 2 hours at 50V (approximately 2A) with a linear ramp temperature, for example from 59-64°C for the *LDLR* exon 3 primers described above or 60-65°C for the *LDLR* exon 8 amplicon described above. Crosscheck ramp control regularly against an external temperature probe calibrated to national standards.
16. Remove bands, seals and cover plates. Stack gels (still anchored on glass and separated by spacers) in 1xTAE buffer containing ethidium bromide for staining. Place on a shaker at minimum speed for 15min.
17. Image on a standard ultraviolet transilluminator with overhead digital camera using an appropriate wavelength filter. Alternatively, Vistra Green™ (Molecular probes, Eugene, OR) can instead be used and gels visualised using a fluorescence scanner such as a Fluorimager™ 595 (Molecular Dynamics, Amersham Pharmacia Biotech, Little Chalfont, UK).
18. Read meltMADGE gel patterns by eye; this is readily done if many tracks show a wild type band and one or two tracks might show a pattern of multiple bands. Common polymorphic bands are also easy to call in this way (provided that the temperature ramp is adequately resolving the homoduplexes of opposite homozygotes) but considerably more attention is then needed to identify patterns not conforming to one of the three common patterns. As in all mutation scanning techniques, recognition of rare variants on a background of common polymorphism is challenging. MADGE image analysis software is available from Phoretix International (Newcastle-Upon-Tyne, UK; <http://www.nonlinear.com>). The Phoretix software includes track alignment and image analysis tools for very fine migration and band-intensity measurements. See examples in Anticipated Results.

TIMING

Steps 1-6: Expect to take 2-8 hours to plan amplicons and order primers (for a medium sized gene and according to experience, software used and difficulties with any melt profiles).

Steps 7-9: Expect to take 2-3 hours to set up and run trial PCRs then 1 day to trial amplicons under several different thermal ramp conditions.

Steps 10-18: For analytical runs, one researcher can handle and run 2 meltMADGE apparatuses in parallel, two runs per day, 10 gels per tank, i.e. approximately 40 gels per day. These might represent 10 384-well plate PCRs generate overnight in advance. This represents a feasible throughput of 10,000 – 20,000 tracks per week per researcher.

Box 1: 3-4 hours.

Box 2: 3-4 hours.

TROUBLESHOOTING

Troubleshooting advice can be found in Table 1

ANTICIPATED RESULTS

Most analytical runs are of large numbers of gels which appear as in Figure 6. However, for the purposes of alignment for illustration of specific features of band patterns in individual tracks, we have either used H-PAGE (horizontal PAGE) gels which use the MADGE gel pouring method but form a horizontal PAGE gel with wells in a traditional row configuration; or tracks have been cut and pasted using MADGE-specific software (Phoretix). A crucial inclusion is a positive control, which can be artificially constructed (see Box 1) if no natural mutants are available for the target amplicon.

Figure 5 shows an example of an artificial positive control. The wild type (WT) and PCR-induced mutant (MUT) allele homoduplexes show different mobilities. The coannealed pair (COANN) shows two additional heteroduplex bands. Note that these controls were loaded on additional tracks provided by the MADGE gel former outside the limits of the 8×12 array which will be loaded from a PCR plate, thus allowing post-PCR add-ins such as artificial controls or re-runs. In this instance, wells were in a traditional alignment but using an 'H-PAGE' (horizontal PAGE) gel former akin to a MADGE gel former.¹⁵

Figure 6 shows two gels (numbered 4 and 15) from a large batch run. Apart from the variant band patterns of the artificial positive control, all tracks just show a wild type band. A little background smear is commonly evident, presumably because in short track lengths, PCR error heteroduplexes are condensed into a relatively short track length. However, heteroduplexes should appear in quantities in near stoichiometry with homoduplex, as observed for a single track (ringed) on gel 15. The two homoduplexes have not resolved on that track.

Figure 7 (again using tracks in an H-PAGE) shows an example of the three band patterns for a common polymorphism. Four bands are evident in heterozygotes (tracks 2 and 6), one band in homozygotes (tracks 1 and 5 representing one homozygous group, tracks 2, 4 and 7 the other)

Figure 8 shows a set of individual track images excised from a full meltMADGE gel image. Each one represents a different mutation in *LDLR* and displays a different band pattern. Although most are transitions, it is clear that position and surrounding sequence context

influence the melting transitions in different ways as apparent in the different final migration patterns.

