A highly accurate, low cost test for *BRCA1* mutations

Nathalie J van Orsouw, Rahul K Dhanda, Youssef Elhaji, Steven A Narod, Frederick P Li, Charis Eng, Jan Vijg

Institute for Drug Development, Cancer Therapy and Research Center, 8122 Datapoint Drive, Suite 700, San Antonio, TX 78229, USA
N J van Orsouw
J Vijg

Mosaic Technologies Inc, 1106 Commonwealth Avenue, Boston, MA 02215, USA
R K Dhanda

Ontario Cancer Institute, Princess Margaret Hospital, 610 University Avenue, Toronto, Ontario, Canada M5G 2M9
Y Elhaji
S A Narod

Program in Population Sciences, Dana-Farber Cancer Institute and Harvard Medical School, 44 Binney Street, Boston, MA 02115, USA
F P Li

Clinical Cancer Genetics and Human Cancer Genetics Programs, Ohio State University Comprehensive Cancer Center, 420 W 12th Avenue, 690c MRF, Columbus, OH 43210, USA
C Eng

Correspondence to:
Dr van Orsouw.
nvorsouw@saci.org

Abstract
The hereditary breast and ovarian cancer syndrome is associated with a high frequency of *BRCA1* mutations. However, the widespread use of *BRCA1* testing has been limited to date by three principal concerns: the fear of loss of health and life insurance, the uncertain clinical value of a positive test result, and the current lack of an inexpensive and sensitive screening test for *BRCA1* mutations. We have developed an inexpensive system for gene mutational scanning, based on a combination of extensive multiplex PCR amplification and two dimensional electrophoresis. The efficiency of this system, as a screening test for *BRCA1* mutations, was evaluated in a panel of 60 samples from high risk women, 14 of which contained a previously identified mutation in *BRCA1*. All 14 mutations were identified, as well as an additional five that had previously escaped detection. In addition to the 19 mutations, a total of 15 different polymorphic variants were scored, most of which were recurring. All were confirmed by nucleotide sequencing. The cost of screening per sample was calculated to be approximately US$70 for the manual technique used in this study, and may be reduced to approximately US$10 with the introduction of commercially available PCR robotics and fluorescent imaging. Implementation of this method of mutation screening in the research and clinical setting should permit rapid accrual of quantitative data on genotype-phenotype associations for the evaluation of diagnostic testing.
(*J Med Genet* 1999;**36**:747–753)

Breast cancer is a common disease with a life time risk in women of 10%. The risk increases substantially when one or more close relatives are affected. The first breast cancer susceptibility gene, *BRCA1*, was cloned in 1994.[1] To date, well over 300 distinct "high penetrance" mutations in this gene have been described by the Breast Cancer Information Core.[2] These confer a breast cancer risk of up to 90% by the age of 70.[3] A second gene involved in hereditary breast cancer syndrome, *BRCA2*, was identified several months later,[4] and at least one more gene remains to be found.[5] Over 80% of families with two or more cases of premenopausal breast cancer and two or more cases of ovarian

cancer are believed to carry a *BRCA1* or *BRCA2* mutation.[6] In addition, other genes may play a role as risk modifiers.[7]

Comprehensive evaluation of breast cancer genes could be used to provide women with information concerning the risk of developing cancer. Much recent work has been directed towards screening women from breast cancer families and other high risk populations to estimate the contribution of *BRCA1* mutations to the overall incidence of breast and ovarian cancers. On the basis of the results of these studies, attempts have been made to estimate the predictive value of a *BRCA1* mutation, that is, whether different mutations have a different risk.[8] Information is now emerging that knowledge of *BRCA1* status is useful in preventive strategies. For example, oral contraceptive use reduces the risk of ovarian cancer by 50% in *BRCA1* carriers.[9]

One of the central themes in cancer risk assessment is the efficiency and accuracy of genetic screening methods. Currently, the ability to conduct large scale, population based studies is constrained by the lack of an accurate and inexpensive method for mutation detection. Nucleotide sequence based screening, which is currently accepted as the gold standard, is offered at US$2400 for screening both *BRCA1* and *BRCA2* by Myriad Genetics (Salt Lake City, UT).

Here, we report on the evaluation of a practical test for *BRCA1* mutation detection, which is based on a combination of extensive multiplex PCR amplification, followed by two dimensional electrophoresis (TDGS, two dimensional gene scanning).[10 11] The results of this study indicate a sensitivity for detecting mutations equal to that of sequencing, but at a much lower cost.

Methods
DNA SAMPLES
DNA samples from patients with known mutations in *BRCA1* were obtained from 60 patients with cancer and unaffected subjects. Mutations had originally been detected for 14 patients by sequence analysis of selected exons (exons 2 and 20) and by the protein truncation test of exon 11. No previous knowledge of mutation status of the panel was known when TDGS was applied.

PCR AMPLIFICATION
Multiplex PCR amplification was carried out in two steps. A 7-plex long distance PCR preamplification from genomic DNA provided the template for the amplification of the 37

DGGE optimised target fragments in four multiplex groups (fig 1). Primers for long and short PCR were obtained from Genosys (primer sequences not shown because of lack of space but will be made available upon request). Long distance PCR reactions were performed as a 7-plex PCR, using 100 ng of total genomic DNA, 2.5 mmol/l MgCl$_2$, 400 µmol/l of each dNTP, and 2.5 units of LA enzyme (LA PCR kit II, Takara) in a total volume of 25 µl in LA buffer. Primer concentrations were 0.125 µmol/l (exons 10-11, 12-13,

18-20), 0.25 µmol/l (exons 14-17, 21-24), and 0.5 µmol/l (exons 1-3, 5-9). Amplification was carried out in a PTC-100 thermocycler (MJ Research) for 60 seconds at 94°C, after which the enzyme was added, followed by 32 cycles at 94°C for 15 seconds, 63°C for 60 seconds, 68°C for 12 minutes, and a final extension at 68°C for 12 minutes.

