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Harmonisation of multi-centre real-time reverse-transcribed PCR results of a candidate prognostic marker in breast cancer: An EU-FP6 supported study of members of the EORTC – PathoBiology Group

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ABSTRACT

Aim: Assessment of intra- and inter-laboratory variation in multi-centre real-time reverse-transcribed PCR (qRT-PCR)-based mRNA quantification of a prognostic marker in breast cancer using external quality assurance (EQA).
Methods: A questionnaire on the methodologies used and EQA calibrators were sent to 5 participating laboratories from 4 European countries, which measured mRNA levels of PITX2 splice variants and reference genes by qRT-PCR.
Results: Differences in the methodology included PCR quantification methodology and equipment, RNA extraction and cDNA synthesis procedures. The intra-laboratory coefficient of variation (CV) ranged from 5 to 23%, and the inter-laboratory CV ranged from 17 to 30%. The inter-laboratory CV was reduced to 13% by using prediluted calibrators and by harmonising the data in the central QA laboratory. Additional normalisation using reference genes did not decrease the variation further.
Conclusions: Both externally provided calibrators and centralised harmonisation are required to reduce the intra-laboratory variation in multi-centre qRT-PCR results to an acceptable level.

1. Introduction

The measurement of biomarkers in tumour tissue is finding increasing use in determining prognosis and therapy prediction in patients with cancer. Two of the earliest biomarkers in breast cancer were the steroid hormone receptors: estrogen receptor and progesterone receptor (ER and PgR). As the degree of benefit from endocrine therapy is directly related to

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the quantity of receptor present in the tumour[1], the quality of the steroid receptor assays is important. Moreover, since patients entering in multi-centre trials are often stratified based on the receptor status, data obtained with steroid hormone receptor assays should be comparable between different institutes. Our laboratory has performed ER and PgR quality assessment studies for almost 20 years for the EORTC Receptor and Biomarker Study Group, now the PathoBiology Group.[2,3] This has resulted in a significant increase in precision of the ER and PgR assays applied by the participating laboratories for the assessment of hormone receptor status for breast cancer patient treatment selection. Similarly, external quality assurance (EQA) has been provided for the measurement of urokinase-type plasminogen activator (uPA) and plasminogen activator inhibitor type 1 (PAI-1) in breast cancer tissue.[4] Both uPA and PAI-1 have reached Level-of-Evidence (LOE) I[5–7] as prognostic biomarkers for breast cancer, having undergone validation using both a randomised prospective trial[8] and a large pooled analysis study.[9] The EQA for both these studies was carried out in our department.[4] The department of Chemical Endocrinology is also responsible for EQA in the Node-Negative Breast Cancer (NNBC)-3-Europe trial, where participating centres perform risk estimation for breast cancer patients by measuring the uPA and PAI-1 concentration in tumour tissue biopsies.

Due to its sensitivity (requiring only a minute amount of tissue), its accuracy and its quantitative characteristics, qRT-PCR is increasingly being used to determine the prognostic value of potential biomarkers in tumour tissues. Not surprisingly, most of the recently identified predictive and prognostic biomarkers are mRNA-based. As part of the validation of prognostic and predictive biomarkers, multi-centre studies enhance the level of evidence of a potential biomarker.[6] Thus, for qRT-PCR of particular biomarkers to achieve a high level of evidence, EQA programmes are required that aid in harmonising results obtained in different centres.

We previously showed in ER-positive breast cancer that DNA methylation of the paired-like homeodomain transcription factor 2 (PITX2) gene was associated with poor outcome in lymph node (LN) negative breast cancer patients whether having received adjuvant endocrine therapy or no adjuvant systemic therapy[10,11], and in LN positive patients who received adjuvant anthracyclin-based chemotherapy (Hartmann et al., submitted for publication). PITX2 is a WNT and/or TGF-beta family driven differentiation marker that contributes importantly to the pituitary gland development[12], including terminal differentiation of the somatotroph and lactotroph lineages.[13] PITX2 is also essential for proper left-right patterning.[14] Three main isoforms PITX2A, -B and –C, which are translated from 3 different mRNA splice variants transcribed from the gene, have been described. Recent results have indicated that PITX2 mRNA expression next to PITX2 DNA methylation is a prognostic marker for breast cancer (Martens et al., in preparation).

