GENETICS

Comparison of *SYBR Green I* and *TaqMan* Real-Time PCR Formats for the Analysis of *her2* Gene Dose in Human Breast Tumors

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We compared two technologies of real-time PCR (with the use of fluorescent *SYBR Green I* dye and specific *TaqMan* probe) for quantification of the dose of *her2* gene in breast tumors. The maximum increase in the gene dose in *TaqMan* and *SYBR Green I* analyses was 10- and 5-fold, respectively. In was found that *TaqMan* and *SYBR Green I* technologies allow detection of the matrix in amounts corresponding to 1-100 and 2.5-40.0 ng genomic DNA, respectively. Tenfold increase in the gene dose leads to incorrect evaluation of multiplication ratio in the *SYBR Green I* analysis. These results suggest that *TaqMan* technology is more preferable for correct evaluation of *her2* gene dose.

Key Words: *her2*, *SYBR Green I*, *TaqMan, breast cancer*

Human epidermal growth factor receptor HER2 is one of the most informative prognostic markers of breast cancer. Hyperexpression of this protein is observed in 25-35% patients. Various clinical parameters (severity of the disease, rate of tumor growth, degree of malignancy, response to immuno- and chemotherapy, *etc*.) depend of the expression of this marker [6]. In most cases, hyperexpression of this receptor is determined by increased dose of *her2* gene in breast cancer cell [3.7]. Low accuracy of immunohistochemical analysis of HER2 status of the tumor, the basic method currently used in clinical practice, dictated the necessity of application of in situ fluorescent hybridization for this purpose. However, wide use of this analysis is limited because it is involved and time consuming, requires expensive equipment and test kits, and preliminary processing of samples is needed. Real-time PCR is a perspective variant for the analysis of *her2* gene dose in the tumor, but the choice of real-time PCR technology in some cases is crucial for obtaining verifiable results.

Here we compared two real-time PCR chemistries (with the use of fluorescent *SYBR Green I* dye and specific *TaqMan* probe) for evaluation of the dose of *her2* gene in breast tumors.

MATERIALS AND METHODS

The dose of *her2* gene was determined in 24 breast tissue samples with histologically documented breast cancer (malignancy grades II and III). Tumor cells constituted not less than 60% sample. Normal tissue samples were obtained from 5 patients during mammary gland plasty.

DNA was isolated using special kits for DNA isolation from animal tissues (V-gene Biotechnology). Relative changes in the dose of *her2* gene were evaluated using the method proposed by Pfaffl

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[5]. The efficiency of the reaction was determined by calibration curves constructed on the basis of the results of PCR with *her2* and *ubc* primers. The dose of the gene in the tumor samples was compared with this value in the control sample. The results were expressed as the ratio of *her2* gene doses in the tumor and normal sample (N_{her2}).

PCR was carried out on an ABI Prism 7000 SDS device (Applied Biosystems). The reaction mixture contained 1x buffer for Taq DNA polymerase; 4.5 mM (for *her2*) or 3.5 mM (for *ubc*) $MgCl_2$ (Ekros), 0.33 mM dTTP, dGTP, dCTP, and dATP, 660 μM dUTP, 0.3 μM each primer, 0.35 μM *TaqMan* probe, 0.5 μM *TAMRA*, and 1 U *Smart Taq* DNA polymerase (Medigen Laboratory). For *SYBR Green I* technology, a similar mixture with *SYBR Green I* (1:80,000) was used (total volume 50 μl). All reagents were at least of chemically pure grade.

Nucleotide sequences of *TaqMan* probes and primers were chosen using Primer Express software (Applied Biosystems). Primers for *SYBR Green I* analysis: for *her* 5'-CCTGTCCCCACTCCTTAATCTC-3' (forward) and 5'-CCGCATTACTTGCAGGTTCTG-3' (reverse); for *ubc* 5'-GTAGGCTTTTCTCCGTCGCA-3' (forward) and 5'-TCACTTATCCCTCCCCTCACC-3' (reverse). Primers and probes for *TaqMan* assay: for *her* 5'-CCAGCGCTTTGTGGTCATC-3' (forward), 5'-GCCCATACTGGACTCATC-3' (reverse) and 5'-*FAM*-TCTGTGCCCCATCCCTGCCTGT-*BHQ1*-3' (probe); for *ubc* 5'-GGAGCGCAGCAAAATGG-3' (forward), 5'-CATTAGCGAAGGCCTCAAGAC-3' (reverse) and 5'-*FAM*-CGGGCT

