Quantification of Chemotherapeutic Target Gene mRNA Expression in Human Breast Cancer Biopsies: Comparison of Real-Time Reverse Transcription-PCR vs. Relative Quantification Reverse Transcription-PCR Utilizing DNA Sequencer Analysis of PCR Products

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The solid tumor mRNA expression of genes related to the mechanism of action of certain antineoplastic agents is often predictive of clinical efficacy. We report here on the development of a rapid and practical real-time RT-PCR method to quantify genetic expression in solid tumors. The genes examined are related to the intracellular pharmacology of gemcitabine and cisplatin, two drugs that are used in the treatment of several types of advanced cancer. We evaluated target gene mRNA levels from breast tumor samples using two quantitative RT-PCR methods: 1) an improved relative RT-PCR method using fluorescence-labeled primers, automated PCR set up, and GeneScan[®] analysis software; and 2) real-time RT-PCR with redesigned primers using an ABI 7900HT instrument, with additional postprocessing of the data to adjust for efficiency differences across the target genes. Using these methods, we quantified mRNA expression levels of deoxycytidine kinase (dCK), deoxycytidylate deaminase (dCDA), the M1 and M2 subunits of ribonucleotide reductase (RRM1, RRM2), and excision cross complementation group 1 (ERCC1) in 35 human

“fresh” frozen breast cancer biopsies. While both assay methods were substantially more rapid than traditional RT-PCR, real-time RT-PCR appeared to be superior to the amplification end-point measurement in terms of precision and high throughput, even when a DNA sequencer was used to assess fluorescence-labeled PCR products. This reproducible, highly sensitive real-time RT-PCR method for the detection and quantification of the mRNAs for dCK, dCDA, RRM1, RRM2, and ERCC1 in human breast cancer biopsies appears to be more informative and less time-consuming

Abbreviations: RT-PCR, reverse transcription polymerase chain reaction; dCK, deoxycytidine kinase; dCDA, deoxycytidylate deaminase; RRM1/RRM2, ribonucleotide reductase M1/M2 subunit; ERCC1, excision repair cross complementation group 1.

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than either classical radioisotope-dependent RT-PCR or the technique utilizing GeneScan[®] analysis described herein. By allowing the measurement of intratumoral target gene expression, these new

methods may prove useful in predicting the clinical utility of gemcitabine- and platinum-containing chemotherapy programs in patients with solid tumors. *J. Clin. Lab. Anal.* 17:184–194, 2003. © 2003 Wiley-Liss, Inc.

Key words: real-time RT-PCR; gene expression; gemcitabine and cisplatin; breast cancer biopsy

Recently, relative quantitation reverse transcriptase polymerase chain reaction (RT-PCR) techniques have been used with human tumor samples to examine the expression of several genes that are involved in the mechanism of action, resistance, or metabolism of a variety of different antineoplastic agents (1,2). These studies have demonstrated that for genes in which mRNA and protein levels are strongly correlated, mRNA expression in tumor tissue examined by RT-PCR prior to drug treatment may be predictive of therapeutic response or resistance (3). In the best-known example, intratumoral levels of thymidylate synthase, the therapeutic target of the fluoropyrimidines, have been significantly correlated with clinical efficacy of 5-fluorouracil-containing regimens (4). Furthermore, in the light of the pleiotropic effects of most cytotoxic antineoplastic agents, it is not surprising that the predictive power of target gene quantitation has been enhanced by determining the expression of multiple therapeutic target genes or of gene products involved in either drug activation or detoxification (5).

Gemcitabine (2',2'-difluorodeoxycytidine) is a new anticancer agent that has significant therapeutic potential against carcinomas of the ovary, lung, pancreas, and breast (6,7). After it enters the cell, gemcitabine must be activated through phosphorylation by the enzyme deoxycytidine kinase (dCK) to produce an active metabolite that is incorporated into DNA or RNA, inhibiting chain elongation (8–12). The drug is inactivated by deoxycytidylate deaminase (dCDA), which reduces therapeutic activity (13–16). Gemcitabine also inhibits the enzyme ribonucleotide reductase (RR), which plays an essential role in regulating intracellular deoxynucleoside triphosphate pools critical for DNA repair (17,18).

Because of their non-overlapping toxicities, gemcitabine is often combined with the anticancer agent cisplatin (cis-diamine-dichloroplatinum [CDDP]) in the treatment of advanced non-small-cell lung cancer or ovarian cancer (6). Cisplatin creates cytotoxic inter-strand DNA cross-links, blocking replication and transcription (19). Overexpression of the DNA repair gene *excision repair cross complementation 1* (*ERCC1*) can decrease the efficacy of cisplatin in human ovarian cancer, and is correlated with clinical resistance to this

drug in patients with carcinoma of the colon treated with cisplatin-containing chemotherapy (20–24).

A quantitative RT-PCR method that was initially developed by Danenberg's group (2) has been widely applied to examine relative levels of therapeutic target gene expression prior to chemotherapy, using a variety of housekeeping genes as standards (3–5). In the course of several ongoing clinical investigations, we have attempted to improve this method by using fluorescence dye-labeled primers, and automated quantification. The PCR products produced from amplification of tumoral mRNAs were run on a DNA sequencer and analyzed with GeneScan[®] software (25). More recently, we have used real-time quantitative RT-PCR detection employing an ABI 7900HT sequence detection system. In the present study, we compare the two quantitative methods using human breast cancer biopsy specimens obtained from patients in an investigational chemotherapy trial. The results demonstrate that real-time RT-PCR of an appropriate design, and with postprocessing of the results, yields higher precision and much faster results for the determination of mRNA expression levels of several target genes compared to other known techniques.

MATERIALS AND METHODS

Tissues

During a phase II clinical trial approved by the institutional review boards of the City of Hope National Medical Center, the University of Southern California/Norris Comprehensive Cancer Center, the University of California–Davis Cancer Center, and the Loyola University Medical Center, patients with advanced breast cancer were treated with the combination of gemcitabine and cisplatin (26). Fresh tumor biopsy samples (10–20 mg wet weight) were obtained from sites of recurrent disease. Fifteen samples were obtained from patients who had received minimal (one or no prior chemotherapy regimens) treatment, and 20 specimens were from the heavily pretreated (two or more prior regimens) patient group. Three patients underwent second biopsies 16 weeks after the treatment had been initiated. All of the samples were sectioned for review by a pathologist, and then placed immediately in liquid nitrogen and kept frozen at –80°C until processed.

