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METHODS

An Improved High Throughput Heteroduplex Mutation Detection System for Screening *BRCA2* Mutations—Fluorescent Mutation Detection (F-MD)

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We describe an improved, fast, automated method for screening large genes such as BRCA2 for germline genomic mutations. The method is based on heteroduplex analysis, and has been adapted for a high throughput application by combining the fluorescent technology of automated sequencers and robotic sample handling. This novel approach allows the entire BRCA2 gene to be screened with appropriate overlaps in four lanes of an ABI 377 gel. The method will detect all types of mutations, especially point mutations, more reliably and robustly than other commonly used conformational sensitive methods (e.g. CSGE). In addition we show that this approach, which relies on band shift detection, is able to detect single base substitutions that have hitherto only been detectable by direct sequencing methods. Hum Mutat 17:220–232, 2001. © 2001 Wiley-Liss, Inc.

KEY WORDS: fluorescent; F-MD; heteroduplex; mutation detection, BRCA2

DATABASES:
BRCA2 – OMIM:600185; GDB:387848; Genbank: U43746, NM_000059; HGMD:BRCA2; http://www.nhgri.nih.gov/Intramural_research/Lab_transfer/Bic/ (Breast Cancer Information Core (BIC)); ftp://genome.wustl.edu/pub/gsc1/brca2/ (BRCA2 sequence) ftp://ftp.sanger.ac.uk/pub/human/sequences/Chr_13 (sequence present in PACs 214K23 and 92M1); http://www.icr.ac.uk/cancgen/cangen/f_md_brca2_primers.htm (Primer Information)

INTRODUCTION

Various techniques are available to detect genomic sequence alterations, many of which rely on alterations in the conformation of the nucleic acid strands. For example, single strand conformational polymorphism (SSCP) analysis [Orita et al., 1989] exploits the conformational changes caused by mutations, and has been used extensively to detect alterations in RNA and single stranded DNA. However, temperature and pH significantly affect the sensitivity of this method, and there is therefore a requirement to optimize the conditions for each amplimer.

A more recent improvement of the approach used in SSCP has been the development of heteroduplex analysis (HA) to detect mutations in double stranded DNA using conformational sensitive gel electrophoresis (CSGE) [Ganguly et al., 1993]. CSGE utilizes the altered mobility of

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heteroduplexed strands in a gel matrix caused by annealing of mutant and wild type strands. The technique eliminates the requirement of conducting separate optimizations for each amplimer, as it is less affected by temperature and pH. Mutated strands that form heteroduplexes with the wild type strands manifest as band shifts from homoduplexes. For these reasons, CSGE has been used extensively in the mutation screening of many genes, including *BRCA2* (MIM# 600185) [Wooster et al., 1995], collagen type XVII (*COL17A1*; MIM# 113811) [Gatalica et al., 1997], Peutz-Jeghers syndrome (*LKB1*, also called STK1; MIM# 602216) [Boardman et al., 2000], and the cylindromatosis gene (*CYLD1*; MIM# 605018) [Bignell et al., 2000]. The CSGE technique has been investigated previously on a fluorescent electrophoresis platform (F-CSGE) [Ganguly et al., 1998]. The report showed it was possible to run three different PCR fragments in one lane of an ABI 377 gel, each labeled with a different colored dye.

Other techniques used for undertaking mutation screening include chemical and enzyme cleavage of mismatches [Cotton, 1999], protein truncation test (PTT) [van der Luijt et al., 1994], denaturing high pressure liquid chromatography (DHPLC) [Arnold et al., 1999; Gross et al., 1999], and direct sequencing. Although each technique has its merits, application of any one of them for high throughput usage is limited by factors such as labor input, speed, expense, and sensitivity.

Abnormalities in the *BRCA2* gene are the cause of a substantial proportion of breast cancer in families with ≥ four cases of young onset disease [Ford et al., 1998] and possibly other inherited cancers such as prostate cancer [Breast Cancer Linkage Consortium, 1999; Gayther et al., 2000]. The BRCA2 protein is involved in the cellular response to DNA damage, forming a protein complex with BRCA1 and Rad51. There is evidence that this complex plays a role in DNA recombination [Chen et al., 1998], however the exact function of the protein is yet to be elucidated.

The *BRCA2* gene has 27 exons and approximately 10 kb of coding sequence. To date, information from the Breast Cancer Information Core (BIC) database describes an array of pathogenic and noncoding *BRCA2* mutations. There are 231 individual frameshift deletions, the majority of these (96%) are ≤ 5 bp, with the largest recorded at 126 bp. The database contains information on 68 separate frameshift insertions, again the majority are ≤ 5 bp (93%). There are three large insertions recorded, one of 71 bp and two with alu repeats inserted. Nonsense and missense mutations account for 93 and 312 separate entries, respectively, and there are 56 polymorphisms recorded. The pattern of alterations in the non coding region is similar, and there is no evidence for large chromosomal rearrangements in this gene [Unger et al., 2000; Peelen et al., 2000]. The spectrum of abnormalities is similar in *BRCA1*, except that there are rarer mutations that result in the duplication or deletion of entire exons, as well gene rearrangements [Unger et al., 2000; Peelen et al., 2000].