Multiplex short PCR was performed in four multiplex groups. Each group required 0.5 µl of long distance PCR products, 3 mmol/l MgCl$_2$, 250 µmol/l of each dNTP, and 5 units of
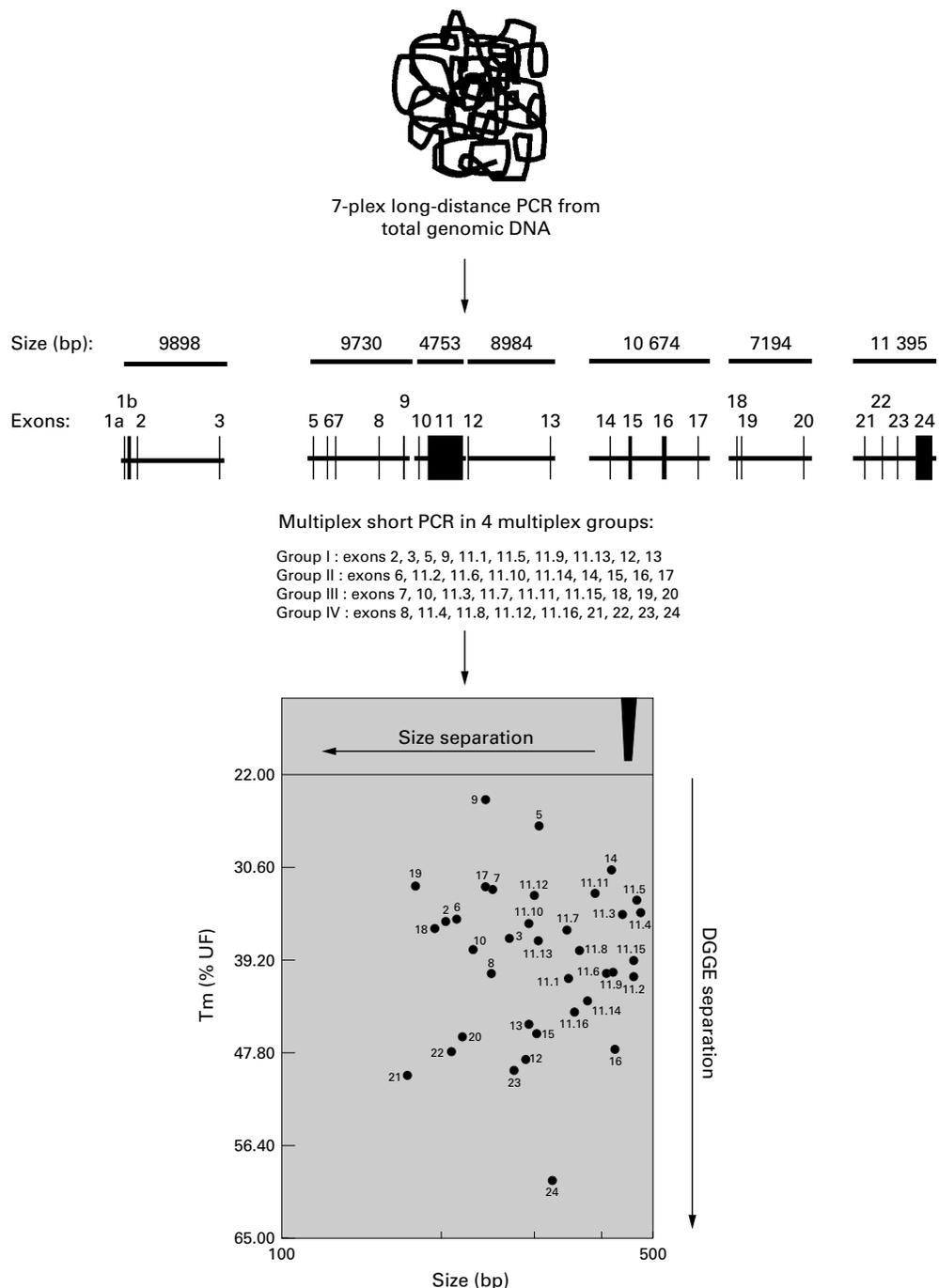

Figure 1   Schematic representation of TDGS as performed for BRCA1 mutation scanning. First, in a single multiplex long distance PCR, over-representation of the target sequences is obtained. Next, individual exons are amplified in four multiplex groups with exon 11 subdivided into 16 overlapping fragments. The products are combined and subjected to 2D electrophoresis, that is, fragments are first separated on the basis of size and subsequently further sorted according to base pair sequence in a denaturing gradient gel.