RNA Isolation and cDNA Synthesis

RNA was isolated from tissues after disruption with a Polytron homogenizer using RNA-zol B (TEL-TEST Inc., Friendswood, TX) according to the manufacturer's instructions. Genomic DNA contamination was removed with DNase I treatment (Ambion, Austin, TX). cDNA was prepared from 3–40 µg RNA in a 100-µL volume, adding 1,000 units MMLV reverse transcriptase enzyme, 20 µL first strand buffer (Life Technologies, Gaithersburg, MD), 100 units RNasin (Promega, Madison, WI), 10 mmol/L dithiothreitol, 4.75 µmol/L random hexamers (Life Technologies) and 600 µmol/L deoxynucleotides (Applied Biosystems, Foster City, CA). The random hexamer primer was annealed for 10 min at 25°C. cDNA synthesis was performed for 45 min at 42°C, followed by 5 min denaturation at 75°C. The reaction was boosted by the addition of 1,000 units MMLV reverse transcriptase enzyme, and the 42°C step was repeated for another 60 min. The enzyme was inactivated for 5 min at 95°C, and the cDNA was stored at –20°C until use.

GeneScan® Method

Dilutions of each cDNA sample in the 5×10^{-1} to 10^{-7} range were constructed in series. For the β -actin housekeeping gene expression analysis, 10^{-3} to 10^{-7} dilutions were used. For the target gene expression studies, 5×10^{-1} to 10^{-5} dilutions were analyzed from each tumor sample. The PCR reactions were performed on an ABI Prism 877 integrated thermal cycler (ABI Turbo PCR version 1.0.1; Applied Biosystems, Foster City, CA) as a single-tube reaction for each primer set, and pooled together in one well after the reaction. The PCR was performed on 0.4 µL diluted cDNA in 5 µL final reaction volume in 15 mM TRIS-HCl pH 8.0, 50 mM KCl buffer, 2.5 mM MgCl₂, 250 µM dNTPs, 0.25 µM primers, and 0.125 U AmpliTaq Gold DNA polymerase (Perkin-Elmer, Foster City, CA) final concentration. The sense primers were labeled with ABI fluorescence dyes: 6-FAM (6-carboxyfluorescein), HEX (4, 7, 2', 4', 5', 7'-hexachloro-6-carboxyfluorescein), and TET (4, 7, 2', 7'-tetrachloro-6-carboxyfluorescein) on the 5' end. After activation of the enzyme at 95°C for 10 min, 30 cycles were performed: 15 sec of denaturation at 95°C followed by 30 sec of primer annealing at 55°C (β -actin, deoxycytidine kinase [dCK], deoxycytidylate deaminase [dCDA], M1 subunit of ribonucleotide reductase [RRM1]) or 61°C (ERCC1, M2 subunit of ribonucleotide reductase [RRM2]), and 60 sec of extension at 72°C. After the last cycle, a 10-min final extension step was added at 72°C. The primer sequences

were as follows:

Gene		Primer Sequence
β -actin	Sense	HEX- 5'-GCGGGAAATCGTGCGTGACATT-3'
	Antisense	5'-CACGAAACTACCTTCAACTCCATC-3'
ERCC1	Sense	TET-5'-CAGGGCCGCCAGCAAGGAAG-3'
	Antisense	5'-AGCCGCCCATGGATGTAGTCTG-3'
dCK	Sense	6-FAM-5'-AACATCGCTGCAGGGAAGTCA-3'
	Antisense	5'-CAAGCTGAGCTCTTATTCGACT-3'
dCDA	Sense	6-FAM-5'-AGTGATGACGTGTTGCCTTGG-3'
	Antisense	5'-TTCGGTATGAATTTCCGGAATGT-3'
RRM1	Sense	TET-5'-TGATCAAGCGAGATGGCCG-3'
	Antisense	5'-CCATCACATCACTGAACACTT-3'
RRM2	Sense	6-FAM-5'-TCCACTATGCTCTCCCTCCGTGT-3'
	Antisense	5'-TGTCTTGAGAGGTCAACCTCCT-3'

From the pooled PCR products, 1–5 µL volume was dried and resuspended in 4 µL formamide loading buffer. GS 500 TAMRA-labeled size marker was added (0.5 µL) to the same tube, and 0.8 µL volume was loaded from each tube on a 4.75% denaturing polyacrylamide gel and run on an ABI Prism 377 DNA sequencer in GeneScan® mode at 3KV constant voltage for 2.5 hr. The gel was analyzed with GeneScan® version 3.1 software, and the peak area was used for further calculations launching to MS Excel®. The fluorescence was plotted against volume. The ratio of the slope of the target gene fluorescence to the slope of the β -actin fluorescence in the same sample was used to express the relative amount of mRNA for each target gene analyzed, modulo PCR efficiency, and dye effects. The PCR reactions were repeated three times, and the samples were run twice from each reaction on the gel. A normal tissue sample was chosen as a calibrator, and the ratio of target gene to β -actin expression for each tumor sample was normalized by the same ratio for the normal tissue sample. Formally, this removes dye and efficiency effects, and provides a reference ratio of one, which represents normal tissue. This is described in more detail in the data analysis section.

TaqMan® Method

The quantification of gene expression levels was also examined using the same cDNA samples as those employed for the GeneScan® experiments using real-time PCR as described previously (27–31). We had to redesign the primers according to the Applied Biosystems guidelines (Primer Express software; Applied Biosystems, Foster City, CA) to fit real-time PCR requirements. Genomic DNA amplification was excluded by designing our primers around the exon-intron

splicing sites. The sequence of primers and probes are as follows:

Gene		Primer Sequence
β -actin	Sense	5'-TGAGCGCGGCTACAGCTT-3'
	Antisense	5'-CCTTAATGTCACACACGATT-3'
	Probe	TET-5'-ACCACCACGGCCGAGCGG-3'TAMRA
ERCC1	Sense	5'-CCAGCAAGGAAGAAATTTGTGAT-3'
	Antisense	5'-GGGCTTGCCACTCCAG-3'
	Probe	6FAM-5'-CCCCTCGACGAGGATGAGGTC-CC-3'TAMRA
dCK	Sense	5'-TCCAGCTTCCTTCTGTCAATTCC-3'
	Antisense	5'-CAACGAAGTGAGAGGCACCA-3'
	Probe	6FAM-5'TCTTTTGTCTTCCTCAGCAGGTT-GGCTTT-3'TAMRA
dCDA	Sense	5'-GCAGAGAATAAGCTGGACACCAA-3'
	Antisense	5'-ATTTTGTTCATGATGGCATTTCAG-3'
	Probe	6FAM-5'TACCCGTACGTGTGCCATGCGG-A-3'TAMRA
RRM1	Sense	5'-CACTAGCTGCGATGCATGTGA-3'
	Antisense	5'-TCGAGATGTAATTTGTCAAACATGA-3'
	Probe	6FAM-5'CAAGCGAGATGGCCGCCAAGA-AC-3'TAMRA
RRM2	Sense	5'-ATGAAAACCTGGTGGAGCGATT-3'
	Antisense	5'-TGGCAATTTGGAAGCCATAGA-3'
	Probe	6FAM-5'CCAAGAAGTTCAGATTACAGAA-GCCCGCTG-3'TAMRA