Ordinarily, screening this gene for all types of mutations in a large population is a laborious undertaking using conventional radioactive or silver stained conformational gel based methods such as SSCP and CSGE. It is certain while using CSGE that some small alterations, such as point mutations, can be missed. Such missense mutations may cause structural changes and consequently, alterations in protein function, and have been shown to be fundamental in the development of a subset of inherited breast cancers [Chapman and Verma, 1996; Roth et al., 1998]. As yet, the majority of missense mutations in *BRCA2* do not have a clearly defined role in oncogenesis, and in order to elucidate their biological significance, techniques will be required that reliably detect all of these alterations. The main aim of this study was to develop a technique for the mutation analysis of genomic DNA, to detect all possible mutations on a single screen, and to generate these data quickly, efficiently, and reliably. To achieve this, we exploited the speed and ease of CSGE, but investigated an alternative gel matrix—MDE (mutation detection enhancing gel) [Prior et al., 1993; Gayther et al., 1996] to improve sensitivity, allied to the potential benefits of laser detection electrophoresis systems, e.g. ABI 377. Additionally we investigated the use of combi-

nations of fluorescent dyes, robotic manipulation of samples, and computerized band shift detection. We have called this approach F-MD—fluorescent mutation detection.

MATERIALS AND METHODS

DNA Isolation and Samples

Lymphocyte DNA was extracted by routine methods [Edwards et al., 1997]. Samples used were from normal individuals and from those with germline *BRCA2* mutations. The total number of positive *BRCA2* mutants investigated comprised eight point mutations and three frameshifts (see Table 1).

PCR

PCR was conducted with 45 *BRCA2* primer pairs (M. Stratton, S. Gayther, personal communication), some of which were also newly designed. Many fragments received individual optimization in order to produce a single, clean PCR product with minimal primer dimer. Primer sequences and PCR conditions are available on request or from the web site—www.icr.ac.uk/cancgen/cangen/f_md_brca2_primers.htm. Sizes of fragments ranged from 199–518 bp. The large exons (10 and 11) were amplified in multiple subfragments. Sufficient overlap was included between subfragments of these and other exons in order to allow for the detection of genetic alterations in the primer regions. Where common polymorphisms were present in primer regions, additional primers were designed for these sequences and mixed in equal ratio with the respective variant primer.

In general, thermocycling was conducted with 'touchdown' protocols. Annealing temperatures of 60–50°C and 55–45°C were chosen. Cycling consisted of an initial denaturation of 95°C for nine min, followed by 94°C for 45 sec, touchdown annealing temperatures for 45 sec, 72°C for 60 sec for 20 cycles. A final round of amplification was conducted at the lowest annealing temperature for 20 cycles. The PCR was terminated with 72°C for 10 min.

A heteroduplex formation step was included at the end of each PCR program consisting of heating the product to 95°C and allowing it to cool to 40°C over a period of 90 min.

Reagents and final concentrations in 15μl PCR reactions were as follows: 50 mM KCl and 10 mM Tris pH 8.3 (Perkin Elmer, Warrington, UK, buffer II), $MgCl_2$ 1.0–3.0 mM (Perkin Elmer), 1.0 mM dNTP (Stratagene UK or ABGene, Epsom, UK, 0.25 mM each), primers at 0.06 OD (approx. 0.5 μM; Perkin Elmer and Oswel DNA Services, Southhampton, UK), 0.12 units Amplitaq Gold (Perkin Elmer), 25 ng ge-

TABLE 1. Description of *BRCA2* Mutations Investigated

Exon/ sub-fragment	Sequence change (nucleotide)	Sequence change (amino acid)	Original detection method in our laboratory	Detected by F-MD	Detected by F-CSGE	Detection method on BIC	Mutation classification
Ex 10.03	1742T>C	I505T	F-MD	Yes	No	DS	UV
Ex 11.16	6893A>G	Y2222C	F-MD	Yes	No	Not on BIC	MS
Ex 11.11	5416A>T	N1730Y	F-MD	Yes	No	Not on BIC	MS
Ex 11.12	5868T>G	N1880K	MDE	Yes	No	DS, DHPLC	UV
Ex 11.05	4035T>C	V1269V	F-MD	Yes	Equivocal[a]	DS, DHPLC	P
Ex 11.15	6631A>C	N2135H	MDE	Yes	Equivocal[a]	Not on BIC	MS
Ex 22	9179C>G	S2984X	CSGE	Yes	Yes	CSGE	NS
Ex 11.12	5972C>T	T1915M	CSGE	Yes	Yes	DS, DHPLC, CSGE	P
Ex 11.13	6174delT	Stop2003	CSGE	Yes	Yes	—	Deletion
Ex 11.13	5909insA	Stop1894	CSGE	Yes	Yes	PTT	Insertion
Ex 11.15	6630delTAACT	—	CSGE	Yes	Yes	—	Deletion

Mutation class on Breast Cancer Information Core (BIC) database (see databases and materials and methods for web address): UV, unclassified variant; P, polymorphism; MS, missense; NS, nonsense mutation.
Detection methods: F-MD, fluorescent mutation detection (using MDE gel; MDE, mutation detection enchancing gel—bands visualized by silver staining or radioactive labelling; F-CSGE, fluorescent conformational sensitive gel electrophoresis; CSGE, conformational sensitive gel electrophoresis—bands visualised by silver staining or radioactive labelling; DS, direct sequencing; DHPLC, denaturing high pressure liquid chromatography; PTT, protein truncation test.
[a]See Figure 3c, F-CSGE panel.

nomic DNA. For some fragments Opti-Prime Buffer 6 (Stratagene, UK) was used as the reaction buffer. Primers were labeled with 5′ FAM (blue), HEX (green), NED (yellow), or ROX (red). More information on NED and the other dyes is available from the ABI website (www.appliedbiosystems.com/MolecularBiology/about/dna/377/377a2c.html).