ampli*taq* Gold *Taq* polymerase (Perkin Elmer) in a total volume of 25 µl in ampli*taq* Gold PCR buffer. Primer concentrations were 0.125 µmol/l (exon 11.1), 0.25 µmol/l (exons 9, 11.2, 11.5, 11.6, 11.7, 11.8, 11.12, 11.15, 12, 13, 17, 18, 19, 21, 22, and 23), 0.375 µmol/l (exons 11.3, 11.9), 0.5 µmol/l (exons 3, 6, 8, 10, 11.14, 14, 16, and 24), 0.75 µmol/l (exons 2, 5, 7, 11.4, 11.10, 11.13, 15, and 20), 0.875 µmol/l (11.11), and 1.25 µmol/l (exon 11.16). Amplification was carried out in a PTC-100 thermocycler (MJ Research) at 95°C for 12 minutes,

followed by 10 cycles at 94°C for 40 seconds, 43°C for one minute minus 0.5°C per cycle, 72°C for one minute 30 seconds, 20 cycles at 94°C for 40 seconds, 40°C for one minute, 72°C for one minute 30 seconds with an increase of one second per cycle, and one cycle at 72°C for 10 minutes. Subsequently, PCR fragments were subjected to one round of complete denaturation and renaturation, that is, 98°C for 10 minutes, 55°C for 30 minutes, and 37°C for 30 minutes, to create heteroduplex molecules.

TWO DIMENSIONAL ELECTROPHORESIS
Five µl of each multiplex group were combined and mixed with loading buffer (0.25% xylene cyanol, 0.25% bromophenol blue, 15% ficoll, and 100 mmol/l Na$_2$ EDTA). The mixture was directly loaded onto the top gel of an automatic 2D electrophoresis apparatus (CBS Scientific Co, Solana Beach, CA)[12], containing 10% polyacrylamide (acrylamide:bisacrylamide 37.5:1) and electrophoresed for four hours at 200 V in 1 × TAE buffer at room temperature. Subsequently, fragments were further separated in the denaturing gradient part of the gel. The gradient used was 22-65% UF (100 UF=7 mol/l urea and 40% formamide) in 10% polyacrylamide and electrophoresis was for 16 hours at 100 V in 1 × TAE buffer at 60°C. The gel was stained with a mixture of SYBR green I and II (Molecular Probes) and photographed under UV light using the Imager gel documentation system (Appligene).

NUCLEOTIDE SEQUENCING
Single fragments were amplified by PCR by using the same primers as used for TDGS, but without the GC clamps and with the exclusion of the heteroduplexing cycle. Twenty µl of PCR product were run on a 2% low melting agarose gel and DNA was purified from the excised bands using the SurePure DNA extraction kit (Embi Tec, San Diego, CA). DNA concentrations were estimated by UV spectrophotometry and by comparison to a size marker in an agarose gel. Sequence reactions contained 90 ng of template, 2 pmol of primer, and 8 µl of Terminator Ready Reaction Mix (Perkin Elmer). Cycling was carried out in a PTC-100 thermocycler for 25 cycles: 96°C for 10 seconds/ 50°C for five seconds/60°C for four minutes. Sequencing products were ethanol precipitated and run in an ABI Prism 310 Genetic Analyzer (Perkin Elmer). Sequencing results were compared to the published sequence of Smith *et al*.[13]

Results
Two dimensional gene scanning (TDGS) is based on a combination of extensive multiplex PCR and two dimensional electrophoresis. The latter comprises a size separation step followed by denaturing gradient gel electrophoresis (DGGE), and allows single base changes to be distinguished among multiple DNA fragments in parallel.[10] [11] PCR amplification of the target sequences is carried out in two steps. First, all coding exons are amplified from genomic DNA by multiplex long distance PCR. Then,
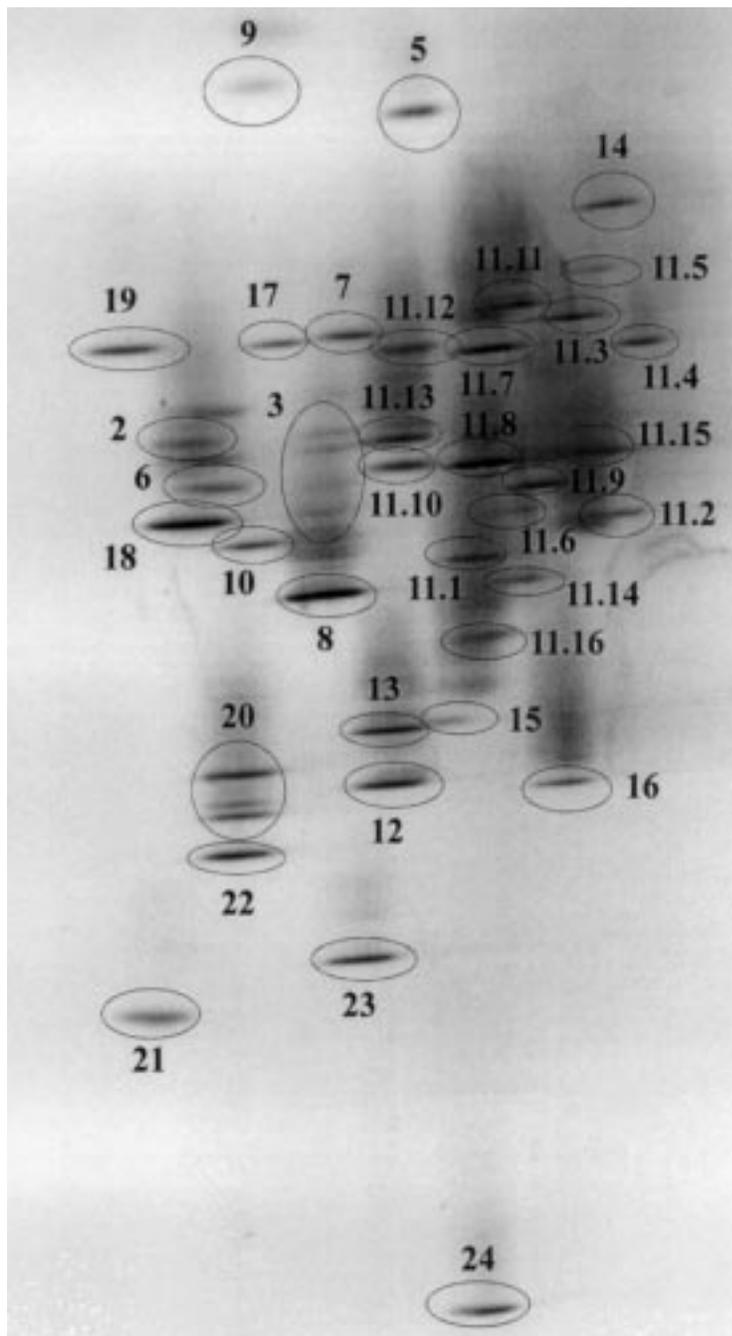

