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## **EMMA, a cost and time-effective diagnostic method for simultaneous detection of point mutations and large-scale genomic rearrangements: application to BRCA1 and BRCA2 in 1,525 patients.**

Virginie Caux-Moncoutier, Laurent Castera, Carole Tirapo, Dorothée Michaux, Marie-Alice Remon, Anthony Laugé, Etienne Rouleau, Antoine de Pauw, Bruno Buecher, Marion Gauthier-Villars, et al.

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**EMMA, a cost and time-effective diagnostic method for simultaneous detection of point mutations and large-scale genomic rearrangements: application to BRCA1 and BRCA2 in 1,525 patients.**

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Manuscripts

1 **EMMA, a cost- and time-effective diagnostic method for simultaneous detection of point**  
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3 **mutations and large-scale genomic rearrangements: application to *BRCA1* and *BRCA2***  
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5 **in 1,525 patients.**  
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## Abstract

The detection of unknown mutations remains a serious challenge and, despite the expected benefits for the patient's health, a large number of genes are not screened on a routine basis. We present the diagnostic application of EMMA (Enhanced Mismatch Mutation Analysis®, Fluigent), a novel method based on heteroduplex analysis by capillary electrophoresis using innovative matrices. *BRCA1* and *BRCA2* were screened for point mutations and large rearrangements in 1,525 unrelated patients (372 for the validation step and 1,153 in routine diagnosis) using a single analytical condition. Seven working days were needed for complete *BRCA1/2* screening in 30 patients by one technician (excluding DNA extraction and sequencing). A total of 137 mutations were found, including a *BRCA2* duplication of exons 19 and 20, previously missed by *Comprehensive BRCAAnalysis*®. The mutation detection rate was 11.9%, which is consistent with patient inclusions.

This study therefore suggests that EMMA represents a valuable short-term and mid-term option for many diagnostic laboratories looking for an easy, reliable and affordable strategy, enabling fast and sensitive analysis for a large number of genes.

### Key words:

*BRCA1*, *BRCA2*, mutation, screening, diagnosis, capillary electrophoresis, EMMA

## INTRODUCTION

The role of genetic screening is becoming increasingly important in cancer diagnosis, prognosis and treatment decisions. Developing fast, reliable and inexpensive methods to detect such mutations is therefore a major challenge for medicine.

Two main strategies are used to search for unknown mutations: direct sequencing and screening. The first strategy is currently considered to be the most reliable, although not totally flawless (Eng, et al., 2001). More specifically, reliable chemistry and software are prominent points to consider.

The second strategy is a two-step strategy involving preliminary screening for the presence of variants on amplicons, followed by sequencing of only those fragment(s) in which a variation was detected. This dramatically reduces the number of fragments that need to be sequenced. These screening strategies are mainly based on heteroduplex analysis (HDA), using either a dedicated liquid chromatographic system (Denaturing High Performance Liquid Chromatography, DHPLC)(Spiegelman, et al., 2000) or a real-time PCR machine (High Resolution Melting curve analysis, HRM)(Wittwer, 2009). During a slow cool-down performed at the end of PCR amplification of a DNA fragment from a heterozygote sample, two homoduplex fragments (corresponding to the normal and mutated allele, respectively) and two heteroduplexes (with mismatched strands due to hybridization of a wild-type strand with a mutant strand) are obtained. The aim of HDA is to check for the presence of heteroduplexes. DHPLC and HRM are widely used in diagnostic laboratories. However, analysis conditions depend on melting domains, and optimization is required for each PCR fragment.

HDA can also be performed by electrophoresis: homoduplexes are separated from heteroduplexes due to differences in electrophoretic mobility. Separations are nowadays

1 mostly performed by multi-capillary electrophoresis, allowing for high automation, low cost  
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3 and high throughput, as recently described in a diagnostic validation of Conformation-  
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5 Sensitive Capillary Electrophoresis (Mattocks, et al.) . A novel HDA method has been  
6  
7 recently developed (Houdayer, et al.2010). This method, called Enhanced Mismatch Mutation  
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9 Analysis (EMMA) is based on the use of innovative matrices increasing the electrophoretic  
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11 mobility differences between homoduplex and heteroduplex DNA (Weber, et al., 2004).  
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13 Sensitivity is further improved by using nucleosides as additives to enhance single-base  
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15 substitution detection. Nucleosides are expected to interact with mismatched bases of  
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17 heteroduplexes, thereby increasing mobility differences with homoduplexes (Weber, et al.,  
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19 2006). Moreover, this method, in combination with adapted semiquantitative PCR conditions,  
20  
21 can be used to simultaneously detect point mutations and large-scale rearrangement in a single  
22  
23 run (Weber, et al., 2007). This feature, combined with the use of a single set of separation  
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25 conditions for all fragments and with the multiplexing capability of the method, leads to a  
26  
27 considerable simplification and cost reduction compared to previous methods.  
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34 The use of EMMA for fast screening of *BRCA1* and *BRCA2* point mutations and large  
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36 rearrangements is described below. Constitutional mutations of the *BRCA1* [MIM 113705]  
37  
38 and *BRCA2* [MIM 600185] genes are associated with a risk of hereditary breast and ovarian  
39  
40 cancer (HBOC). It is essential to identify *BRCA1/2* mutations to provide appropriate  
41  
42 counseling to patients and relatives, but this represents a challenging and time-consuming  
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44 task, as the vast majority of mutations are unique and spread over the entire coding sequence.  
45  
46 In this paper, we report the results of *BRCA1* and *BRCA2* screening for point mutations and  
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48 large rearrangements in 1,525 unrelated patients (372 for the validation step and 1,153 in  
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50 routine diagnosis), and discuss the performance of this new strategy.  
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## PATIENTS AND METHODS

### Patients

Genetic testing for *BRCA1* and *BRCA2* was proposed to women based on individual and/or family history (herein defined as index cases i.e. the first family member in whom complete *BRCA1/2* gene screening was performed). Individual inclusion criteria included: i) breast adenocarcinoma before the age of 36, ii) medullary adenocarcinoma without age limitation, iii) breast adenocarcinoma and ovarian cancer. Male breast cancer was also considered for genetic testing. Family history was defined as either i) 3 breast cancer cases in first- or second-degree relatives in the same lineage, ii) 2 breast cancer cases in first- or second-degree relatives (with a transmitting male), with one cancer before the age of 40 or one cancer before 50 and the other before 70 iii) 1 breast cancer case and one first- or second-degree relative (with a transmitting male) with ovarian cancer. During the course of this study, and in line with French recommendations, inclusion criteria were recently extended to women with isolated ovarian adenocarcinoma before the age of 70 (<http://www.e-cancer.fr/les-soins/oncogenetique>). A consecutive series of 1,525 ascertained cases, mostly of Caucasian descent, were studied. All patients attended a visit with a geneticist and a genetic counselor in a family cancer clinic, mostly at the Institut Curie, Paris, France. Patients gave their informed consent for *BRCA1/2* gene analyses. .

### Nucleic acid extraction

For mutation scanning purposes, DNA was extracted from 2 ml whole blood samples collected on EDTA using a modified perchlorate/chloroform procedure (first 465 samples), as

1 previously described (Johns and Paulus-Thomas, 1989), or the NucleoSpin blood L kit from  
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3 Macherey Nagel (following 849 samples) or the Quickgene 610-L automated system from  
4  
5 FujiFilm (last 211 samples) according to the manufacturer's instructions. Three different  
6  
7 extraction procedures were used during this study in the context of a constant effort to reduce  
8  
9 the time and labor involved, finally leading to the semi-automated solution from FujiFilm.  
10  
11 DNAs were calibrated to 50 ng/ $\mu$ l by UV spectrophotometric assay (Nanodrop). Absorbance  
12  
13 ratios (260/280) and (260/230) had to be in the 1.8-2.0 and 2.0-2.2 ranges, respectively.  
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### 22 **Enhanced Mismatch Mutation Analysis (EMMA)**