The optimal PCR primer and probe concentrations for each target gene and the β -actin housekeeping gene were determined to reach maximum efficiency during the amplification. The PCR reaction was determined in a 20- μ L final volume adding 1 μ L cDNA from each sample, using TaqMan[®] Universal PCR mix (Applied Biosystems, Foster City, CA). For each target gene, the probe concentration was 0.3 μ mol/L, and the primer concentrations for the detection of the dCK, dCDA, RRM1, and RRM2 genes were 0.4 μ mol/L, 0.2 μ mol/L for *ERCC1*, and 0.8 μ mol/L for the β -actin. The PCR amplification was performed in 384-well plates using the following cycling conditions: 2 min at 50°C for the AmpErase uracil-N-glycosylase reaction to remove any cross contamination, 10 min at 95°C to activate the AmpliTaq Gold DNA polymerase, followed by 40 cycles at 95°C for 15 sec and at 60°C for 1 min. During the PCR reaction, the Taq DNA polymerase cleaved the probe and released the 5' end reporter fluorescence dye (TET or 6-FAM), the fluorescence of which was detected by the ABI 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA). The number of cycles to reach the fluorescence threshold was defined as the cycle threshold (Ct value). For the PCR efficiencies, Ct values were observed for

a serial dilution from a MCF-7 human breast cancer cell line, and graphed against the base 10 log of the pg of cDNA, demonstrating an extremely good fit to the expected linear relationship. The slopes and r^2 values were: β -actin (−3.91,0.994), dCK (−3.39,0.994), dCDA (−4.07,0.992), RRM1 (−4.02,0.992), RRM2 (−4.23,0.998), and ERCC1 (−3.45,0.997). The slope was used to estimate the efficiency of each gene amplification according to the formula:

$$\text{efficiency} = 10^{(-1/\text{slope})} - 1 \quad (1)$$

The resulting efficiencies were: β -actin (80%), dCK (97%), dCDA (76%), RRM1 (77%), RRM2 (72%), and ERCC1 (95%). The MCF-7 cell line was used as a proxy for the PCR efficiency in the tumor samples due to limitations in the quantity of biopsy material. The MCF-7 breast cancer cell line was chosen by comparing 18 different cell lines with the GeneScan[®] method, and selecting the cell line closest in expression levels to the target genes as observed in the biopsy samples (unpublished data). Relative gene expression was determined as the ratio of the gene of interest to the internal reference gene expression based on the Ct values, and normalized by the same calibrator used in the GeneScan[®] method. By combining this normalization with an adjustment for variations in efficiency across genes, we arrive at a sensible estimate for expression ratios standardized to the normal tissue, as described earlier for the GeneScan[®] method (see the following section). Contamination of the RNA samples by genomic DNA was excluded by an analysis of samples without prior cDNA synthesis.

DATA ANALYSIS

For the GeneScan[®] method, we used the linear range of the PCR amplification curve. The fluorescence is proportional to the number of moles of target at the end of the 30 cycles of PCR reaction, with the constant of proportionality identified as the dye constant:

$$F = d * N \quad (2)$$

Assuming that the initial number of moles of target gene prior to the PCR reaction is proportional to the volume, we note that the ending number of moles of target is also proportional to the volume, regardless of efficiency. As a result, we can write:

$$\frac{N}{V} * d = \frac{\Delta F}{\Delta V} = \text{slope} \quad (3)$$

From here, we obtain the ratio of slopes for a target gene to the housekeeping gene. For example, for dCK (which uses the 6-FAM dye) vs. β -actin (using

the HEX dye), we note:

$$\text{ratio of slopes} = \frac{d_{6-FAM}}{d_{HEX}} * \frac{N_{dCK}/V_{dCK}}{N_{\beta-actin}/V_{\beta-actin}} \quad (4)$$

By obtaining the ratio for a tumor sample q , and dividing by the same ratio for the normal tissue used as a standard (s), the dye ratios cancel, and we obtain:

$$\begin{aligned} & \text{ratio of slopes for sample } / \\ & \text{ratio of slopes for standard (normal tissue)} \\ & = \frac{\left(\frac{N_{dCK}^q/V_{dCK}^q}{N_{\beta-actin}^q/V_{\beta-actin}^q} \right)}{\left(\frac{N_{dCK}^s/V_{dCK}^s}{N_{\beta-actin}^s/V_{\beta-actin}^s} \right)} \end{aligned}$$

This provides a measure of the target gene expression relative to β -actin, and normalized by the standard. Hence, a value of one indicates equal expression of dCK relative to β -actin for the biopsy material in sample q as observed in the normal tissue. A similar construct applies for the other target genes of interest.

To obtain a similar estimate using the TaqMan[®] method requires a slightly different analysis, and both the changes in efficiency and dye effects between the target gene (x) and β -actin (r) must be accommodated. To this end, let $X_{N,q}$ represent the ratio of the initial number of moles of target gene x to β -actin in sample q , and let $X_{N,s}$ represent the same quantity for the standard, taken as before as the normal tissue in this case. We then obtain (Frankel et al., unpublished results):

$$\frac{X_{N,q}}{X_{N,s}} = \frac{(1 + \text{efficiency}_{\beta-actin})^{C_{T,r,q} - C_{T,r,s}}}{(1 + \text{efficiency}_x)^{C_{T,x,q} - C_{T,x,s}}} \quad (6)$$

where the efficiency is estimated using the MCF-7 cells and applying Eq. [1]. As with the GeneScan[®] method, this ratio provides a measure of the target gene expression relative to β -actin and normalized by the standard. Again, a value of one indicates, as before, equal expression of the target gene relative to β -actin for the biopsy material in sample q as observed in the normal tissue. We note that in the ideal case, in which the efficiencies are both perfect and identical, this reduces to the standard formula in the ABI instructions:

$$\frac{X_{N,q}}{X_{N,s}} \approx 2^{-\Delta\Delta C_T} \quad (7)$$

In both methods, then, the ratios of target gene expression to that of the reference gene (β -actin) relative to normal tissue were taken as the fundamental estimates.