Representative PCR fragments were run on a variety of gels (agarose (National Diagnostics Laboratories, Hull, UK), ABI 377 (San Francisco, CA) using a standard genotyping gel and run module, Genescan followed by Genotyper analysis and F-MD, see below) to establish signal strength and mobility prior to full analysis. Signal strength was measured in order to obtain a minimum intensity of 1000 ABI fluorescent units for each fragment in the mixture of up to 12 fragments. Fragments were combined and mixed (post PCR multiplexing) on a Biomek 2000 robot (Beckman, High Wycombe, UK) prior to analysis. On average, fragments were diluted by between 1/12 and 1/36 prior to full analysis.

F-MD Analysis

The modified MDE gel matrix consisted of 1X MDE (FMC Corporation—Flowgen, Staffordshire), 10% ethylene glycol, 15% formamide in 0.6X Tris Borate buffer (TBE -53.4 mM tris base, 53.4 mM boric acid, 1.2 mM EDTA, pH 8.4). The gel was polymerized with ammonium peroxydisulphate (0.046% w/v final) and TEMED (0.034% v/v final).

An ABI 377 DNA sequencer was modified and used for DNA heteroduplex fragment separation and analysis**.** A modified Genescan run module was written for data collection (available on request). Electrophoresis conditions were 500 V, for 18 hr at 13°C in 0.6X TBE. Gels were cast to a thickness of 0.2 mm in 12 cm well-to-read plates with 64 well combs. Cooling was achieved by connecting the ABI 377 to a cooling water bath (2209 Multitemp, LKB, Amersham/Pharmacia, Little Chalfont, UK), the default heating command being disabled in the run module and replaced by a 'chiller' command. Water vapor was removed from the air with a portable dehumidifier (Ebac, UK), to prevent condensation on the cooled glass plates during the run. Water condensation interfered with laser detection of products. Filter set D was used for data collection. The ABI 377 was set at 1200 scans per hr.

Prior to loading, samples were mixed with an equal volume of loading buffer (blue dextran 50 mg/ml in 25 mM EDTA—pH 8.0 mixed equally with freshly deionized ultrapure formamide (99.5%; Sigma F9037)).

Data collection was conducted with Genescan version 3.0 software (ABI) using a peak half-width of 5 with no smoothing and no split-peak correction. Data analysis was performed by Genotyper version 2.1 software (ABI). Heteroduplexes were identified by the presence of more than one peak for a specific PCR product. All experiments were conducted at least twice.

F-CSGE Analysis

This was conducted essentially as per Ganguly et al. [1998]. Gels consisted of acrylamide (4% final concentration), 15% formamide, in 1X TBE. The stock acrylamide used (40%) was mixed with bis-acrylamide (BAP) in the ratio 99:1. The gel was polymerized with ammonium peroxydisulphate (0.045% w/v final) and TEMED (0.080% v/v final). Gels (36 cm well-to-read) were run with or without ethylene glycol at 10% final concentration. The ABI 377 was not cooled and set to 2400 scans per hour. Gels were run at 1800V and reached temperatures of 33°C on average. All experiments were conducted at least twice. Data collection and analysis was performed as for F-MD.

Sequencing

Sequencing was conducted to confirm mutations using dRhodamine dye terminator cycle sequencing (ABI) with both forward and reverse PCR primers.

Database—BIC

The Breast Cancer Information Core (BIC) database is accessible via the World Wide Web for registered users. It catalogs *BRCA1* and *BRCA2* mutations submitted from international sources (www.nhgri.nih.gov/Intramural_research/

Lab_transfer/Bic. This may require a password). The database was used to identify and analyze the type, detection method, and depositor source of *BRCA2* mutations.

Mutation nomenclature. Mutations are descibed by standard nomenclature (ariel.ucs.unimelb.edu.au /~cotton /antonara.htm).

RESULTS

Figure 1 shows a gel image of four lanes that contain all 45 fragments of the *BRCA2* gene. These were post PCR multiplexed with up to 12 fragments per lane. The figure shows that there is sufficient space between all the fragments for detection of any band shifts. One such band shift can be seen in 'lane 4' (arrowed)—this is a shift in the FAM (blue fluorescence) labeled exon 2, caused by a common polymorphism. This mutation is a noncoding 203G>A polymorphism and is present in the population at a frequency of 26% [Wagner et al., 1999]. Although some of the shift is obscured by the ROX (red fluorescence) fragment above exon 2, when only the signal from the blue channel is examined, the shift becomes very obvious. This is illustrated in Figure 2, panel d, where the additional band produced by the polymorphism can be clearly seen (arrowed).