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27 *Primer design.* Primers were purchased from Fluigent, Paris, France. *BRCA1* and  
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29 *BRCA2* coding sequences were divided into 81 amplicons analyzed in 24 multiplex PCRs  
30  
31 including one internal control for large-scale rearrangement analysis (Primer sequences are  
32  
33 available from Fluigent). Primers were designed to include flanking intronic sequences  
34  
35 containing recognized splice sites and avoiding known polymorphism to prevent mispriming.  
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37 Due to the well-known limitations of capillary electrophoresis (Rozycka, et al., 2000) and  
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39 topological effects (Weber, et al., 2004), false-negatives can occur in the 70bp from both  
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41 extremities of the amplicon. As a result, larger and overlapping amplicons were designed so  
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43 that the sequence of interest always fell outside these 70bp in at least one amplicon (Weber, et  
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45 al., 2006). One primer of each pair was labeled with 6-carboxyfluorescein (6-FAM).  
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53 *Amplicon synthesis.* Primer mixes were prepared according to the manufacturer's  
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55 instructions (Fluigent) and all multiplex PCRs were performed using a single condition with  
56  
57 the Qiagen Multiplex PCR kit (Qiagen, Courtaboeuf, France). Briefly, samples were  
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59 generated in a 10- $\mu$ l reaction volume containing 100 ng of genomic DNA, 1 $\mu$ l i.e. 0.3  $\mu$ M of  
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1 primer mix (Fluigent), 2µl of water and 5µl i.e. 1X Qiagen Multiplex PCR Master mix  
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3 supplied by the manufacturer (Qiagen). The PCR programs were run in a GeneAmp 9700  
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5 thermocycler (Applied Biosystems, Courtaboeuf, France) and consisted of a first denaturation  
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7 step at 95°C for 15 min, followed by 23 cycles of denaturation at 94°C for 30 s, annealing at  
8  
9 58°C for 90 s and extension at 72°C for 90 s, followed by a final extension at 72°C for 10  
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11 min. PCR products were denatured for 5 min at 96°C then gradually reannealed at -1°C/min  
12  
13 to reach 25°C. On completion of PCR, PCR products were stored at -20°C until migration.  
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20 **Analysis.** PCR tube volume was completed with pure water up to 20µL. Wells in  
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22 columns A and H were filled with 1 µL EMMA buffer 10x to compensate for the higher  
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24 intensity of the capillaries at the extremities of the area. PCRs were electrophoresed with a  
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26 single analytical condition (15kV at 30°C and a fifty cm length-to-detector array) on an  
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28 ABI3100 (Applied Biosystems) using EMMA polymer and according to the manufacturer's  
29  
30 instructions (Fluigent, Paris, France). Data were analyzed using dedicated software  
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32 (Emmalys, Fluigent). Electrophoregrams were examined and scored by two operators.  
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39 **Point mutations.** PCR products showing abnormal EMMA profiles (e.g. multiple peaks,  
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41 shouldering or peak widening) were re-amplified, purified and sequenced in both directions  
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43 using the BigDye Terminator Cycle Sequencing V1.1 Ready Reaction kit (Applied  
44  
45 Biosystems) with incorporation of the PCR oligonucleotides as extension primers, followed  
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47 by electrophoresis in an ABI PRISM 3130XL Genetic Analyzer with analysis using the  
48  
49 Collection and Sequence Analysis software package (Applied Biosystems). Nucleotide  
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51 position was numbered on the basis of the coding sequence NM\_007294.2 and NM\_000059.3  
52  
53 for *BRCA1* and *BRCA2*, respectively. Nucleotide numbering reflects cDNA numbering with  
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55 +1 corresponding to the A of the ATG translation initiation codon in the reference sequence  
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58 according to recommended guidelines (available at <http://www.emqn.org/emqn.php> and  
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1 www.hgvs.org/mutnomen). The initiation codon is codon 1. Electrophoregrams were  
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3 examined and scored by two operators. All mutations were confirmed on a second blood or  
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5 buccal swab sample.  
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10 **Large rearrangements.** EMMA and quantitative multiplex PCR of short fluorescent  
11 fragments (QMPSF) were both used to screen for *BRCA1* and *BRCA2* rearrangements because  
12 automated profile analysis was easier with QMPSF (see below). QMPSF was used as  
13 previously described (Casilli, et al., 2006) . All rearrangements were confirmed on a second  
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15 blood sample and using another technique e.g. long range PCR, transcript analysis or a  
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17 dedicated array-CGH (Rouleau, et al., 2007) . Spurious single-exon deletions due to  
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19 mispriming were therefore unambiguously excluded.  
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### 29 **Unknown Variants (UVs) interpretation**

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31 ***In silico analysis.*** Unfortunately, one half of the variations observed in the *BRCA1/2*  
32 genes are UVs (Hofstra, et al., 2008), making biological and clinical interpretation a  
33 challenging task and consequently leading to clinically difficult situations. To facilitate  
34 subsequent genetic counseling, all identified UVs were submitted to *in silico* analysis using  
35 Alamut (Interactive Biosoftware), a decision-support system for mutation interpretation that  
36 integrates a splice prediction module. Apart from the impact on splicing, unknown variants  
37 were also analyzed for their putative “protein-based” impact by cross-species and Grantham  
38 score (Grantham, 1974) comparisons, and were then scored using an in-house model (Supp.  
39 Figure S1) validated on a series of 378 hereditary breast/ovarian cancer patients and previous  
40 literature [unpublished data, available on request]. Variants were classified as neutral or of  
41 little clinical significance below 6 and worthy of complementary investigations (e.g.  
42 cosegregation analyses or functional studies) above 12. In such cases, the clinical context was  
43 also thoroughly discussed with the clinical geneticist.  
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4        **Transcript analysis.** Patients harboring unknown variants putatively leading to splice  
5 defects following Alamut *in silico* analyses were further investigated at the cDNA level.  
6 Based on previous knowledge (Houdayer, et al., 2008), defects were defined as a minimum  
7 10% decrease of the wild-type score. Emergence of a cryptic splice site was considered  
8 significant and worthy of RNA study when it scored at least 50% of the corresponding wild-  
9 type score. ESEs were not considered for routine purposes.

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RNA was extracted from lymphoblastoid cell lines with and without puromycin treatment. RNA was reverse-transcribed (Houdayer, et al., 2004) and the *BRCA1/2* coding sequence surrounding the region of interest was amplified (primer sequences available on request). Amplicons were purified and sequenced in both directions using the BigDye Terminator Cycle Sequencing V1.1 Ready Reaction kit (Applied Biosystems) with incorporation of the PCR oligonucleotides as extension primers, followed by electrophoresis in an ABI PRISM 3130XL Genetic Analyzer with analysis using the Collection and Sequence Analysis software package (Applied Biosystems). Normal controls were always included in these experiments.

## RESULTS

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6 An EMMA-based strategy was designed for fast screening of *BRCA1* and *BRCA2*  
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8 point mutations and large rearrangements in 1,525 unrelated hereditary breast and/or ovarian  
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10 cancer (HBOC) patients: 1,153 patients were screened in a routine setting following a  
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12 validation step enrolling 372 patients.  
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### Validation step

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22 **Point mutations.** Before implementing EMMA as a routine diagnostic technique, a  
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24 panel of known *BRCA* mutations was tested (Weber, et al., 2004; Weber, et al., 2006) [and  
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26 author's unpublished data]. A series of blind studies were then performed using DHPLC and  
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28 QMPSF as reference techniques for point mutation and large rearrangement screening,  
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30 respectively. DHPLC was chosen as a reference because it is considered to be the gold  
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32 standard for mutation prescreening with more than 1,000 PubMed references  
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34 [http://www.ncbi.nlm.nih.gov/sites/entrez]. Moreover, our laboratory has an extensive  
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36 experience with this technique, with more than 4,000 patients screened for *BRCA1/2* and *RBI*  
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38 point mutations (Houdayer, et al., 2004; Wagner, et al., 1999) and, more recently, large-scale  
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40 rearrangements using the DHPLC-derived technique called MP/LC (Dehainault, et al., 2004).  
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42 The blind studies involved a total of 372 patients. Two hundred and seventy nine cases were  
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44 first screened on *BRCA1* by Fluigent in their mutation detection facility, and 93 cases were  
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46 then screened on both *BRCA1* and *BRCA2* in our laboratory. PCR conditions and analyses  
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48 were as described above. An equivalent sensitivity was demonstrated, as 127 variations  
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50 (excluding polymorphisms) were found with both techniques. Actually, one variation was  
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52 missed by EMMA (*BRCA2*, c.9364G>A/p.Ala3122Thr), but another variation missed by  
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54 DHPLC was detected by EMMA (*BRCA1*, c.2311T>C/p.Leu771Leu).  
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4        ***Large rearrangements.*** The principle of the method consists of quantitative evaluation  
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6 of the relative proportion of a given fragment in the unknown sample with respect to a normal  
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8 control. In order to compare two fragments amplified in two different tubes, peak intensities  
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10 and areas are normalized to a non-mutated DNA fragment used as an internal control.  
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12 Fluorescence intensities were then normalized by adjusting the peaks and areas obtained for  
13  
14 the control amplicons to the same level and the yield of each amplicon in the various samples  
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16 was evaluated. As some profiles exhibit variations due to polymorphisms, rare variants and  
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18 mutations, Emmalys analyzes variant and non-variant profiles separately and deletions are  
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20 detected by a 50% decrease in peak intensity and area, while duplications are detected by a  
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22 1.5-increase in profile area. Firstly, a 30-sample dedicated large rearrangement panel was  
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24 blindly tested using this procedure. This panel included 20 *BRCA1* large rearrangements  
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26 (deletions and duplications) and 10 normal controls. All samples were correctly scored. The  
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28 second step of the validation consisted of identification of 3 large rearrangements present in  
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30 the 372 patients. Two deletions encompassing exons 15 and 16 and 13 to 15, respectively,  
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32 were correctly detected. The third deletion, a deletion of *BRCA1* promoter, was not detected  
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34 because the current EMMA primer panel does not explore this part of the gene (which is  
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36 covered by our QMPSF assay).  
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43        ***Interpretation of polymorphisms according to their profiles.***

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45        One of the main challenges with prescreening methods is the correct identification of  
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47 polymorphisms. This does not constitute an issue for genes with a very low polymorphic  
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49 content such as *RBI* but is a major hurdle for *BRCA* genes, which exhibit a large number of  
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51 polymorphisms throughout their sequence. In this case, the advantages of prescreening  
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53 methods are lost if each variant peak has to be sequenced (Mattocks, et al. 2010). The  
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55 situation becomes much simpler if the method allows correct recognition of polymorphisms  
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57 according to their profile. This requires a high profile specificity and reproducibility. Profiles  
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1 of distinct mutations from the same fragment must therefore be distinguished, and for the  
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3 same variant, the same profile should be observed in independent experiments.

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6 Reproducibility was evaluated within series and between series, by superimposition of  
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8 profiles from the same variant. Profiles matched very accurately (Figures 1A, B). A high  
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10 profile specificity was also observed, as illustrated e.g. Figure 2 illustrating 2 polymorphic  
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12 profiles and the co-occurrence of these two polymorphisms in the same patient.