The above-mentioned results were obtained within an EORTC-PathoBiology Group, EU funded FP6 study 'DNA Methylation'. In the current study, 3 rounds of EQA were used to quantify and if possible reduce the inter- and intra-assay variability of qRT-PCR in and between 5 laboratories from 4 European countries that participated in this FP6 study. We

Table 1 – Methods of participants.

	Participant	1	2	3	4	5
RNA extraction	*Method*					
	Trizol				X	
	RNABee	X				
	RNeasy		X	X		X
	DNase		X		X	X
RT reaction	*Enzyme*					
	M-MLV			X		X
	Superscript	X	X		X	
	Primers					
	Random hexamers	X	X	X	X	X
	Oligo-dT	X				X
	RNAse-H treatment	X				X
PCR reaction	*Apparatus*					
	AbiPrism	X	X	X	X	
	Mx3000P	X				
	LightCycler					X
	Mix					
	Stratagene	X				
	Applied Biosystems	X	X	X	X	
	Qiagen					X
	Protocol	Denaturation, 35 cycles: 15 s 95 °C 30 s 62 °C 20 s 72 °C 20 s 79 °C Melting curve	Denaturation, 40 cycles: 15 s 95 °C 60 s 60 °C Melting curve	Denaturation, 40 cycles: 15 s 95 °C 60 s 60 °C Melting curve	Denaturation, 50 cycles: 15 s 95 °C 60 s 65 °C	Denaturation, 35 cycles: 15 s 95 °C 30 s 60 °C 30 s 72 °C Melting curve

quantified by qRT-PCR the potential biomarker PITX2 and its splice variants, and three reference genes, B2M, HPRT1 and HMBS, in externally provided biological samples. The main objective of the first EQA round was to monitor adherence to established consensus protocols and consistency in assay methodologies, and to determine base-line inter- and intra-assay variability. The second EQA round was used to obtain data to investigate the beneficial effect of the use of calibrators. In the last round, the effect of normalisation of the results was studied using internal reference genes and by centrally harmonising the qRT-PCR results.

2. Material and methods

2.1. EQA materials: cell lines, RNA, cDNA

The EQA samples that were distributed to the participants consisted of vials of total RNA, isolated from different cell lines (SW480 (colon), HEPG2 (liver), JEG3 (trophoblast), JAR (trophoblast), MCF7 (breast), MDA-MB175VII (breast), OVCAR3 (ovary)) and/or vials of cDNA, prepared from the RNA mentioned above by the central QA laboratory (see below). The calibrator for the PITX2A variant was the breast cancer cell line MDA-MB175VII; for the PITX2B variant, the ovarian cancer cell line OVCAR-3; for the PITX2C variant, the breast cancer cell line MCF7; and for all variants the trophoblast cell line JEG3 was used. As calibrator for the 3 reference genes, B2M, HPRT1 and HMBS, RNA and cDNA prepared from MCF7 cell line were used. Negative controls included genomic DNA and samples without template (NTC).

2.2. RNA extraction

For the calibrators, RNA extraction from the cell lines was performed by the central QA laboratory. Total RNA was isolated using the RNeasy mini kit (Qiagen, Hilden, Germany) with on-column DNase-I treatment. Quality of the RNA was checked by examining ribosomal RNA bands after agarose gel electrophoresis. RNA concentrations were determined from the spectrophotometric absorption at 260 nm using the Genequant (Amersham, Eindhoven, The Netherlands). Similarities and dissimilarities between the RNA extraction methods used by the participants in this study are listed in Table 1.

2.3. RT reaction

cDNA that was sent as EQA sample or as calibrator was prepared by the central QA laboratory. For this, purified total RNA (1.0 μg) was denatured for 10 min at 70 °C, and immediately cooled on ice. Reverse transcription was performed using the reverse transcription system (Promega Benelux B.V., Leiden, The Netherlands) according to the manufacturers' protocol. After annealing of random hexamers for 10 min at 20 °C, cDNA synthesis was performed for 60 min at 42 °C followed by an enzyme inactivation step for 5 min at 95 °C. In the first EQA round, the participants were provided with reverse transcription kits by the QA laboratory (reverse transcription System, Promega Benelux). Details of the different RT reactions employed in a later round by the participants are listed in Table 1.