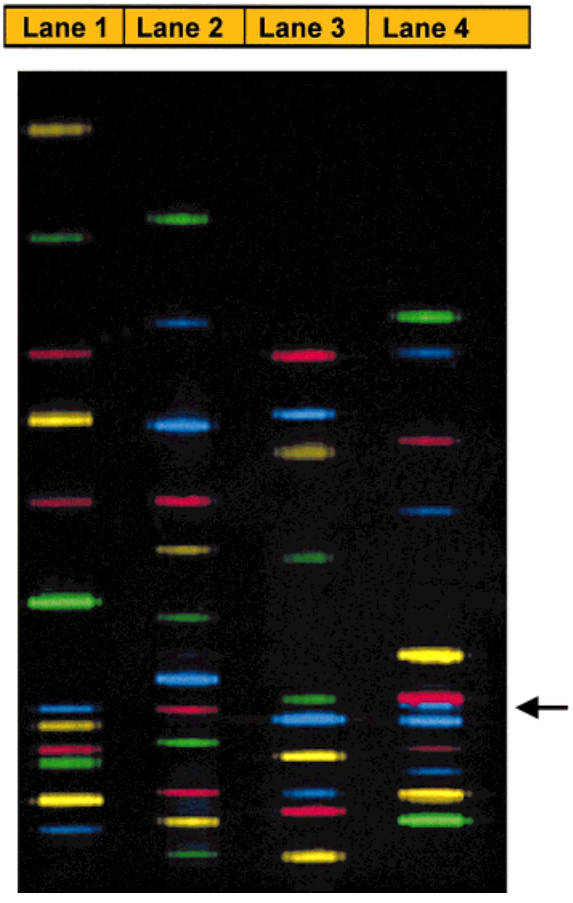


FIGURE 1. **F-MD gel image showing all 45 *BRCA2* fragments in four lanes of the gel. Polymorphism in Ex 2 arrowed. Lane 4 (blue).**

The four panels in Figure 2 show ABI 'Genotyper' chromatograms produced by analyzing each fluorochrome of the gel image shown in Figure 1 separately. The separation was done with the aid of the ABI Data Collection software which creates a correction factor or 'matrix.' The 'matrix' mathematically eliminates spectral overlap or 'bleed-through.'

It was found that fragments do not strictly migrate according to their molecular weight in MDE gel matrix. This effect is due to the inherent conformation and secondary structure of different sequence stretches. An example of this is shown in Figure 2. Exons 23-24 and 11.09 differ in size by approximately 100 bp, yet they migrate at the same rate (approximately 16K scans).

The F-MD gels were run for a total of 18 hours, which allowed sufficient time for the potential heteroduplexes of fragments to be detected. At 1200 scans per hour, the largest fragment, exon 11.07 (18.8K scans) took 15 hours to pass the laser. During development of the technique, on rare occasions, it was noted that the heteroduplexes which were produced by some larger fragments did not emerge from the MDE gel in the run times available. This is in contrast to conventional radioactive or silver stained conformational sensitive gels, where the whole gel is visualized for band shifts. However, as development proceeded, it became evident that the fluorescent MDE gel was able to resolve homoduplexes very efficiently. Therefore, in situations where heteroduplexes did not pass the detection laser due to the severe conformational changes caused by larger deletions and insertions, the homoduplexes of such mutations were easily detectable. To show this and to make fur-