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15 Polymorphisms were consequently expected to be identified from their variant profile. To  
16  
17 validate this hypothesis, all variant profiles interpreted as polymorphisms in this series of 93  
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19 patients were sequenced (for a total of 1,200 sequences), and sequencing results were  
20  
21 compared to EMMA interpretation. In every case, the expected polymorphism was found and  
22  
23 no extra polymorphism was found in the remaining variant profiles, corresponding to a  
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25 sensitivity and specificity of 100% in this series. To ensure correct interpretation,  
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27 polymorphisms should be interpreted together and compared between each other instead of  
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29 being compared to a normal control.  
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### 36 **Mutations Identified in Routine Screening**

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41 DNAs from 1,153 hereditary breast/ovarian cancer patients were subjected to routine  
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43 EMMA screening followed by sequencing of variant fragments. An average of 10 to 15% of  
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45 PCRs had to be re-amplified due to low signal-to-noise ratio (see discussion section) and 3%  
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47 of variant amplicons were sequenced. The 137 mutations found are reported in Supp. Tables  
48  
49 S1A-B. The overall mutational detection rate according to our inclusion criteria was 11.9%  
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51 i.e. 6% and 5.9% on *BRCA1* and *BRCA2*, respectively. The unknown variant detection rate  
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53 (Supp. Table S2) was 33.5% i.e. higher than previously reported because of the length of the  
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55 intronic sequences analyzed that includes a 70bp “safe zone” (see “primer design”). This  
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57 increased number of intronic UVs may represent a drawback of the design which is why  
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1 subsequent *in silico* splice analysis is recommended to confidently predict their putative  
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3 impact on splicing. UV detection rate was approximately twofold higher in *BRCA2* (23.8%)  
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5 than in *BRCA1* (9.7%). This figure is explained by the respective length of their coding  
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7 sequences, which in turn means that fewer mutations are found in *BRCA2* as compared to  
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10 *BRCA1*. Mutations were similar in type and relative proportion in *BRCA1* and *BRCA2* (Table  
11  
12 1). They mainly resulted in truncated proteins or RNA decay. *BRCA2* yielded a slightly  
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14 higher rate of frameshift mutations, due to a greater number of frameshift deletions (51.5% vs.  
15  
16 33.3% of the mutational spectrum for *BRCA2* and *BRCA1*, respectively). Frameshift  
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18 mutations are by far the largest class of mutations, followed by nonsense and splice mutations  
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20 (average 20% and 9% for both genes). Other mutations comprised rare mutations, i.e.  
21  
22 missense, in-frame and large rearrangements. Four large rearrangements were found on  
23  
24 *BRCA1* and 2 on *BRCA2*. In other words, point mutations and large rearrangements accounted  
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26 for 94.2% and 5.8% of the mutational spectrum of *BRCA1*, respectively. For *BRCA2*, point  
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28 mutations accounted for 97.1% of the mutational spectrum whereas large rearrangements  
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30 represented 2.9% of the mutational spectrum  
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36 The most frequent mutations found were c.5266dupC on *BRCA1* and c.2808\_2811del on  
37  
38 *BRCA2* (each found 6 times) followed by *BRCA2* c.5946delT (4 times). Four other mutations  
39  
40 were each found 3 times, i.e. c.1A>G, c.68\_69del and c.4986+6T>C on *BRCA1* and  
41  
42 c.1773\_1776del on *BRCA2*. This lack of frequent mutations probably reflects the mixed  
43  
44 ethnic origin of the study population derived from Paris and suburbs.  
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47  
48 According to our criteria, 18 UVs putatively led to a splice anomaly. Lymphoblastoid cell  
49  
50 lines were available in 10 cases and another blood sample has yet to be obtained from the  
51  
52 remaining 8 cases. Two UVs on both *BRCA1* and *BRCA2* actually lead to a splice defect. The  
53  
54 c.547+3A>T / IVS08+3A>T and c.4484G>T / p.Arg1495Met on *BRCA1* showed decrease of  
55  
56 the donor site with subsequent exon 8 and exon 14 skipping, respectively. The c.316+5G>C /  
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1 IVS03+5G>C and the c.7975A>G / p.Arg2659Gly on *BRCA2* also showed decrease of the  
2  
3 donor site with subsequent exon 3 and exon 17 skipping.  
4

5 Apart from splicing, 3 other UVs were prominent protein-damaging candidates with scores  
6  
7 greater than 16 i.e. c.92T>A/p.Ile31Asn and c.122C>T/p.Pro41Leu, c.280C>T/p.Pro94Ser on  
8  
9 *BRCA1* and *BRCA2*, respectively. They are included in class 4 of the recently proposed  
10  
11 classification system (Plon, et al., 2008).  
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### 17 **Throughput**

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19 Patients were initially analyzed by series of 96, according to the same organization as  
20  
21 previously used with DHPLC. Following a learning curve period, series of 30 patients were  
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23 subsequently analyzed. It now takes seven working days for complete *BRCA1/2* screening in  
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27 30 patients by one technician (excluding DNA extraction and sequencing).  
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## DISCUSSION

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8 One thousand five hundred and twenty five unrelated breast and/or ovarian cancer  
9 patients were studied, as part of the routine clinical management provided by the Institut  
10 Curie, Paris, France. To our knowledge, this is the largest complete mutational screening of  
11 *BRCA1/2* genes reported to date in Europe.  
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## EMMA

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25 This is the first report of a large-scale study using EMMA, some aspects of this  
26 methodology therefore need to be discussed. Capillary electrophoresis was developed as an  
27 attractive strategy for mutation detection in the early 1990's (Khrapko, et al., 1994). However,  
28 its broad diffusion was hampered by design constraints and lack of sensitivity (Rozycka, et  
29 al., 2000). The recently described Heteroduplex Analysis by Capillary Array Electrophoresis  
30 (Perez-Cabornero, et al., 2009), also called Conformation-Sensitive Capillary Electrophoresis  
31 (CSCE)(Mattocks, et al. 2010) represents a real improvement, but it also presents certain  
32 disadvantages. It uses a home-made polymer recipe that does not allow batch-to-batch  
33 reproducibility. Consequently, all fragments with altered peak patterns need to be sequenced,  
34 dramatically increasing the amount of sequencing needed for genes such as *BRCA1/2*, which  
35 involve frequent polymorphisms; large rearrangements cannot be detected and mutational  
36 screening must be completed by another method.. EMMA retains the simplicity and  
37 throughput of CSCE, but as a result of a series of additional developments comprising  
38 industrialized ready-to-use separation matrix and specific software, it overcomes the above  
39 limitations. It allows easy and reliable recognition of polymorphisms according to their  
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1 profile, and it is the first technique able to detect large rearrangements and point mutations in  
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3 a single run (Weber, et al., 2007).  
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5 . Some technical remarks and guidelines for implementation and use, based on our  
6  
7 experience, are listed below. Regarding DNA extraction, 3 different procedures were used  
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9 during the course of the study (see Materials and Methods” section) without any incidence on  
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11 data quality. Due to its high sensitivity, in order to take full advantage of the potential of the  
12  
13 technique for direct polymorphism identification, specific PCR amplifications must be used  
14  
15 since nonspecific products could generate shouldering for a wild-type sample, making the  
16  
17 profile difficult to interpret. Nonspecific products may also be generated by primer  
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19 degradation with time, but this is easily detected because profiles lose their reproducibility.  
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21 Capillary electrophoresis always involves slight run-to-run and capillary-to-capillary mobility  
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23 shifts, so it is therefore important to compare peaks after correct peak alignment, which can be  
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25 tricky with conventional software in the case of complex profiles. The Emmalys software  
26  
27 appeared to effectively deal with this difficulty, as suggested by the robustness of  
28  
29 polymorphism analysis.  
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32 Strikingly, heteroduplexes could be either slower or faster than homoduplexes and no rule  
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34 was found that could link one type of nucleotide substitution to one type of mobility  
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36 difference or profile shape. Electrophoretic mobility does not only depend on the mismatch  
37  
38 bases, but also on the local sequence environment of this mismatch, since distortions of the  
39  
40 DNA helix at one base pair are propagated to the neighboring bases(Weber, et al., 2004). As a  
41  
42 consequence of the above, a heteroduplex can exceptionally comigrate with the peak  
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44 corresponding to the following/preceding peak in the multiplex electrophoregram. Therefore,  
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46 if an amplicon from a patient shows an abnormal profile, but does not show any variation on  
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48 sequencing, it could indicate that the altered amplicon is actually a neighboring amplicon.  
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50 This can be easily confirmed by observing the result of large rearrangement analysis, which  
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52 should in turn mimic a deletion on the neighboring peak. Similarly, the quantitative analysis  
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1 used to detect large rearrangements can also be helpful to detect allele drop-out due to  
2 mispriming. This information is important in order to detect situations in which one allele  
3 would not be amplified and therefore not analyzed. Moreover, the nucleotide variation  
4 inducing the mispriming can also be deleterious. Obviously, in such a case another primer  
5 pair must be used to distinguish this allelic drop-out from a *bona fide* deletion.  
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8 Finally, to ensure reliable and easy interpretation of polymorphisms, it is recommended to  
9 work on series of 30 samples or more. Below this range, it may prove difficult to correctly  
10 interpret polymorphism profiles because of an insufficient number of reference profiles.  
11

12 Distinct injection protocols are available to ensure optimum results (EMMA high-low). In a  
13 first, “low” injection mode, samples are injected for 10s - 20s at 1kV – 4kV then  
14 electrophoresed at 15kV for 45 min. This injection mode has a sufficiently low consumption  
15 to allow a second run to be performed from the same sample using a “higher injection”  
16 protocol, in the event of occasional unexpected problems (missed injection, capillary failure,  
17 microbubble, etc). All of these “tricks”, recommendations and checkpoints are summarized in  
18 the Analysis Pipeline, Figure 3.  
19

