An improved method of competitive PCR for quantitation of gene copy number

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Previous studies using differential PCR to quantitate amplification of a target gene in human tumor tissue utilized co-amplification of a second nonamplified gene as an internal standard (1). However, this approach does not take into account the problem that PCR amplification can be problematic when more than one set of primers are amplified in the same sample. More recently, fragments which are constructed so that they can be amplified with the same primers as the target gene have been used as an internal standard when quantitating gene copy number by competitive PCR (2–4). When these internal standard fragments (here abbreviated 'Std_i') are mixed with DNA before PCR, the ratio of target to Std_i after PCR will indicate the relative amount of target sequence. Determination of gene copy number for a given DNA sample requires comparison with a second source of DNA containing a known copy number for the target gene. For this technique to be accurate, equivalent amounts of genomic DNA from the source of DNA with known copy number and test DNA must be added to these two separate PCR mixture. However, when the test DNA is derived from tumor specimens, the DNA amount judged by optical density does not necessarily represent the amount of target sequence added to the PCR mixture because tumor-derived DNA preparations frequently are contaminated with RNA, and often show variable degrees of DNA degradation. Thus, the copy numbers determined by competitive PCR could be inaccurate.

In this report, we circumvent the problems associated with tumor-derived DNA by adding a second PCR reaction which amplifies a second gene which is not amplified in tumor DNA and is similar in size to the target fragment so that it should have the same degree of degradation as the target sequence. Thus, the PCR product of this reaction control should reflect the amount of the target used in PCR. For more accurate measurement of the amount of control DNA, another internal standard fragment (abbreviated 'Std_c'), constructed so that it amplifies with the same primers as the control gene, was added to the second PCR reaction. After the target and reaction control sequences were amplified by PCR separately, the quotient of the ratio of target

to Std_i divided by the ratio of reaction control to Std_c will represent the relative copy number of the target gene in the cells:

$$\text{Target Gene Copy Number} = (\text{Target}/\text{Std}_i)/(\text{Reaction control}/\text{Std}_c)$$

For our studies, the target gene to be quantified was the *her-2/neu* oncogene (abbreviated 'HER') and the reaction control was placental-like alkaline phosphatase gene (abbreviated 'PLAP'). The following primers (5' ends are on the left) were used in these PCR.

HER primers were used for amplifying the *her-2/neu* fragment (200 bp in length, codons 832–883). PLAP primers were for a fragment (156 bp) of PLAP gene (located at chromosome 2q34–37). Std_i and Std_c primers were for making the standard molecules Std_i (427 bp) and Std_c (419 bp) respectively. Std_i and Std_c were obtained by PCR using 25 pmol of the corresponding primers and 100 ng of plasmid pBLPLAP512 (5), and purified by electrophoresis. For competitive PCR (for both HER and PLAP), 100 ng of the genomic DNA, primers (25 pmol of each), internal standard (1.0 amol) were used in a total volume of 25 µl. Five µl of each competitive PCR product were electrophoresed on a 2% agarose gel. After taking pictures of the EtBr-stained gel, the relative amounts of PCR products were determined with a densitometer. The ratios of HER to Std_i in DNA from tumor and normal tissues were 2.2 and 0.8 respectively (2.4 times in tumor as in normal), while the ratios of PLAP to Std_c in tumor and normal DNA were 1.2 and 1.5 (0.80 times in tumor as in normal) indicating that the amount of target sequence in tumor DNA that was added to the competitive PCR is only 80% of that from normal. Thus, the *her-2/neu* copy number in the tumor DNA is 3.0 times (2.4 divided by 0.80) of that in normal tissue.

Our other data showed that the amounts of genomic DNA added to PCR is linearly proportional to the ratios of target product to internal control over a wide range (from 100 ng to 5000 ng of genomic DNA). Since 100 ng is used per reaction, a 50-fold amplification of the target gene would still be in the linear range of the assay.

	Forward reaction	Reverse reaction
HER	CTACATGGGTGCTTCCCAT	TGTCCTTGGTCCTTCACCT
Std _i	CTACATGGGTGCTTCCCATTCAGAACAGAGCCTGG	TGTCCTTGGTCCTTCACCTGCGGAAATCCCAGGT
PLAP	CACCTGTCCAGGCCAA	GAGAGGAGGGGAGGCT
Std _c	CACCTGTCCAGGCCAACAGAACAGAGCCTGG	GAGAGGAGGGGAGGCTGCGGAAATCCCAGGT

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