2.4. PCR

SYBR green-based real-time qRT-PCR assays were set up for 3 PITX2 variants, and one assay (PITX2-all) was developed that does not discriminate between the variants. For the PITX2-all assay, the forward primer was 5'-GAG CTG GAG GCC ACT TTC-3', and the reverse primer was 5'-CCG AAG CCA TTC TTG CAT AG-3'. The PITX2A variant was amplified using the same reverse primer, and 5'-GCG TGT GTG CAA TTA GAG AAA G-3' as forward primer. For PITX2B, the specific forward primer was 5'-GCC GTT GAA TGT CTC TTC TC-3'; and for PITX2C, 5'-CTC ATC TTC CTG TCA CCA TC-3'. For both PITX2B and PITX2C, 5'-CCT TTG CCG CTT CTT CTT AG-3' was used as reverse primer. In addition, the EQA programme included 3 reference genes: hypoxanthine ribosyltransferase (HPRT1), ß-2-microglobulin (B2M) and hydroxymethylbilane synthase (HMBS, formerly porphobilinogen deaminase, PBGD).[15] Forward and reverse primers for HPRT1 were 5'-TAT TGT AAT GAC CAG TCA ACA G-3' and 5'-GGT CCT TTT CAC CAG CAAG-3', respectively; for B2M, 5'-CTT TGT CAC AGC CCA AGA TAG-3' and 5'-CAA TCC AAA TGC GGC ATC TTC-3' and for HMBS, 5'-CAT GTC TGG TAA CGG CAA TG-3' and 5'-GTA CGA GGC TTT CAA TGT TG-3'. Details of the different PCR machines, temperature-, duration- and threshold-settings used by the participants are listed in Table 1. Several manufacturers produce similar SYBR Green I mixes that can be used for real-time PCR. However, in a preliminary experiment differences between the Applied Biosystems SYBR Green QPCR master mix and the Stratagene Brilliant SYBR Green QPCR master mix were detected (data not shown). Depending on the gene of interest, either mix could yield lower Ct values. Therefore, the optimal SYBR Green PCR master mix was provided to all participants in order to assure uniformity of results.

2.5. Quantification, normalisation and statistics

Detailed protocols necessary to be able to complete this EQA round were provided. These consisted of protocols describing how the materials and reagents should be used, and how the data should be collected and interpreted. The central QA laboratory sent prediluted calibrators to the participants for quantification of the unknown samples. The amount allocated to these calibrators was in arbitrary units. The unknown samples were quantified against the calibrators for all the three reference genes and for the PITX2 assays. All samples were measured in triplicate so that inter-laboratory CVs could be quantified. All raw data were sent to the central QA laboratory. For all EQA rounds, the partners were designated a different participant number, and reports on the results were returned to the partners together with the disclosure of the particular partner number for that round. Harmonisation was performed by correcting the data from a participant using the ratio of the average of all the data from a participant over the average of all the data from all the participants.

3. Results

3.1. First EQA round

3.1.1. Inventory of consensus protocols, procedures and equipment of participants

To determine sources of variation among participants a questionnaire on the procedures, equipment and protocols used by the participants was sent out. These participants were from Rotterdam (Department of Medical Oncology, Erasmus Medical Center Rotterdam, The Netherlands), Dublin (Department of Pathology and Laboratory Medicine, St. Vincent's University Hospital, Dublin, Ireland), St Cloud (Laboratoire d'Oncogénétique, Centre René Huguenin, St-Cloud, France), Basel (Stiftung Tumorbank Basel, Riehen, Switzerland) and Nijmegen (Department of Chemical Endocrinology, Radboud University Nijmegen Medical Centre, Nijmegen, The Netherlands).