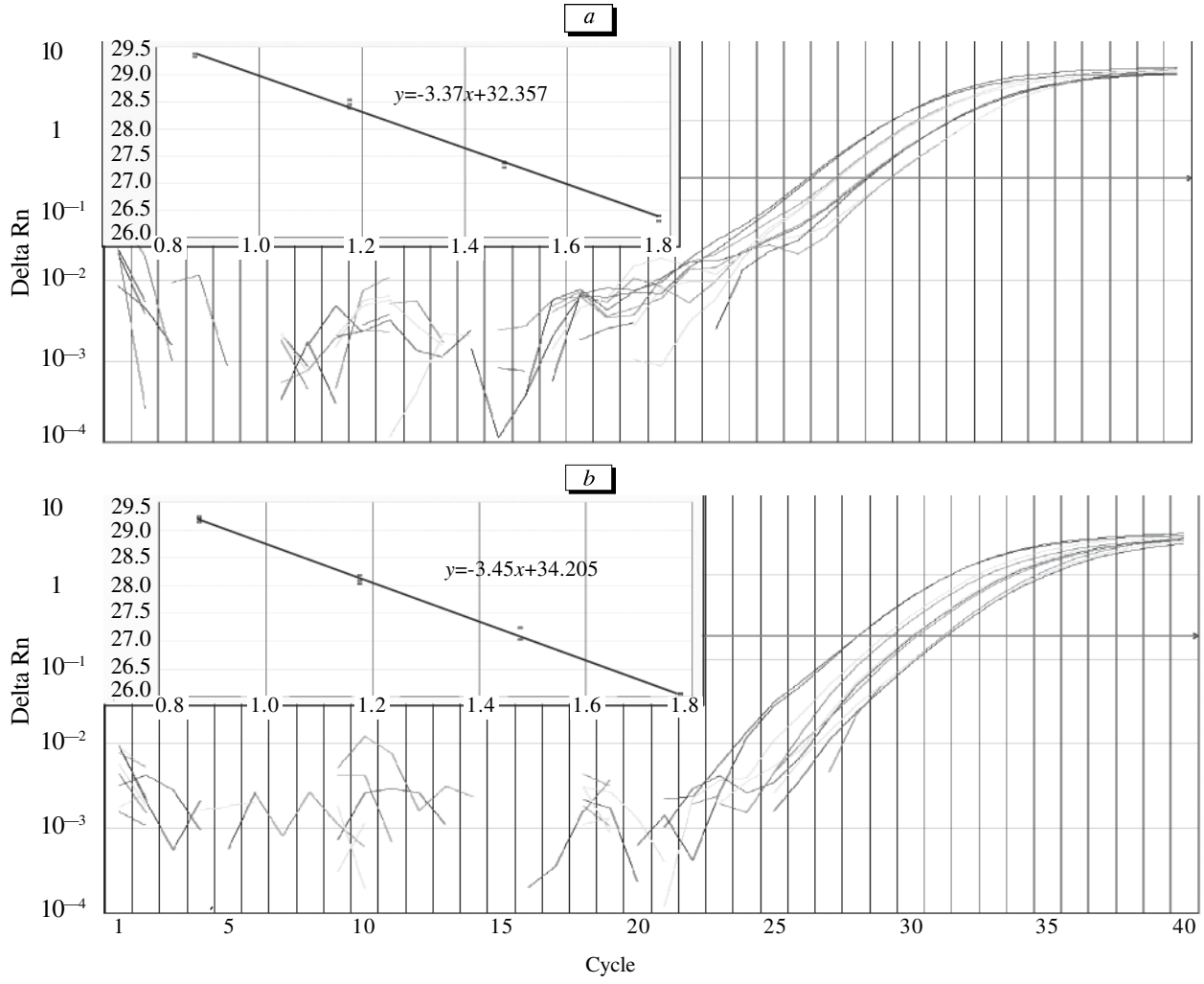

Fig. 1. Real-time PCR of *her2* (*a*) and *ubc* (*b*) gene fragments (300 nM) using *TaqMan* probes. Amplification of serial 2-fold dilutions of genomic DNA (4.5-36.0 ng) with a pair of primers complimentary to gene fragments. The plots show changes in fluorescence intensity as a function of PCR cycle number and calibration curves in coordinates "logarithm of matrix DNA quantity — *Ct*". *a*) efficiency of PCR was 98%, slope -3.37, r^2>0.99; *b*) efficiency of PCR was 95%, slope -3.45, r^2>0.99. Here and on Figs. 2, 3: Delta Rn is the difference between fluorescence intensities in the studied sample and negative control standardized relative to internal control of the reaction.

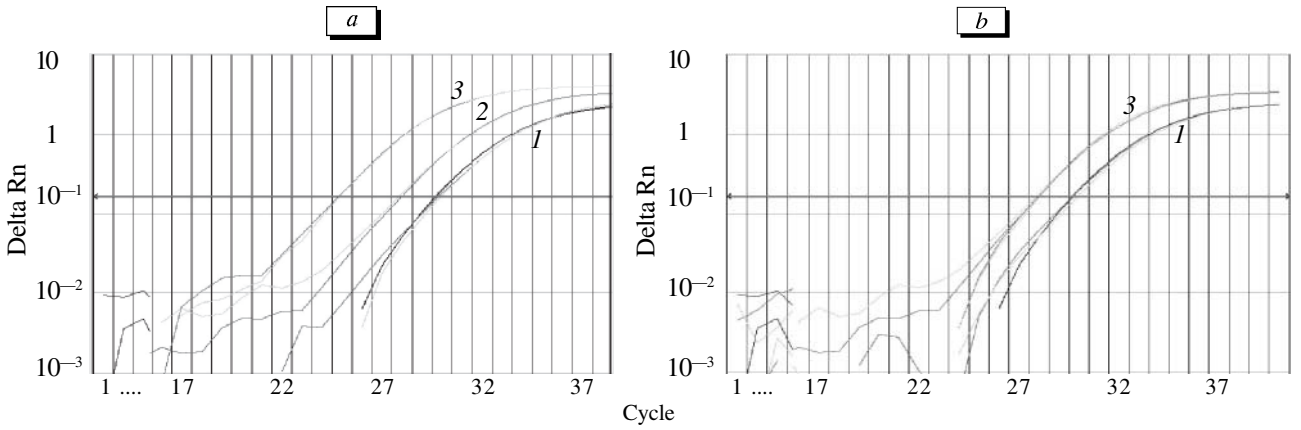

Fig. 2. *TaqMan* amplification with *her2* and *ubc* primers for samples with maximum (*a*) and not increased (*b*) levels of *her2* gene. *1*) pool of amplification curves with *ubc* primers for the tumor and control tissue. Coincidence of amplification curves for both matrices indicates similar initial quantity of tumor and control DNA. *2*) amplification with *her2* primers in the control; *3*) experiment.

GTGAGGTCGTTGAAACAAG-*BHQ1*-3' (probe), where *FAM* is 5-carboxyfluorescein and *BHQ1* fluorescence quencher.

Temperature regimen: initial denaturation at 95°C for 5 min, then 40 cycles at 95°C for 15 sec and 60°C for 1 min. After reaction, an aliquot of the amplification mixture was applied on 8% PAAG (Sigma), stained with ethidium bromide, and documented using Gel Doc 1000 system (Bio-Rad). For the analysis of PCR products in the *SYBR Green I* assay, a melting curve was constructed for each sample after amplification. Calibration curves were constructed using 2-fold dilutions of genomic DNA isolated from normal mammary gland tissue (4.5-72.0 ng DNA in the sample). Each point is the mean of three experiments.

RESULTS

Using *TaqMan* probe we showed that *Ct* value (a point where fluorescence curve crosses the fixed fluorescence threshold on the fluorescence intensity — PCR cycle number curve) strictly linearly depended on the logarithm of initial copy number of genomic DNA (r^2>0.99). The efficiency of reactions (*E*) was calculated by the formula: $E=10^{1/|m|}-1$, where *m* is the slope of the curve, was 92-100% for all test samples (Fig. 1).