Figure 2   Empirical BRCA1-TDGS pattern of a control sample, containing polymorphisms in exons 3 and 20. Because of the heteroduplex step at the end of the PCR amplification, heterozygous variants are shown by the appearance of four rather than one spot: one homoduplex wild type (WT), one homoduplex mutant (MU), and two heteroduplex molecules (HE), each comprising one wild type and one mutant strand. The less stable heteroduplex molecules will melt earlier than the two homoduplex molecules (note the non-separated heteroduplexes in the exon 20 polymorphism).
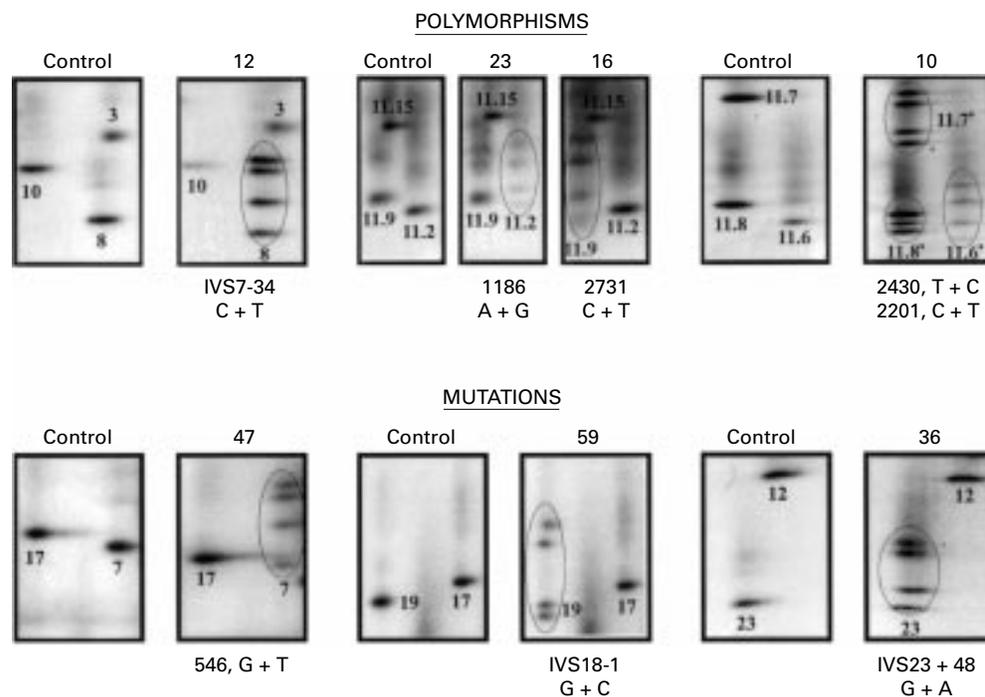
Figure 3 Examples of heterozygous polymorphisms and mutations in BRCA1 as detected by TDGS. The top panels represent different frequently observed polymorphisms in exons 8, 11.2, 11.6, 11.7, 11.8, and 11.9 and the bottom panels represent different mutations in exons 7, 19, and 23 (tables 1 and 2).

the mixture of long fragments is used as the enriched template for the multiplex amplification of the relatively short (150 to 500 bp) DGGE optimised target fragments.[14] PCR amplification of the *BRCA1* gene required a multiplex of seven long fragments serving as template for a total of 37 short PCR fragments. These fragments covered all coding exons of the gene (the non-coding exons 1a, 1b, and the non-coding part of exon 24 were excluded); 16 overlapping fragments were needed to cover the large exon 11 (fig 1). Two dimensional separation of the mixture of all *BRCA1* fragments simultaneously in a DGGE gel allowed detection of all mutational variants (fig 2). Heterozygous mutations or polymorphisms are detectable as four spots rather than one spot, representing the two homoduplex and the two heteroduplex variants. In some cases, the two heteroduplex variants are not well separated, resulting in the appearance of three instead of four spots. Examples of heterozygous mutations and polymorphisms are shown in fig 3.

To evaluate the sensitivity and specificity of this test, a panel of 60 DNA samples, previously screened for mutations in *BRCA1* using the protein truncation test, alone or in combination with partial nucleotide sequencing, was rescreened by TDGS (table 1). In 14 of these samples a *BRCA1* mutation had been identified. Twenty-seven samples were from family members of the mutation positive cases, but sequencing had found them to be non-carriers. A total of 19 samples were from familial cancer patients that had previously tested negative for exon 11 with the protein truncation test. Of the 14 previously identified mutations, 11 were in exon 11, and one mutation each was in exons 2, 8, and 20. All mutations

but one were predicted to lead to a truncated protein. Included were seven deletions of one to 40 nucleotides, four insertions of one nucleotide, and three base substitutions. TDGS was applied to the panel in a blinded fashion and all variants were detected (detection rate of 100%, table 1). No false negatives were found.