The feedback on this questionnaire is summarised in Table 1. There were significant differences in the standard protocols between the laboratories. RNA was extracted using Trizol, RNABee or RNeasy mini columns, and RNA quality control was performed by agarose gel electrophoresis (3 of 5 participants) or Agilent Bioanalyzer (2/5). The RT reaction was done using M-MLV (2/5) or superscript II RNase H-reverse transcriptase kit from Invitrogen (3/5). All participants used random hexamers, whereas 2 laboratories also included oligo-dT primers. Two of the participants applied an RNAse-H treatment to the obtained cDNA. All participants had at least yearly PCR apparatus calibration and maintenance scheme. Four of the 5 participants used the Applied-Biosystems Abi-Prism (7000/7700) PCR apparatus, 1 participant used in addition the Mx3000P Real-Time PCR System from Stratagene, whereas 1 participant used the LightCycler from Roche.

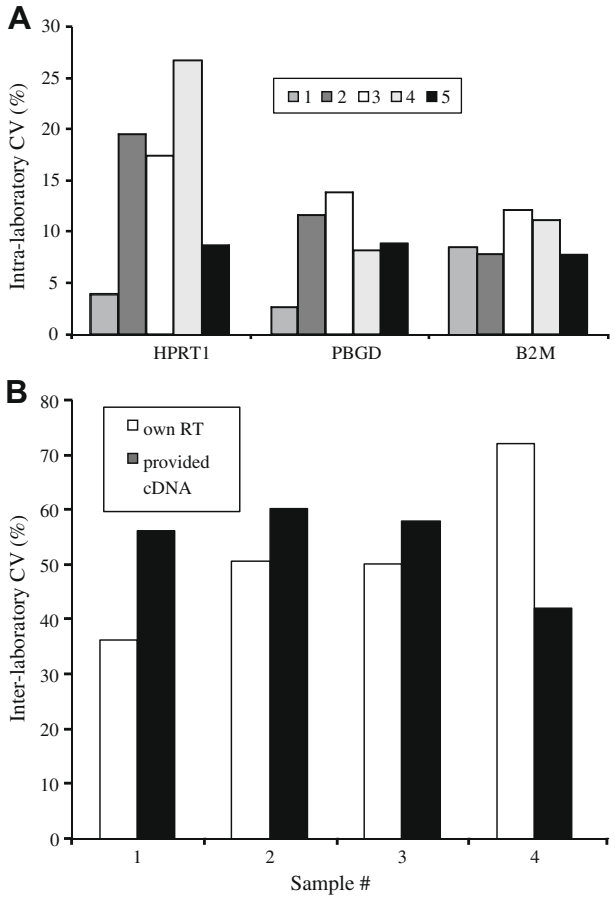

Fig. 1 – First EQA round. (A) Intra-laboratory CV for each participant per assay. (B) Inter-laboratory CV of samples that were reverse-transcribed by the participants according to their in-house RT protocol or by the central EQA laboratory.

3.1.2. Establishment of inter- and intra-laboratory variation among participants

To determine the inter- and intra-laboratory variation, EQA samples were prepared by the central QA laboratory and distributed to all partners. The samples consisted of 4 vials of total RNA, isolated from different cell lines (MDA-MB175VII, OVCAR-3, MCF7 and JEG-3) and 4 vials of cDNA, prepared from the RNA mentioned above by the central QA laboratory. In addition, one no-template control (NTC) sample was sent. The samples were sent on dry-ice to ensure integrity. Reagents necessary to perform the RT and PCR reactions for this EQA round were also distributed to the appropriate partners. This was done to establish whether differences in the data obtained between the partners could be attributed to the differences in reagents. For this first EQA round, primers for real-time PCR for 3 reference genes (B2M, HPRT1 and HMBS) were distributed among the participants.

In this first QA round, the intra-laboratory CV was 3–26 (mean approximately 10%, Fig. 1A), while the inter-laboratory CV was 36–70% (Fig. 1B). This large inter-laboratory CV was not explained by the RT-reaction as it was similar when the participants performed the RT reaction themselves (using the RNA that was received from the central laboratory) or when externally provided cDNA was used (Fig. 1B).