Figure 9 shows a single heterozygote gel track analysed in Phoretix software (Figure 9a); and a Phoretix automatic extraction and rearraying of 96 meltMADGE tracks which contained a polymorphic amplicon (Figure 9b). The trained eye can recognize band patterns at a glance but image analysis can help in case of doubt. MADGE-specific Phoretix software can be used to measure precisely migration (x axis) and to quantify band intensities (y axis).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Box 1**Generation of artificial positive controls and test sample heteroduplexes**

When developing new assays for population scanning for identification of unknown mutations, the initial absence of natural positive controls is a concern. Generate an artificial positive control and test sample heteroduplexes as follows:

1. Using a 'wild type' DNA template, set up PCR reactions as outlined in steps 8 and 9 of the main procedure. Use a 'wild type' primer pair (perfectly matched to the template) to obtain a 'wild type' PCR product (WT). Obtain a 'mutant' PCR product (MUT) by substituting one of the wild type primers with a primer containing a one base chemical mutation at position -4 from 3' end. TEST PCR products are obtained by amplifying test samples with wild type primers.
2. Check the quantity of MUT and WT PCR products on a standard agarose gel. Estimate the relative concentrations of the PCR products and adjust so that the concentrations are approximately equal.
3. Mix an equal volume of the MUT PCR product with the WT PCR product. Co-anneal this mixture of PCR products to generate positive control heteroduplexes (Figure 5) by heating at 95°C for 3min and then 40°C for 5min. Possible heteroduplexes in TEST sample PCR products, generated with wild type primers, are maximised as described in procedure step 9 PCR.
4. Proceed from step 10 of the main Procedure to analyse the samples by meltMADGE.

Box 2**Optimizing PCR conditions**

In the first instance, if available, use a 96-well gradient block with a thermal gradient of annealing temperature on the long axis of the microplate and a MgCl_2 gradient (1-4mM) on the short axis of the plate, all applied to the same template DNA (which can be pooled DNA to preserve individual stocks), to optimise the reaction.

1. Transfer the products to a standard polyacrylamide MADGE gel and evaluate the overall temperature-magnesium dependence of the reaction following electrophoresis and ethidium bromide staining¹⁵ (steps 10-18 of the main Procedure).
2. Proceed from step 7 of the main Procedure, using the test samples and the optimized temperature and Mg^{2+} conditions determined in step 2 above.

Figure 1. Microplate array diagonal gel electrophoresis

In the most minimal format for gel setup, the two dimensional gel former contains 96 cuboid ‘teeth’ arranged in a microplate compatible format (9mm pitch between wells). Acrylamide gel mix is poured into the former (a) and a glass plate silanised with γ -methacryloxypropyltrimethoxysilane laid onto the former (b,c,d) – the glass touches the teeth directly, excludes air and once the gel is set the glass plate is prised away and acts as the carrier on which the open faced gel is anchored.

Figure 2. Observed versus predicted T_m

This plot shows the correlation between T_m values predicted by the Melt87 algorithm and the temperature at which amplimers melt during melt-MADGE with 4M urea is almost perfect; more than 98% of the variance is explained by linear regression. The intercept of regression indicates that 1 M urea reduces the predicted melting point of a sequence by ~ 2.5°C. The statistics are based on theoretical and experimental analysis of 35 BRCA1 amplimers.

Figure 3. MeltMADGE apparatus

3A. Tank with electrodes; glass serpent in base with connectors for external silicon tubing from recirculating water bath (protective cover for serpent removed – see Fig 3c and Supplementary Figure 16) ; and housing with impellor for vigorous mixing of tank buffer. Glass serpent dimensions given in the text.

3B. Schematic overview of a meltMADGE tank. A more detailed set of schematics is provided in the Supplementary files which should be sufficient to enable a workshop to build the apparatus. Refer to text ‘Equipment Setup’ section for further details.

3C. Full apparatus assemblies: meltMADGE tank (i); electrophoresis power supply (ii); external temperature monitor, with probe suitable to rest in the electrophoresis buffer (iii); and programmable recirculating water bath (iv).

Figure 4. MeltMADGE gel preparation and loading

4A. Single meltMADGE gel pouring. In this format, gel mix is poured into the MADGE gel former and then a sticky silanised glass plate (treated side facing gel) overlaid, but leaving a small gap at one end (glass shorter than former). The closure excludes air and once the gel is set, the glass plate with gel attached, is prised away from the gel former by using a spatula at the end where there is a gap.