Among the 19 samples previously tested negative for exon 11 and rescreened by TDGS, none contained a truncating mutation in exon 11, which was expected. However, five variants were identified by TDGS. One of these was an unclassified variant in exon 11, but this was a single T to G nucleotide substitution, which leads to a Asn to Lys amino acid substitution. Two additional unclassified variants were detected. The first was a T to C at position 172, which leads to a Met to Thr substitution in exon 2. The second was a G to C in intron 18, which may influence splicing. Two pathogenic mutations were detected. The first was a G to T at nucleotide 546 in exon 7, which leads to a premature stop codon. The second was a single base pair substitution in intron 5, which leads to aberrant splicing and premature chain termination. These mutations have been described previously.[2 15]

In addition to the mutations and variants described above, a number of polymorphic variants were detected in the 60 samples. Many of these were identical, as indicated by their characteristic spot configurations. This was confirmed by individual analysis of each variant by 1D DGGE (results not shown). On the basis of different spot configurations, a total of 18 TDGS variants in 12 TDGS fragments were distinguished and confirmed by direct nucleotide sequencing (table 2). None of the variations was false positive. Comparison to the *BRCA1* sequence[13] showed that these 18

Table 1   Mutational analysis of the BRCA1 gene in 60 subjects by TDGS and sequence analysis

Sample No	Cancer*	Mutation in TDGS fragment	Exon	Nucleotide change	Additional heterozygous variants in TDGS fragments†
1	Br (bilat)	20	20	insC, 5382	11.6[a], 11.7[a], 11.8[a], 11.9, 11.11[a], 11.13, 11.14, 13, 16[a]
2	Br, Ov	2	2	delAG, 185	11.6[a], 11.7[a], 11.8[a], 11.9, 11.11[a], 11.13, 11.14, 13, 16[a]
3	Ov				
4					
5					
6		11.2/11.3	11	del40, 1294	11.6[a], 11.7[b], 11.8[b], 11.9, 11.11[a], 11.13, 11.14, 13, 16[a]
7					
8	Br, Ov	11.10	11	T→G, 3053	11.6[a], 11.7[a], 11.8[a], 11.9, 11.11[a], 11.13, 11.14, 13, 16[b]
9					
10	(Male)				11.6[a], 11.7[a], 11.8[a], 11.9, 11.11[a], 11.13, 11.14, 13, 16[a]
11					11.6[a], 11.7[a], 11.8[a], 11.9, 11.11[a], 11.13, 11.14, 13, 16[a]
12					8
13					11.6[a], 11.7[a], 11.8[a], 11.9, 11.11[a], 11.13, 11.14, 13, 16[a]
14	(Male)	11.14/11.15	11	insT, 3769	11.6[a], 11.7[a], 11.8[a], 11.9, 11.11[a], 11.13, 13, 16[a]
15					8
16	Br	11.3/11.4	11	insA, 1559	11.6[b], 11.7[a], 11.8[a], 11.9, 11.11[a], 11.13, 11.14, 13, 16[a]
17					8, 11.6[a], 11.7[a], 11.8[a], 11.9, 11.11[a], 11.13, 11.14, 13, 16[a]
18	Br	8	8	delC, 633	11.6[b], 11.7[a], 11.8[a], 11.9, 11.11[a], 11.13, 11.14, 13, 16[a]
19					8, 11.6[a], 11.7[a], 11.8[a], 11.9, 11.11[a], 11.13, 11.14, 13, 16[a]
20	Ov, Tong				8
21	Mel				11.6[b], 11.7[a], 11.8[a], 11.9, 11.11[a], 11.13, 11.14, 13, 16[a]
22	(Male)				11.6[b], 11.7[a], 11.8[a], 11.9, 11.11[a], 11.13, 11.14, 13, 16[a]
23					8, 11.2
24	Br	11.14/11.15	11	G→T, 3867	11.9
25	Br				11.6[b], 11.7[a], 11.8[a], 11.9, 11.11[a], 11.13, 11.14, 13, 16[a]
26	Br				11.6[b], 11.7[a], 11.8[a], 11.9, 11.11[a], 11.13, 11.14, 13, 16[a]
27	Br, Endo	11.4/11.5	11	C→T, 1806	8, 11.6[a], 11.7[a], 11.8[a], 11.9, 11.11[a], 11.13, 11.14, 13, 16[a]
28					8, 11.9
29	Ov	11.15	11	insT, 3879	
30					
31	Br	11.9	11	delAA, 2800	
32	Br, Ov	11.3	11	delAG, 1479	11.6[a], 11.7[a], 11.8[a], 11.9, 11.11[a], 11.13, 11.14, 13, 16[a]
33	Br	11.6	11	delA, 2190	
34	Ov	11.5/11.6	11	delGAAA, 2072	11.7[a], 11.8[a], 11.9, 11.11[a], 11.13, 11.14, 13, 16[a]
35	Br				
36	Br				11.6[a], 11.7[a], 11.8[a], 11.9, 11.11[a], 11.13, 11.14, 13, 16[a], 23
37	Br				11.6[a], 11.7[a], 11.8[a], 11.9, 11.11[a], 11.13, 11.14, 13, 16[a]
38	Ov				8
39	Ov				8, 11.11[b]
40	Ov				
41	Ov				8, 11.6[a], 11.7[a], 11.8, 11.9, 11.11[a], 11.13, 11.14, 13, 16[a]
42	Br	6	Intron 5	T→G, IVS5-11	8
43	Cx, Br (bilat)				8, 11.6[a], 11.7[a], 11.8[a], 11.9, 11.11[a], 11.13, 11.14, 13, 16[a]
44	Br				
45	Cx, Br, Ov				
46	Br				
47	Br (bilat)	7	7	G→T, 546	
48	Cx, Br				
49	Br				8, 11.2
50	Br				11.6[a], 11.7[a], 11.8[a], 11.9, 11.11[a], 11.13, 11.14, 13, 16[a]
51	(Male)				
52	Br				11.6[a], 11.7[a], 11.8[a], 11.9, 11.11[a], 11.13, 11.14, 13, 16[a]
53	Br				11.6[c]
54	Ov	11.14/11.15	11	T→G, 3827	11.6[b], 11.7[a], 11.8[a], 11.9, 11.11[a], 11.13, 13, 16[a]
55	Br, Ov				11.6[c]
56	Ov	2	2	T→C, 172	8, 11.6[a], 11.7[a], 11.8[a], 11.9, 11.11[a], 11.13, 11.14, 13, 16[a]
57	Br (male)				8, 11.2
58	Br				11.2
59	Br	19	Intron 18	G→C, IVS18-1	8, 11.2, 11.6[a], 11.7[a], 11.8[a], 11.9, 11.11[a], 11.13, 11.14, 13, 16[a]
60	Br, Ov				

