### RNA isolation and cDNA synthesis used for Stage 2 and 3 assays.

### LEUs

### Whole blood was drawn from healthy control donors (collected in EDTA tubes). Blood samples were kept ice-cold for a maximum of 30 minutes before RNA extraction. Total RNA was extracted from whole blood (200µL) using a MagnaPure Compact workstation and MagnaPure RNA Isolation Kit according to the manufacturer’s protocol. The integrity of isolated RNA was checked with a Bioanalyzer. After quantification with a NanoDrop spectrophotometer (NanoDrop Technologies, Inc. Wilmington, DE, USA), 300-700ng of total RNA was reverse transcribed with Prime-Script RT kit (TaKaRa Biotechnology, Japan) following manufacturer’s protocol (the kit includes a mixture of random and Oligo(dT) primers)

### PBMCs

### Whole blood with EDTA was drawn from healthy control donors. Peripheral Blood Mononuclear Cells (PBMCs) were isolated by density gradient centrifugation using Ficoll-Paque TM PLUS (GE Healthcare, Uppsala, Sweden). RNA was extracted by using TRIzol reagent (Invitrogen, Paisley, United Kingdom) according to the manufacturer’s protocol. Isolated RNA was further processed on columns from RNeasy Kit and RNase-Free DNase set to cleanup RNA and remove any DNA traces, according to the protocols provided by the manufacturer (Qiagen Sciences, Maryland, USA). The integrity of RNA purified was checked in 1.5% agarose gel stained with ethidium bromide. After quantification with a NanoDrop spectrophotometer (NanoDrop Technologies, Inc. Wilmington, DE, USA), 1000 ng of total RNA was reverse transcribed using High Capacity RNA-to-cDNA Master Mix with a mix of random and Oligo(dT) primers according to the manufacturers’ protocols (Applied Biosystems, Life Technologies, Foster City, USA).

**PBLs**

Depending on the contributing laboratory, two different protocols were followed (see below). Since no significant differences were observed regarding *BRCA1* splicing, all data has been pooled together in the results section.

Protocol 1

1 ml of peripheral blood (collected in Sodium Heparin Tube) was cultured in 9 ml of RPMI medium containing 20% of FBS, 1% penicillin-streptomycin and phytohemagglutinin (PHA, 100 μg per ml) at 37ºC and 5%CO2. After 72 hours, 3 ml of Cell Lysis Solution (Promega) was added for each ml of sample, and the sample incubated for 10 minutes at room temperature, spun at 13,000 rpm for 2 minutes, and the supernatant discarded. This last step was repeated as necessary to ensure the pellet was nearly white. For purification of total RNA, RNeasy Mini Kit (Qiagen) was used according to manufacturer’s protocols (Qiagen Sciences, Maryland, USA). After quantification with a NanoDrop spectrophotometer (NanoDrop Technologies, Inc. Wilmington, DE, USA), 1000 ng of total RNA was reverse transcribed using SuperScript II Reverse Transcriptase with random primers according to the manufacturers’ protocols (Invitrogen, Life Technologies, Foster City, USA). To ensure the appropriate RNA transcription into cDNA, real time PCR was performed using the TaqMan® Gene Expression Assay (Applied Biosystems, Life Technologies, Foster City, USA) to detect the constitutive expression of the ABL gene.

Protocol 2

White blood cells were isolated from fresh whole blood (collected in EDTA tubes) and used either fresh or frozen in FCS with 10% of DMSO for subsequent culture in a complete medium consisting of RPMI 1640 supplemented with L-glutamine (Gibco) and 12.5% FCS, 1x L-glutamine, 0.8 mM sodiumpyruvate (Gibco), 17 mM Hepes buffer (Gibco), 4.2x10-2 mM 2-mercaptoethanol (Gibco), 42 units/ml penicillin–streptomycin and 0.21 g/ml amphotericin B solution (Sigma). Lymphocyte growth was stimulated with 50 µl/ml PHA (Gibco) and 10 units/ml of IL-2 (Roche). Cells were harvested at day 7. Total RNA was subsequently isolated using TRIzol (Invitrogen) or TRIpure (Roche) reagent, quantified with a NanoDrop spectophotometer, and first-strand cDNA was obtained from 500 ng of RNA with reverse transcriptase II (Invitrogen) using a combination of random hexamers and oligo-dT primers (Invitrogen) or random hexamers alone, according to the manufacturers’ instructions.

