

## Quantification of BRCA1 Expression Level Using Standard RT-PCR Reagents – a Sensitive Method for the Detection of Low Amounts of Transcripts

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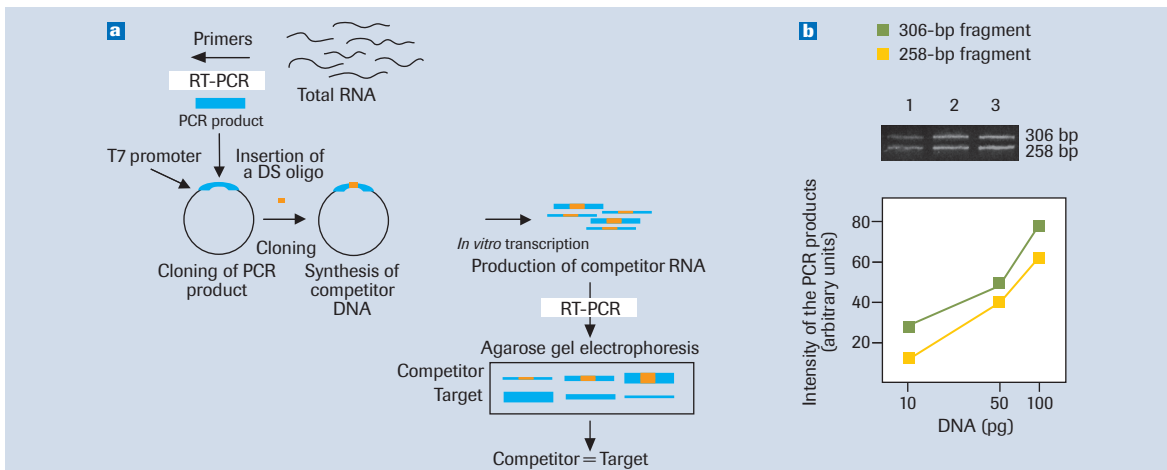
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**Mutations in the breast cancer susceptibility gene, BRCA1, occur in the majority of families with multiple members affected with breast or ovarian cancer. Women who inherit a BRCA1 mutation have 40%–80% risk of developing breast cancer or ovarian cancer. However, up to 90% of breast cancers are sporadic, and mutations in BRCA1 seem to be a rare event in sporadic breast tumors.**

### Introduction

BRCA1 is expressed at a low level in a wide range of normal and pathological human tissues, suggesting a generic activity in cell homeostasis. The BRCA1 tumor suppressor gene encodes a 220-kDa nuclear phosphoprotein which participates in the DNA damage response and acts to maintain the integrity of the genome. Additionally, BRCA1 has been found to broadly regulate gene transcription. The broad



**Figure 1: Design and validation of a competitive RT-PCR assay. (a)** A cDNA encompassing exon 4 to 6 of the human BRCA1 gene was modified by integration of a double strand oligonucleotide. After cloning, the chimerical fragment was transcribed *in vitro* and the resulting RNA was used for a quantitative RT-PCR assay. **(b)** The efficiency of amplification of the synthetic RNA is similar to the rate of amplification of the endogenous wild-type BRCA1 cDNA fragment in a range of concentrations (10 pg to 100 pg of cDNA).

caretaker function of BRCA1 does not explain why mutations lead to cancer only in certain organs, suggesting the involvement of tissue-specific coregulators. Therefore, we hypothesized that dysregulation of BRCA1 gene expression in human samples, especially sporadic breast cancer, might also account for the pathogenesis. In order to determine the putative variations of BRCA1 expression levels, we set up a quantitative RT-PCR based on a competition of amplification between a chimerical BRCA1 synthetic transcript and the endogenous messenger RNA.

## Materials and Methods

### Production of competitor RNA

A BRCA1 cDNA fragment (position 390–647, exons 4–6) was amplified by RT-PCR using C3 primers: 5'-TGTGCTTTTCAGCTTGACACAGG-3' and 5'-CGTCTTTTGAGGTTGTATCCGCTG-3' [1]. After cloning into a pGEM-T vector (Promega), the respective insert was digested with *Xba*I at position 112 and a blunt-end oligonucleotide was ligated at this site. The vector contains a promoter site for the T7 RNA polymerase, and the corresponding chimerical RNA was produced according to the instructions of the manufacturer. After purification, this competitor RNA was quantitated by spectrophotometry, and aliquots were diluted in the presence of yeast tRNA as a carrier (Figure 1a).

