Inter-laboratory quality control for hormone-dependent gene expression in human breast tumors using real-time reverse transcription-polymerase chain reaction

*P de Cremoux*¹, *I Bieche*^{2,3}, *C Tran-Perennou*¹, *S Vignaud*⁴, *E Boudou*¹,
*B Asselain*⁷, *R Lidereau*², *H Magdelénat*¹, *V Becette*⁵, *B Sigal-Zafrani*⁶
and *F Spyrtatos*⁴

¹Unité de Pharmacologie, Institut Curie, 26 rue d'Ulm, 75005 Paris, France

²Laboratoire INSERM E0017, Centre René Huguenin, Saint Cloud, France

³Laboratoire de Génétique Moléculaire, UPRES EA 3618, Faculté des Sciences Pharmaceutiques et Biologiques de Paris, Paris, France

⁴Laboratoire d'Oncobiologie, Centre René Huguenin, Saint Cloud, France

⁵Département d'Anatomo-Pathologie, Centre René Huguenin, Saint Cloud, France

⁶Service d'Anatomo-Pathologie, Institut Curie, Paris, France

⁷Service de Biostatistiques, Institut Curie, Paris, France

(Requests for offprints should be addressed to P de Cremoux; Email: patricia.de-cremoux@curie.net)

Abstract

Quantitative reverse transcription-polymerase chain reaction (RT-PCR) used to detect minor changes in specific mRNA concentrations may be associated with poor reproducibility. Stringent quality control is therefore essential at each step of the protocol, including the PCR procedure.

We performed inter-laboratory quality control of quantitative PCR between two independent laboratories, using in-house RT-PCR assays on a series of hormone-related target genes in a retrospective consecutive series of 79 breast tumors. Total RNA was reverse transcribed in a single center. Calibration curves were performed for five target genes (estrogen receptor (ER) α , ER β , progesterone receptor (PR), CYP19 (aromatase) and Ki 67) and for two reference genes (human acidic ribosomal phosphoprotein PO (RPLPO) and TATA box-binding protein (TBP)). Amplification efficiencies of the calibrator were determined for each run and used to calculate mRNA expression. Correlation coefficients were evaluated for each target and each reference gene.

A good correlation was observed for all target and reference genes in both centers using their own protocols and kits ($P < 0.0001$). The correlation coefficients ranged from 0.90 to 0.98 for the various target genes in the two centers. A good correlation was observed between the level of expression of the ER α and the PR transcripts ($P < 0.001$). A weak inverse correlation was observed in both centers between ER α and ER β levels, but only when TBP was the reference gene. No other correlation was observed with other parameters.

Real-time PCR assays allow convenient quantification of target mRNA transcripts and quantification of target-derived nucleic acids in clinical specimens. This study addresses the importance of inter-laboratory quality controls for the use of a panel of real-time PCR assays devoted to clinical samples and protocols and to ensure their appropriate accuracy. This can also facilitate exchanges and multicenter comparison of data.

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Introduction

Reverse transcription-polymerase chain reaction (RT-PCR) methods have become widely used over recent years in the context of basic research and are now increasingly used in the field of medical diagnostics. However, the use of quantitative PCR in clinical fields (clinical microbiology, virology or oncology) requires accurate controls in order to be routinely used in clinical samples, particularly for low level mRNA targets. Over the last few years, the absence of standardized kits for commercially available attractive targets has led to the development of numerous in-house PCR assays. A review of the literature shows that the reported performances, both for the sensitivity and specificity of these assays, are often divergent.

An analysis of PCR techniques shows that false-positive results due to carry-over and false-negative results due to PCR inhibition are not systematically controlled. These shortcomings of 'classical' PCR should be resolved with the development of standardized real-time PCR assays. Inter-laboratory comparisons should then be possible and regular quality controls will be necessary to ensure the reliability of real-time PCR assays.