For evaluation of the reproducibility of the results of *her2* gene dose measurements in human breast tumors, we determined N_{her2} for 5 normal human mammary gland samples. To this end, 4.5, 9.0, 18.0, and 36.0 ng DNA from all 5 samples was amplified with *her2* and *ubc* primers. The ratio N_{her2} was within 0.73-1.08 (mean 0.95±0.14), N_{her2}> 1.37 attested to increased dose of *her2* gene (according to the formula *M+3SD*).

Increased dose of *her2* gene was detected in 6 of 24 human breast tumor samples: 2 samples with 1.5- to 2-fold increased gene dose, 3 samples with 4-5-fold increased gene dose, and 1 sample with 10-fold increased gene dose compared to normal mammary gland tissue (Table 1). When equal initial amounts of DNA from the tumor and normal tissue were used, *her2* amplification curves for the tumor attained the fixed fluorescence threshold earlier than the curve for the normal tissue (Fig. 2, *a*), which attested to increased *her2* gene dose in the tumor, or were close to the control, which reflected unchanged *her2* gene dose in the sample (Fig. 2, *b*).

Reaction conditions for *SYBR Green I* assay were optimized according to recommendations of

TABLE 1. Increase in *her2* Gene Dose in Breast Tumors According to *TaqMan* Assay

Sample	her2		ubc	
	Ct	$(E_{her})^{\Delta Cther}$ (control—sample)	Ct	$(E_{her})^{\Delta Cther}$ (control—sample)
Normal tissue	28.09	1	29.78	1
her2 gene dose not increased	28.16	0.95	29.92	0.92
Maximum level of *her2* gene	24.92	10.28	30.07	0.83