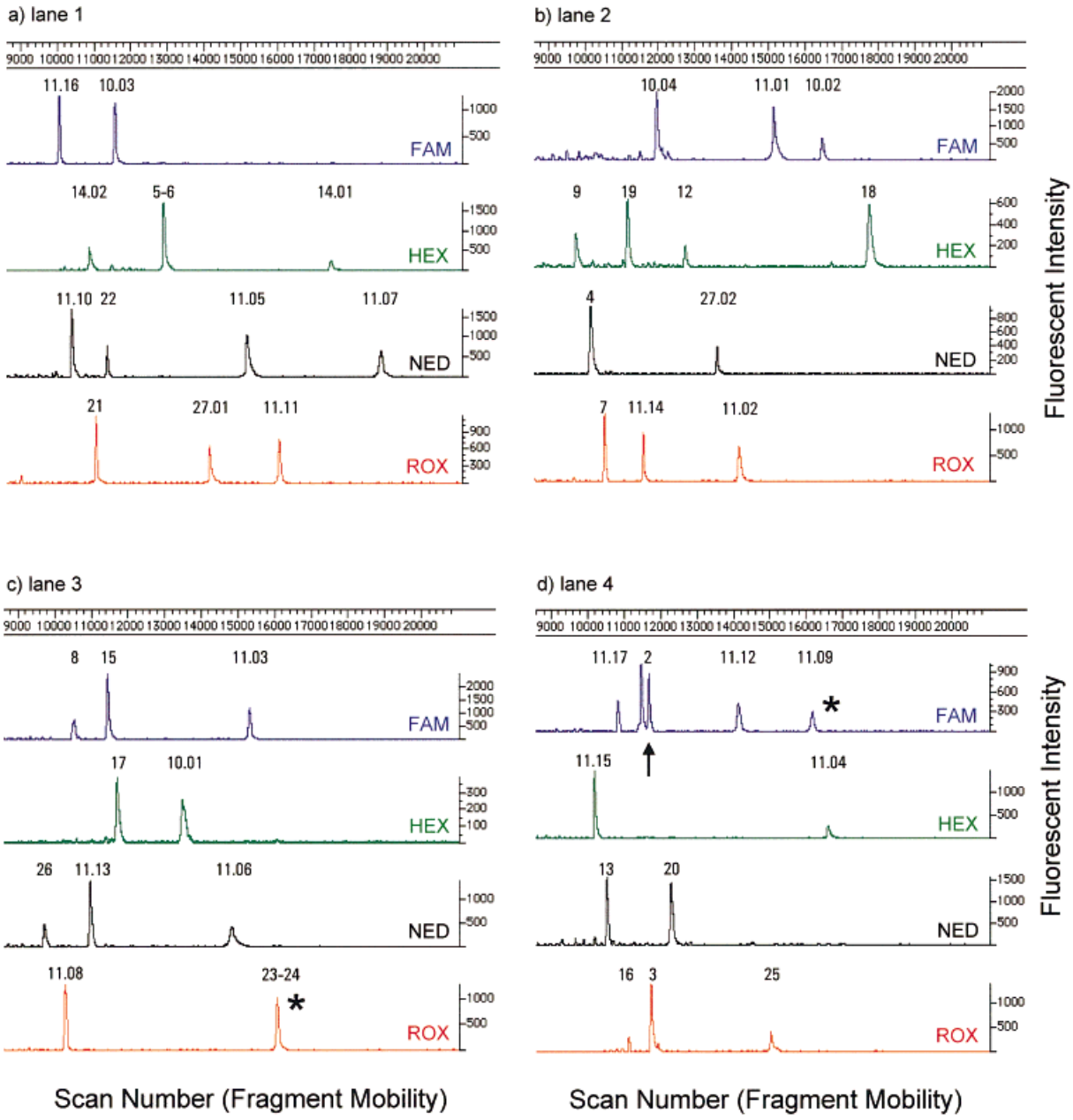


FIGURE 2. **Chromatograms produced from the gel in Figure 1. Panels a–d represent lanes 1–4, respectively. Traces produced with ABI 'Genotyper' software. Blue = Fam; Green = Hex; Black = Ned (yellow in Fig. 1); Red = Rox. Panel d shows a polymorphism in Ex 2 (arrowed). Exons and sub-fragments shown above peaks. *Panel c, Ex 23-24 (Rox), and d, Ex 11.09 (Fam), have similar migration (16.2K scans) although they vary in size by 100 bp.**

ther comparisons with CSGE, experiments were conducted on identical known mutant samples.

Figure 3 shows representative results for the comparison between F-MD and F-CSGE [Ganguly et al., 1998], for mutant and wild type sequences. Mutant samples were obtained from individuals with germline *BRCA2* mutations and confirmed by sequencing. A total of 11 mutant samples was used for the comparison.

Panels 3a and 3b illustrate that two point mutations, easily detectable on F-MD, were not visible when run on a CSGE gel matrix. Two other

mutations behaved similarly (data not shown). F-CSGE results were unchanged when gels were repeated with the addition of ethylene glycol (data not shown). The F-MD results obtained were routinely reproducible from gel to gel and at least two gels were run for each mutant.

Examining the band shifts caused by the T to C transition in exon 11.05 (panel 3c 4035T>C), shows that the band shift on the F-MD gel, although not as pronounced as in other panels, exhibited completely separate peaks. This is in contrast to the F-CSGE pattern, where the shift is more of a shoulder. Notably, the F-CSGE shoulder in this fragment was not reproducible from gel to gel (data not shown). Small shoulders were observed in two of the 11 fragments examined by F-CSGE. This feature was in contrast to F-MD, where a separate peak was always seen and there was no ambiguity in signal. In addition, band shifts were always reproducible on F-MD gels.

The results obtained from F-MD and F-CSGE analysis of the 11 mutations are summarized in Table 1. In general band shifts on F-MD gels were more pronounced than those on F-CSGE gels, making them far easier to detect. Figure 3 panels d and e illustrate that band shifts on F-CSGE gels can be unambiguous. However, the resolution of mutant fragments is inferior to F-MD. This was the situation for five of the 11 mutations screened by F-CSGE where the bandshifts were unambiguous.

Figure 3e illustrates that heteroduplexes are retarded to a greater extent in F-MD, and also illustrates that, as with all other frameshifts mutations, both homoduplex (wt/wt and mut/mut) fragments are resolved. However, with some larger fragments, heteroduplexes (wt/mut, mut/wt) formed because of gross alterations retarded to such an extent by the MDE matrix that they were unable to pass the laser. This was not considered to be a drawback for screening, as the presence of both homoduplex peaks was sufficient to detect any mutation. An example of this type of alteration is shown in Figure 3f. The panel shows that both heteroduplexes produced by a 5 bp deletion (6630delTAACT) were completely retained by the F-MD gel during its run, whereas they were able to pass the laser and be detected on the F-CSGE gels.

