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An Improved Method for Semiquantification of Gene Amplification from Archival Material

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A differential PCR-based assay is presented that increases the accuracy of quantification of *C-erbB-2* gene-copy number in DNA extracted from archival tumors. The *C-erbB-2* gene is amplified in a high percentage of human adenocarcinomas arising at numerous sites, including breast, lung, and stomach. A number of studies have correlated *C-erbB-2* with poor prognosis. Gene copy number may be relevant in identifying patients with different clinical outcomes. In this study a target gene and a single copy reference gene were coamplified in the same reaction tube. The level of target gene amplification was reflected by the ratio of the two resulting PCR products. Cell lines exhibiting variable copies ranging from 1 to >8 of the *C-erbB-2* gene were used as quality controls. This technique can reliably show a single copy difference between cell lines and can be used to semiquantitatively estimate gene copy number in DNA extracted from archival paraffin-embedded samples.

Gene amplification is an important event in the induction and/or progression of many tumors. As more data become available, the clinical consequences of those genotypic changes become evident. In neuroblastoma the copy number of the N-*myc* gene needs to be assessed accurately to predict severity of disease and, therefore, treatment response.[1,2] In consequence, the need for accurate reproducible and quantitative data using molecular biology techniques needs to parallel that seen with other routine biochemical parameters.

An increasing number of articles have appeared in the literature that have semiquantitatively estimated gene copy number and correlated this to known clinical parameters. The variations seen between these results can make interpretation difficult. How accurate gene copy number needs to be is open to debate, and whether semiquantitative data give as much prognostic information over more accurate methods remains to be shown.

Southern blotting is the most commonly used technique for the analysis of gene copy number. Improved methods for Southern blotting to increase simplicity and accuracy are now being used. This, in some ways, attempts to overcome the problems associated with inaccurate DNA loading, tissue degradation, and differing relative intensities, sizes, and, therefore, efficiencies of probes.[3] Good-quality, high-molecular-weight DNA is required for Southern blots, requiring extraction from fresh tissue. Often, however, fresh tissue is not available in sufficient quantity for analysis; therefore a method that would allow quantitation of gene copy number from small amounts of fixed tissues would be beneficial. In addition, tumor samples often contain high proportions of stromal contamination. This can be corrected using microdissection of tumor tissue.

The detection of *C-erbB-2* gene amplification in archival tissue by differential PCR has been described.[4,5] A semiquantitative estimation of results was proposed using the cell line SKBR3 as a quality control. This technique claimed to be capable of detecting amplification in specific target genes as low as two-fold. The differential PCR involves the coamplification of a single-copy reference gene and target gene of unknown amplification within the same reaction tube. Following completion of the reaction, the relative ratio of intensity of target gene to single-copy reference gene can be calculated. These ratios can then be analyzed against those of the quality controls, and an estimation of gene copy number can therefore be made. We have extended this technique to include other cell lines with a known copy number of *C-erbB-2*, thereby improving the accuracy of quantification.

The aim of this study was to assess the intra-assay variation of results for *C-erbB-2* amplification from a DNA pool in a number of cell lines known to harbor different copies of the *C-erbB-2* gene. This would enable us to determine the consistency of our results from the same 10 μm section and, therefore, the efficiency and reproducibility of our measurements. Also, by increasing the number of cell lines used as quality controls, we aimed to improve on the semiquantitative methods already available for *C-erbB-2* gene copy number estimation.

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MATERIALS AND METHODS

Cell Lines

The mammary carcinoma cell lines SKBR3, MDA-MB-361, MDA-MB-453, and BT20 were acquired from the American Type Culture Collection. The N87 gastric cell line was provided by Dr. C.R. King (Molecular Oncology, Inc., Gaithersburg, Maryland 20878).

C-erbB-2 gene amplification in the cell lines used in this study had been estimated by Southern hybridization and dot–blot analysis: N87, >8 copies;[6] SKBR3, 4–8 copies;[7] MDA-MB-361, 2–4 copies;[7] MDA-MB-453, 2 copies;[7] and BT20, 1 copy.[7]

Cell Culture

All cells were cultured using the appropriate media: 10% heat-inactivated fetal calf serum (MDA-MB-361, 15%), 100 U/ml of penicillin, and 100 μg/ml of streptomycin (GIBCO). Cells were grown in 5% CO_2 in air at 37°C in a humidified incubator. Media used for culture were Leibovitz's L-15 (MDA-MB-453 and MDA-MB-361), McCoy's 5a medium (SKBR3), RPMI 1640 (N87) and Eagle's minimal essential medium with nonessential amino acids, and Earle's BSS (BT20). Cells were grown to confluence and harvested using a cell scraper from a 175-cm^2 flask into sterile PBS. The cell suspension was centrifuged at 900*g* for 10 min. Cell pellets were resuspended in 1–2 drops of 1% agarose, fixed in 10% phosphate-buffered formalin for 48 hr, and then embedded in paraffin wax. Sections of this fixed material (10 μm) were processed to provide controls for PCR analysis.