20 The viscosity of EMMA separation medium is higher than that of other solutions  
21 conventionally used in ABI instruments. This requires certain modifications of the capillary  
22 filling and apparatus operation sequences (provided by Fluigent), compared to standard  
23 procedures. For instance, this involves longer array filling time, capillary rinsing with buffer  
24 every 100 runs, and capillary array exchange every 400 runs. Replacing EMMA buffer 1x  
25 daily is also recommended to avoid loss of resolution. System performance and integrity  
26 should be checked once a week using a control sample (a 4-plex PCR with variant profiles for  
27 each peak, available from Fluigent). As for any heteroduplex analysis, homozygous mutations  
28 cannot be detected by EMMA on the direct sample, but this can be circumvented by adding a  
29 wild-type DNA in the sample to force heteroduplex formation, as previously demonstrated  
30 with DHPLC (Ferec, et al., 2004).  
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1 In addition to the above precautions and issues, which require the level of care typically  
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3 required by molecular diagnosis, EMMA uses standard and well-known sequencers and a set  
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5 of ready-to-use reagents, thereby facilitating upgrading to this new technology.  
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8 The cost of consumable items used to screen *BRCA1* and *BRCA2* by EMMA is estimated to  
9  
10 be less than 100 euros per patient (based on price list). This estimation excluded the costs of  
11  
12 DNA extraction and sequencing, purchase of the sequencer, instrument depreciation and  
13  
14 labor. The markedly reduced need for downstream sequencing (typically 3%), as a result of  
15  
16 polymorphism identification, is also a factor for cost reduction (Sevilla, et al., 2002).  
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19 This technology allows a high throughput and our laboratory now generates close to 9000  
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21 amplicons per month for diagnostic purposes; it should be stressed that the management of  
22  
23 such a large volume of data requires a robust data management system. We have therefore  
24  
25 constructed a FileMakerPro® database which integrates information from sampling to the  
26  
27 final report, but which also interacts with the Institute's databases. This allows careful follow-  
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29 up of the entire analysis [Laugé A et al., poster 664 Assises de Génétique 2010].  
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34 The advantages of EMMA compared to our previous DHPLC-based strategy are  
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36 obvious: they comprise a single condition for amplification and run, a higher throughput and  
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38 lower costs (lab technician time decreased to one quarter and overall cost decreased to one  
39  
40 third, compared to DHPLC in our hands). The simultaneous detection of point mutations and  
41  
42 large rearrangements is another advantage. However promoter regions are not covered and  
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44 this must be taken into account, since deletions of the promoter regions are a probably rare but  
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46 significant cause of HBOC (Brown, et al., 2002; Caux-Moncoutier, et al., 2009). Although  
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48 technical performance was acceptable, the complexity of the currently available Emmalys  
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50 interface prevents routine use of large rearrangement detection. On the other hand, Emmalys  
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52 was found to be robust and user-friendly for point mutation detection.  
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1 More generally, we believe EMMA should be considered as an alternative to the High  
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3 Resolution Melting curve analysis (HRM) technique. HRM is a very fast and elegant tube-  
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5 method also allowing simultaneous detection of point mutations and large rearrangements in  
6  
7 one experiment (Coulet, et al., 2010 ; Rouleau, et al., 2009). Its main advantage over EMMA  
8  
9 is the absence of post-PCR manipulation. However, based on the recent literature, HRM  
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11 appears to have a number of drawbacks (which are overcome by EMMA), notably:

12  
13 i) its sensitivity depends on the nucleotide composition of the amplicon (melting domains).

14  
15 HRM diagnostic guidelines for *BRCA1* screening recommend extreme caution when  
16  
17 analyzing high-GC, low-GC contents and fragments containing many different variants (van  
18  
19 der Stoep, et al., 2009; Wittwer, 2009)

20  
21 ii) interpretation of polymorphisms according to their profile would need probes or sequence  
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23 analysis to exclude the presence of a mutation with an identical melt profile (Nguyen-Dumont,  
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25 et al., 2009).  
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### 34 **Spectrum of Mutations**

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39 The mutation detection rate in *BRCA* genes largely depends on the patient inclusion  
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41 criteria and selecting high-risk patients from breast and ovarian families would provide the  
42  
43 geneticist with a higher mutation detection rate than including patients based exclusively on  
44  
45 their personal history. That being said, the sensitivity of EMMA was 11.9% in our series of  
46  
47 1,153 patients, which is in line with expectations with regards to patient inclusions and  
48  
49 compared to previous results using DHPLC as well as data from French laboratories  
50  
51 (<http://www.e-cancer.fr>).  
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54  
55 Considering the mutational spectrum, the recurrent so-called Ashkenazim mutations (*BRCA1*  
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57 68\_69del, 5266dupC and *BRCA2* 5946delT) represented 9.5% of our identified mutations,  
58  
59 which is why prescreening for these 3 mutations is proposed during genetic counseling to  
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1 patients reporting an Eastern European origin. Apart from these mutations, we did not observe  
2  
3 a larger panel of recurrent mutations which would justify a first-line screening procedure, as  
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5 described in other populations (Revillion, et al., 2004). Few deleterious inframe/missense  
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7 mutations were found but this could actually reflect an interpretation bias in view of the fact  
8  
9 that 386 unknown variants were detected (detection rate 33.5%). In other words, these 386  
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11 variants no doubt included deleterious neutral/missense/intronic mutations, which is why  
12  
13 scoring variants to highlight prominent candidates for future collaborative studies is of special  
14  
15 relevance for optimization of molecular diagnosis. There is considerable debate about the  
16  
17 usefulness of large rearrangement screening in *BRCA* genes. The main argument against such  
18  
19 screening, is that the time and efforts needed for such screening might be used more  
20  
21 efficiently by screening more patients but for point mutations only. Previous reports (Engert,  
22  
23 et al., 2008; Mazoyer, 2005) suggested that large rearrangements accounted for an average of  
24  
25 10% of the *BRCA1* mutational spectrum. Although our present results are lower (5.8%), we  
26  
27 can confidently confirm that it is worth searching for *BRCA1* large rearrangements in genetic  
28  
29 diagnosis, regardless of the patient's family history. The situation is less clear for *BRCA2*  
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31 because previous studies, including those from our team (Casilli, et al., 2006; Tournier, et al.,  
32  
33 2004), were based on smaller series and selected high-risk families (Agata, et al., 2005;  
34  
35 Engert, et al., 2008; Woodward, et al., 2005). These studies led to the overall conclusion that  
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37 *BRCA2* genomic rearrangements are worth investigating in high-risk families. This does not  
38  
39 mean that these mutations are associated with a higher risk, but that due to the very low  
40  
41 contribution of large rearrangements in the *BRCA2* mutational spectrum, the search for these  
42  
43 mutations should be limited to patients with a severe family history associated with a high  
44  
45 *BRCA2* mutation detection rate. Two *BRCA2* large rearrangements, one complete deletion and  
46  
47 one duplication of exons 19, 20 (Figure 4) were found in the present series of 1,153  
48  
49 consecutive patients. The deletion was found in a young breast cancer patient with no family  
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51 history i.e. a low-risk case. Conversely, the duplication was found in a high-risk family: the  
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1 index case and her older sister developed bilateral breast carcinoma at 48, 64 and 57, 63 years  
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3 of age, respectively. Their paternal aunt and grandmother also developed breast cancer at 45  
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5 and 61 years of age, respectively. The overall probability of identifying a *BRCA* mutation in  
6  
7 our index case was 59.4% (24.6% for *BRCA1* and 34.9% for *BRCA2*) according to the  
8  
9 BRCAPRO model (Parmigiani, et al., 1998) and 57.9%(12.1% for *BRCA1* and 45.8% for  
10  
11 *BRCA2*) according to the BOADICEA model (Antoniou, et al., 2008). Interestingly, her older  
12  
13 affected sister was found to be negative using a combination of direct sequencing for  
14  
15 *BRCA1/2* and a limited 5-site rearrangement panel for *BRCA1*(*Comprehensive*  
16  
17 *BRCAAnalysis*®, Myriad® Genetics laboratories Inc. Salt Lake City, USA). Following  
18  
19 identification of the *BRCA2* duplication in our index case, genetic testing and appropriate  
20  
21 genetic counseling were made available to the relatives e.g. 6 unaffected females (at least) and  
22  
23 the affected sister. As expected, the affected sister carried the *BRCA2* duplication. More  
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25 generally, and as this kind of mutation might also be found in low-risk situations, this study  
26  
27 argues in favor of methods allowing simultaneous and convenient detection of point  
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29 mutations and large rearrangements to enable clinical geneticists to meet the constraints of  
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31 routine genetic testing.  
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### 41 **General conclusions and perspectives**

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46 The main advantages of EMMA are that it provides a marked increase of throughput  
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48 (or cost reduction at a given throughput) compared to DHPLC and is based on standard  
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50 sequencers routinely used in laboratories. It can therefore be easily implemented in  
51  
52 laboratories familiar with capillary electrophoresis, and allows screening and sequencing on  
53  
54 the same platform. Due to its flexibility, EMMA could also be used for rapid *BRCA* screening  
55  
56 of a subset of patients e.g. before poly(ADP-ribose) polymerase inhibitor treatment (Audeh, et  
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58 al., 2010 ; Tutt, et al. 2010).  
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1 Finally, the future of EMMA should be considered in the context of growing  
2  
3 sequencing capacities provided by the next-generation-sequencing (NGS) platforms. Since 2  
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5 SOLiD NGS platforms (ABI-Life technologies) are available in our Institute, we tested this  
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8 technology for *BRCA* diagnostic applications [Houdayer C et al., European Society of Human  
9  
10 Genetics meeting 2010, P11.089]. The SOLiD chemistry appears to be reliable for diagnostic  
11  
12 purposes, but implementation of a SOLiD diagnostic pipeline remains complex at the present  
13  
14 time for reasons of sample preparation, bioinformatics, fast-evolving protocols and costs.  
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17 EMMA obviously cannot compete with SOLiD in terms of throughput, but indeed only a  
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19 small number of diagnostic laboratories actually need such amazing sequencing capacities.  
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22 The throughput and therefore data handling constraints of the 454 NGS machine (Roche) are  
23  
24 still enormous, although lower than those of SOLiD, but well known sensitivity/specificity  
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26 problems in homopolymer runs have to be taken into account (Emrich, et al., 2007; Wicker, et  
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28 al., 2006). The present study therefore suggests that EMMA represents a valuable short-term  
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30 and medium-term option for many diagnostic laboratories looking for an easy, reliable and  
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32 affordable strategy, enabling fast and sensitive analysis for a large number of genes.  
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#### Commercial interest:

The EMMA technology is dependent on Curie Institute/CNRS/University Pierre et Marie

Curie joint patents

DSL and JLV are shareholders of Fluigent

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## FIGURE LEGENDS

**Figure 1A. Reproducibility between series.**

Screenshot from Emmalys showing superimposition of 5 different samples harboring the same polymorphism (*BRCA2* exon 2), from 5 different series of 93 patients.