To compare the two methods, the difference in the logarithm of the values obtained from the two different methods is the natural measure of their relative difference (32). The additivity and symmetry properties

of this scale are desirable for the usual applications of these measurements to comparative investigations. Base 2 logarithms were used for ease of interpretation.

Agreement between the GeneScan[®] and TaqMan[®] methods was assessed by plotting the difference vs. the sum of log of the fundamental estimates (33). A standard deviation (SD) of 1 of these differences on the base 2 log ratio scale would imply that the methods may differ by a factor of 2, and occasionally by a factor of 4. The mean difference is also of interest, since we attempted to correct for systematic bias and differences in primer efficiency. We did not use correlation as primary summaries of these data because correlations are strongly influenced by the range of available sample values.

RESULTS AND DISCUSSION

Quantitative PCR techniques are the most sensitive methods to measure differential gene expression, and have become common diagnostic tools in clinical practice. These techniques employ a variety of different methods to measure the amount of PCR product after amplification, including detection by flow cytometry (34), electrochemiluminescence (35), or agarose or polyacrylamide gel electrophoresis using isotope (³²P or ³³P)-labeled amplicons (1,2). Unfortunately, such methods are labor-intensive and time-consuming (36). Clinical investigators need a more rapid method to evaluate changes in expression for several genes from the same sample.

Danenberg's laboratory (1,2) developed an improved quantitative RT-PCR method to examine target gene expression in tumor biopsy samples, employing a variety of housekeeping genes (particularly β -actin) as internal reference standards. This method does not require gene cloning, it provides a relative level of quantitation by expressing the ratio of the gene of interest to a housekeeping gene from the same tissue (same cDNA sample is used for both), and it is easy to perform in clinical laboratories. For the ratio calculation, the linear region of the PCR amplification curve is used, dividing the slope of the target gene mRNA fluorescence with respect to volume by the slope of β -actin (housekeeping gene) from the same sample to obtain an estimate of relative expression with a constant bias within a target gene.

We attempted to improve upon this method by using a PCR robot (ABI Prism 877 integrated thermal cycler [ABI Turbo PCR version 1.0.1]). This allowed us to perform reactions in parallel for many genes with different PCR conditions from the same cDNA dilutions, thereby reducing reagent costs and increasing accuracy. Isotope labeling was replaced with ABI

fluorescence dyes, which are stable for months. Viewing the PCR products on the polyacrylamide gel in the same lane enables us to compare the level of expression for many genes immediately (Fig. 1), and, by normalizing, the constant bias can be removed. In an attempt to further improve the speed at which these analyses can be performed, we subsequently developed real-time PCR methods for quantitating the expression of the same group of genes relevant to the mechanism of action, detoxification, or resistance to the antineoplastic agents gemcitabine and cisplatin. In this study, we compared the relative quantitation of dCK, dCDA, RRM1, RRM2, and *ERCC1* in human breast cancer biopsies obtained immediately prior to the initiation of combination chemotherapy treatment with those agents. β -Actin was used as the housekeeping gene (Fig. 1A). mRNA expression levels for dCK, dCDA, RRM1, and RRM2 have not been previously studied in biopsy samples taken from human solid tumors.

Figure 1A demonstrates the position and length of the PCR products as the GeneScan[®] software displays the data on the chromatograph. The software from the GS-500 marker automatically calculates the size of the PCR products with 1 base pair (bp) accuracy. Our amplicons were β -actin 233 bp, dCK 246 bp, dCDA 292 bp, RRM1 289 bp, RRM2 341 bp, and *ERCC1* 430 bp. For quantitative analysis, the software calculates the peak area as tabular data, which can be opened in Microsoft Excel[®] for further analysis (37–39). Table 1 shows the comparison of the data for gene expression using GeneScan[®] and real-time PCR techniques. The results are represented as an average of data from three PCR experiments.

During the GeneScan[®] analysis, we used six dilutions from the cDNA to draw the amplification curve. The ratio of target gene fluorescence to β -actin was calculated from the slope of the linear part of the PCR amplification graph (Fig. 1B). Our experiments showed a day-to-day variability of approximately 30–70%. The difference primarily represents changes in PCR efficiency over time, well-to-well amplification variability in the thermal cycler, and pipetting errors. β -Actin is amplified from a high dilution of cDNA (10^{-3} to 10^{-7}), and the low amount of target makes the amplification reaction volatile. The gel-to-gel variability from the same PCR experiment was found to be lower (30–40%) (data not shown).

We repeated measurements of gene expression levels from the same patient samples using the real-time PCR method. The gene of interest/ β -actin ratio represents the relative expression of each gene studied for all of the clinical specimens, and this was normalized by a standard (normal tissue) as described in the previous section. As seen in Table 1, gene expression varied over

a wide (often more than 10-fold) range in the tumor biopsy specimens studied.

When we compared the results with the two methods, the SD of the difference of log ratios was similar across all genes, ranging from 1.1 to 1.7. The pooled SD was 1.5, using a one-way analysis of variance (ANOVA) to adjust for gene-specific (primer) effects. This implies that the two methods may differ by more than a factor of 2.8. However, in all genes except RRM2, the zero point marking no difference was well within the first SD, and even for RRM2 it was right on the edge of the first SD. The plots of difference vs. sum appeared to be reasonably free of trends in bias or variance, suggesting that this assessment applies over the observed range of expression levels (see Fig. 2).

Variations in cycling conditions, errors in the estimate of efficiencies for the TaqMan[®] analysis, and the high standard error rate of the GeneScan[®] method may explain the differences that we observed between the two methods. During the real-time PCR analysis, we found the error rate to be much lower and the results highly replicable. Our experience (Table 1) agrees with that of other laboratories (27), suggesting that the SD value of the triplicates within the same run using real-time PCR is often <5%, with an average coefficient of variation of 10.3% (range: 9.21–12.40%), and that precision is high between runs (coefficient of variation <20% in our laboratory [unpublished data]). For the GeneScan[®] method, the average coefficient of variation was 44% (range: 39.32–50.55%).