*Br = breast, Ov = ovarian, Tong = tongue, Mel = malignant melanoma, Endo = endometrial, Cx = cervix.
†Sequence analysis is depicted in table 2.
[a]–[c] See table 2.

different spot configurations comprised 15 different sequence variants (table 2).

Of the 15 variants confirmed by sequence analysis, 10 were known polymorphisms, three were unclassified variants, and two were novel variants not found in the BIC database (table 2). Two of the three unclassified variants occurred in subjects in whom a mutation in the gene had also been identified (samples 6 and 8, tables 1 and 2). The third unclassified variant was found in a patient with ovarian cancer for whom no *BRCA1* mutation had been identified (sample 39 in table 1). One of the two novel variants (seen in one of 60 subjects), an A to T transversion at position 3107 resulting in a Lys to Asn amino acid change, was found in the same fragment (fragment 11.11), which already contained an unclassified variant. Neither nucleotide sequencing nor PTT had previously detected this variant in this patient with ovarian cancer (sample 39, tables 1 and 2). The second novel variant was also found in only one subject, in exon 23 (sample 36). Interestingly, this IVS23+48G/A occurred in a patient with breast cancer in which no other *BRCA1* mutation could be detected.

As mentioned above, the characteristic spot configuration of recurrent mutations and polymorphisms allows their identification once they have been sequenced. Fig 4 shows three different spot configurations for fragment 11.6 with some of the surrounding fragments as refer-

Table 2   Sequence analysis of polymorphic variants in BRCA1 detected in the panel of 60 subjects

Exon	TDGS fragment	Nucleotide position*	Nucleotide change	Type of variant	Codon	Amino acid change	Type of polymorphism†
8	8	IVS7-34	C→T	Transition	—	—	Polymorphism
11	11.2	1186‡	A→G	Transition	356	Gln→Arg	Polymorphism
	11.6[a]	2201‡	C→T	Transition	694	Ser→Ser	Polymorphism
	11.6[b]	2196‡	G→A	Transition	693	Asp→Asn	Polymorphism
		2201‡	C→T	Transition	694	Ser→Ser	Polymorphism
	11.6[c]	2196‡	G→A	Transition	693	Asp→Asn	Polymorphism
	11.7[a] = 11.8[a]	2430‡	T→C	Transition	771	Leu→Leu	Polymorphism
	11.7[b] = 11.8[b]	2434	T→C	Transition	772	Val→Ala	Unclassified variant
	11.9	2731‡	C→T	Transition	871	Pro→Leu	Polymorphism
	11.11[a]	3232	A→G	Transition	1038	Glu→Gly	Polymorphism
	11.11[b]	3107	A→T	Transversion	996	Lys→Asn	Not reported
		3238‡	G→A	Transition	1040	Ser→Asn	Unclassified variant
	11.13 = 11.14	3667‡	A→G	Transition	1183	Lys→Arg	Polymorphism
13	13	4427‡	T→C	Transition	1436	Ser→Ser	Polymorphism
16	16[a]	4956‡	A→G	Transition	1613	Ser→Gly	Polymorphism
	16[b]	5075	G→A	Transition	1652	Met→Ile	Unclassified variant
23	23	IVS23+48	G→A	Transition	—	—	Not reported

*Nucleotide numbering starts at the first transcribed base according to GenBank entry U14680.
†Named according to BIC as of 10.2.99.
‡Common polymorphisms defining haplotypes (see also table 3).