**Figure 1B. Reproducibility within series.**

Screenshot from Emmalys showing superimposition of 33 different samples harboring the same polymorphism (*BRCA1* exon 13), from a series of 93 patients.

**Figure 2. Profile specificity**

Screenshots from Emmalys with 3 polymorphic profiles from 2 adjacent codons (*BRCA1* exon 11). In grey: normal monomorphic profile, in black: polymorphic profiles. From left to right: c.2077G>A/p.Asp693Asn, c.2082C>T/p.Ser694Ser and the combination of both (c.2077G>A/p.Asp693Asn plus c.2082C>T/p.Ser694Ser). All polymorphic profiles are clearly distinct.

**Figure 3. EMMA : analysis pipeline**

Schematic representation of a routine EMMA analysis.

**Figure 4. Duplication of *BRCA2* exons 19 and 20.**

Three screenshots from Emmalys showing the 3 multiplex PCRs incorporating *BRCA2* exons 19 and 20 (panels A, B and C). Exons under study and the amplicon number (when the exon is investigated in overlapping amplicons) are indicated at the top of the corresponding peaks. The electrophoregrams in grey and black represent a normal control and the duplicated patient, respectively. Profiles are superimposed then normalized using the internal control. Two ratios are used to determine the presence or absence of a large-scale rearrangement: R1 is the ratio between peak intensity of the fragment of interest over peak intensity of the internal control. R2 (indicated for each peak) is the ratio of R1 of the sample of interest over control R1 defined as a mean R1 value based on several control samples. Duplicated exons are indicated by an arrow. Exon 19 is investigated in 2 overlapping amplicons (upper panels A, B), while exon 20 is investigated with a single amplicon (bottom panel C).

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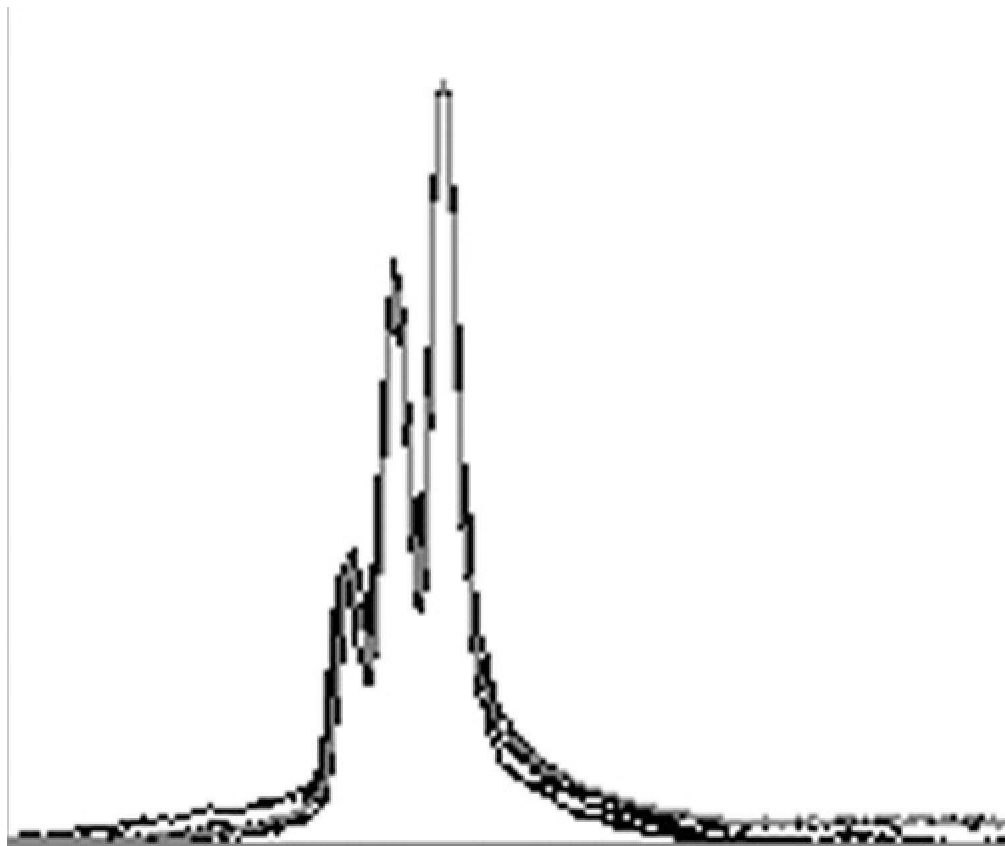
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3 **Tables**  
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5 **Table 1.** Deleterious mutations found according to their type.  
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	BRCA1 (number and %)	BRCA2 (number and %)
Frameshift	37 (53.6%)	47 (69.1%)
Nonsense	16 (23.2%)	13 (19.1%)
Splice	8 (11.6%)	4 (5.9%)
Large rearrangements	4 (5.8%)	2 (2.9%)
Missense	4 (5.8%)	1 (1.5%)
In frame	0 (0%)	1 (1.5%)
<b>Total</b>	<b>69 (100%)</b>	<b>68 (100%)</b>
Mutation detection rate (1153 patients)	6%	5.9%