### Extraction of RNA and RT-PCR

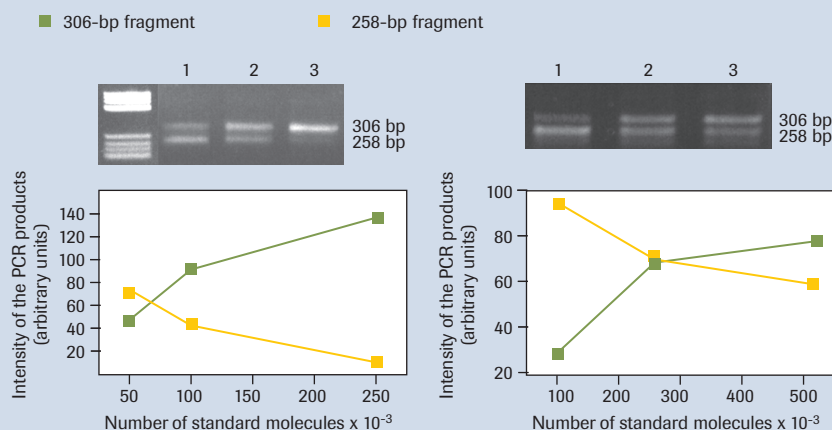
RNA extraction was performed on cultured cells or human biopsies snap frozen in liquid nitrogen using a standard procedure [2, 3]. Serial dilutions of total RNA were quantitated by spectrophotometry and electrophoresis on agarose gels in comparison with known amounts of standard RNA. Reverse transcription followed by polymerase chain reaction (RT-PCR) was performed with 0.3  $\mu$ g or 0.6  $\mu$ g of total RNA in 100  $\mu$ l

containing standard Taq polymerase buffer 3.0 mM MgCl<sub>2</sub>, 200  $\mu$ M each of the 4 deoxyribonucleoside triphosphates and C3 primers. After initial denaturation at 92°C for 2 minutes, 6 units of M-Mulv reverse transcriptase were added to the reaction mixture and incubated for 35 minutes at 42°C. Reverse transcriptase was then inactivated by heating (94°C, 3 minutes) and after cooling to 4°C, PCR amplification of the cDNA product was carried out by adding 0.6 units of Taq DNA polymerase after 35 cycles in a commercial thermocycler in the following conditions: 1 minute denaturation at 94°C, annealing at 55°C for 2 minutes, and extension for 3 minutes at 72°C. With these conditions, heterodimers between PCR products derived from mRNA and the competitor were not detected. Aliquots were then analyzed on 2% agarose gels containing 0.1  $\mu$ g/ml ethidium bromide. Photographs of the gels were scanned and the intensity of the two bands corresponding to PCR products was determined using image analyzer software (Wayne Rasband, NIH). The amount of PCR products corresponding to BRCA1 mRNA and to synthetic RNA was plotted against the initial number of synthetic RNA added to the test tubes (Figure 1a). The abscissa at the intersection of the curves represents an estimate of the equivalence point between the initial amount of the competitor molecules and the number of copies of BRCA1 mRNA in 0.3  $\mu$ g of sample RNA.

## Results and Discussion

### Validation of the competitive RT-PCR assay

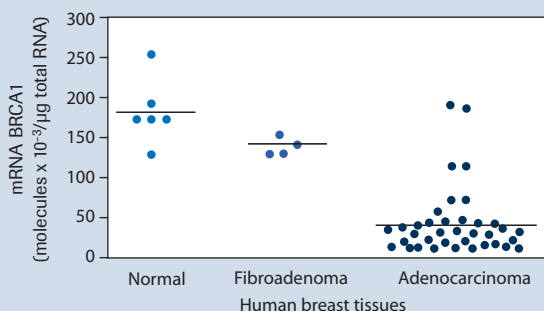
In order to evaluate the sensitivity of the RT-PCR, serial dilutions of the chimerical competitor and wild-type BRCA1 cDNA were amplified (Figure 1b). The amount of PCR product was then determined by densitometry on ethidium-bromide-stained agarose gels and plotted against the initial concentration of material. Data obtained show that the