3.2. Second EQA round

Participants received prediluted calibrators for PITX2 and its three splice variants and 5 unknowns to be measured in triplicate and quantified using the calibrators. The calibrators consisted of dilutions of cDNA from cell lines that highly expressed the gene(s) of interest. The average intra-laboratory CVs for all 5 samples are shown in Fig. 2A for each separate assay, whereas Fig. 2B shows the average intra-laboratory CVs for all samples and all assays. Clearly, laboratories 1, 2 and 5 showed acceptable variations (<15%), whereas laboratories 3 and 4 had higher CVs ranging from 12 to 30%, depending on the assay. The average intra-laboratory CV ranged from 7 to 21% (Fig. 2B). For the PITX2, PITX2A and PITX2C assays, the inter-laboratory variation ranged from 19 to 24% (Fig. 2C), with the variation for PITX2B assay being as high as 53%. This was caused by the consistently higher values for PITX2B reported by participant 5. Excluding the results from participant 5, the inter-laboratory CV for the PITX2B assay was 28% (not shown).

3.3. Third EQA round

For the final round, both prediluted calibrator curves and cDNAs from 8 unknown samples (obtained from SW480,

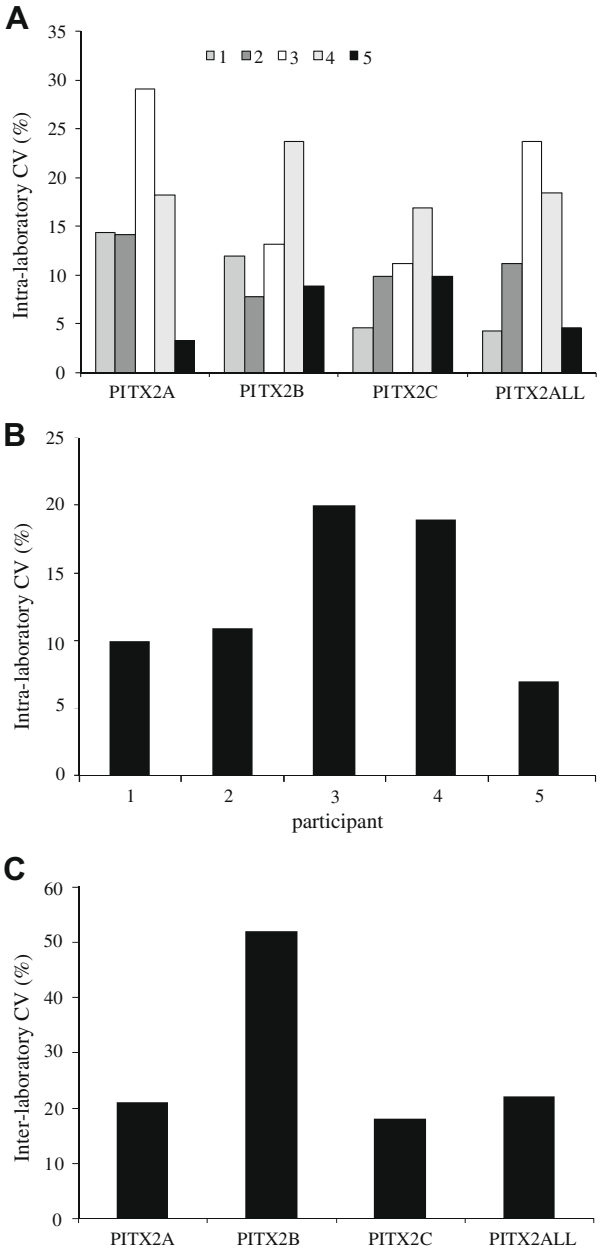

Fig. 2 – Second EQA round: intra-laboratory CV for each participant, either per assay (A) or on average for all assays (B). 1–5 denote the different participants. (C) Inter-laboratory CVs per assay.