Inter-laboratory quality assurance programs for diagnostic tests based on nucleic acid amplification are not widely implemented in clinical laboratories and remain limited to a few tests, mainly in hematology, microbiology and virology. The development of specific inter-laboratory quality assurance trials based on methodologic proficiency testing and directed to the evaluation of any diagnostic molecular target may be useful, particularly in breast cancer patients treated by hormone therapy. Endocrine manipulations are the most effective and least toxic form of systemic therapy currently available for the management of hormone-dependent breast cancer. The panel of available compounds has grown considerably over recent years, with the addition to the older agents, tamoxifen and high-dose progestogens, of type I and II aromatase inhibitors, pure anti-estrogens and, for premenopausal women, luteinizing hormone-releasing hormone analogs. The question of the determinants of resistance or sensitivity to treatment therefore needs to be considered. The estrogen receptor (ER) assay was first proposed by Jensen et al. (1971) to determine which patients are likely to respond to endocrine therapy. It was subsequently established that patients with ER-rich tumors respond to endocrine therapy, whereas patients with ER-negative tumors are unlikely to respond (McGuire *et al.* 1975). Although response rates of up to 70% are achieved with endocrine therapy in patients with tumors expressing ER and progesterone receptor (PR), most responsive tumors eventually acquire resistance. The mechanisms responsible for the resistance of tumors

expressing ER and/or PR may be multifactorial. Molecular biotechnology offers a great potential for developing multiparametric analysis of mRNA expression of clinically relevant biological parameters in estrogen-dependent breast tumors.

We first evaluated endogenous controls for gene expression studies in breast cancer (F Spyrtatos, unpublished observations). The present study was designed to evaluate the accuracy of quantitative analyses of endocrine-related gene expression in breast cancer using real-time RT-PCR so that they can be used in prospective multicenter clinical trials. Quantitative RT-PCR assays may be particularly useful for quantitative determination of weakly expressed targets such as CYP19 (aromatase) and ER β mRNA for which no consensus immunohistochemistry assay is available.

This quality control was performed between two independent laboratories (Centre René Huguenin (CRH) and Institut Curie (IC)) on a series of 79 breast cancer samples. The endocrine-related target genes were in-house assays designed by each laboratory (ER α , ER β , PR and CYP19). Similar Ki 67 and references gene (human acidic ribosomal phosphoprotein PO (RPLPO) and TATA box-binding protein (TBP)) designs were used in both centers.

Materials and methods

Human breast tumor samples

A panel of 79 untreated residual breast tissue samples from informed patients undergoing primary surgery for breast cancer were retrospectively analyzed: 39 were provided by the CRH and 40 were provided by the IC. Tumor sampling was performed extemporaneously by a pathologist. Samples were frozen in liquid nitrogen within 20 min after resection using the same procedure in each center. The residual fragment was stored in liquid nitrogen in RNase-free conditions until use. We retrospectively analyzed this residual tissue for ER α , ER β , PR, CYP19 and Ki 67 mRNA expression. Adjacent fragments of frozen material were fixed in alcohol-formalin-acetic acid (AFA) and stained with hematoxylin and eosin in order to determine the proportion of tumor cells in each sample. Clinical staging and histological diagnosis were routinely determined in each case.

Calibration curves

Calibration curves were determined for each target gene using a unique mix of relevant total RNA including: a pool of total RNA from breast tumors known to have high levels of ER α transcripts and proteins, a pool of total RNA from breast tumors known to have high levels of PR transcripts and proteins (Bieche *et al.* 2001, de Cremoux *et al.* 2002),

an ovarian total RNA, known to express high levels of ER β transcripts (Moore *et al.* 1998, Pujol *et al.* 1998) (human ovary total RNA reference 735260 (Stratagene, Amsterdam, The Netherlands)) and a placental total RNA (Moore *et al.* 1998) (human placenta total RNA reference 64024-1 (Clontech, USA)) known to express high levels of CYP19 gene transcripts. Six-fold diluted cDNA (1:25 to 1:800 for ER β , PR, Ki 67, TBP and RPLPO; 1:25 to 1:3200 for ER α and CYP19) were obtained after reverse transcription of the mix of these relevant total RNA and were amplified to obtain a standard curve and to assess PCR efficiency. The highest level of this calibration curve was used as calibrator in each experiment for each target gene.