DNA Extraction

A 10-μm section from each block was taken and placed in a sterile microcentrifuge tube. The blade was changed between blocks, and the work surfaces were cleaned continually with xylene to prevent cross-contamination. The tissue was dewaxed by adding 1 ml of xylene and then pelleted by centrifuging at 13,000*g* for 15 min. This step was repeated. The xylene was decanted off between steps as waste. The tissue was then washed twice using 100% alcohol and pelleted, and the alcohol was decanted. The Eppendorf tube was covered with a piece of perforated parafilm, and the pelleted tissue was dried in a 300-position vacuum centrifuge at 37°C.[8] Care was taken to ensure a minimum of five spaces between samples on the rotor to reduce the risk of any cross-contamination of the samples.

Tissue DNA was extracted in 200–300 μl of digestion buffer (50 mM Tris at pH 8.5, 1 mM EDTA, 0.5% Tween 20, and 500 μg/ml of proteinase K) (Boehringer Mannheim) over 5 days at 37°C. The proteinase K was then inactivated by boiling the sample at 94°C for 8 min. Any remaining tissue was pelleted and the sample stored at −20°C.[8–10]

PCR

Oligonucleotides

Oligonucleotides were selected for the target gene *C-erbB-2* generating a 98-bp fragment and for reference genes as defined by Neubauer et al.[5] Sequences of the oligonucleotides used as PCR primers in this study are shown in Table 1.

PCR Conditions

Competitive PCRs were carried out in a total volume of 50 μl. For individual amplimer pairings, see Analytical Algorithm, below. Prior to PCR, 0.5 μM oligonucleotides each (4 in total) were combined with 10 μl of archival DNA in a final volume of 25 μl, heated to 95°C for 10 min, and cooled rapidly to 4°C on a thermocycler (Biometra). Samples were pulse-centrifuged, and the tubes were placed on ice. Twenty-five microliters of Mastermix comprising 2 mM each dATP, dTTT, and dGTP, 0.5 mM dCTP (Pharmacia), 1 μCi of either [α-^{32}P]dCTP or [α-^{33}P]dCTP, 1 unit of *Taq* DNA polymerase, and 10× reaction buffer (Boehringer Mannheim) was added. The reaction mixture was overlaid with 2 drops of mineral oil. PCR conditions were 94°C for 1 min, 51°C for 1 min, and 72°C for 1 min for cycles 1–24 and 94°C for 1 min, 51°C for 1 min, and 72°C for 11 min for cycle 25, followed by incubation at 60°C for 30 min.

The radiolabeled PCR products generated were resolved by electrophoresis on a 10% polyacrylamide gel. The gel was transferred onto Whatman 3MM paper and dried under vacuum for 50 min. The products were visualized by autoradiography with Min-R PE (Kodak) film at −70°C.

Analytical Algorithm

To analyze gene copy number for *C-erbB-2*, an analytical algorithm as described by Neubauer et al.[5] was used (Fig. 1). In this algorithm a series of competitive PCRs are performed to confirm gene amplification and to exclude spurious results.

Reaction 1: γ IFN 150 and γ IFN 82 Competitive PCR

DNA extracted from archival material is known to be fragmented.[8–10] This reaction was carried out to confirm that extracted DNA would be of sufficient quality to allow further analysis. In any competitive PCR, preferential amplification of shorter PCR products occurs. Highly fragmented DNA would contain significantly more targets for the γ-interferon (IFN) 82 primers than the γ-IFN 150 primers and could also give spurious results in subsequent competitive PCRs.