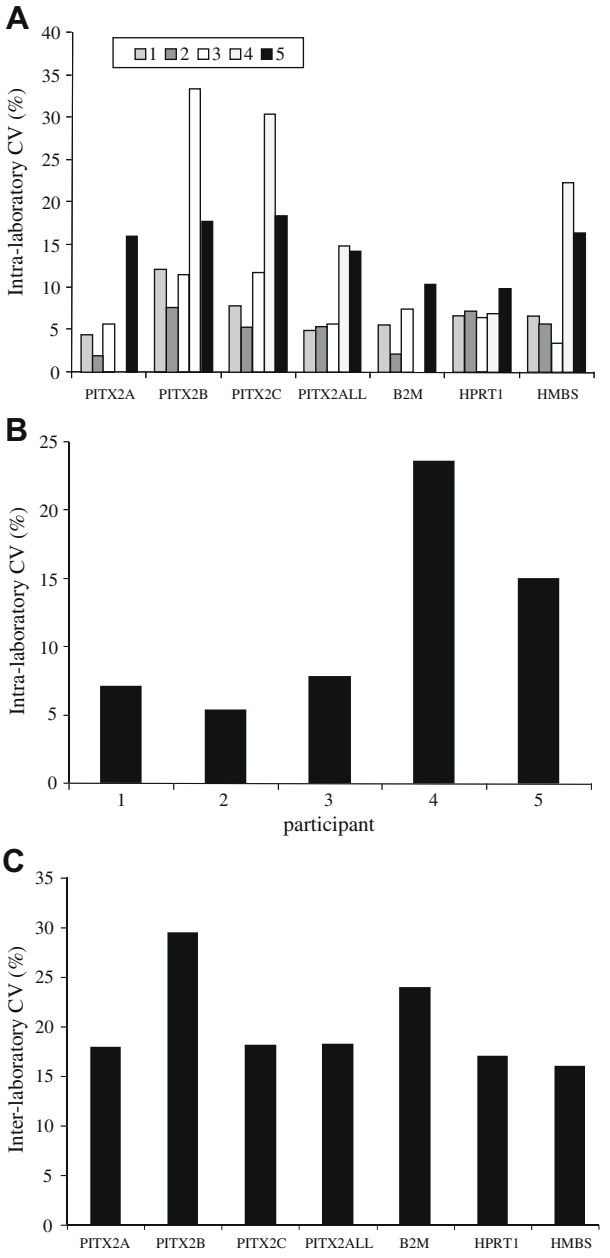

Fig. 3 – Third EQA round: intra-laboratory CV for each participant, either per assay (A) or on average for all assays (B). 1–5 denote the different participants. (C) Inter-laboratory CVs per assay. PITX2A and B2M values for participant 4 are missing.

HEPG2, JEG3, JAR, MCF7 (2x), MDA-MB175VII and OVCAR3 cell lines) were sent to all participants. The average intra-laboratory CV for the final EQA round for all samples are shown in Fig. 3A, for each separate assay, whereas Fig. 3B shows the average intra-laboratory CV for all samples and all assays in this EQA round. For this analysis, samples with levels <10 (AU) were considered negative. The intra-laboratory CV ranged from 7 to 23% depending on the participant. The inter-laboratory CV ranged from 15 to 29%, with a mean inter-laboratory CV of 20% (Fig. 3C). Subsequently, all data were normalised with a harmonisation procedure. For this, the data from a participant were corrected using the ratio of the average of all the data from a participant over the average of all the data from all the participants. If a particular participant reported consistently higher (or lower) results, this procedure corrected for that. The harmonisation factors per assay and per participant are shown in Fig. 4A. Especially for PITX2B, the differences between laboratories were high (nearly 30%). For the other assays, the inter-laboratory CV was 15–23%. Some participants exhibited consistently lower values (as exemplified by a harmonisation factor >1, e.g. for partner 3), or higher values (e.g. partner 5), requiring a harmonisation factor <1 (Fig. 4A).