RNA extraction

Total RNA was extracted from crushed tumor samples by the RNA plus kit (Bioprobe, Saint Quentin, France) at IC and by RNABle (Eurobio, Les Ulis, France) at CRH, according to the manufacturer's instructions. The RNA samples were stored in RNase-free distilled water, at -80°C . The quality of RNA samples was determined by electrophoresis on 2% agarose gel with ethidium bromide staining. The 18S and 28S RNA bands were visualized under u.v. light. All samples presented a high quality RNA profile. The quantity of RNA was measured by spectrophotometry at 260 nm.

cDNA synthesis

RNA was reverse transcribed in one center (CRH) to avoid possible differences related to variable efficiency of reverse transcriptases. Total RNA was reverse transcribed in a final volume of 20 μl containing $1\times$ reverse transcriptase buffer (500 mM of each deoxynucleotide triphosphate, 3 mM MgCl_2 , 75 mM KCl and 50 mM Tris-HCl (pH 8.3)), 10 units RNase inhibitor (Promega, Madison, WI, USA), 10 mM dithiothreitol, 50 units Superscript II RNase H $^{-}$ reverse transcriptase (Invitrogen), 1.5 mM random hexamers (Pharmacia, Uppsala, Sweden) and 1 μg total RNA (patient samples and calibrator). Samples were incubated at 20°C for 10 min and 42°C for 30 min, reverse transcriptase was then inactivated by heating at 99°C for 5 min and cooling for 5 min.

Real-time PCR amplification

ER α , ER β , PR, CYP19 and Ki 67 transcripts were quantified using real-time quantitative RT-PCR assays. Primers and probes were selected by Oligo 4.0 (National Biosciences, Plymouth, MN, USA) (CRH) or Primer Express (Applied Biosystems, Foster City, CA, USA) (IC) software. The nucleotide sequences were then blasted

against dbEST, htgs and nr (the non-redundant set of the GenBank, European Molecular Biology Laboratory (EMBL) and DNA Data Bank of Japan (DDBJ) database sequences) to confirm the total gene specificity of the nucleotide sequences chosen as primers and probes. To avoid amplification of contaminating genomic DNA, one of the two primers was placed in a different exon.

Each laboratory used its own design for ER α , ER β , PR and CYP19 (Bieche *et al.* 2001, de Cremoux *et al.* 2002) gene transcript analysis. Common primers and probes designed by CRH were used for Ki 67, TBP and RPLPO (Bieche *et al.* 2000, Spyrtatos *et al.* 2002). The nucleotide and probe sequences are shown in Table 1.

PCR reactions were performed using an ABI Prism 7700 Sequence Detection System (Applied Biosystems) at IC. Real-time detection was performed under the usual conditions for each center: at CRH, they were performed using the SYBR Green Core Reagent kit or the Taqman PCR Core Reagent kit (Applied Biosystems) and at IC they were performed using qPCR Core Reagent Kit (Eurogentec, Senaing, Belgium). Fluorescent probes were synthesized by Applied Biosystems, and primers were synthesized by Invitrogen (Paisley, Strathclyde, UK). The thermal cycling conditions comprised an initial denaturation step at 95°C for 10 min, then 40 cycles at 95°C for 15 s and 1 min at an annealing temperature depending upon the target, as shown in Table 1. Transcripts of TBP (Spyrtatos *et al.* 2002) and RPLPO (Bieche *et al.* 2000, Spyrtatos *et al.* 2002) were quantified as endogenous RNA of reference genes to normalize the expression of the genes of interest. They were chosen according to a quality control study assessing the most adapted reference genes in breast tumor assays (F Spyrtatos, unpublished observations).

All measurements were performed in duplicate under blind conditions. Two non-template controls were included in each amplification run.

Results are expressed as n -fold differences in target gene expression relative to reference gene (either TBP and RPLPO) and the calibrator was defined as the 'N target'. It was determined as follows: $N_{\text{target}} = 1 + \frac{[E^{\text{(C}_{\text{calibrator}} - \text{C}_{\text{sample}})}]_{\text{target}}}{[E^{\text{(C}_{\text{calibrator}} - \text{C}_{\text{sample}})}]_{\text{reference gene}}}$, where E is the efficiency of PCR measured using the slope of the calibration curve and Ct is the cycle threshold.