TABLE 1 Sequences of Oligonucleotides Used in This Study

Amplimer size	5′ Primer	3′ Primer
C-erbB-2 98 bp 2122–2219[11]	5′-CCTCTGACGTCCATCATCTC-3′	5′-ATCTTCTGCTGCCGTCGCT-3′
γ-IFN 150 bp 4582–4731[12]	5′-TCTTTTCTTTCCCGATAGGT-3′	5′-CTGGGATGCTCTTCGACCT C-3′
γ-IFN 85 bp 4647–4731[12]	5′-AGTGATGGCTGAACTGTCGC-3′	5′-CTGGGATGCTCTTCGACCT C-3′
γ-IFN 82 bp 2012–2093[12]	5′-GCAGAGCCAAATTGTCTCCT-3′	5′-GGTCTCCACACTCTTTTGC A-3′
n-Ras 110 bp 1–111[13]	5′-ATGACTGAGTACAAACTGGT-3′	5′-CTCTATGGTGGGATCATAT T-3′

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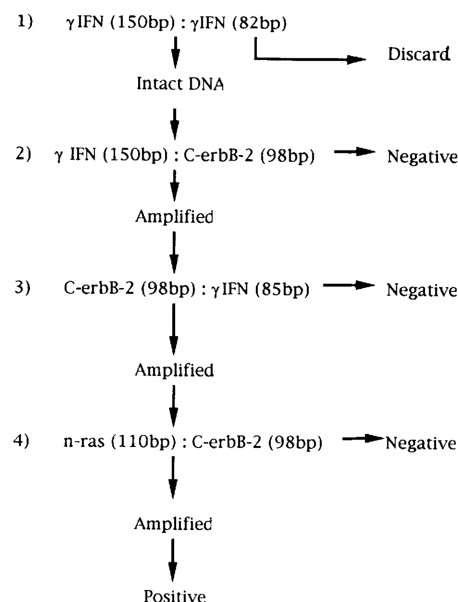

FIGURE 1 Analytical algorithm used to analyze gene copy number for *C-erbB-2*.

The reaction generates two different-sized fragments, each from a different exon of the γ-IFN gene. Differences in signal intensity of the two bands could not be attributed to gene amplification but would result from DNA fragmentation. The GC content, and, therefore, the labeling index, differ significantly (40% vs. 23%, γ-IFN 150 vs. γ-IFN 82) for these products; after correction for this factor, DNA exhibiting a ratio of γ-IFN 82 to γ-IFN 150 of >3:1 was excluded from further analysis. Neubauer et al.[5] found DNase-digested genomic DNA resulting in γ-IFN 150 versus γ-IFN 82 ratios of 4 still able to be analyzed for *C-erbB-2* gene amplification using the *C-erbB-2* 98-bp to γ-IFN 85 ratio); however, results were more variable when employing DNA that was further degraded by formalin fixation.

Reactions 2 and 3: γ-IFN 150 and C-erbB-2 *98 and γ-IFN 85 and* C-erbB-2 *98 Competitive PCR*

These reactions provide the screen for gene amplification of the target *C-erbB-2* using γ-IFN as a reference gene. The assumption was that γ-IFN was not amplified in any of the tissue samples. Reaction 2 screened *C-erbB-2* amplification against the larger amplimer γ-IFN 150. Potentially, this reaction may produce false-positive results should the smaller *C-erbB-2* PCR product undergo a more efficient process of amplification than the larger γ-IFN. Reaction 3 was performed to eliminate this possibility. The γ-IFN 85 amplimer amplifies the same exon of the γ-IFN gene, using the same 3′ primer as the γ-IFN 150 reaction. This reduces variation between these reactions that might otherwise complicate the analysis. The combination of these two reactions provides a stringent screening procedure for amplification of *C-erbB-2* in relation to the reference gene γ-IFN.

Reaction 4: n-RAS 110 vs. C-erbB-2 *98 competitive PCR*

Genetic instability is a function of many tumors. The potential for chromosomal deletion or replication involving the γ-IFN reference gene used above therefore exists. To exclude the possibility that deletion or replication of the γ-IFN gene might bias results in some aneuploid tumors, a further reference gene from a different chromosome was used. Competitive PCR with n-RAS 110 versus *C-erbB-2* 98 was performed on all amplified tumors and a proportion of nonamplified tumors.

Only tumors that showed amplification in all three test reactions (reactions 2, 3, and 4) were scored as amplified.

Estimation of Copy Number

Quantitation of Copy Number

Copy number was estimated by densitometric analysis of the reaction products from PCR 3. The densitometric ratio of *C-erbB-2* 98 to γ-IFN 85 was calculated for all samples. All densitometry was performed by one observer using a Joyce Loebel Digitizer and a Sony CCD video camera module. To allow quantification of signal, a linear relationship of optical density versus concentration of isotope (nCi/g) for the autoradiograph was established. ^{14}C standards (Amersham) were used to give an estimate of the linear range for quantification and an upper limit of optical density for the film. The autoradiographic exposure times were varied between 1 and 4 hr to ensure that saturation of the film did not occur.

