RNA extraction, complementary cDNA synthesis and qRT-PCR

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Abstract

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Protocol

RNA extraction

Step 1.

Total RNA is extracted from breast specimens by using the acid-phenol guanidium method (RNABLE, Eurobio) by following the protocol of the supplier. The quality of the RNA samples is determined by electrophoresis through agarose gels and staining with ethidium bromide, and the 18S and 28S RNA bands are visualized under UV light.

Complementary cDNA synthesis

Step 2.

RNA is reverse transcribed in a final volume of 20 µL containing 1X RT buffer, 10mM of each dNTP, 20 units of rRNasinTM RNase inhibitor (Promega, Madison, WI), 10 mM DTT, 100 units of Superscript II RNase H-reverse transcriptase (Life Technologies, Inc., Gaithersburg, MD), 0.15 µg/µL random primers (Life Technologies, Inc., Gaithersburg, MD), and 1 µg of total RNA. The samples are incubated at 25°C for 10 min and 42°C for 30 min, and reverse transcriptase is inactivated by heating at 99°C for 5 min and cooling at 4°C for 5 min.

qRT-PCR

Step 3.

Quantitative values are obtained from the cycle number (Ct value) at which the increase in the fluorescence signal associated with exponential growth of PCR products began to be detected by the laser detector of the ABI Prism 7900 Sequence Detection System (Perkin-Elmer Applied Biosystems, Foster City, CA), using PE biosystems analysis software according to the manufacturer's manuals.

The precise amount of total mRNA added to each reaction mix (based on optical density at 260 nm) and its quality (*i.e.*, lack of significant degradation) are both difficult to assess. Transcripts of the *TBP* gene (Genbank accession NM_003194) encoding the TATA box-binding protein (a component of the DNA-binding protein complex TFIID) are therefore also quantified as an endogenous RNA control, and

each sample are normalized on the basis of its *TBP* content. *TBP* is selected as endogenous control because of the moderate prevalence of its transcripts, and because there are no known *TBP* retropseudogenes (retropseudogenes lead to co-amplification of contaminating genomic DNA and consequently interfere with qRT-PCR, despite the use of primers in separate exons).

Results, expressed as N-fold differences in target gene expressions relative to the *TBP* gene (and termed “NTARGET”), are determined as NTARGET = $2^{\Delta Ctsample}$, where the DCt value of the sample is determined by subtracting the Ct value of the specific target gene from the Ct value of the *TBP* gene. The primers for genes are chosen with the assistance of the Oligo 6.0 software (National Biosciences, Plymouth, MN). The dbEST database is scanned to confirm the total gene specificity of the nucleotide sequences chosen for the primers and the absence of single nucleotide polymorphisms. To avoid amplification of contaminating genomic DNA, one of the two primers is placed at the junction between two exons. Agarose gel electrophoresis is used to verify the specificity of PCR amplicons.