Statistical analysis

Data are expressed as ratios between the quantity of target transcripts and the quantity of the reference genes TBP and RPLPO, in arbitrary units. Non-parametric tests were used. Spearman's rank correlation was used to evaluate the relationship between relative mRNA expression of target genes at IC and CRH, relative to both reference genes.

Table 1 Primers and probe sequences for RT-PCR amplification

Gene	Annealing °C	Product size (nt)	Primers	Probe
ER α *	57	155	agcaccagtggaagctact tgaggcacacaaactcct	tggtacatcatctctcggttccga
ER β *	65	143	aagaatatctgtgtcaaggccatg ggcaatcacccaaaccaaag	ttgctgaacgccgtgaccgatg
PR*	60	122	gaaccagatgtgatctatgcagga cgaaaacctggcaatgatttagac	acctgacacctccagttctttgctgacaag
CYP19*	65	73	tggaatacaactcgaccttct cacagactgtgaccatacgaacaa	tgaaagctctgtcaggccccgg
ER α †	65	108	ccaccaaccagtgcaccatt ggcttttctgtatccaccttc	
ER β †	65	143	agatgccctgggtgaagcaag gacagcgcagaagtgcacac	
PR†	65	121	cgcgctctaccctgcactc tgaatccggcctcaggtagtt	
CYP19†	65	93	tgtggacgtgtgacccttct accacgatagcatttctgcca	atgctggacaccttaacacgctctcttga
Ki 67†	65	105	attgaacctcggaagagctga ggagcgcaggatattccctta	acgacatgaaaaccaacaaaga
TBP†	65	89	cacgaaccacggcactgatt tttctgtgcccagctcggac	tgtgcacaggagccaagagtgaaga
RPLPO†	65	149	ggcgacctggaagtccaact ccatcagcaccacagccttc	atctgctgcatctgcttgagccca

Primers and probes are designed by *IC and †CRH.

Results

The clinical and pathological characteristics of the tumors are shown in Table 2. The ER α and PR protein content were initially measured by enzyme immunoassay or immunohistochemistry (IHC). The distribution of ER/PR content was as follows: 43 (54%) samples were ER positive and PR positive, 14 (18%) samples were ER negative and PR negative, 17 (22%) samples were ER positive and PR negative and five (6%) samples were ER negative and PR positive.

PCR optimization

PCR optimization had been performed previously in each laboratory using similar rules as described (Bieche *et al.* 2001, Spyrtos *et al.* 2002, de Cremoux *et al.* 2003).

The specificity of all PCR products was also initially determined in both centers by the presence of a single band of the expected size after migration on agarose gels.

The linearity for each target gene and reference gene was observed for more than 5 log dilutions, including the observed samples values.

A calibration curve was generated by analyzing serial dilutions of the pool of RNA, as described in Materials and methods for each target and reference gene, to assess PCR efficiency. The PCR efficiency for ER α , ER β , PR, CYP19, Ki 67, RPLPO and TBP was above 90% for all

genes. The intra-assay coefficient of variation (CV) of Ct values was <2% and the interassay CV was <5% as calculated by the standard error.

Correlation of target gene mRNA transcript levels between the two centers for each reference gene

All results are expressed as continuous variables. For each parameter, and for both reference genes, the mean and standard deviation are presented in Table 3 for TBP and for RPLPO as reference genes. The levels of expression were comparable for all target genes in both centers. As shown in Tables 4 and 5, a statistically significant correlation ($P < 0.0001$) was demonstrated between the level of gene expression between the two centers for all target genes, considering either TBP or RPLPO as the reference gene.