Use of Cell Lines as External Standards

Although the densitometric technique described above could provide data on the relative degree of gene amplification between tumor samples, external standards of known amplification would be required to allow a degree of quantitative analysis to be performed. To this end, cell lines were selected with increasing copy numbers of the *C-erbB-2* gene and included in each PCR assay as external standards. By analyzing these samples in an identical manner, it would be possible to produce a quantitative estimate of the degree of *C-erbB-2* gene amplification for each individual tumor sample. These external standards were also used to assess the reproducibility, sensitivity, and accuracy of this method in multiple experiments.

RESULTS

DNA Extraction

To assess the efficiency of DNA extraction from tissue sections, samples were incubated for either 24 or 120 hr (5 days) prior to performance of PCR for a 137-bp fragment of h-γ-actin on 10-μl aliquots of extracted DNA. Insufficient DNA was extracted over 24 hr to allow consistent detection of γ-actin using the PCR protocol described above. Following extraction of DNA over 5 days, all samples showed strong signals for γ-actin (data not shown).

Cycle Number

The ratios of target gene to reference gene are relatively insensitive to cycle number.[14] This was confirmed by taking DNA from the BT20 cell line and performing differential PCR for a variable number of cycles (Fig. 2). The relative ratios for the products of PCR reaction 3 (*C-erbB-2* and γ-IFN 85) did not alter significantly with cycle numbers between 25 and 40 (see Table 2). For each cycle number, six PCR reactions have been performed. The relative ratios for the products are shown along with mean ratios and 95% confidence limits.

Ratio of *C-erbB-2* 98 to γ-IFN 85 vs. Copy Number

The ratio of *C-erbB-2* 98 to γ-IFN 85 reaction product to gene copy number was established using the cell lines described (see Materials and Methods). A linear relationship between these parameters was

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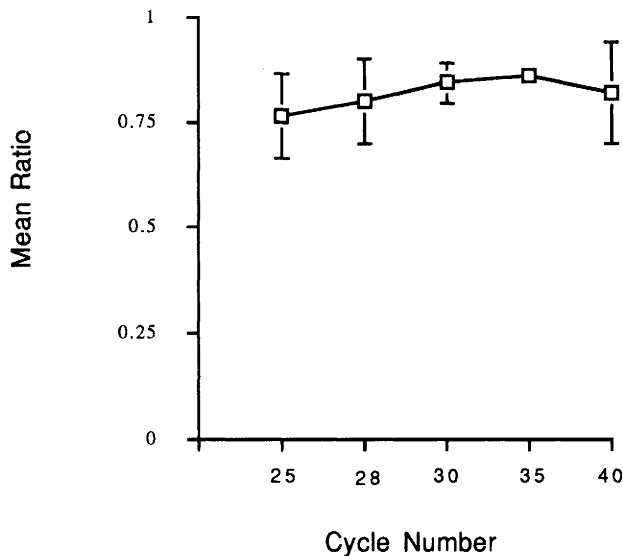

FIGURE 2 Evidence showing that the ratio (□) of target and reference gene are preserved through a range of PCR cycles.

observed when multiple samples from each cell line were analyzed (Fig. 3).

Sensitivity

To determine the sensitivity of the assay system in detecting differences in copy number, samples of the cell lines BT-20 (1 copy *C-erbB-2*), MDA-MB-453 (2 copies), MDA-MB-361 (2–4 copies), SKBR3 (4–8 copies), and N87 (>8 copies) were included in the assays, using both ^{32}P and ^{33}P and performed over a 3-day period. In all cases, the mean±2 S.D. were estimated to provide the 95% confidence intervals for each of the cell lines (and, therefore, the relative copy number for *C-erbB-2*). These results are shown in Table 3.

Samples with ratios for PCR reaction 3 that fell within the range of one of the above cell lines were assigned a copy number identical to the cell line, those with ratios falling between the ranges of two cell lines were assigned a copy number between the copy numbers of the two cell lines. For example, in assay 1 above, a sample with a ratio of 1.24 would fall within the range found for MDA-MB-453 and would be assigned a copy number of 2, whereas a sample with a ratio of 1.6 would fall between the ratios of MDA-MB-453 and SKBR3 and be assigned a copy number of 2–4. By increasing the number of cell lines as quality controls, a more accurate assessment of *C-erb-B2* copy number can be established.