4B. Multiple simultaneous meltMADGE gel pouring. In this format, gels and glass plates, each set of former and plate arranged as in 4A but then stacked together, are placed on edge in a pouring tank. Gel mix is poured into the tank at one end where there is a small space available. As the gel level rises, it enters the gap between former and glass plate for each set simultaneously.

4C. A passive replicator is used to load 96 wells at once directly from a PCR microplate.

4D. After loading, the gel is covered with a second glass plate (i). The glass–gel–glass ‘sandwich’ is secured with two stationary rubber bands (ii). The long edges of the gel are

sealed with ~15cm-long pieces of silicon rubber tubing forced between the glasses to touch the gel (iii,iv).

Figure 5. Artificial positive control in meltMADGE

The wild type (WT) and PCR-induced mutant (MUT) allele homoduplexes show different mobilities. The coannealed pair (COANN) shows two additional heteroduplex bands. Note that these controls were loaded on additional tracks provided by the MADGE gel former outside the limits of the 8×12 array which will be loaded from a PCR plate, thus allowing post-PCR add-ins such as artificial controls or re-runs. In this instance, wells were in a traditional alignment but using an 'H-PAGE' (horizontal PAGE) gel former akin to a MADGE gel former (AB ref).

Figure 6. Images from two gels from a large scale meltMADGE run

The figure shows two gels (numbered 4 and 15) from a large batch run. Apart from the variant band patterns of the artificial positive control, all tracks just show a wild type band in gel 4. A little background smear is commonly evident, presumably because in short track lengths, PCR error heteroduplexes are condensed into a relatively short track length. However, heteroduplexes should appear in quantities in near stoichiometry with homoduplex, as observed for a single track (ringed) on gel 15. The two homoduplexes have not resolved on that track.

Figure 7. Bands patterns from a thermal ramp analysis of a SNP (using tracks in an H-PAGE gel)

The image represents an example of the three band patterns for a common polymorphism. Four bands are evident in heterozygotes (tracks 2 and 6), one band in homozygotes (tracks 1 and 5 representing one homozygous group, tracks 2, 4 and 7 the other)

Band Pattern code	(1)	Artificial +ve Control	(2)	(3)	(4)	(5)	(6)	(7)
Amino acid change	Wild type	N/A	(Splice site)	C68Y	D69G	E80K	fsE80	W66G
Nucleotide change	Wild type	313+33 T>C	313+1 G>A	266 G>A	269 A>G	301 G>A	301delG	259 T>G
Real gel band pattern

Figure 8. A set of individual track images excised from a full meltMADGE gel image
 Each track represents a different mutation in *LDLR* and displays a different band pattern. Although most are transitions, it is clear that position and surrounding sequence context influence the melting transitions in different ways as apparent in the different final migration patterns.

Figure 9. Phoretix software analysis of meltMADGE images

A) shows a single heterozygote gel track analysed in Phoretix software; B) shows a Phoretix automatic extraction and rearranging (ii) of 96 tracks in a meltMADGE gel (i) in which a polymorphic amplicon had been electrophoresed. The trained eye can recognize band patterns at a glance but image analysis can help in case of doubt. MADGE-specific Phoretix software can be used to measure precisely migration (x axis) and to quantify band intensities (y axis).

Table 1**Troubleshooting**

Step	Problem	Possible reason	Possible solution
PCR	PCR failure	Primer problem or reagent failure	Try known good primers with the other reagents
PCR	Low yield	Suboptimal reaction conditions	Try optimisations altering MgCl ₂ (gradient on a plate) or annealing temperature.
MeltMADGE	No difference of band mobility in different temperature ramp runs.	Working in an unsuitable temperature range	Recheck predicted melting temperature. Check temperatures being used. Try constant temperature runs over a wider range of temperatures to identify the mobility transition temperature.
MeltMADGE	Positive control (heteroduplexes) not showing band resolution	Flawed positive control, or unsuitable thermal ramp conditions.	Check that the PCR and reannealing process was properly followed. Recheck thermal ramp conditions are appropriate.
MeltMADGE	Loss of control of the thermal ramp (rising too quickly)	Too high a current (amperometric heating)	Lower the voltage setting.
MeltMADGE	Glass serpent breakage	The glass serpent is the most vulnerable part of the apparatus.	Always leave the protective plastic cover in situ. Take special care if ever connecting/disconnecting the serpent.