Correlation between target gene mRNA transcript levels

We observed a positive correlation between ER α and PR transcript levels in both centers and with both reference genes, with correlation coefficients of 0.49 and 0.47 at IC, and 0.41 and 0.42 at CRH, using TBP and RPLPO ($P < 0.001$) respectively. No correlation was observed in this limited series between ER α and Ki 67 and aromatase transcripts. A weak inverse correlation was observed

Table 2 Clinical and pathological characteristics of the samples

Characteristics	Number (%)
Patients and clinical tumor characteristics	
Age	
<50 years	16 (20)
≥50 years	63 (80)
Tumour size	
T1	17 (21)
T2	48 (61)
T3/T4	14 (18)
Nodal status	
N0	55 (70)
N1	22 (28)
N2	1
Unknown	1
Histological characteristics	
Macroscopic tumor size	
<30 mm	48 (61)
≥30 mm	31 (39)
Pathological nodal status	
pN ⁻	36 (46)
pN ⁺	41 (52)
No axillary dissection	2
Histological type	
Infiltrating ductal	64 (81)
Infiltrating lobular	9 (11)
Others	6 (8)
Grade (SBR)*	
I	13 (16)
II	43 (54)
III	20 (25)
Unknown	3 (4)
Hormone receptor status	
ER Positive	60 (76)
ER negative	19 (24)
PR positive	48 (61)
PR negative	31 (39)

*Scarfi, Bloom and Richardson

between ER α and ER β only with TBP as reference gene at IC ($r = -0.33$) and CRH ($r = -0.40$) ($P < 0.003$ and $P < 0.0004$ respectively).

Interpretation of discrepancies

The expression of all target genes was concordant for all samples but one: for example, samples expressing high levels of ER α and PR transcripts in one center expressed high levels of ER α and PR mRNA transcripts in the other center, except in one case where a 5Ct difference was observed between the ER α measurement performed at CRH and IC. This sample clearly expressed low levels of ER α mRNA at IC and high levels of ER α mRNA at CRH, using both TBP and RPLPO as reference gene. Sequencing of the two ER α amplicons demonstrated that this sample presented a rare polymorphism in 1559 nt (gtg > ggg) in position 10 of the IC upper primer.

In conclusion, no other major discrepancy was observed in any of the target or reference genes.

Discussion

This study was designed to develop the first inter-laboratory quality control to assess the accuracy and conditions of real-time RT-PCR assays for use in prospective clinical protocols in primary breast cancer.

For mRNA detection, we used a quantitative RT-PCR method based on real-time analysis of PCR amplification. This assay is sensitive and allows accurate quantification of mRNA expression, as previously demonstrated for ER α and PR transcripts by both laboratories (CRH and IC) (Bieche *et al.* 2001, de Cremoux *et al.* 2002, 2003, Spyrtos *et al.* 2002). The real-time PCR method has several advantages over classical RT-PCR-based methods. It does not require post-PCR sample handling, thereby avoiding problems related to carry-over. It also has a wide dynamic range with very low intra-assay and interassay CV values (<5%).

Real-time RT-PCR specific errors in the quantification of mRNA transcripts are usually correlated to possible variations in starting material which may be a major problem for patient samples for which sampling is uncertain (Freeman *et al.* 1999). To compensate for

Table 3 Expression level of target gene transcripts in both centers, using TBP and RPLPO as reference genes. Genes transcripts are expressed as means $\pm$ S.D.

Target gene	TBP		RPLPO	
	IC	CRH	IC	CRH
ER α	572 $\pm$ 533	482 $\pm$ 454	565 $\pm$ 618	606 $\pm$ 997
ER β	39 $\pm$ 46	47 $\pm$ 55	34 $\pm$ 37	53 $\pm$ 58
PR	358 $\pm$ 419	511 $\pm$ 709	317 $\pm$ 400	581 $\pm$ 915
CYP19*	5361 $\pm$ 5568	7678 $\pm$ 7563	5346 $\pm$ 6801	9894 $\pm$ 11 003
Ki 67	2127 $\pm$ 1877	1453 $\pm$ 1359	2167 $\pm$ 3332	1658 $\pm$ 2037

*, $\times 1000$.