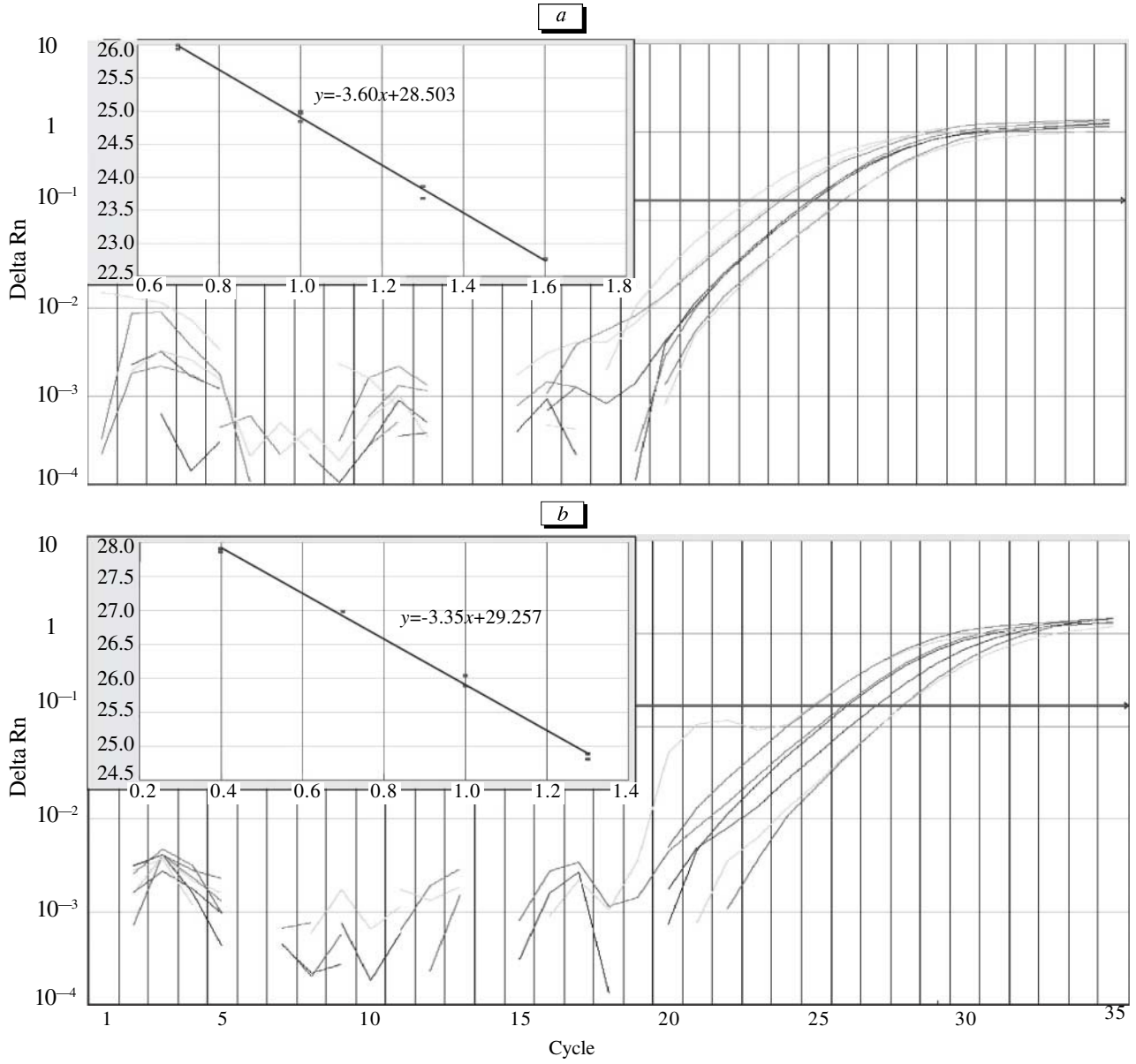

Fig. 3. Real-time PCR of *her2* (*a*) and *ubc* (*b*) gene fragments (300 nM) using *SYBR Green I* technology. Amplification of serial 2-fold dilutions of genomic DNA (5-40 ng) with pairs of primers complimentary to *her2* (*a*) and 2.5-20.0 ng genomic DNA for *ubc* (*b*) gene fragments. The plots show changes in fluorescence intensity as a function of PCR cycle number and calibration curves in coordinates "logarithm of matrix DNA quantity — *Ct*". *a*) efficiency of PCR was 90%, slope -3.60, r^2>0.99; *b*) efficiency of PCR was 99%, slope -3.35, r^2>0.99.

Applied Biosystems company. Analysis of calibration curve showed that the experimental results were well reproduced for 50 ng genomic DNA, while increasing the amount of genomic DNA worsened reproducibility. Therefore, 5, 10, 20 and 40 ng genomic DNA from normal human mammary gland tissue were used for the construction of calibration curve. The efficiency of amplification in all cases was comparable with that for *TaqMan* technology (87-99%, Fig. 3). The reproducibility of the analysis was verified using 5 normal mammary gland tissue samples as described above. Despite the fact the total number of patients with increased dose of *her2* gene was 6 of 24 (similarly to *TaqMan* analysis), the recorded multiplicity of the gene dose depen-

TABLE 2. Distribution of Patients within the Group with Increased *her2* Gene Dose for Two Real-Time PCR Formats

PCR format	Increase in the dose of *her2* gene		
	1.5-2-fold	4-5-fold	>10-fold
TaqMan	2	3	1
SYBR Green I	2	4	0

ded on the assay (Table 2). For instance, according to *SYBR Green I* and *TaqMan* assays the increase in the number of *her2* gene copies in the sample with its maximum content was 5- and >10-fold, respectively.

Real-time PCR meets all requirements for the analyses used in clinical practice: short time of analysis, automation, use of small amounts of biopsy material, reliability, and reproducibility of the results. This comparative study of two real-time PCR formats for measurement of *her2* gene dose can be crucial for the choice of correct methods of diagnostics of HER2 status of breast tumors in clinical practice. The interval for the initial amount of the matrix for *TaqMan* technology is 1-100 ng DNA with normal *her2* gene dose, while *SYBR Green I* has a narrower interval of the initial matrix amount 2.5-40.0 ng. In further studies we recommend to use *TaqMan* technology for measurement of *her2* gene dose in breast tumors, because this assay makes it possible to analyze samples with broader interval of the initial quantity of DNA matrix with is important for evaluation of the increase in *her2* gene dose.

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