**LCLs.**

Lymphoblastoid cell-lines (LCLs) from healthy control individuals were generated by the Kathleen Cuningham Consortium for Research into Familial Breast Cancer (kConFab). The cells were routinely maintained at 37°C in 5% CO2, in RPMI 1640 (PAA Laboratories GmbH, Pasching, Austria) containing 10% heat-inactivated foetal bovine serum (PAA Laboratories GmbH, Pasching, Austria) and 1% penicillin/streptomycin (PAA Laboratories GmbH, Pasching, Austria). The cells were cultured for one month replacing media as necessary. RNA was extracted from cyclohexamide-untreated LCLs, using TRIzol reagent (Invitrogen, Paisley, United Kingdom) according to the manufacturer’s instructions. Each RNA sample was further processed on columns from RNeasy Mini Kit and RNase-Free DNase set to cleanup RNA and to remove any DNA traces, according to the protocols provided by the manufacturer (Qiagen Sciences, Maryland, USA). After quantification with a NanoDrop spectrophotometer (NanoDrop Technologies, Inc. Wilmington, DE, USA), the integrity of RNA purified was checked in a 1.5% agarose gel stained with ethidium bromide. 300-700ng of total RNA was reverse transcribed with Prime-Script RT kit (TaKaRa Biotechnology, Japan) following manufacturer’s protocol (the kit includes a mixture of random and Oligo(dT) primers)

**BREAST**

A normal breast tissue sample from a healthy woman was obtained after cosmetic breast reduction surgery (reduction mammaplasty). RNA was extracted from an epithelial enriched area (selected by a pathologist) by using TRIzol reagent (Invitrogen, Paisley, United Kingdom) according to the manufacturer’s protocol. Isolated RNA was further processed on columns from RNeasy Kit and RNase-Free DNase set to cleanup RNA and remove any DNA traces, according to the protocols provided by the manufacturer (Qiagen Sciences, Maryland, USA). 800ng of total RNA was reverse transcribed with Prime-Script RT kit (TaKaRa Biotechnology, Japan) following manufacturer’s protocol (the kit includes a mixture of random and Oligo(dT) primers)

**BRCA1-IRIS detection assay.**

We performed RT-PCR assays using 3 PCR primers in a single tube as follows: one forward primer at the end of BRCA1 exon 11 (5’-GACTGCAAATACAAACACCCA-3’), and two reverse primers located at intron 11 IRIS specific region (3’- GTGAAGCAGCATCTGGGTG-5’), and exon 12 (3’-CTGGTGGACTTACTTCTGGTTTC-5’). The exon 12 primers allow amplifying the full-length reference transcript as well as gDNA contamination (if any). Lack of genomic contaminations was considered as a proof that the intron 11 primer was detecting BRCA1-IRIS expression, with expected PCR products of 198bp (full-length), 421bp (IRIS isoform), and 600bp (genomic contamination). We performed 30-cycle PCRs with extended elongation times (1 minute) to favor amplification of genomic contamination (if any).

**Primers used in Stage 2(and 3) exon scanning.**

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
|  | **Forward primers** | | **Reverse primers (FAM-labelled)** | |
| **Exon** | **c.DNA** | **Sequence** | **c.DNA** | **Sequence** |
| (1B) | - | TCCGTGGCAACAGTAAAGC | - | - |
| 1A | c.-92 | GACAGGCTGTGGGGTTTCT | - | - |
| 3 | c.92 | TCAAGGAACCTCTCTCCACA | c.113 | TTTGTGGAGACAGGTTCCTTGA |
| 6 | - | - | c.299 | TCCAAACCTGTGTCAAGCTG |
| 7 | c.372 | CATCCAAAGTATGGGCTACAG | - | - |
| 8 | c.459 | TGTCCAACTCTCTAACCTTG | c.518 | GGTTGTATCCGCTGCTTTGT |
| 10 | c.635 | CCAGGGATGAAATCAGTTTGGA |  | - |
| 11q | - | - | c.819 | TGGCTCCACATGCAAGTTTG |
| 12 | c.4130 | GCGTCTCTGAAGACTGCTCA | c.4164 | CTGAGAGGATAGCCCTGA |
| 13 | c.4262 | ATGGGAGCCAGCCTTCTAAC | c.4295 | ATGGAAGGGTAGCTGTTAGAAGG |
| 14 | c.4438 | TCTGCAGATAGTTCTACCAG | c.4417 | AAAGGCCTTCTGGATTCTGG |
| 16 | c.4943 | AAAGAATGTCCATGGTGGTG | c.4898 | CTCACACTTTCTTCCATTGC |
| 20 | c.5230 | AGAAACCACCAAGGTCCAAAG |  | - |
| 22 | - | - | c.5360 | CACAGCTGTACCATCCATTC |
| 24 | - | - | c.5498 | ACCACAGGTGCCTCACACAT |