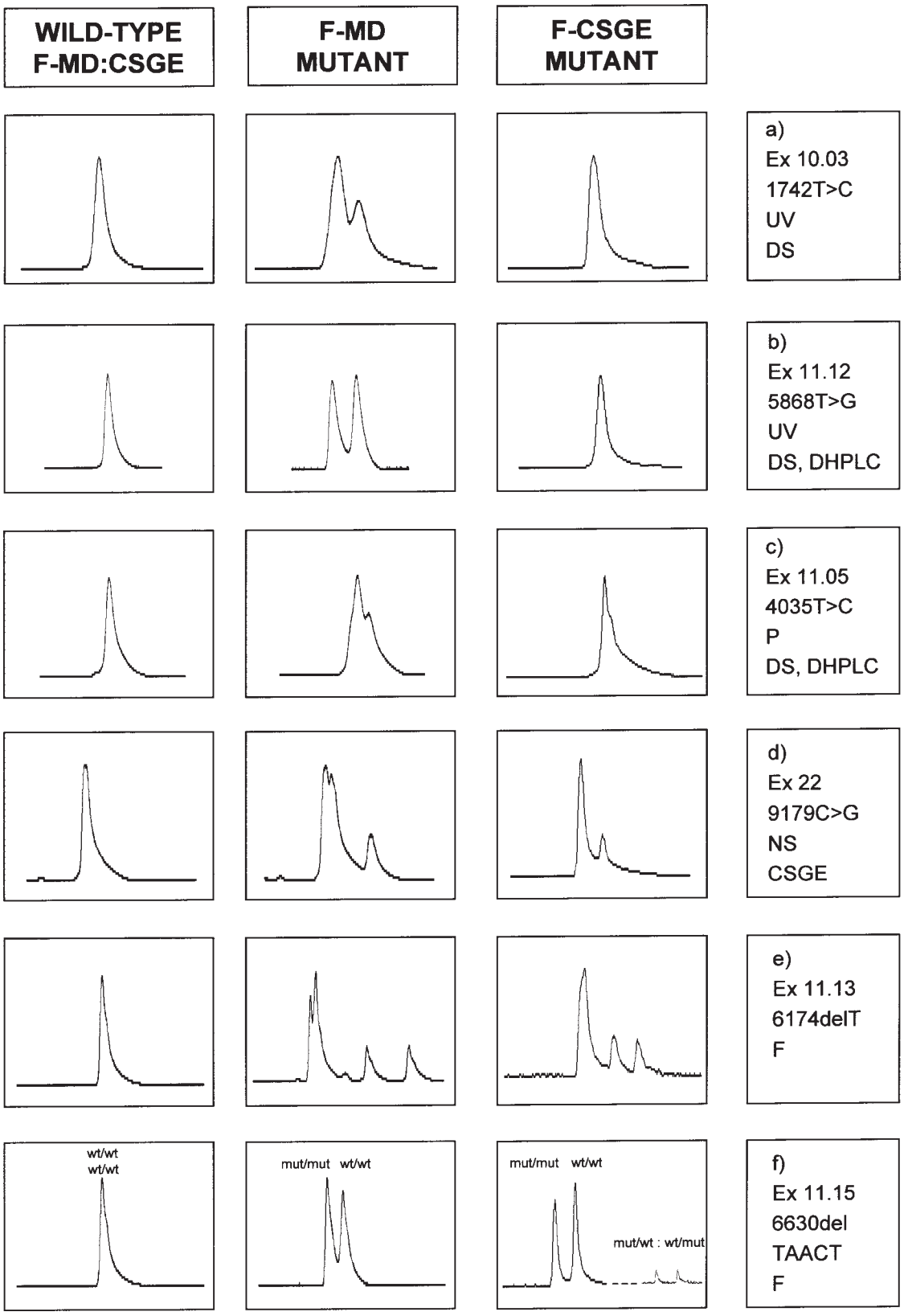
Mutant fragments were also run on F-MD gels in the absence of ethylene glycol. The band shifts observed were identical to those produced with ethylene glycol present. However, when present, ethylene glycol retarded fragments. This feature was beneficial when up to 12 fragments were mixed in a lane, as it allowed more space for resolution of bands and for detection of shifts. Fragments on gels with ethylene glycol ran on average 1.7 times slower. If there was no requirement to mix multiple fragments, omitting ethylene glycol resulted in a significant time saving.

To investigate the effects of temperature on mutant detection, F-MD gels were run at 13°C, 18°C, and 23°C. Fragments migrated in a temperature dependent manner, migrating on average 1.4 times faster at 23°C than at 13°C (data not shown). In contrast, the resolving power of the gels decreased as temperature increased. Some mutant fragments were affected more by temperature than others (data not shown). Taking into consideration these two factors, a running temperature of 13°C, which required access to a cooling unit, was found to be optimum.

DISCUSSION

The method described may be of interest to those who need to conduct high throughput mutation analyses with a high degree of sensitivity. We suggest that for screening *BRCA2*, F-MD can detect point mutations more reliably and robustly than the commonly used conformation gel based band shift assay CSGE, and that F-MD will de-

FIGURE 3. **Samples from normal individuals and *BRCA2* carriers. Traces: left column shows wild type trace obtained on F-MD and F-CSGE. Middle columns show mutant traces obtained with both techniques, respectively. For illustration, traces were scaled to wild type peak width for both techniques. Samples' details are shown for each panel (a–f)—exon, sequence change, mutation type, and detection technique on BIC database. UV, unclassified variant; P, polymorphism; NS, nonsense mutation; F, frameshift; DS, direct sequencing; DHPLC, denaturing high pressure liquid chromatography. Panel f displays a F-CSGE trace with all the possible mutant (mut) and wild type (wt) DNA duplex strands (homoduplex: mut/mut and wt/wt; heteroduplex: mut/wt and wt/mut). The heteroduplex peaks are not shown to scale. The F-MD shows only homoduplexes, as the heteroduplexes were retarded by the gel and did not pass the laser during the run time. On average, normal peak widths for F-MD were 13.5 min, and for F-CSGE, 1.3 min (scan number and intensity are not shown).**


WILD-TYPE
F-MD:CSGE
F-MD
MUTANT
F-CSGE
MUTANT
a)
Ex 10.03
1742T>C
UV
DS
b)
Ex 11.12
5868T>G
UV
DS, DHPLC
c)
Ex 11.05
4035T>C
P
DS, DHPLC
d)
Ex 22
9179C>G
NS
CSGE
e)
Ex 11.13
6174delT
F
wt/wt
wt/wt
mut/mut wt/wt
mut/mut wt/wt
mut/wt : wt/mut
f)
Ex 11.15
6630del
TAACT
F

tect alterations as reliably as DHPLC and direct sequencing (see Table 1 for summary).

Sensitivity has been increased by virtue of the fact that every fragment loaded in the gel has to migrate through the entire gel in order to be detected, a distance of approximately 12 cm. This is in contrast to a conventional gel where, in a mix of fragments, the larger ones may travel only a short distance into the gel in order to retain shorter fragments for visualization. The additional distance traveled on the ABI platform allows any conformational variants to be resolved. For screening purposes, the use of larger fragments is beneficial in terms of consumables and labor costs. Restricting fragments to 200–300 bp for *BRCA2*, while increasing detection sensitivity, would increase the number of fragments for screening from 45 to 65.