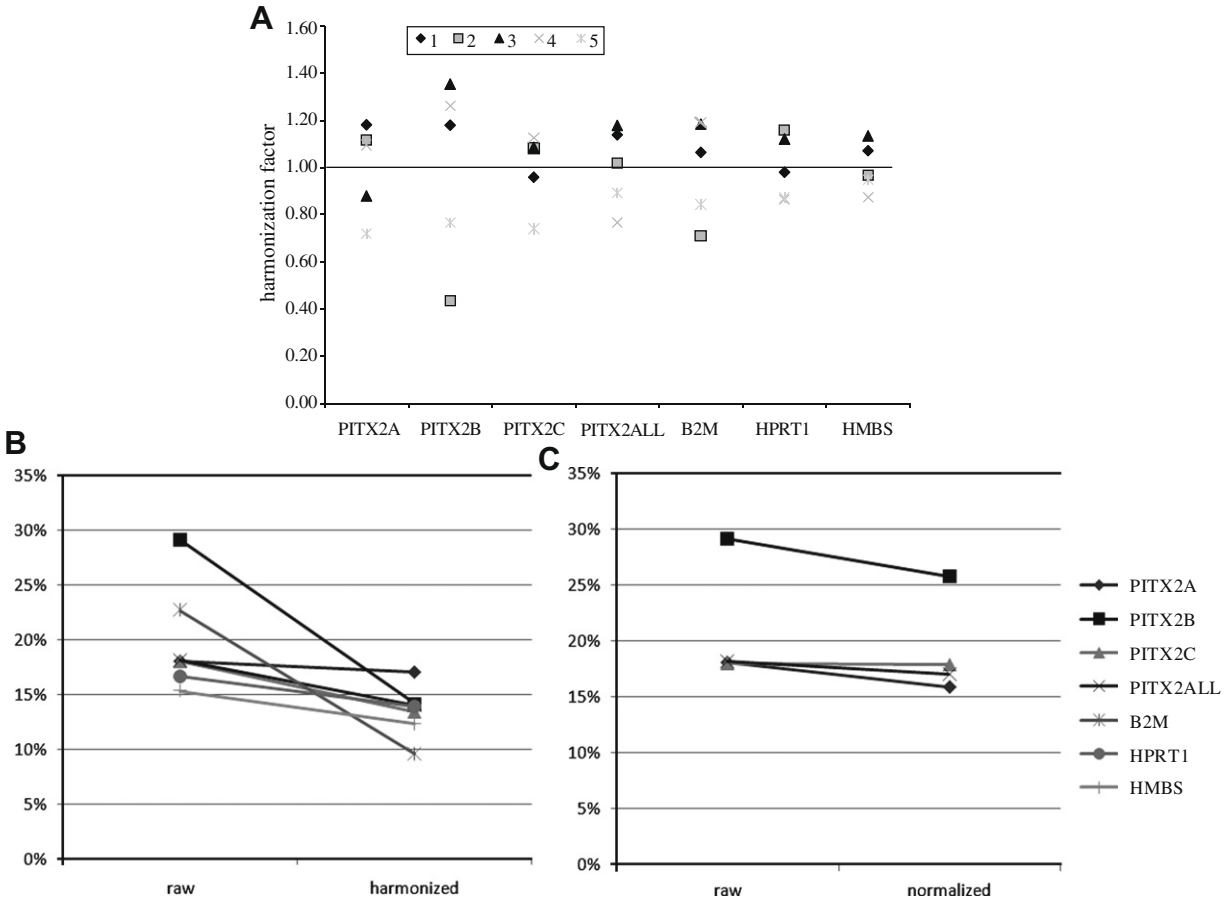

Fig. 4 – (A) Harmonisation factors for the different assays and participants. (B) Effect of harmonisation of data on inter-laboratory CV per assay. (C) Effect of normalisation – using reference gene expression – of data on inter-laboratory CV per assay.

As a result, the inter-laboratory CV of assays for which such consistent deviations occurred was diminished by harmonisation. As can be seen in Fig. 4B, the inter-laboratory CV of PITX2B and B2M was significantly attenuated by harmonisation. For PITX2A there was no effect, whereas the other assays showed a minor improvement in the inter-laboratory CV. Normalisation of the data using reference gene expression alone had no significant effect on the between-laboratory variation (Fig. 4C). In conclusion, while normalisation of the data using reference gene expression was ineffective our harmonisation procedure reduced the average between-laboratory variation from 25% (range 15–29%) to an acceptable 13% (range 10–17%).

4. Discussion

One of the aims of our 6th FP: 'DNA-methylation' project was to quantify at the mRNA level multiple potential prognostic and predictive epigenetic markers in breast cancer. One of the mRNA species investigated was PITX2, the epigenetic modulation of which was shown to be a strong prognostic marker[10,11] (Hartmann et al., submitted for publication). Prior to the various participating centres embarking on the measurement of this as well as other mRNA transcripts, we carried out EQA on the qRT-PCR procedure for quantifying PITX2 and its variants. Our attempts to harmonise the results obtained in the different centres are described in this report. Intra-laboratory CVs ranged between 5 and 23%, and the inter-laboratory CV was 17–30%. Differences between laboratories could not be normalised by correcting for reference gene expression. However, harmonisation of data further attenuated the between-laboratory variation to 13%, which seems excellent given the use of different protocols and PCR platforms.