Exon 11, Fragment 6

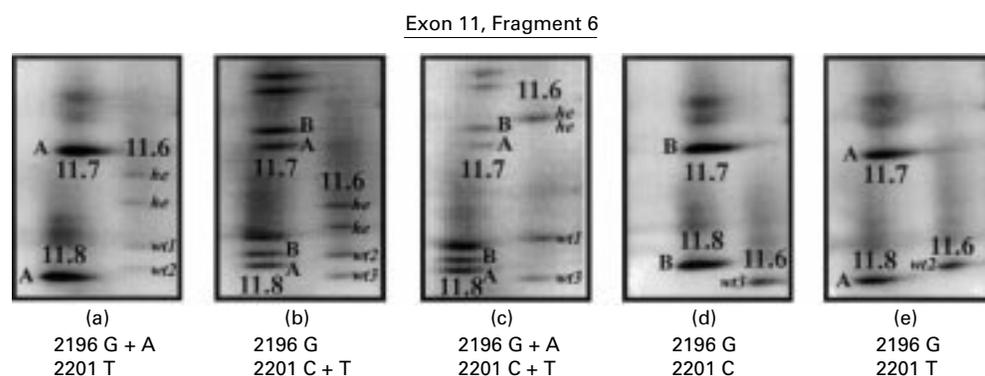| (a)	(b)	(c)	(d)	(e)
2196 G + A	2196 G	2196 G + A	2196 G	2196 G
2201 T	2201 C + T	2201 C + T	2201 C	2201 T

Figure 4   Examples of the effect of different variations in the same TDGS fragment. Panels a-c show three different patterns of heterozygosity in fragment 11.6, whereas panels d and e each show a different pattern of homozygosity.

ence points. Each of these three configurations represents a frequently observed heterozygous variant associated with a specific polymorphism. Sequence analysis indicated that two positions in this fragment, 2196 and 2201, are polymorphic. The fragment in fig 4a is heterozygous at position 2196 (G + A) and homozygous at position 2201 (T), the fragment in fig 4b is homozygous at position 2196 (G) and heterozygous at position 2201 (C + T), and the fragment in fig 4c is heterozygous at both positions. So far, for fragment 11.6, a total of three alleles were identified, that is, *wt1-3* (fig 4a-c).

The corresponding homozygous situations were more difficult to recognise from the 2D pattern solely by eye. The use of the surrounding spots as markers was complicated by the frequent occurrence of variations in these fragments as well (see, for example, the variation in fragments 11.7 and 11.8 in fig 4b and c). Nevertheless, the fragment 11.6 homozygous spots in fig 4d and e could be identified as alle-

les *wt3* and *wt2* respectively, which was confirmed by a subsequent 1D DGGE experiment (results not shown).

A recurrent pattern of identical spot configurations, which occurred in exon 8, seven of the fragments of exon 11, exon 13, and exon 16, was also noted. These variants represented polymorphisms that could be arranged into haplotypes (table 3). One of the benefits of parallel screening by TDGS is that each pattern shows all heterozygous variants in the entire gene.

Discussion

This study shows that *BRCA1* mutation screening by TDGS is an accurate method of mutation detection. Previous evaluations of TDGS tests for other large human disease genes support the claim that DGGE based tests are as accurate as nucleotide sequencing.[16–18] It may be that heterozygous mutations are even easier to detect on the basis of the DGGE principle (that is, three or four spots rather than one) than by sequencing (that is, two overlapping peaks). TDGS shows all heterozygous variations in the entire gene, as illustrated by the high number of variants detected. A total of 34 mutations and variants were observed in a population of 60 subjects. This technique will enable epidemiologists to record all variations within the gene for the establishment of genotype-phenotype correlations.

Table 3   Polymorphisms defining haplotypes

	Exon 11						Exon 13	Exon 16	
Type	2196	2201	2430	2731	3232	3667	4427	4956	%*
A	G	C	T	C	A	A	T	A	63
B	G	T	C	T	G	G	C	G	28
C	A	T	C	T	G	G	C	G	7
D	G	C	T	T	A	A	T	A	2

*Occurrence in 126 chromosomes.

In TDGS, recurrent polymorphisms can be recognised based on their unique spot distribution pattern, but new variants must be sequenced to determine the exact nature of the observed variation. Theoretically, 300 single base substitutions are possible along a 100 base pair DNA fragment. However, so far, an average of only 14 variations has been observed per *BRCA1*-TDGS fragment (information derived from the BIC). Thus, the possibility that a mutation in a given fragment will show the exact same spot configuration as a known polymorphism is small. However, it has become clear that for a gene containing many polymorphic sites, unequivocal identification of homozygously occurring alleles is only possible by careful examination of the variant in the context of the surrounding spots, which can also represent polymorphic fragments. Increased standardisation and the application of automated image analysis systems containing information on the spot positions of all known variations should greatly facilitate spot identification for this purpose.

During the course of this study, attention was given to the actual costs of the test. It has previously been shown that the maximum throughput of TDGS for a typical large disease gene, for example, the retinoblastoma susceptibility gene *RB1*, is 60 tests per technician per week using manual procedures.[19] The costs for *BRCA1* are higher, because of the need for four instead of only one multiplex groups, and was calculated to be about US$70 per test. The most prominent cost components in the manual procedure involve labour and PCR enzymes. The costs of PCR primers were low since these were based on the total number of reactions possible with the lowest volume that could be ordered. It should be noted that had each fragment been separately amplified rather than coamplified the costs associated with enzymes and labour would have been significantly higher.

The costs of the assay can be dramatically reduced by automation. PCR robotics reduces both labour and reagent costs (smaller PCR volumes), while fluorescent imaging using dye labelled primers obviates the need to stain the gels. The costs per sample are then mainly determined by the primers that have to be labelled with a fluorophore, while labour and enzyme costs become vanishingly small. According to our calculations, these proposed automation steps will drive the costs down to about US$10 per test, which is approximately one to two orders of magnitude lower than current sequencing protocols.[19] This anticipated increase in cost-efficiency is based solely on commercially available instrumentation and not on technological improvement.