Correlations of gemcitabine and cisplatin target gene expression with response to treatment in patients receiving this combination await completion of the therapeutic phase of this clinical trial. However, we are encouraged that the large dynamic range in gene expression ratios observed with real-time PCR and the enhanced precision vs. the GeneScan[®] analysis may facilitate prediction of drug resistance and/or sensitivity, as has been the case for prior investigations of the predictive power of tumoral thymidylate synthase expression in patients treated with a fluoropyrimidine (3,4). While correlative analysis of our data awaits completion of the clinical trial, the enhanced precision of efficiency adjusted real-time PCR shows added promise for uncovering such correlative relationships. For example, with the GeneScan[®] method, due to the large coefficients of variation, no statistically significant relationship was observed between dCK and *ERCC1* ($P=0.42$). With the TaqMan[®] method, without adjusting for efficiency differences, the statistics were improved but not significant ($P=0.12$). Finally, with efficiency adjustment, we obtained statistical significance ($P=0.03$). While this particular result is only marginally interesting, considering our current understanding of

(A)

(B)

(C)

Fig. 1. Determination of relative gene expression of chemotherapeutic target genes in breast cancer biopsy sample using GeneScan[®] software. **A:** GeneScan[®] 3.1 electrophoretogram display of the RT-PCR amplicons for one patient sample in one lane of the gel file from the ABI 377 DNA sequencer. The y-axis is the relative intensity of fluorescence, and the x-axis represents the designate size of the fragments in base pairs (bp) as shown on the bottom of the chromatograph. The small peaks in this lane are the GeneScan[®] 500 ROX internal lane size standards. The large peaks are the following: β -actin 233 bp (HEX labeled), the 6-FAM-labeled dCK (246 bp), dCDA (292 bp) and RRM2 (341 bp), the TET-labeled RRM1 (289 bp), and *ERCC1* (430 bp). **B:** The amplification level is shown from the same patient sample on the MS Excel[®] graph. This graph summarizes the data from six lanes of the gel file. Each gene is amplified from six dilutions of the cDNA and gives a saturation curve. **C:** The linear part of graph B is used for further calculations. dCDA and β -actin are shown as examples. The ratio of the target gene/ β -actin is calculated from the slopes, with dye effects accommodated by normalization. The data with r^2 values <0.9500 were discarded.

TABLE 1. Taq/Man[®]/GeneScan[®] data comparison using β -actin as the housekeeping gene