Table 4 Correlation between target gene transcript levels between IC and CRH, using TBP as reference gene

Target gene	Correlation*	
	<i>r</i>	<i>P</i>
ER α	0.93	<0.0001
ER β	0.92	<0.0001
PR	0.98	<0.0001
CYP19	0.90	<0.0001
Ki 67	0.97	<0.0001

*, evaluated by Spearman's rank test.

sample-to-sample variations of mRNA content, each target gene was normalized with endogenous reference genes (RPLPO, TBP). The RT step also largely contributes to the overall variation of quantitative RT-PCR (Bustin 2000, Ovstebo *et al.* 2003). However, in this study, we used a common cDNA sample to avoid variations due to this step. Consequently, only the PCR step was evaluated in this quality control.

To compensate for any possible inter-PCR variation between runs, the expression of each target gene was calibrated. In these assays, designed to be used in clinical trials, the standard curve for the calibrator was prepared as a mix of human breast tumor samples and two marketed ovarian and placental RNAs known to present high levels of ER β and CYP19 mRNA. The mix was used in all experiments, allowing us to control the reliability of the method over time. A calibration curve was included in each experiment and analyzed under the same conditions as for unknown samples. PCR efficiency was also calculated from the calibration curve in each experiment and for each transcript. The possible variation from one run to the next was therefore taken into account (as described in Materials and Methods). The use of a calibration curve for quantitative gene expression studies is not widely applied, but has been previously described to be a useful tool (Ovstebo *et al.* 2003).

Real-time RT-PCR analyses of tumor mRNA in both centers revealed a good correlation for each target gene and each control gene. We used different ER α , ER β , PR and CYP19 specific primer sets and probes and, as expected, we did not observe any differences due to the localization of the amplicon analyzed, except in one case for ER α , corresponding to the presence of a rare polymorphism. The real-time PCR procedure and the PCR kit used did not influence the quantification of mRNA. The amplification conditions of the PCR assay described here were previously optimized and validated by each center (Bieche *et al.* 2001, de Cremoux *et al.* 2002). This collaborative study showed a high reproducibility of the PCR assay between two independent centers when

Table 5 Correlation between target gene transcript levels between IC and CRH, using RPLPO as reference gene

Target gene	Correlation*	
	<i>r</i>	<i>P</i>
ER α	0.97	<0.0001
ER β	0.95	<0.0001
PR	0.98	<0.0001
CYP19	0.91	<0.0001
Ki 67	0.92	<0.0001

*, evaluated by Spearman's rank test.

identical cDNA were analyzed regardless of the PCR kit and design used.

Furthermore, this good correlation was not only found for genes with high mRNA expression, such as ER α , PR, Ki 67, but also for genes with low mRNA expression, such as CYP19 gene transcripts and ER β . This represents an additional relevant tool for analysis of hormone-dependent gene expression assays in clinical samples, where no other routinely adapted tool is currently available.

Multicenter validation and standardization of PCR have been previously described for the detection of food-borne bacteria transferred from animal to food products, by semiquantitative PCR, using a unique primer design for five pathogens (Hoorfar & Cook 2003, Malorny *et al.* 2003). The present study represents the first real-time PCR quality control in breast cancer.

In another field, a multicenter European prospective study of the candidate reference genes for diagnosis and residual disease detection in leukemic patients by quantitative RT-PCR was recently published (Beillard *et al.* 2003). This study initially selected three reference genes from a total of 14 (Abelson, β 2 microglobulin and β glucuronidase) and prospective and retrospective studies on more than 400 samples confirmed that these three genes had a stable mRNA expression. However, the authors of this study propose that only the Abelson gene should be used as a reference gene for quantitative RT-PCR analysis of residual disease, as its expression is not significantly different between normal and leukemic samples at diagnosis, confirming the major importance of these quality control studies before using these tools in prospective clinical trials. The results obtained in this study on snap-frozen material allowed us to use our protocol in a recent multicenter prospective clinical trial of neoadjuvant endocrine therapy whose preliminary results were presented in the last San Antonio Breast Cancer Symposium (Tubiana-Hulin *et al.* 2003).

In conclusion, we have developed the first independent quality control of mRNA expression of estrogen-

dependent target genes in breast carcinoma by quantitative RT-PCR. The good correlation between two independent centers using a different design of PCR analysis allows these assays to be used for prospective clinical trials.

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