Of concern when using fluorescent detection platforms is the amount of color bleed-through of one dye signal into that of another. Such a phenomenon can produce spurious peaks and create the illusion of band shifts. Figure 2 clearly shows that there is no color bleed-through with the ABI filter set D. Investigation into the older ABI color set (Fam, Hex, Tet, and Tamra) showed that levels of bleed-through were unacceptable when attempting to analyze many fragments in a lane (data not shown).

Cooling had a significant effect on gel resolution and sensitivity. Cooling of the gel was achieved with a chiller unit connected into the existing cooling system of the ABI 377. Band shifts caused by point mutations were detected with higher sensitivity at lower run temperatures. The optimum temperature for running was found to be 13°C. In general, the reduced temperature did not affect detection of frameshift mutations, so it would be feasible to run F-MD gels at higher temperatures in order to detect gross abnormalities. Buffer strength also influenced run time. We chose a tris borate buffer strength of 0.6X (see materials and methods), as this decreased run speed and increased resolution (data not shown).

When considering a method for mutation analysis, many of those available have a trade off between sample throughput and sensitivity. In general, techniques that have a high throughput are compromised by their sensitivity and vice versa. For this reason CSGE has been the favored method of screening in our laboratories. The method has been amenable to processing large sample sets with acceptable sensitivity. CSGE has been used extensively for the characterization of various genes—*BRCA2* [Wooster et al., 1995], *COL17A1* [Gatalica et al., 1997], and *CYLD* [Bignell et al., 2000]. Investigation of the F-MD method has highlighted the possible limitations of CSGE for screening and detection point mutations. In addition the limitations of CSGE also have been observed and discussed in the identification of the cylindromatosis tumor-suppressor gene [Bignell et al., 2000], whereby three families were positively linked to this gene, but no band shifts were detected.

A panel of mutants was selected from those detected in our laboratory to represent the different types of alteration seen in *BRCA2*. Of the eight point mutations studied, two were found originally by conventional CSGE, two by conventional MDE, and four by F-MD. The three frameshift mutations were detected originally by CSGE. All were confirmed subsequently by direct sequencing.

One of the main aims of this study was to discover how sensitive CSGE was at detecting point mutations. In no case did CSGE detect a mutation when F-MD did not. Of more importance was the observation that F-MD found mutations not detected by F-CSGE (4/8 point mutations). We believe that F-MD is a major improvement on CSGE and will become the favored gel based method for screening multiplexed PCR products in our laboratory.

The efficiency of heteroduplex analysis can depend on amplimer design. Heteroduplex formation is compromised if mutations are at the beginnings or ends of amplimers. In order to allow for this, we designed amplimers with 30–50 bp overlaps between adjacent amplimers. This is a common strategy, however we acknowledge that this cannot guarantee detection of all mutations.

As with any heteroduplex method, homozygous base substitutions are unlikely to be found. These are likely to be extremely rare in *BRCA2*, although we have found no reference to a frequency. This is similar to the germline *P53* situ-

ation where the frequency is believed also to be zero (IARC TP53 Mutation Database, see references). In general this type of alteration can only be found reliably by direct sequencing. It may also be possible to detect such alterations by mixing in samples with known normal DNA samples in order to produce heteroduplexes artificially. We did not investigate this methodology as it can potentially reduce the overall sensitivity of detection. Homozygous insertions and deletions that are difficult to detect on CSGE gels can be detected reliably by F-MD. To illustrate this, the mutation shown in Figure 3e is a deletion of a 'T.' This gives rise to two homoduplex bands that are resolved by F-MD, but not by F-CSGE. The separation on F-MD is sufficient that if both alleles had lost the 'T', the band produced would be discernible by migration from the wild type amplimer.

With this fluorescent detection technique, heteroduplexes that are produced by larger alterations, such as insertions and deletions, are less likely to pass the laser for detection. This is especially true of heteroduplexes from the larger amplimers. It is thought that the secondary structure of these heteroduplexes may cause them to be 'retarded' by this particular gel matrix to a greater extent than the unmodified polyacrylamide used in F-CSGE. We found that this retardation did not hinder mutation detection, as the homoduplexes produced by insertions and deletions were always seen using F-MD. The largest deletion we investigated was 5 bp (Fig. 3f), which is representative of deletions seen in *BRCA2* (221 of the total of 232 frameshift deletions submitted to the Breast Cancer Information Core (BIC) database are ≤ 5 bp), and was easily detectable by F-MD. The largest frameshift deletion deposited on BIC for *BRCA2* to date was a 126 bp (9200del126). Rare deletions such as this will be detectable on F-MD by virtue of the shorter mutated allele.

Large insertions are also rare in *BRCA2* (63 of a total of 68 frameshift insertions are ≤ 5 bp; 56 of the 68 are 1 bp insertions). One of largest frameshift insertions deposited on BIC for *BRCA2* to date is 71 bp (8506ins71). Detection of such large insertions will depend on the initial size of the amplimer, in this particular instance the amplimer corresponding to this region (ex 18) migrates with a run time that is sufficient for a mutated 571 bp product to be detected. This is the case for any amplimer, within our set, that is extended by a similar size. Additionally two large alu repeat insertions have been described. One alu insertion contains a repeat of 346 bp, the other is not described in detail as it was detected by Southern blotting. Insertions of this type may not be found by heteroduplex analysis.