TABLE 2 Relative Ratios of PCR Products for Reaction 3

	Cycles				
	25	28	30	35	40
	0.68	0.72	0.87	0.85	0.78
	0.75	0.87	0.83	0.86	0.92
	0.8	0.82	0.84	0.87	0.84
	0.75	0.83	0.87	0.86	0.85
	0.83	0.78	0.85	0.88	0.81
	0.78	0.78	0.81	0.86	0.74
Mean	0.765	0.8	0.845	0.86	0.82
S.D.	0.05	0.05	0.02	0.01	0.06
Mean + 2 S.D.	0.86	0.9	0.89	0.88	0.94
Mean − 2 S.D.	0.66	0.69	0.79	0.84	0.69
Variation (%)	6.75	6.47	2.78	1.20	7.55

PCR reaction 3 (*C-erbB-2* and γ-IFN 85). This did not alter significantly between 25 and 40 cycles.

Intra-assay Variation

Intra-assay variation was assessed using multiple samples from each cell line in single assays. The standard deviation from the mean was calculated as a percentage of the mean ratio for all samples and the intra-assay variation assessed using this percentage. The results for each of the cell lines used are shown in Table 4.

Figure 4, A and B, demonstrates the intra-assay variation for the cell lines BT20, MDA-MB-453, and SKBR3 using [^{32}P]dCTP. Figure 4C demonstrates the intra-assay variation for four cell lines, BT20, MDA-MB-453 SKBR3 and N87, using [^{33}P]dCTP.

Inter-assay Variation

Inter-assay variation was also estimated for each cell line. The mean ratios, standard deviations from the mean, and inter-assay variations for each cell line are shown in Table 5.

DISCUSSION

Producing quantitative data using PCR is complicated by the exponential nature of the reaction. Small changes in amplification efficiency can result in dramatic changes in product yields. In addition, the amount of product-generated plateaus during the latter stages of the reaction as a result of the consumption of necessary components and the generation of enzyme inhibitors. These characteristics of PCR can obscure differences in the initial amounts of target sequences during the course of amplification. These problems can be overcome with the use of an internal control such as, γ-IFN.[15]

Although coamplification of a control gene can correct for variation in tube-to-tube amplification efficiency, these data must be obtained during the exponential phase of the reaction. Obtaining data during the exponential phase of the reaction can be particularly difficult to achieve if the control gene is expressed at a different level than the target gene. In addition, multiple sets of primers in the same reaction vessel may interfere with either target, reference gene, or both. Therefore, the use of quality controls as external reference points becomes all the more important.

A further requirement for quality con-

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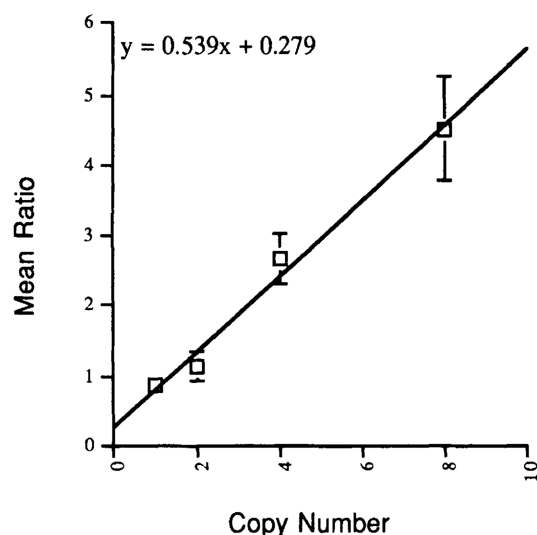

FIGURE 3 A standard curve of ratio (□); *C-erbB-2*/INF 85 of intensity of target and reference bands against copy number for the four cell lines BT20, MDA-MB-453, SKBR3, and N87. Data generated from [^{33}P]dCTP (day 3).

TABLE 3 Sensitivity of Competitive PCR for *C-erbB-2* Copy Number

Assay	Lower limit (95% CI)[a]	Mean (±2 S.D.)[b]	Upper limit (95% CI)
Day 1 using ^{32}P			
BT20[c]	0.828	0.995 ± 0.166	1.16
MDA453[d]	1.169	1.318 ± 0.148	1.467
SKBR3[e]	1.949	2.31 ± 0.36	2.669
Day 1 using ^{33}P			
MDA453	0.655	0.772 ± 0.118	0.899
MDA361[f]	1.106	1.168 ± 0.062	1.23
SKBR3	1.037	1.237 ± 0.2	1.437
N87[g]	2.141	2.493 ± 0.352	2.845
Day 2 using ^{32}P			
BT20	0.663	0.736 ± 0.072	0.809
MDA453	0.822	0.932 ± 0.11	1.042
MDA361	1.14	1.348 ± 0.208	1.556
Day 2 using ^{33}P			
MDA361	1.35	1.535 ± 0.18	1.71
SKBR3	1.655	2.004 ± 0.35	2.353
N87	2.783	3.317 ± 0.534	3.851
Day 3 using ^{32}P			
SKBR3	1.272	1.691 ± 0.418	2.109
N87	1.489	2.683 ± 1.194	3.876
Day 3 using ^{33}P			
BT20	0.788	0.863 ± 0.076	0.939
MDA453	0.930	1.138 ± 0.208	1.346
SKBR3	2.315	2.683 ± 0.368	3.051
N87	3.774	4.514 ± 0.74	5.254