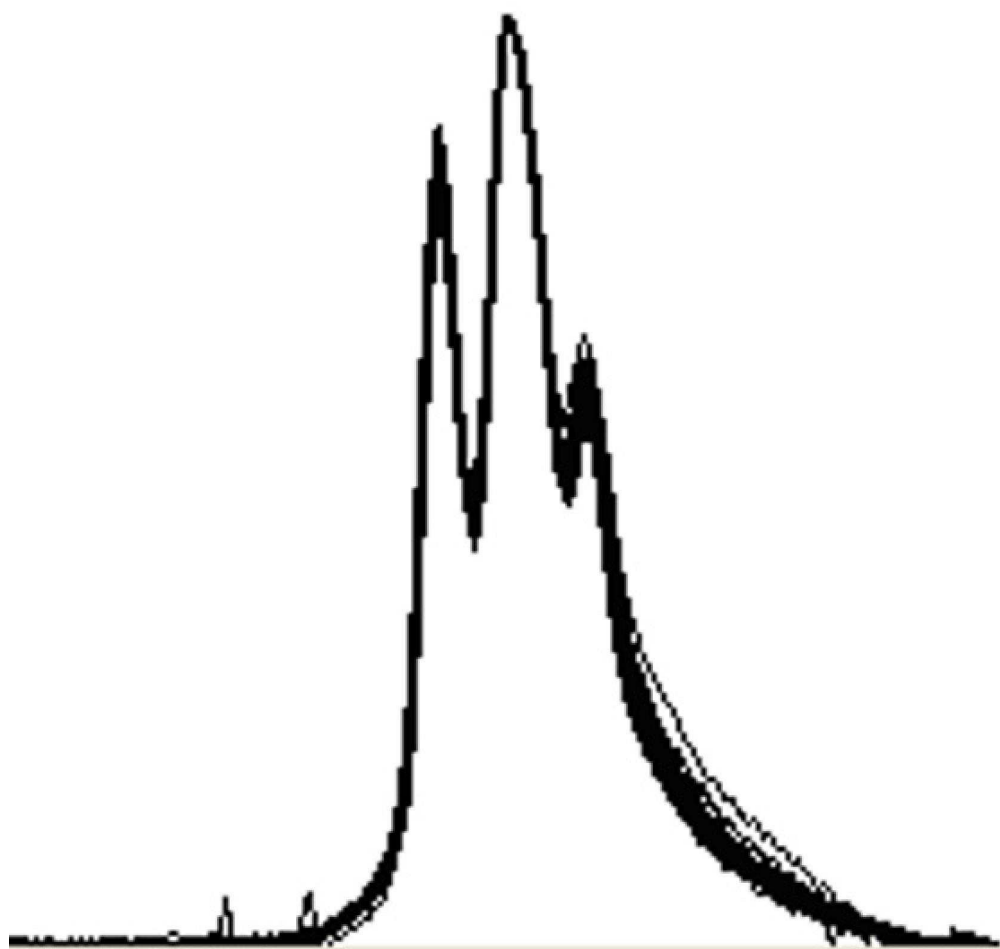


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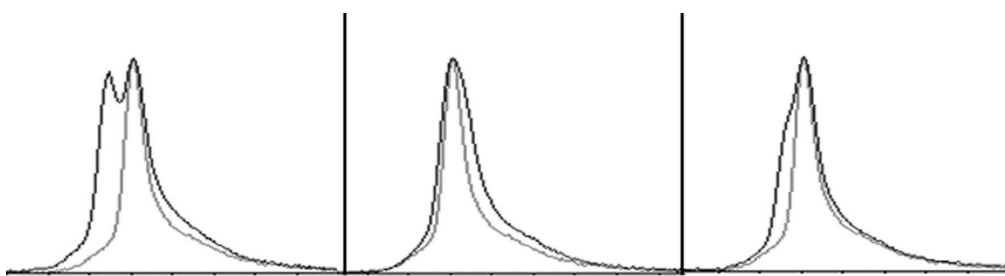
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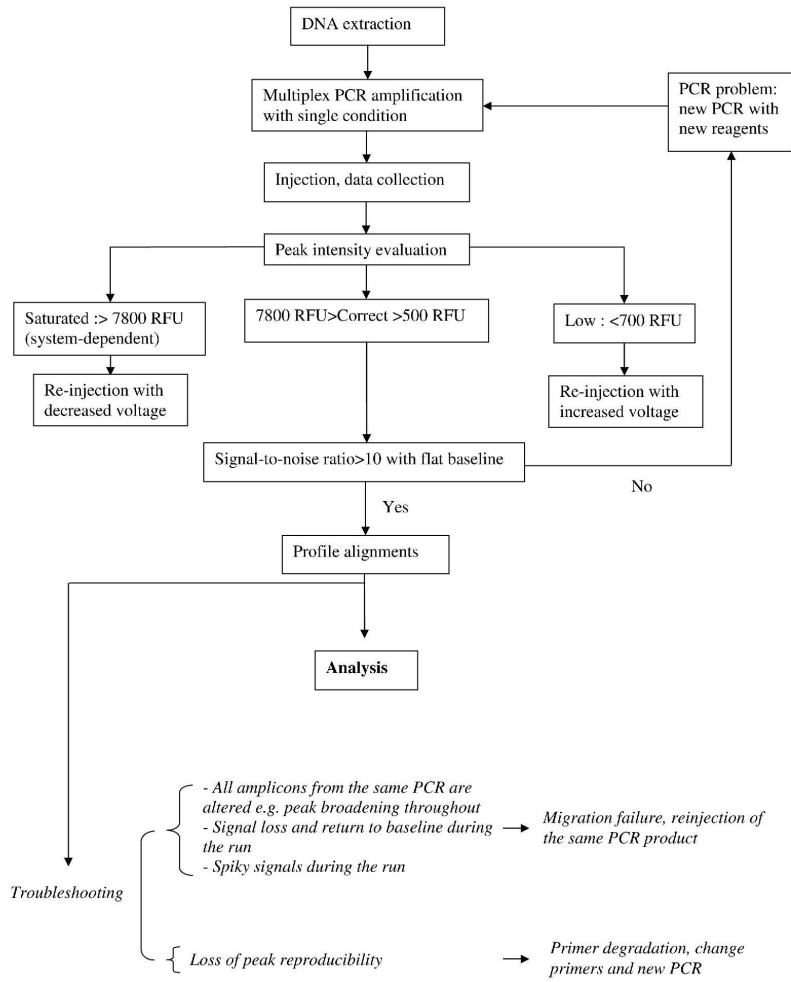
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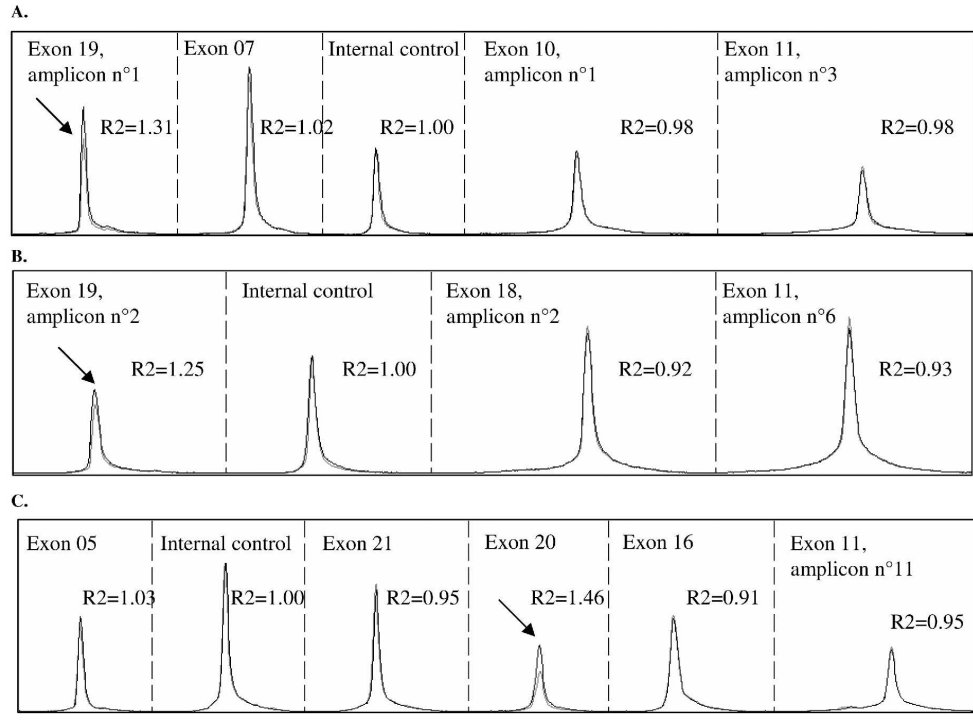
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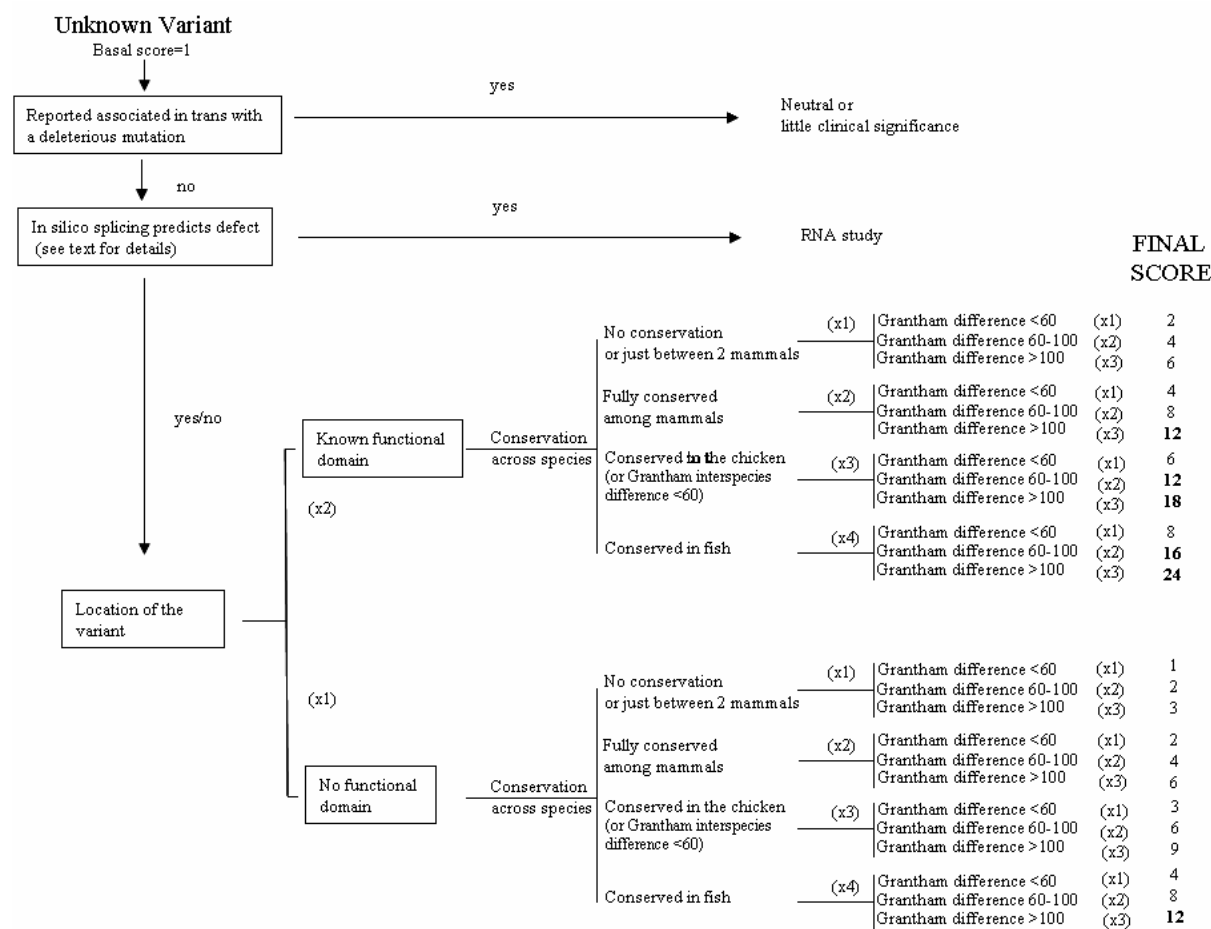


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Review

**Supplementary Figure S1. In-house algorithm for UV scoring**

A restrictive definition of functional domains was used to avoid redundancy between “conservation across species” and “known functional domain” items. The amino-acid positions 24-64 (RING finger), 503-508 (SLN1), 607-615 (SLN2), 1189, 1457, 1524, 1542 (serine phosphorylation sites), 1649-1736 (BRCT1) and 1756-1855 (BRCT2) were therefore considered as functional domains for BRCA1. Functional domains for BRCA2 were as follows: amino-acid positions 18-105 (transactivation), 987-1069, 1198-1293; 1407-1498 ; 1501-1589; 1649-1735; 1822-1914; 1955-2035; 2036-2112 (BRC repeats), 3263-3269 (SLN1) and 3381-3385 (SLN2). Scores from 12 and above are considered “likely deleterious”.



**Supplementary Table S1A. Deleterious mutations found on *BRCA1***

Recurrence numbers are in brackets. Nucleotide position was numbered on the basis of the coding sequence NM\_007294.2. Nucleotide numbering reflects cDNA numbering with +1 corresponding to the A of the ATG translation initiation codon in the reference sequence. The initiation codon is codon 1.