The robustness of the results obtained in qRT-PCR of RNA obtained from tumour tissue samples largely depends on the quality of the starting material used. Therefore, before the harmonisation of results experiments started, all participants of the 6th FP: 'DNA-methylation' were trained in tissue handling, and DNA and RNA isolation. Uniformity of protocols was ascertained during training of technical laboratory personnel of each of the participating institutes at the bench in the quality assurance laboratory at the start of the project (Workshop Nijmegen, 'Nucleic Acid extraction and Quantification', May 17–18, 2004). At this workshop, consensus protocols for tissue, RNA and cDNA handling, quality control, and real-time PCR performance and data handling were developed and distributed among the participants. Most impor-

tantly, crucial steps in the RNA and DNA handling procedures were identified. Recommendations were made to use tissue sections for morphological examination and to establish the percentage tumour cells, on the procedure used to extract DNA from the samples, and to use column-based RNA extraction procedure if possible. Furthermore, spectrophotometry and gel electrophoreses, or if possible a Bioanalyser, should be used to assess RNA quality. DNAse treatment is considered an option only if the PCR is sensitive for genomic DNA and the primers cannot be changed. On column DNAse treatment would then be preferable. The best choice of house-keeper(s) is at least HPRT and PBGD, and a third housekeeper depending on tissue. The participants were made aware that several methods for normalisation of data exist. When using housekeepers, absolute quantification (using e.g. plasmid constructs) of both the gene of interest and the housekeeper is best. Finally, the so-called Delta-Ct method can only be used when the target and housekeepers are amplified with the same efficiency.

Previously, an Italian inter-laboratory comparison from 42 laboratories[16,17] identified high variability in performance among laboratories using TaqMan probes in conjunction with a variety of real-time PCR platforms. Similarly, EQUAL-quant is a European Union funded multidisciplinary EQA scheme that included 137 participating laboratories from 29 countries.[18] Both these extensive EQA programmes showed that results from some participating laboratories fell well out of the range of the other partners. The conclusion was that these EQA schemes demonstrated both the requirement and demand for external assessment of technical standards in real-time PCR. The data obtained during our EQA scheme, although only encompassing 5 laboratories, were encouraging. Despite differences in analysis methodology and PCR equipment, the inter-laboratory CV could be reduced to approximately 13% using prediluted calibrators and by harmonising the data in a central QA laboratory. Given the high sensitivity and dynamic range of real-time RT-PCR, we feel that these variations are low. For ER protein measurements, inter-laboratory CVs of approximately 30% (using Enzyme-Immuno Assays) to 40% (using Ligand Binding Assays) were found[2], which could be attenuated to 10–15% by harmonisation.[3] Similarly, with ELISAs for uPA and PAI-1 the inter-laboratory CVs of 40–60% were found that could be reduced to 15–20% using common external standards sent out by the central QA laboratory.[4]

In conclusion, for multi-centre validation studies of biomarkers quantified by qRT-PCR, it is necessary to use externally provided calibrators for the genes of interest to obtain comparable results between different laboratories. Subsequent harmonisation will further reduce variation in qRT-PCR results, and both are required to reduce the variation to an acceptable level for multi-centre measurement of prognostic and predictive ribonucleotides in breast cancer. With many RNA-based prognostic markers being revealed by us (e.g.[19–21]) and others in the current omics-era, EQA programmes as the encouraging one performed here will be needed to lift mRNA-based markers to a higher level of evidence needed for them to initiate prospective validation, which is required for actual clinical application. Based on the current promising results and preliminary data that PITX2 at the mRNA levels is a prognostic marker (manuscript in preparation), the involved laboratories have initiated a multi-centre validation of PITX2 mRNA measurement as a potential new prognostic marker in breast cancer.

Conflict of interest statement

None declared.

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