[a](CI) Confidence interval.
[b]S.D. Standard deviation.
[c](BT20) 1 copy.
[d](MDA453) 2 copies.
[e](SKBR3) 4–8 copies.
[f](N87) >8 copies.

trols when calculating gene copy number becomes apparent when reviewing the mathematical analysis of differential PCR. The ratio of the PCR products reflects the ratio of the original copy numbers of the reference and target genes when the efficiencies of primers for both sets of genes become equal. This can be achieved by varying the concentrations of reference and target gene primers and by changing the DNA segment to be amplified. Therefore, when establishing a ratio of target to reference gene for the cell lines and assuming that the PCR reaction has generated PCR product with the same efficiency for tumor DNA, a direct semiquantitative comparison can be made between tumor DNA and cell line quality control.[14] The use of a second reference gene would overcome a potential problem with nondiploid tumors and the possibility of underestimating gene amplification.[5]

Some studies have performed differential PCR directly on the tissue section.[4,5] DNA extracted from these sections, however, would allow us to perform multiple analyses and also to investigate other molecular markers without the requirement to take multiple sections from a block.

Proteinase K has been shown to stablize over a 5-day extraction period, and digestion of archival sections provided a better quality and quantity of DNA over boiling or incubation with SDS.[9] It is important to show consistently that this technique can demonstrate a difference between cell lines used as controls and that the intra-assay variation remains within acceptable limits.

The *C-erbB-2* 98-bp product approached saturation with increasing copy number, and the use of optical density for quantification became less reliable. It was not possible to dilute our PCR products, as this had the undesired effect of decreasing the intensity of our reference band, making densitometric interpretation difficult.

We have been unable to show with this data strict control of our inter-assay variations, which may reflect the extreme sensitivity of this reaction. Our intra-assay data remain within acceptable limits suggesting that DNA extracted from a single 10-μm section may be suitable for multiple analyses. For accurate quality control data to be generated when analyzing tumor DNA, we suggest that quality controls are run in triplicate.

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TABLE 4 Intra-assay Variation for Ratio of *C-erbB-2*/γ-IFN 85 for Control Cell Lines

Cell lines (copy number)	BT20 (1)	MDA453[a] (2)	MDA361[a] (2–4)	SKBR3 (4–8)	N87 (>8)
Intra-assay variation for day 1					
using ^{32}P	8.3% (18)	5.6% (20)		7.8% (11)	
using ^{33}P		7.6% (10)	2.6% (10)	8% (10)	7% (10)
Intra-assay variation for day 2					
using ^{32}P	4.9% (10)	6% (10)	7.7% (17)		
using ^{33}P			6% (9)	8.7% (10)	8% (9)
Intra-assay variation for day 3					
using ^{32}P				12% (8)	22% (8)[b]
using ^{33}P	4.4% (5)	9.1% (5)		7% (5)	8% (5)
Mean[c]	5.8 ± 1.6	7.1 ± 2.5	5.4 ± 2.5	8.7 ± 1.9	7.75 ± 0.5

[a](M453) MDA-MB-453; (M361) MDA-MB-361. Numbers in parentheses refer to number of samples per cell line for each assay.
[b]Not included for calculations of mean because of overlap in sensitivity between SKBR3 and N87.
[c](Mean) Mean intraassay variation for each cell line.