Site	Description	Expected Consequence
Exons 1-2	c.-200-?_80+?del (deletion of exons 1 and 2)	
Exon 2	c.1A>G (3)	p.Met1?
	c.19_47del29	p.Arg7_Asn16>CysfsX24
	c.68_69delAG (3)	p.Glu23ValfsX17
Intron 2	c.81-2A>G	
Exon 5	c.140G>A	p.Cys47Tyr
Intron 5	c.212+3A>G	
	c.213-02A>C	
Exon 7	c.342_343delTC	p.Pro115X
Exons 8 to 13	c.442-?_4357+?del (deletion of exons 8 to 13)	
Exon 9	c.569_570insAACG	p.Val191ThrfsX3
Exon 11	c.800C>G	p.Ser267X
	c.815_824dup10	p.Thr276AlafsX14
	c.843_846del4	p.Ser282TyrfsX15
	c.928C>T	p.Gln310X
	c.1016dupA	p.Val340GlyfsX6
	c.1121delC	p.Thr374AsnfsX2
	c.1953_1956del4	p.Lys653SerfsX47
	c.2197_2201del5	p.Glu733ThrfsX5
	c.2269delG	p.Val757PhefsX8
	c.2308delT	p.Ser770HisfsX22
	c.2561_2565del5	p.Ala854ValfsX47
	c.2952delT	p.Ile986SerfsX14
	c.3285delA	p.Lys1095AsnfsX14
	c.3377delC	p.Pro1126HisfsX3
	c.3403C>T	p.Gln1135X
	c.3417delT	p.Ser1139ArgfsX16
	c.3428delCinsTA	p.Ser1143LeufsX6
	c.3481_3491del11	p.Glu1161PhefsX3
	c.3593T>A	p.Leu1198X
	c.3648dupA	p.Ser1217IlefsX2
	c.3700_3704del5	p.Val1234GlnfsX8
	c.3748G>T	p.Glu1250X
	c.3753T>A	p.Cys1251X
	c.3756_3759del4	p.Ser1253ArgfsX10
	c.3771_3778del8	p.Glu1257AspfsX7
	c.3839_3843del5ins4	p.Ser1280X
c.3841C>T	p.Gln1281X	
c.3937C>T	p.Gln1313X	
c.4065_4068del4	p.Asn1355_Gln1356>LysfsX10	
c.671-?_4185+?del (deletion of exons 11 and 12)		

Site	Description	Expected Consequence
Exon 12	c.4165_4166delAG	p.Ser1389X
Exon 13	c.4201C>T	p.Gln1401X
	c.4258C>T	p.Gln1420X
	c.4327C>T	p.Arg1443X
Exon 14	c.4372C>T	p.Gln1458X
	c.4484G>T (2)	p.Arg1495Met
Exon 16	c.4810C>T	p.Gln1604X
	c.4945_4947delAGAinsTTTT (2)	p.Arg1649PhefsX30
Intron 16	c.4986+6T>C (3)	
Exon 17	c.5071dupA	p.Thr1691AsnfsX4
Exon 20	c.5266dupC (6)	p.Gln1756ProfsX74
Exons 21 to 24	c.5278-?_*1381+?del (deletion of exons 21 to 24)	
Exon 21	c.5307T>A	p.Tyr1769X
Exon 24	c.5503C>T	p.Arg1835X
	c.5541C>A	p.Cys1847X



**Supplementary Table S1B. Deleterious mutations found on *BRCA2***

Recurrence numbers are in brackets. Nucleotide position was numbered on the basis of the coding sequence NM\_000059.3. Nucleotide numbering reflects cDNA numbering with +1 corresponding to the A of the ATG translation initiation codon in the reference sequence. The initiation codon is codon 1.

Site	Description	Expected Consequence
Exons 1 to 27	c.(?-227)_(*902_?)del (complete deletion)	
Exon 3	c.145G>T (2)	p.Glu49X
	c.289G>T	p.Glu97X
Intron 6	c.516+1G>T	
Exon 10	c.1238delT	p.Leu413HisfsX17
	c.1310_1313del4	p.Lys437IlefsX22
	c.1511_1512delCT	p.Ser504TyrfsX9
	c.1593dupA	p.Glu532ArgfsX3
	c.1773_1776del4 (3)	p.Ile591MetfsX22
	c.1813dupA	p.Ile605AsnfsX11
Exon 11	c.1929delG	p.Arg645GlufsX15
	c.2588dupA	p.Asn863LysfsX18
	c.2612C>A	p.Ser871X
	c.2808_2811del4 (6)	p.Ala938ProfsX21
	c.2899_2900delCT	p.Leu967ArgfsX14
	c.3195delT	p.Asn1066IlefsX11
	c.3645_3646del2ins7	p.Phe1216LysfsX14
	c.3744_3747del4 (2)	p.Ser1248ArgfsX10
	c.4136dupA	p.Ile1380AspfsX2
	c.4284dupT	p.Gln1429SerfsX9
	c.4965C>A	p.Tyr1655X
	c.5197dupT	p.Ser1733PhefsX10
	c.5576_5579del4	p.Ile1859LysfsX3
	c.5616_5620del5	p.Lys1872AsnfsX2
	c.5645C>A	p.Ser1882X
	c.5682C>G	p.Tyr1894X
	c.5909C>A (2)	p.Ser1970X
	c.5946delT (4)	p.Ser1982ArgfsX22
	c.6079dupA	p.Arg2027LysfsX22
	c.6082_6086del5	p.Glu2028LysfsX19
	c.6275_6276delTT (2)	p.Leu2092ProfsX7
	c.6359C>G	p.Ser2120X
	c.6373dupA	p.Thr2125AsnfsX4
	c.6405_6409del5	p.Asn2135LysfsX3
	c.6644_6647del4	p.Tyr2215SerfsX13
	c.6656C>G	p.Ser2219X

Site	Description	Expected Consequence
Exon 14	c.7069_7070delCT	p.Leu2357ValfsX2
Exon 15	c.7480C>T	p.Arg2494X
	c.7612A>T	p.Lys2538X
Intron 15	c.7617+1G>T	
Exon 16	c.7680dupT	p.Gln2561SerfsX5
	c.7795_7797delGAA	p.Glu2599del
	c.7805G>C	p.Arg2602Thr
Exon 18	c.8021delA	p.Lys2674ArgfsX2
	c.8029_8030delGA	p.Glu2677LysfsX3
	c.8032-8033dupAG	p.Asp2679GlyfsX16
	c.8207delT	p.Leu2736ProfsX2
Exons 19 to 20	c.8332-?_8632+?dup (duplication of exons 19 and 20)	
Exon 20	c.8548_8551del4	p.Glu2850GlnfsX12
Exon 23	c.9026_9030del5	p.Tyr3009SerfsX7
	c.9097delA	p.Thr3033LeufsX29
Exon 24	c.9154C>T	p.Arg3052Trp
	c.9253dupA	p.Thr3085AsnfsX26
Intron 24	c.9257-1G>A	

**Supplementary Table S2A. Unknown variants found on *BRCA1***

Recurrence numbers are in brackets. Nucleotide position was numbered on the basis of the coding sequence NM\_007294.2. Nucleotide numbering reflects cDNA numbering with +1 corresponding to the A of the ATG translation initiation codon in the reference sequence. The initiation codon is codon 1.

Site	Description	Expected Consequence
Intron 1	c.-19-24A>G	
Intron 2	c.81-178_167del12 (*)	
	c.81-14C>T	
Exon 3	c.92T>A	p.Ile31Asn
Intron 3	c.134+149A>C	
	c.135-27T>A	
Exon 5	c.199G>T	p.Asp67Tyr
Intron 6	c.301+55G>A	
	c.302-24_22delAAT	
Exon 7	c.314A>G	p.Tyr105Cys
	c.425C>A	p.Pro142His
Intron 7	c.441+52del12 (2)	
Exon 8	c.448A>G	p.Thr150Ala
	c.456C>T (2)	p.Leu152Leu
	c.466C>A	p.Leu156Ile
	c.536A>G (2)	p.tyr179Cys
Intron 8	c.547+3A>T (2)	
	c.548-34T>C	
	c.548-58dupT	
	c.548-65A>C	
Intron 9	c.593+76C>G	
	c.594-34T>C	
Intron 10	c.670+85T>C (2)	
Exon 11	c.981A>G (4)	p.Thr327Thr
	c.1456T>C (2)	p.Phe486Leu
	c.1487G>A	p.Arg496His
	c.1541C>G	p.Pro514Arg
	c.1648A>C (2)	p.Asn550His
	c.1866G>A	p.Ala622Ala
	c.1940G>A	p.Ser647Asn
	c.2352G>A	p.Ser784Ser
	c.2458A>G	p.Lys820Glu
	c.2477C>A (2)	p.Thr826Lys
	c.2518A>T	p.Ser840Cys
	c.2733A>G (4)	p.Gly911Gly
	c.2942C>T	p.Pro981Leu
	c.3296C>T	p.Pro1099Leu
c.3302G>A	p.Ser1101Asn	

	c.3305A>G	p.Asn1102Ser
	c.3418A>G (4)	p.Ser1140Gly
	c.3600G>T	p.Gln1200His
<b>Site</b>	<b>Description</b>	<b>Expected Consequence</b>
Exon 11	c.3608G>A	p.Arg1203Gln
	c.3640G>A	p.Glu1214Lys
	c.3804T>C	p.Asn1268Asn
	c.3823A>G (4)	p.Ile1275Val
Exon 12	c.4113G>A (2)	p.Gly1371Gly
Intron 12	c.4185+3A>T	
	c.4185+21delTG	
	c.4185+21_22dupTG	
Exon 13	c.4255G>C	p.Glu1419Gln
Intron 13	c.4358-37G>A	
Exon 14	c.4445A>G	p.Asp1482Gly
Exon 15	c.4636G>A	p.Asp1546Asn
Intron 14	c.4485-32C>T	
	c.4485-57T>C	
Intron 15	c.4675+31C>T	
	c.4675+81T>C	
Exon 16	c.4691T>C	p.Leu1564Pro
	c.4812A>G	p.Gln1604Gln
	c.4840C>T	p.Pro1614Ser
Exon 17	c.4993G>C	p.Val1665Leu
Intron 16	c.4987-20A>G	
	c.4987-25A>G	
Intron 17	c.5074+107C>T	
	c.5074+108G>A (3)	
	c.5075-74C>T	
Exon 19	c.5177G>T	p.Arg1726Ile
Intron 19	c.5193+64T>G	
	c.5194-14T>A	
	c.5194-165_162dup	
Exon 20	c.5203G>A	p.Glu1735Lys
	c.5213G>A	p.Gly1738Glu
Intron 20	c.5277+55A>T	
Intron 21	c.5332+182dupA (*)	
	c.5332+78C>T	
	c.5333-130T>C (2)	
	c.5333-44A>T	
	c.5333-61G>C	
	c.5333-134C>A (4)	
Intron 22	c.5406+63G>T	
Exon 23	c.5412C>T (2)	p.Val1804Val