The TDGS test is ideal for large studies to establish the potential value of screening for mutations in *BRCA1* as an aid to breast cancer management. However, the hereditary breast cancer syndrome is genetically heterogeneous and other susceptibility genes have been identified, including *BRCA2*, *TP53*, *PTEN*, and, arguably, *ATM*.[20–24] In order to use genetic screening as an aid for risk assessment of breast cancer, a battery of genetic tests should be applied. TDGS is flexible since its design permits the rapid expansion of a test to include additional genes. TDGS tests, which screen multiple genes in a single assay, can and have been developed.[10]

1 Miki Y, Swensen J, Shattuck-Eidens D, *et al*. A strong candidate for the breast and ovarian cancer susceptibility gene BRCA1. *Science* 1994;**266**:66-71.
2 Breast Cancer Information Core Electronic Database. http://www.nhgri.nih.gov/Intramural_research/Lab_transfer/Bic
3 Ford D, Easton DF, Bishop DT, Narod SA, Goldgar DE. Risks of cancer in BRCA1-mutation carriers. *Lancet* 1994;**343**:692-5.
4 Wooster R, Bignell G, Lancaster J, *et al*. Identification of the breast cancer susceptibility BRCA2. *Nature* 1995;**378**:789-92.
5 Ford D, Easton DF, Stratton M, *et al* and the Breast Cancer Linkage Consortium. Genetic heterogeneity and penetrance analysis of the BRCA1 and BRCA2 genes in breast cancer families. The Breast Cancer Linkage Consortium. *Am J Hum Genet* 1998;**62**:676-89.
6 Narod SA, Ford D, Devilee P, *et al*. An evaluation of genetic heterogeneity in 145 breast-ovarian cancer families. Breast Cancer Linkage Consortium. *Am J Hum Genet* 1995;**56**:254-64.
7 Friend SH. Breast cancer susceptibility testing: realities in the post-genomic era. *Nat Genet* 1996;**13**:16-17.
8 Gayther SA, Warren W, Mazoyer S, *et al*. Germline mutations of the BRCA1 gene in breast and ovarian cancer families provide evidence for a genotype-phenotype correlation. *Nat Genet* 1995;**11**:428-33.
9 Narod SA, Risch H, Moslehi R, *et al*. Oral contraceptive use and the risk of hereditary ovarian cancer. *N Engl J Med* 1998;**339**:424-8.
10 van Orsouw NJ, Vijg J. Design and application of 2-D DGGE-based gene mutational scanning tests. *Genet Anal* 1999;**14/5-6**:205-13.
11 van Orsouw NJ, Dhanda RK, Rines DR, *et al*. Rapid design of denaturing gradient-based two-dimensional electrophoretic gene mutational scanning tests. *Nucleic Acids Res* 1998;**10**:2398-406.
12 Dhanda RK, Smith WM, Scott CB, Eng C, Vijg J. A simple system for automated two-dimensional electrophoresis: applications to genetic testing. *Genet Test* 1998;**2**:67-70.
13 Smith TM, Lee MK, Szabo CI, *et al*. Complete genomic sequence and analysis of 117 kb of human DNA containing the gene BRCA1. *Genome Res* 1996;**6**:1029-49.
14 Li D, Vijg J. Multiplex co-amplification of 24 retinoblastoma gene exons after pre-amplification by long-distance PCR. *Nucleic Acids Res* 1996;**24**:538-9.
15 Friedman LS, Ostermeyer EA, Szabo CI, *et al*. Confirmation of BRCA1 by analysis of germline mutations linked to breast and ovarian cancer in ten families. *Nat Genet* 1994;**8**:399-404.
16 van Orsouw NJ, Li D, van der Vlies P, *et al*. Mutational scanning of large genes by extensive PCR multiplexing and two-dimensional electrophoresis: application to the RB1 gene. *Hum Mol Genet* 1996;**5**:755-61.
17 Rines RD, van Orsouw NJ, Sigalas I, Li FP, Eng C, Vijg J. Comprehensive mutational scanning of the p53 coding region by two-dimensional gene scanning. *Carcinogenesis* 1998;**19**:979-84.
18 Smith WM, van Orsouw NJ, Fox EA, Kolodner RD, Vijg J, Eng C. Accurate, high-throughput "snapshot" detection of hMLH1 mutations by two-dimensional DNA electrophoresis. *Genet Test* 1998;**2**:43-53.
19 Dhanda RK, van Orsouw NJ, Sigalas I, Eng C, Vijg J. Critical factors in the performance and cost of two-dimensional gene scanning: RB1 as a model. *Biotechniques* 1998;**25**:664-75.
20 Li FP, Fraumeni JF Jr, Mulvihill JJ, *et al*. A cancer family syndrome in twenty-four kindreds. *Cancer Res* 1988;**48**:5358-62.
21 Sidransky D, Tokino T, Helzlsouer K, *et al*. Inherited p53 gene mutations in breast cancer. *Cancer Res* 1992;**52**:2984-6.
22 Nelen MR, Padberg GW, Peeters EAJ, *et al*. Localization of the gene for Cowden disease to chromosome 10q22-23. *Nat Genet* 1996;**13**:114-16.
23 Liaw D, Marsh DJ, Li J, *et al*. Germline mutations of the PTEN gene in Cowden disease, an inherited breast and thyroid cancer syndrome. *Nat Genet* 1997;**16**:64-7.
24 FitzGerald MG, Bean JM, Hegde SR, *et al*. Heterozygous ATM mutations do not contribute to early onset of breast cancer. *Nat Genet* 1997;**15**:307-10.