TABLE 5 Interassay Variation for Each Cell Line

Cell line	Mean	S.D.	Variation (%)
BT-20	5.86	2.12	36
MDA-MB-453	7.07	1.6	22
MDA-MB-361	5.36	2.57	47
SKBR3	8.7	1.94	22
N87	7.75	0.57	6

From our data, the intra-assay variations for those cell lines exhibiting up to 8 copies with ^{32}P remained within acceptable limits (3–12%). This was not the case for N87, and the intra-assay variation broadened. This probably reflected the limit of sensitivity for the X-ray film. Preflashing the film produced little improvement in the range of intra-assay variation (data not shown). Therefore, by using ^{32}P we were unable to reliably show a difference between N87 exhibiting >8 copies and SKBR3 with 4–8 copies, as the relative ranges for the two standard deviations overlapped. The film saturation problem, however, was overcome by using a lower energy isotope. [α-^{33}P]dCTP was substituted at the same concentration and was not only found to give tighter bands but, more importantly, could consistently show a difference between all the cell lines.

To date, the tumor DNA that we have analyzed from transitional carcinomas of the bladder has not exceeded a copy number of 8.[16] As stated, however, densitometric analysis may be limited using ^{32}P by our X-ray film, but by using a lower energy isotope such as ^{33}P, an extended range for quantification may be gained. A gene copy number possibly in excess of our quality controls may need to be determined by Cherenkoff counting.

Slamon and colleagues have postulated that disease behavior differs for breast tumors with *C-erbB-2* gene copy numbers of 1, 2–5, 5–20, and >20. To date, *C-erbB-2* amplification and protein overexpression correlate strongly with node-positive breast carcinoma and overall reduction in disease-free interval.[17–20] Therefore, use of these assays as prognostic indicators in conjunction with other parameters makes accurate assessment of gene copy number paramount, and the use of differential PCR will allow us to perform quite extensive retrospective studies. Whether quality controls are taken from already established cell lines or are constructed using varying copies of a target gene, a standard curve can be constructed, thereby providing semiquantitative results.

We could not assume that our cell line DNA would give us the same range of ratios each day, and, therefore, when assessing tumor DNA, one would need to run the quality controls with each reaction.

This study has shown, for the first time, that the use of external standards, such as cell lines with known copy number, can facilitate the accurate assessment of gene copy number by differential PCR. Intra-assay variations of <10% reflect the high degree of reproducibilty of this technique. The combination of differential PCR with appropriate quality controls therefore represents a powerful tool for the assessment of gene copy

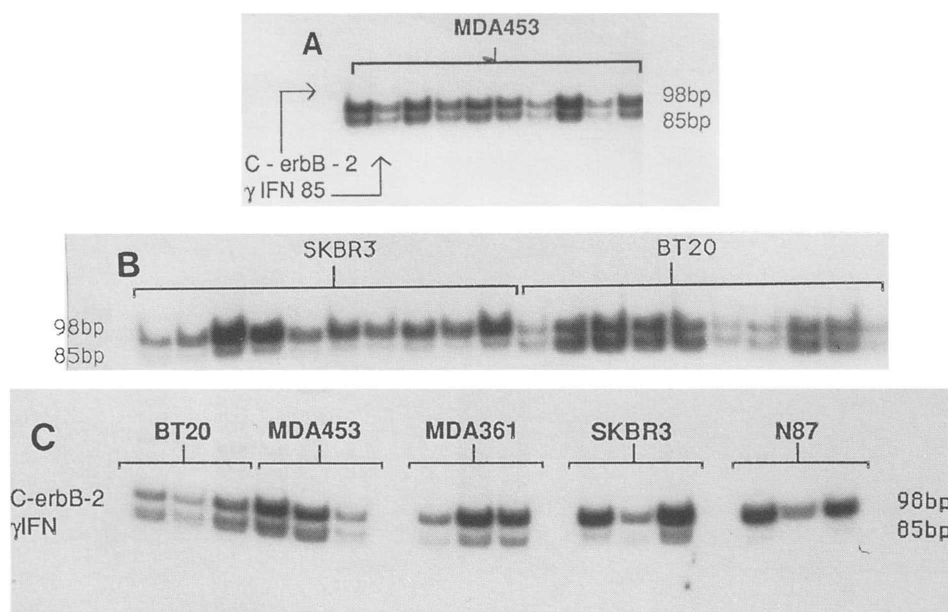

FIGURE 4 (*A*) Intra-assay variation for cell line MDA453, using [^{32}P]dCTP; (*B*) Intra-assay variation for two cell lines, BT20 and SKBR3, using [^{32}P]dCTP; (*C*) Intra-assay variation for four cell lines, BT20, MDA453, SKBR3, and N87, using [^{33}P]dCTP.

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number in both fresh and archival materials.