**Figure 2: Quantification of BRCA1 expression using competitive RT-PCR.** Intensity of the bands corresponding to the PCR products plotted against the initial amount of competitor RNA. Fifteen microliters of the reaction (total volume 100  $\mu$ l) performed from serial dilutions of chimerical BRCA1 RNA and fixed amount of total RNA were analyzed on agarose gels; the 306-bp band corresponds to the expected size of the chimerical PCR product, and the 258-bp band to wild-type BRCA1 cDNA. **(a)** Amplification in low-expressing samples. **(b)** Quantification in samples expressing BRCA1 at a high level.

### Figure 3: Analysis of BRCA1 gene expression.

Quantification was performed as described in 6 normal breast samples, 4 fibroadenomas and 37 breast cancers. For each group, the horizontal line corresponds to the median value of expression level.



signal was proportional to the amount of cDNA added to the reaction mixture, indicating that endogenous BRCA1 mRNA and synthetic mRNA are amplified at the same rate (Figure 1b). The signal is proportional to the log of the amount of synthetic RNA within a wide range of concentrations, from  $3 \times 10^3$  to  $10^6$  copies per assay (data not shown). Furthermore, the quantification of BRCA1 mRNA/ $\mu$ g of total RNA is not modified by the amount of total RNA analyzed [4]. No PCR by-products or primer dimers were seen in the assays, even with a very low amount of target.

Following these preliminary validations, the amount of BRCA1 mRNA was determined in several human cell lines from 0.3  $\mu$ g of total RNA and from 0.6  $\mu$ g of total RNA extracted from human biopsies. The assays were performed with serial dilutions of the competitor and a constant amount of sample RNA. The quantities of PCR products were plotted against the initial number of synthetic RNA added. The abscissa at the intersection of the curves represents an estimate of the equivalence point between the initial amount of the competitor molecules and the number of copies of BRCA1 mRNA per cell, considering that each cell contains 25 pg

of total RNA. This method allowed us to measure the level of BRCA1 transcript in a wide range of values and could even be applied to samples containing only a few copies of BRCA1 mRNA with the same sensitivity (Figure 2). The values obtained using this technique were consistent with the protein levels analyzed by western blots [4].

The steady-state level of BRCA1 mRNA was investigated in normal and pathological breast tissues using the competitive RT-PCR method (Figure 3). Data obtained are summarized in Figure 3, and indicate that the BRCA1 mRNA level is significantly decreased ( $p = 0.0003$ ) in malignant specimens ( $37.054 \pm 4.24 \times 10^3$  copies/ $\mu$ g of total RNA) compared with normal breast tissues (and  $179.2 \pm 40.8 \times 10^3$  copies/ $\mu$ g of total RNA). In the fibroadenoma samples, the expression level of BRCA1 was similar to the level measured in normal breast samples ( $134.7 \pm 10.44 \times 10^3$  copies/ $\mu$ g of total RNA). In conclusion, the majority of the breast cancer samples analyzed exhibit a 10- to 12-fold decrease in BRCA1 mRNA level compared with normal breast tissues or benign lesions. However, this low level of BRCA1 mRNA does not seem to be associated with a specific histological type of cancer. Therefore, these results suggest that the down regulation of BRCA1 is a feature of sporadic breast cancer.

## Conclusions

Although real-time quantitative RT-PCR is a very powerful tool for studying the expression of genes, the technology is not always accessible. The technique described in this report can be set up easily in any laboratory possessing standard molecular biology equipment such as a thermocycler, agarose gel electrophoresis equipment, and a desktop image scanner. Furthermore, this technique can be applied for small amounts of material with high reproducibility. ■

## References

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Product	Pack Size	Cat. No.
<b>T7 RNA Polymerase</b>	1,000 U	10 881 767 001
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<b>Taq DNA Polymerase, 1 U/<math>\mu</math>l</b>	250 U	11 647 679 001
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<b>Deoxynucleoside Triphosphate Set, PCR Grade, Na-Salt</b>	4 x 250 U	11 969 064 001
<b>Taq DNA Polymerase (1 U/<math>\mu</math>l), dNTPack</b>	250 U	04 738 225 001
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