Patient	Source	dCK ^a			dCDA			RRM1			RRM2			ERCCI		
		TaqMan Mean $\pm$ S.D.	GeneScan Mean $\pm$ S.D.	TaqMan Mean $\pm$ S.D.	TaqMan Mean $\pm$ S.D.	GeneScan Mean $\pm$ S.D.	GeneScan Mean $\pm$ S.D.	TaqMan Mean $\pm$ S.D.	TaqMan Mean $\pm$ S.D.	GeneScan Mean $\pm$ S.D.	TaqMan Mean $\pm$ S.D.	TaqMan Mean $\pm$ S.D.	GeneScan Mean $\pm$ S.D.	TaqMan Mean $\pm$ S.D.	TaqMan Mean $\pm$ S.D.	GeneScan Mean $\pm$ S.D.
1-A ^b	Post. cervical node	1.43 $\pm$ 0.05	2.09 $\pm$ 0.71	0.37 $\pm$ 0.02	0.68 $\pm$ 0.25	0.63 $\pm$ 0.02	0.73 $\pm$ 0.25	0.63 $\pm$ 0.02	0.73 $\pm$ 0.25	1.67 $\pm$ 0.11	5.86 $\pm$ 2.33	0.53 $\pm$ 0.02	0.74 $\pm$ 0.16	0.53 $\pm$ 0.02	0.74 $\pm$ 0.16	0.53 $\pm$ 0.02
1-B	Cervical node	1.29 $\pm$ 0.07	0.62 $\pm$ 0.24	0.36 $\pm$ 0.02	0.17 $\pm$ 0.07	0.51 $\pm$ 0.04	0.35 $\pm$ 0.14	0.51 $\pm$ 0.04	0.35 $\pm$ 0.14	1.51 $\pm$ 0.08	1.39 $\pm$ 1.06	0.47 $\pm$ 0.03	0.28 $\pm$ 0.05	0.47 $\pm$ 0.03	0.28 $\pm$ 0.05	0.47 $\pm$ 0.03
2	R. breast biopsy	2.37 $\pm$ 0.09	4.05 $\pm$ 1.54	0.76 $\pm$ 0.06	2.11 $\pm$ 1.09	1.27 $\pm$ 0.04	2.05 $\pm$ 0.22	1.27 $\pm$ 0.04	2.05 $\pm$ 0.22	4.20 $\pm$ 0.13	9.27 $\pm$ 2.53	0.70 $\pm$ 0.04	1.23 $\pm$ 0.79	0.70 $\pm$ 0.04	1.23 $\pm$ 0.79	0.70 $\pm$ 0.04
3	R. chest wall	0.95 $\pm$ 0.06	1.00 $\pm$ 0.29	0.70 $\pm$ 0.05	0.42 $\pm$ 0.18	1.34 $\pm$ 0.08	1.75 $\pm$ 0.33	1.34 $\pm$ 0.08	1.75 $\pm$ 0.33	1.96 $\pm$ 0.13	5.92 $\pm$ 1.93	0.73 $\pm$ 0.05	0.54 $\pm$ 0.20	0.73 $\pm$ 0.05	0.54 $\pm$ 0.20	0.73 $\pm$ 0.05
4-I-A ^c	L. ant. chest wall	0.11 $\pm$ 0.02	1.22 $\pm$ 0.32	2.15 $\pm$ 0.16	1.33 $\pm$ 0.47	1.81 $\pm$ 0.13	0.99 $\pm$ 0.41	1.81 $\pm$ 0.13	0.99 $\pm$ 0.41	0.49 $\pm$ 0.05	11.10 $\pm$ 7.05	1.79 $\pm$ 0.12	1.00 $\pm$ 0.23	1.79 $\pm$ 0.12	1.00 $\pm$ 0.23	1.79 $\pm$ 0.12
4-I-B	L. lat. chest wall	1.41 $\pm$ 0.71	1.21 $\pm$ 0.39	1.93 $\pm$ 0.87	0.64 $\pm$ 0.11	2.25 $\pm$ 1.07	0.85 $\pm$ 0.28	2.25 $\pm$ 1.07	0.85 $\pm$ 0.28	1.97 $\pm$ 1.03	2.62 $\pm$ 1.19	1.70 $\pm$ 0.79	1.47 $\pm$ 0.35	1.70 $\pm$ 0.79	1.47 $\pm$ 0.35	1.70 $\pm$ 0.79
4-II-A	L. Inf. chest wall	0.02 $\pm$ 0.00	0.52 $\pm$ 0.36	4.76 $\pm$ 0.46	0.77 $\pm$ 0.24	2.67 $\pm$ 0.30	0.58 $\pm$ 0.41	2.67 $\pm$ 0.30	0.58 $\pm$ 0.41	0.14 $\pm$ 0.01	2.94 $\pm$ 2.21	3.82 $\pm$ 0.32	1.67 $\pm$ 0.93	3.82 $\pm$ 0.32	1.67 $\pm$ 0.93	3.82 $\pm$ 0.32
4-II-B	L. sup. chest wall	0.08 $\pm$ 0.02	1.14 $\pm$ 0.35	2.93 $\pm$ 0.35	0.56 $\pm$ 0.42	2.09 $\pm$ 0.19	0.45 $\pm$ 0.12	2.09 $\pm$ 0.19	0.45 $\pm$ 0.12	0.16 $\pm$ 0.03	1.39 $\pm$ 1.49	2.38 $\pm$ 0.22	1.59 $\pm$ 0.07	2.38 $\pm$ 0.22	1.59 $\pm$ 0.07	2.38 $\pm$ 0.22
5-I ^d	Chest wall skin	0.20 $\pm$ 0.01	1.80 $\pm$ 0.80	11.42 $\pm$ 0.75	1.67 $\pm$ 0.87	8.20 $\pm$ 0.52	2.61 $\pm$ 0.92	8.20 $\pm$ 0.52	2.61 $\pm$ 0.92	0.13 $\pm$ 0.10	4.12 $\pm$ 1.12	8.87 $\pm$ 0.70	1.70 $\pm$ 0.54	8.87 $\pm$ 0.70	1.70 $\pm$ 0.54	8.87 $\pm$ 0.70
5-2	Chest wall skin	0.00 $\pm$ 0.00	1.65 $\pm$ 0.58	38.01 $\pm$ 4.57	1.09 $\pm$ 0.47	15.88 $\pm$ 1.28	1.95 $\pm$ 0.39	15.88 $\pm$ 1.28	1.95 $\pm$ 0.39	0.00 $\pm$ 0.00	4.61 $\pm$ 1.03	25.40 $\pm$ 1.61	1.70 $\pm$ 0.52	25.40 $\pm$ 1.61	1.70 $\pm$ 0.52	25.40 $\pm$ 1.61
6-A	R. sup.clavicular node A	2.50 $\pm$ 0.15	0.84 $\pm$ 0.30	0.48 $\pm$ 0.02	0.42 $\pm$ 0.15	1.06 $\pm$ 0.07	0.99 $\pm$ 1.15	1.06 $\pm$ 0.07	0.99 $\pm$ 1.15	3.01 $\pm$ 0.13	10.43 $\pm$ 8.64	0.68 $\pm$ 0.03	1.34 $\pm$ 1.10	0.68 $\pm$ 0.03	1.34 $\pm$ 1.10	0.68 $\pm$ 0.03
6-B	R. sup.clavicular node B	1.95 $\pm$ 0.17	1.73 $\pm$ 0.22	0.27 $\pm$ 0.02	0.77 $\pm$ 0.26	0.89 $\pm$ 0.09	0.94 $\pm$ 0.53	0.89 $\pm$ 0.09	0.94 $\pm$ 0.53	2.91 $\pm$ 0.35	14.51 $\pm$ 5.66	0.58 $\pm$ 0.08	N/A	0.58 $\pm$ 0.08	N/A	0.58 $\pm$ 0.08
6-C	R. sup.clavicular node C	1.17 $\pm$ 0.01	0.96 $\pm$ 0.00	0.34 $\pm$ 0.00	0.63 $\pm$ 0.13	0.74 $\pm$ 0.01	1.08 $\pm$ 0.41	0.74 $\pm$ 0.01	1.08 $\pm$ 0.41	2.16 $\pm$ 0.09	13.61 $\pm$ 8.50	0.55 $\pm$ 0.01	N/A	0.55 $\pm$ 0.01	N/A	0.55 $\pm$ 0.01
7-A	R. chest wall sup.	2.15 $\pm$ 0.52	N/A	4.44 $\pm$ 1.07	N/A	3.90 $\pm$ 0.96	N/A	3.90 $\pm$ 0.96	N/A	3.54 $\pm$ 1.16	N/A	2.32 $\pm$ 0.61	N/A	2.32 $\pm$ 0.61	N/A	2.32 $\pm$ 0.61
7-B	R. chest wall inf.	1.58 $\pm$ 0.12	N/A	1.32 $\pm$ 0.09	N/A	1.86 $\pm$ 0.15	N/A	1.86 $\pm$ 0.15	N/A	2.24 $\pm$ 0.17	N/A	1.04 $\pm$ 0.07	N/A	1.04 $\pm$ 0.07	N/A	1.04 $\pm$ 0.07
8-I-A	R. breast at medial	0.91 $\pm$ 0.07	1.31 $\pm$ 0.37	1.12 $\pm$ 0.08	0.50 $\pm$ 0.26	1.04 $\pm$ 0.05	0.38 $\pm$ 0.13	1.04 $\pm$ 0.05	0.38 $\pm$ 0.13	0.88 $\pm$ 0.04	2.59 $\pm$ 0.25	0.88 $\pm$ 0.06	0.85 $\pm$ 0.11	0.88 $\pm$ 0.06	0.85 $\pm$ 0.11	0.88 $\pm$ 0.06