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Intron 23	c.5467+148delT	
	c.5468-10C>A (2)	

(\* ) these UVs were detected following allele drop-out (see text)

For Peer Review

**Supplementary Table S2B. Unknown variants found on *BRCA2***

Recurrence numbers are in brackets. Nucleotide position was numbered on the basis of the coding sequence NM\_000059.3. Nucleotide numbering reflects cDNA numbering with +1 corresponding to the A of the ATG translation initiation codon in the reference sequence. The initiation codon is codon 1.

Site	Description	Expected Consequence
Exon 2	c.-11C>T (5)	
	c.-12T>C	
	c.64G>A	p.Ala22Thr
Intron 2	c.67+62T>G (12)	
	c.67+82C>G (14)	
	c.68-7T>A (7)	
Exon 3	c.122C>T	p.Pro41Leu
	c.198A>G	p.Gln66Gln
	c.223G>C (3)	p.Ala75Pro
	c.280C>T	p.Pro94Ser
Intron 3	c.316+5G>C	
	c.316+6T>C	
	c.316+12A>G	
	c.316+13A>G	
	c.316+57C>T	
	c.317-12G>A	
	c.317-92delA	
Exon 4	c.324T>C	p.Asn108Asn
	c.400C>A	p.Leu134Ile
Intron 4	c.425+52A>G	
Intron 5	c.475+3A>T	
	c.476-5C>T	
	c.476-24A>G	
Intron 6	c.516+21A>T (4)	
	c.516+54T>C	
	c.517-19C>T (4)	
	c.517-23_22delTA	
	c.517-74G>C	
	c.517-89G>A	
Exon 7	c.532A>C	p.Lys178Gln
Intron 7	c.631+18G>A	
	c.631+25C>T	
	c.631+66C>T	
	c.632-16A>C	
	c.632-75T>C	
Intron 8	c.682-30A>C	
	c.682-32A>G	
Exon 10	c.943T>A	p.Cys315Ser
	c.978C>A	p.Ser326Arg

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3		c.986G>C	p.Arg329Thr
4		c.1096T>G	p.Leu366Val
5		c.1181A>C	p.Glu394Ala
6	Exon 10	c.1216G>A	p.Ala406Thr
7		c.1219C>G	p.Gln407Glu
8		c.1272A>G	p.Ser424Ser
9		c.1275A>G	p.Glu425Glu
10		c.1281C>A	p.Asp427Glu
11		c.1395A>C	p.Val465Val
12		c.1514T>C	p.Ile505Thr
13		c.1561T>G	p.Ser521Ala
14		c.1786G>C	p.Asp596His
15		c.1788T>C (5)	p.Asp596Asp
16		c.1792A>G (2)	p.Thr598Ala
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18	Intron 10	c.1910-43T>C	
19	Exon 11	c.1964C>G	p.Pro655Arg
20		c.1966A>G	p.Thr656Ala
21		c.2330A>G	p.Asp777Gly
22		c.2350A>G	p.Met784Val
23		c.2477A>G	p.Glu826Gly
24		c.2538A>C (2)	p.Ser846Ser
25		c.2550A>G (2)	p.Gln850Gln
26		c.2635T>A	p.Ser879Thr
27		c.2803G>A (4)	p.Asp935Asn
28		c.2803G>C	p.Asp935His
29		c.2817C>T	p.Thr939Thr
30		c.3097G>T	p.Asp1033Tyr
31		c.3152T>C	p.Leu1051Ser
32		c.3264T>C (2)	p.Pro1088Pro
33		c.3445A>G	p.Met1149Val
34		c.3515C>T (2)	p.Ser1172Leu
35		c.3516G>A (3)	p.Ser1172Ser
36		c.3723T>G	p.Phe1241Leu
37		c.3749A>G	p.Glu1250Gly
38		c.3869G>A	p.Cys1290Tyr
39		c.3949A>T (2)	p.Thr1317Ser
40		c.4090A>C (5)	p.Ile1364Leu
41		c.4164T>C	p.Thr1388Thr
42		c.4199A>G	p.His1400Arg
43		c.4241C>T	p.Thr1414Met
44		c.4242G>A	p.Thr1414Thr
45		c.4271C>G (2)	p.Ser1424Cys
46	c.4614T>C (2)	p.Ser1538Ser	
47	c.4677T>C	p.Phe1559Phe	
48	c.4686A>G	p.Gln1562Gln	
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	c.4850G>A	p.Ser1617Asn
	c.4903T>C	p.Leu1635Leu
	c.5070A>C	p.Lys1690Asn
Exon 11	c.5268A>G	p.Val1756Val
	c.5312G>A (2)	p.Gly1771Asp
	c.5418A>G (2)	p.Glu1806Glu
	c.5503A>G	p.Asn1835Asp
	c.5634C>T	p.Asn1878Asn
	c.5688A>G	p.Ala1896Ala
	c.5704G>A (2)	p.Asp1902Asn
	c.5741G>C	p.Ser1914Thr
	c.5745G>A	p.Thr1915Thr
	c.5778T>G	p.Ser1926Arg
	c.5785A>G	p.Ile1929Val
	c.5852G>A	p.Ser1951Asn
	c.5896C>T	p.His1966Tyr
	c.5928G>T	p.Gly1976Gly
	c.5937C>G	p.Ser1979Arg
	c.5985C>T	p.Asn1995Asn
	c.6215C>G	p.Ser2072Cys
	c.6295A>G	p.Arg2099Gly
	c.6323G>A (3)	p.Arg2108His
	c.6338A>G (2)	p.Asn2113Ser
	c.6347A>G (3)	p.His2116Arg
	c.6553G>T	p.Ala2185Ser
Intron 11	c.6842-20T>A	
Exon 12	c.6853A>G (2)	p.Ile2285Val
Intron 12	c.6938-26T>C	
Exon 13	c.6972T>C	pHis2324His
Intron 13	c.7007+18T>A	
	c.7007+53G>A	
	c.7008-44A>G	
Exon 14	c.7017G>C (2)	p.Lys2339Asn
	c.7052C>G	p.Ala2351Gly
	c.7082A>G	p.His2361Arg
	c.7242A>T	p.Ser2414Ser
	c.7319A>G (3)	p.His2440Arg
Exon 15	c.7469T>C	p.Ile2490Thr
	c.7478T>G	p.Met2493Arg
	c.7534C>T	p.Leu2512Phe
	c.7559G>T	p.Arg2520Leu
	c.7565C>T	p.Ser2522Phe
Intron 15	c.7618-74A>G	
Intron 16	c.7805+46C>T	
	c.7805+47A>G	



	c.7806-40A>G	
Exon 17	c.7975A>G	p.Arg2659Gly
Intron 17	c.7976+35C>A (2)	
Intron 17	c.7976+57G>C	
Exon 18	c.7994A>G (2)	p.Asp2665Gly
	c.8149G>T (2)	p.Ala2717Ser
	c.8182G>A (2)	p.Val2728Ile
Intron 18	c.8331+109G>A (4)	
Exon 19	c.8460A>C (2)	p.Val2820Val
Intron 19	IVS19+19A>G	
	c.8487+47C>T (8)	
	c.8487+82G>A (3)	
	c.8488-52A>G	
Exon 20	c.8503T>C	p.Ser2835Pro
	c.8567A>C (3)	p.Glu2856Ala
	c.8592C>T	p.Ala2864Ala
Intron 20	c.8633-4T>A	
Intron 21	c.8754+75A>G	
Exon 22	c.8850G>T (2)	p.Lys2950Asn
	c.8904C>T	p.Thr2968Thr
Exon 23	c.9038C>T	p.Thr3013Ile
Intron 23	c.9117+44G>T	
Exon 24	c.9216G>A	p.Val3072Val
Exon 25	c.9275A>G	p.Tyr3092Cys
	c.9292T>C	p.Tyr3098His
	c.9371A>T	p.Asn3124Ile
	c.9373C>T	p.Leu3125Phe
Intron 25	c.9501+4A>G	
Exon 26	c.9586A>G	p.Lys3196Glu
	c.9606G>C	p.Pro3202Pro
	c.9610A>G	p.Thr3204Ala
	c.9613_9614delGCinsCT	p.Ala3205Leu
	c.9613G>A	p.Ala3205Thr
Intron 26	c.9648+106delT (4)	
	c.9648+64T>C	
	c.9648+84G>A (3)	
	c.9649-20C>T (2)	
	c.9649-65_62del4	
Exon 27	c.9730G>A (2)	p.Val3244Ile
	c.9738C>A	p.Ala3246Ala
	c.9972A>G	p.Pro3324Pro
	c.10045A>G	p.Thr3349Ala
	c.10078A>G	p.Lys3360Glu