In early reports on mutation detection in *BRCA1* and *BRCA2*, the most commonly detected abnormalities were truncating mutations, such as deletions and insertions, and were detected by PTT [Hogervorst et al., 1995] or SSCP [Lancaster et al., 1996]. As these were the major class of mutations detected, they were arguably regarded as being the main causal type of abnormalities leading to cancer predisposition in individuals carrying these genes. This belief may have resulted in publication bias, whereby only major sequence alterations were reported. The bias may have been compounded further, as mentioned earlier, by the use of techniques that generally only detected major alterations. Recently, the detection of point mutations has increased. This can be seen in the BIC database, where, due to an increase in demand for genetic testing, the number of point mutations submitted to the database by Myriad Genetics Inc. and others has increased significantly.

Although great emphasis has been placed on being able to detect point mutations in our study, the significance of those that are not obvious polymorphisms, in terms of altering protein structure and function, remains unknown. There is mounting evidence that missense mutations can have significant functional effects and predispose to familial breast cancer in both the *BRCA1* [Chapman and Verma, 1996; Humphrey et al., 1997; Zhang et al., 1998; Scully et al., 1999] and *BRCA2* genes [Roth et al., 1998; Chen et al., 1999], thus making it unwise to disregard all such alterations as non-pathogenic.

The true significance of these mutations will become evident as more are found and their functional effects are elucidated. Many laboratories are reporting the discovery of these muta-

tions and Myriad Genetics Inc. has become the biggest contributor to BIC because of their 'sequencing-only' screening policy. We conducted a survey of the BIC database and found that, as a proportion of all coding point mutations submitted, Myriad Genetics has reported 61% (225/367) of the total. In contrast, the proportion submitted by them is far lower for coding frameshifts at 42% (114/271) as there are many other methods that find this type of mutation. Direct sequencing is by far the favored method for detecting point mutations. Analysis of BIC to examine the frequency of frameshifts and point mutations detected in the *BRCA2* gene, and the methods used to detect these mutations, revealed that for frameshifts the proportion found by direct sequencing versus all other methods was 38.0%. For point mutations, the proportion found by direct sequencing was significantly higher at 58.5%. This observation again highlights the potential advantages of any gel based technique that can provide a similar sensitivity for detection of point mutations to direct sequencing.

When screening large genes in large populations, the cost and throughput considerations of the screening method become paramount. Although F-MD requires the gel to be run for 18 hours, the method consistently assays up to 600 fragments per gel. This is equivalent to an output of around 30 fragments/hr. This is the screen rate for band shift detection and a small allowance has to be made for the time taken to verify the mutation by subsequent direct sequencing. This screening rate compares very favorably with both DHPLC and direct sequencing where approximately 7–8 fragments per hr can be screened. The screen rates have been calculated for DHPLC performed on a Transgenomics WAVE (D. Cuthbert-Heavens, personal communication) system and for direct sequencing using a 64 lane gel on an ABI 377 (R. Hamoudi, personal communication). In addition, if direct sequencing is used solely for mutation detection, substantial additional time may be required to analyze the sequence in detail to find sequence variations.

DHPLC is becoming a popular method for mutation screening, however there are some considerations that may currently preclude the method from high throughput usage. In order to achieve maximum detection, each fragment injected into the separation column requires a custom temperature profile that matches the sequence melting temperature. This factor may hinder post PCR mixing of samples and restrict the sample throughput. In addition, the purchase costs of a DHPLC system are significant. Many laboratories may already operate an automated analyzer such as the ABI 377, and would be able easily to modify an existing machine to conduct F-MD screening. These analyzers are used routinely to sequence any band shifts detected by DHPLC, and can now conduct all aspects of the mutation detection process, eliminating the need for additional equipment.

Ignoring labor costs, direct sequencing is by far the most expensive method for screening, and for the purposes of comparison we made this the unit cost per fragment. Much of this cost is due to the commercial charges levied for sequencing kits. In comparison, F-MD costs about 0.07U per fragment, i.e. 14 fragments can be screen for the price of one direct sequence analysis. Therefore, the entire *BRCA2* gene can be screened for the cost of approximately three direct sequence analyses. At commercial rates of charge, for example those levied by diagnostic testing companies, 35 *BRCA2* genomes could be screened by F-MD for the cost of one commercial genome screen.

In view of the above considerations, F-MD could be considered as a method for genetic analysis of large genes in a diagnostic setting. Whereas it is common in diagnostic services to screen large genes in fragment batches, if results are required urgently, it is feasible to automate fragment generation for a gene such as *BRCA2* for a single sample. This would simply require the 45 pairs of fluorescent primers to be placed in a 96 well tray, enabling all fragments to be generated in one day. Subsequent checks, adjustments, and runs would yield a turnaround time of approximately 96 hr. Such a turnaround time should be applicable to many other genes, and could ultimately speed up test times.

In conclusion, we have illustrated how F-MD is able to detect mutations in the *BRCA2* gene

with better resolution than the conformation based gel method, CSGE. In particular, point mutations were detected with a higher level of sensitivity. F-MD detected many point mutations that have been found previously only by DHPLC and direct sequencing. These point mutations are shown in Figure 3a-c, and were deposited on the BIC database, and described as having been found by DHPLC and direct sequencing or by direct sequencing alone. These observations suggest that F-MD may have the detection capability of these two alternative methods. However, in order to investigate this hypothesis, a large comparative study would have to be undertaken to evaluate the sensitivities of all three methods. In terms of cost and speed, F-MD has significant advantages, which make it ideal for high throughput mutation screening. The improvement in detection ability is attributed to the unique combination of mixing fluorescent dyes, a modified gel matrix, and running conditions that allow all fragments to travel the whole length of the gel and robotic manipulation. This method is not only of value to those screening *BRCA2* for mutations, but is easily applicable to any other gene of interest (for example *BRCA1* and *P53)* that shows a similar spectrum of alteration.

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