8-I-B	R. breast at lateral	0.64 $\pm$ 0.04	0.75 $\pm$ 0.40	0.74 $\pm$ 0.04	0.37 $\pm$ 0.29	0.57 $\pm$ 0.03	0.87 $\pm$ 0.27	0.57 $\pm$ 0.03	0.87 $\pm$ 0.27	0.91 $\pm$ 0.06	4.13 $\pm$ 3.06	0.58 $\pm$ 0.05	0.33 $\pm$ 0.22	0.58 $\pm$ 0.05	0.33 $\pm$ 0.22	0.58 $\pm$ 0.05
9-1	lymph node	1.78 $\pm$ 0.11	1.05 $\pm$ 0.14	0.20 $\pm$ 0.01	0.51 $\pm$ 0.25	0.70 $\pm$ 0.04	0.65 $\pm$ 0.29	0.70 $\pm$ 0.04	0.65 $\pm$ 0.29	1.49 $\pm$ 0.13	1.26 $\pm$ 0.37	0.40 $\pm$ 0.02	0.85 $\pm$ 0.57	0.40 $\pm$ 0.02	0.85 $\pm$ 0.57	0.40 $\pm$ 0.02
9-2	lymph node	1.82 $\pm$ 0.10	2.21 $\pm$ 1.37	0.20 $\pm$ 0.03	0.38 $\pm$ 0.23	0.75 $\pm$ 0.04	0.30 $\pm$ 0.12	0.75 $\pm$ 0.04	0.30 $\pm$ 0.12	1.53 $\pm$ 0.07	10.33 $\pm$ 3.31	0.47 $\pm$ 0.03	1.14 $\pm$ 0.03	0.47 $\pm$ 0.03	1.14 $\pm$ 0.03	0.47 $\pm$ 0.03
9-3	lymph node	2.85 $\pm$ 0.88	1.10 $\pm$ 0.22	0.52 $\pm$ 0.15	1.13 $\pm$ 0.30	1.58 $\pm$ 0.46	4.10 $\pm$ 1.35	1.58 $\pm$ 0.46	4.10 $\pm$ 1.35	2.91 $\pm$ 0.97	6.21 $\pm$ 1.41	0.97 $\pm$ 0.29	0.71 $\pm$ 0.06	0.97 $\pm$ 0.29	0.71 $\pm$ 0.06	0.97 $\pm$ 0.29
10-I-A	L. chest wall	1.12 $\pm$ 0.11	1.47 $\pm$ 0.73	0.26 $\pm$ 0.02	0.42 $\pm$ 0.18	0.68 $\pm$ 0.04	0.67 $\pm$ 0.44	0.68 $\pm$ 0.04	0.67 $\pm$ 0.44	1.21 $\pm$ 0.07	1.37 $\pm$ 0.64	0.43 $\pm$ 0.03	0.85 $\pm$ 0.61	0.43 $\pm$ 0.03	0.85 $\pm$ 0.61	0.43 $\pm$ 0.03
10-I-B	L. chest wall	0.93 $\pm$ 0.08	1.36 $\pm$ 0.17	0.74 $\pm$ 0.05	0.39 $\pm$ 0.23	0.78 $\pm$ 0.07	0.31 $\pm$ 0.10	0.78 $\pm$ 0.07	0.31 $\pm$ 0.10	0.65 $\pm$ 0.04	0.43 $\pm$ 0.08	0.96 $\pm$ 0.06	0.29 $\pm$ 0.11	0.96 $\pm$ 0.06	0.29 $\pm$ 0.11	0.96 $\pm$ 0.06
10-II	L. ant. chest	0.84 $\pm$ 0.06	1.19 $\pm$ 0.71	0.59 $\pm$ 0.04	0.34 $\pm$ 0.17	0.58 $\pm$ 0.04	0.35 $\pm$ 0.15	0.58 $\pm$ 0.04	0.35 $\pm$ 0.15	0.61 $\pm$ 0.04	0.42 $\pm$ 0.19	0.63 $\pm$ 0.08	0.43 $\pm$ 0.08	0.63 $\pm$ 0.08	0.43 $\pm$ 0.08	0.63 $\pm$ 0.08
11	L. chest wall	0.50 $\pm$ 0.06	1.49 $\pm$ 0.69	0.51 $\pm$ 0.03	0.81 $\pm$ 0.44	1.16 $\pm$ 0.08	0.80 $\pm$ 0.28	1.16 $\pm$ 0.08	0.80 $\pm$ 0.28	0.87 $\pm$ 0.08	5.07 $\pm$ 2.96	0.59 $\pm$ 0.04	0.86 $\pm$ 0.63	0.59 $\pm$ 0.04	0.86 $\pm$ 0.63	0.59 $\pm$ 0.04
12	R. chest wall	1.37 $\pm$ 0.08	1.24 $\pm$ 0.46	0.50 $\pm$ 0.03	0.57 $\pm$ 0.12	0.79 $\pm$ 0.04	0.46 $\pm$ 0.26	0.79 $\pm$ 0.04	0.46 $\pm$ 0.26	1.78 $\pm$ 0.14	1.01 $\pm$ 0.43	0.39 $\pm$ 0.02	0.07 $\pm$ 0.02	0.39 $\pm$ 0.02	0.07 $\pm$ 0.02	0.39 $\pm$ 0.02
13	R. chest wall	0.61 $\pm$ 0.07	0.75 $\pm$ 0.32	0.31 $\pm$ 0.02	0.52 $\pm$ 0.30	0.48 $\pm$ 0.05	0.58 $\pm$ 0.12	0.48 $\pm$ 0.05	0.58 $\pm$ 0.12	1.02 $\pm$ 0.07	4.77 $\pm$ 2.60	0.23 $\pm$ 0.02	0.40 $\pm$ 0.25	0.23 $\pm$ 0.02	0.40 $\pm$ 0.25	0.23 $\pm$ 0.02
14	L. breast tissue	0.52 $\pm$ 0.02	1.40 $\pm$ 0.70	0.27 $\pm$ 0.02	0.76 $\pm$ 0.27	0.51 $\pm$ 0.02	0.93 $\pm$ 0.20	0.51 $\pm$ 0.02	0.93 $\pm$ 0.20	1.90 $\pm$ 0.07	9.56 $\pm$ 7.47	0.31 $\pm$ 0.01	1.45 $\pm$ 0.48	0.31 $\pm$ 0.01	1.45 $\pm$ 0.48	0.31 $\pm$ 0.01
15-I-A	R. chest wall sup.	3.56 $\pm$ 0.40	0.97 $\pm$ 0.80	0.71 $\pm$ 0.04	1.49 $\pm$ 1.44	2.07 $\pm$ 0.11	1.66 $\pm$ 1.24	2.07 $\pm$ 0.11	1.66 $\pm$ 1.24	5.43 $\pm$ 0.27	26.60 $\pm$ 30.51	0.68 $\pm$ 0.05	1.53 $\pm$ 1.07	0.68 $\pm$ 0.05	1.53 $\pm$ 1.07	0.68 $\pm$ 0.05
15-I-B	R. chest wall inf.	1.48 $\pm$ 0.13	1.51 $\pm$ 0.60	0.93 $\pm$ 0.06	1.64 $\pm$ 0.42	2.51 $\pm$ 0.10	1.13 $\pm$ 0.39	2.51 $\pm$ 0.10	1.13 $\pm$ 0.39	2.44 $\pm$ 0.12	14.86 $\pm$ 7.67	1.14 $\pm$ 0.09	0.68 $\pm$ 0.24	1.14 $\pm$ 0.09	0.68 $\pm$ 0.24	1.14 $\pm$ 0.09
15-II-A	R. upper back superior	1.56 $\pm$ 0.13	1.09 $\pm$ 0.49	0.81 $\pm$ 0.04	1.30 $\pm$ 0.89	1.59 $\pm$ 0.14	1.80 $\pm$ 0.68	1.59 $\pm$ 0.14	1.80 $\pm$ 0.68	3.11 $\pm$ 0.25	11.01 $\pm$ 3.65	0.68 $\pm$ 0.03	0.05 $\pm$ 0.05	0.68 $\pm$ 0.03	0.05 $\pm$ 0.05	0.68 $\pm$ 0.03
15-II-B	R. upper back inferior	1.80 $\pm$ 0.23	1.96 $\pm$ 0.64	0.69 $\pm$ 0.08	0.98 $\pm$ 0.32	1.58 $\pm$ 0.16	1.03 $\pm$ 0.36	1.58 $\pm$ 0.16	1.03 $\pm$ 0.36	3.29 $\pm$ 0.32	23.13 $\pm$ 12.02	0.63 $\pm$ 0.06	0.99 $\pm$ 0.79	0.63 $\pm$ 0.06	0.99 $\pm$ 0.79	0.63 $\pm$ 0.06
16-A	L. breast inf.	1.85 $\pm$ 0.12	N/A	0.51 $\pm$ 0.03	N/A	2.51 $\pm$ 0.12	N/A	2.51 $\pm$ 0.12	N/A	2.55 $\pm$ 0.12	N/A	0.63 $\pm$ 0.03	N/A	0.63 $\pm$ 0.03	N/A	0.63 $\pm$ 0.03
16-B	L. sup.clavicular lymph node	4.25 $\pm$ 0.45	N/A	0.76 $\pm$ 0.03	N/A	6.56 $\pm$ 0.22	N/A	6.56 $\pm$ 0.22	N/A	5.76 $\pm$ 0.53	N/A	1.71 $\pm$ 0.12	N/A	5.76 $\pm$ 0.53	1.71 $\pm$ 0.12	N/A
16-C	L. axillary lymph node	1.00 $\pm$ 0.05	N/A	0.23 $\pm$ 0.02	N/A	1.08 $\pm$ 0.07	N/A	1.08 $\pm$ 0.07	N/A	1.45 $\pm$ 0.11	N/A	0.27 $\pm$ 0.01	N/A	1.45 $\pm$ 0.11	0.27 $\pm$ 0.01	N/A
17	L. breast tissue	0.22 $\pm$ 0.05	N/A	0.92 $\pm$ 0.18	N/A	0.99 $\pm$ 0.21	N/A	0.99 $\pm$ 0.21	N/A	0.69 $\pm$ 0.22	N/A	3.61 $\pm$ 0.75	N/A	0.69 $\pm$ 0.22	3.61 $\pm$ 0.75	N/A