ACKNOWLEDGMENTS

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REFERENCES

1. Seeger, R.C., G.M. Brodeur, and H. Sather. 1985. Association of multiple copies of the N-myc oncogene with rapid progression of neuroblastoma. *N. Engl. J. Med.* **313:** 1111–1117.
2. Brodeur, G.M., R.C. Seeger, M. Schwab, H.E. Varmus, and J.M. Bishop. 1984. Amplification of N-myc in untreated neuroblastomas correlates with advanced disease stage. *Science* **224:** 1121–1124.
3. Farkas, D.H., L.W. McMahon, P. Cai, and R.M. Umek. 1993. Improved quantitation of HER-2/neu gene copy number in breast tumor-derived DNA samples. *Anat. Pathol.* **100:** 444–450.
4. Frye, R.A., C.C. Benz, and E. Liu. 1989. Detection of amplified oncogenes by differential polymerase chain reaction. *Oncogene* **4:**(9) 1153–1157.
5. Neubauer, A., B. Neubauer, M. He, P. Effert, D. Iglehart, R.A. Frye, and E. Liu. 1992. Analysis of gene amplification in archival tissue by differential polymerase chain reaction. *Oncogene* **7:**(5) 1019–1025.
6. Kasprzyk, P.G., S.U. Song, P.P. Di Fiore, and C.R. King. 1992. Therapy of an animal model of human gastric cancer using a combination of anti-erbB-2 monoclonal antibodies. *Cancer Res.* **52:** 2771–2776.
7. Kraus, M.H., N.C. Popescu, S.C. Ambsaugh, and C.R. King. 1987. Overexpression of the EGF receptor related proto-oncogene erbB-2 in human mammary tumor cell lines by different molecular mechanisms. *EMBO J.* **6:**(3) 605–610.
8. Wright, D.K. and M.M. Manos. 1990. Sample preparation from paraffin embedded tissues. *PCR protocols: A guide to methods and applications* (ed. M.A. Innes), Chapter 19, pp. 153–158. Academic Press, San Diego, CA.
9. Jackson, D.P., F.A. Lewis, G.R. Taylor, A.W. Boylston, and P. Quirke. 1990. Tissue extraction of DNA and RNA and analysis of the polymerase chain reaction. *J. Clin. Pathol.* **43:** 499–504.
10. Provan, A.B., E. Hodges, A.G. Smith, and J.L. Smith. 1992. Use of paraffin wax embedded bone marrow trephine biopsy specimens as a source of archival DNA. *J. Clin. Pathol.* **45:** 763–765.
11. Yamamoto, T., S. Ikawa, T. Akiyama, K. Semba, N. Nomura, N. Miyajima, T. Saito, and K. Toyoshima. 1986. Similarity of protein encoded by the human C-erbB-2 gene to epidermal growth factor receptor. *Nature* **319:** 230–234.
12. Gray, P.W. and D.V. Goeddel. 1982. Structure of the human immune interferon gene. *Nature* **298:** 859–863.
13. Taparowsky, E., K. Shimizu, M. Goldfarb, and M. Wigler. 1983. Structure and function of the human N-ras gene. *Cell* **34:** 581–586.
14. Lubin, M.B., J.D. Elashoff, S.J. Wang, J.I. Rotter, and H. Tarada. 1991. Precise gene dosage determination by polymerase chain reaction: Theory, methodology, and statistical approach. *Mol. Cell. Probes* **5:** 307–317.
15. Siebert, P.D. and J.W. Larrick. 1992. Competitive PCR. *Nature* **359:**(6395) 557–558.
16. Underwood, M.A., J.M.S. Bartlett, D.S. Gardiner, J. Reeves, R. Scott, and T.G. Cooke. (in prep.).
17. Slamon, D.J., G.M. Clark, S.G. Wong, W.J. Levin, A. Ullrich, and W.L. McGuire. 1987. Human breast cancer: Correlation of relapse and survival with amplification of the HER-2/neu oncogene. *Science* **235:**(4785) 177–182.
18. Tiwari, R.K., P.I. Borgen, G.Y. Wong, C.C. Cordon, and M.P. Osborne. 1992. HER-2/neu amplification and overexpression in primary human breast cancer is associated with early metastasis. *Anticancer Res* **12:**(2) 419–425.
19. Slamon, D.J., W. Godolphin, L.A. Jones, J.A. Holt, S.G. Wong, D.E. Keith, W.J. Levin, S.G. Stuart, J. Udove, and A. Ullrich. 1989. Studies of the HER-2/neu proto-oncogene in human breast and ovarian cancer. *Science* **244:**(4905) 707–712.
20. Slamon, D.J. 1990. Studies of the HER-2/neu proto-oncogene in human breast cancer. *Cancer Invest.* **8:**(2) 373–379.

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