^aAll values standardized to normal tissue.^bCapital letters after the patient number indicates that multiple biopsies were obtained from different sites at the same time.^cRoman numeral after the patient number indicates: I- first biopsy, II, second biopsy 16 weeks later.^dSecond arabic number after the patient number indicates different segments of the same surgical sample.

N/A, not available.

Fig. 2. Comparison of real-time (TaqMan[®]) and endpoint (GeneScan[®]) RT-PCR. Each figure (A–E) represents a comparison between the two different methods for each gene. Values plotted are the log of the values in Table 1 for TaqMan[®] minus the log of the values in Table 1 for GeneScan[®], plotted against the sum of the same two logs. The dotted line represents the first SD, and the dashed line represents the mean difference between the two methods. For low signal, the dCK (A) measurements appear to have bias, which corrects to near 0 for higher expression ratios. For all other genes, there is no suggestion of a magnitude-created bias between the two methods (B–E, corresponding to dCDA, RRM1, RRM2, and *ERCC1*).

these two genes, it demonstrates the importance of efficiency-adjusted real-time PCR in obtaining accurate quantification of gene expression with high precision. It may also be a critical factor in finding a statistically significant result for the role of the studied genes in the tumors of patients treated with gemcitabine and cisplatin.

Our data also demonstrate the potential for intra-patient variation in mRNA levels. Multiple biopsies from different areas from the same patient may have different gene expression levels. For example, if we examine the real-time PCR data for patient 4 (4-I-A to 4-I-B), the RRM2 and dCK results showed fourfold and >10-fold differences, respectively. Even the segments of the same surgical sample (patients 5 and 9) showed

considerable variability—often more than a fivefold difference. This suggests the importance of the development of computer-aided laser capture microdissection, to “pick” only cancer cells, prior to gene expression analysis. However, this is still a major technical challenge, since it is not routinely applicable to “fresh” frozen tumor biopsy samples.

Our data suggest that the real-time PCR (TaqMan[®]) method is more accurate than the GeneScan[®], possibly because the PCR products are relatively short fragments in the 80–200 bp range, which increase the efficiency of amplification. For the GeneScan[®] analysis, the length of the amplicon starts from 130–150 bp to exclude the fluorescence primer dimers from the gel, and the size goes up to 500 bp. In real-time PCR, the specificity is

improved with the hybridization step, when the labeled probe anneals to the amplicons, and the released fluorescence signal is measured. The real-time PCR method gives results over a 5 log range without any additional dilution of the cDNA; for the GeneScan[®] analysis, we must dilute the cDNA serially to find the linear range of the amplification curve. The ABI 7900 HT DNA sequence detection instrument can analyze up to 100 samples on one plate (384 well) or fewer samples for two or more genes at the same time. On the ABI 377 DNA sequencer, we can examine eight samples for six dilutions of each on the same gel. The real-time PCR method required 2 weeks to analyze the samples, which is more than 10 times less than the GeneScan[®] method. The GeneScan[®] method is useful for research purposes, and for laboratories that have DNA sequencers but no Taqman[®] instrument. Real-time PCR with ABI 7700 or 7900HT DNA sequence detection system (TaqMan[®]) is affordable for reference laboratories, research institutes, and large clinical laboratories. At present, however, a comparison of real-time PCR data from different laboratories is difficult because of the lack of universal standards for each gene of interest. For absolute quantification purposes, and to calculate absolute copy number, the cloned gene of interest must be available in plasmid form (31).

In conclusion, a comparison of gene expression data from breast cancer biopsy samples with two quantitative RT-PCR techniques shows that the precision of the TaqMan[®] method is superior to the GeneScan[®] analysis, as shown in Table 1. Furthermore, the GeneScan[®] technique is more labor-intensive, and the error rate may be increased because of the large number of analytical steps involved and the inability to run large numbers of samples simultaneously. The large coefficients of variation in the GeneScan[®] method obfuscate important biological signals, such as a correlation between dCK and ERCC1 expression levels ($P=0.42$ with GeneScan[®], and $P=0.03$ with efficiency-adjusted TaqMan[®]). The real-time TaqMan[®] PCR method, when adjusted for differences in efficiency, provides a more rapid and precise quantification, and is preferable in this setting.

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