Introduction

One of the important hallmarks of cancer is an aberrant growth control, resulting, for example, from genetic mutations in proto-oncogenes and tumor-suppressor genes, in cell-cycle control genes, or in specific genes that are linked to signal-transduction pathways, differentiation and apoptosis. Associated with aberrant growth control, most of the cancer cells have another important feature that distinguishes them from normal somatic cells, i.e. extended, or even unlimited, replicative capacity. Telomeres, the DNA-protein structures comprising the physical terminus of eukaryotic chromosomes, seem to play a crucial role in this process.

Telomeres protect chromosome ends from exonucleolytic degradation, end-to-end fusion, and irregular recombination. In addition, in somatic cells, telomeres progressively shorten due to the inability of the conventional DNA replication machinery to replicate the extreme 5’ terminus of linear DNA [end-replication problem, reference 1]. Once telomeres shorten below a critical threshold, they lose the capacity to cap chromosomes effectively and activate a damaged-DNA response pathway that causes cell-cycle arrest.

In contrast, tumor cells, as well as certain normal cells like germ-line cells and stem cells, are capable of proliferating with a prolonged or even infinite life span by maintaining constant telomere lengths [2]. The maintenance of stable telomere length is associated with the activation of telomerase, an enzyme that compensates for the loss of telomeric DNA by adding repeat sequences to the chromosome ends [3]. Telomerase is a ribonucleoprotein consisting of a catalytic protein component [hTERT; human Telomerase Reverse Transcriptase; 4, 5] and a protein-associated RNA [hTR; human telomerase-associated RNA; reference 6]. Telomerase thereby acts as a reverse transcriptase that uses part of its intrinsic RNA component as template for telomeric repeat synthesis. Both subunits are essential for restoring telomerase activity in vitro, and introduction of these genes into normal human cells can extend the life span of these otherwise mortal cells [7, 8].

Using the PCR-based TRAP method, telomerase activity has been detected in most neoplastic lesions and appears to be necessary for the sustained proliferation of most advanced cancers [9,10]. However, the direct analysis of telomerase activity requires enzymatically active specimens, which makes accurate quantification more difficult. Therefore, determination of expression levels of telomerase-encoding mRNA hTERT and protein-associated RNA hTR as an indirect method for detecting telomerase represents an alternative approach that overcomes these technical limitations.

Initial research studies using a qualitative RT-PCR assay have shown a significant correlation between telomerase activity and hTERT/hTR expression, although in some instances hTR may also be expressed at a lower level or even independent of enzyme activity, indicating the need for a quantitative approach for assessing hTR [11].

In this article we report on real-time kinetic RT-PCR assays for the quantitative detection of the telomerase catalytic subunit hTERT mRNA and the telomerase-associated RNA hTR using the LightCycler instrument.

Materials and Methods

RNA isolation from cell lines

Total RNA was prepared using the High Pure RNA Isolation Kit according to the manufacturer’s instructions in the corresponding package insert.

Detection of telomerase components hTERT and hTR

Quantitative detection of the telomerase components hTERT and hTR was performed with the LightCycler Instrument using the TeloTAGGG hTERT Quantification Kit and the TeloTAGGG hTR Quantification Kit according to the corresponding package inserts. For all RT-PCRs the correlation coefficient (r) was > 0.99, indicating a precise log-linear relation in the range of 10^7 to 10^1 copies per reaction.
Results and Discussion

Selection of primers and Hybridization Probes

Telomerase hTERT mRNA has been shown to undergo alternate splicing in several cell lines and tissues, whereby several of the splice sites remove critical reverse transcriptase motifs [12]. In order to detect only those splice variants that could be translated into a functionally active telomerase enzyme, primers and hybridization probes have been designed for the specific detection of hTERT-RNA transcripts containing the β-region of the hTERT mRNA.

A pseudogene-free housekeeping control gene PBGD [porphobilinogen deaminase; reference 13] with a low expression level similar to hTERT and hTR was selected as a control for the amount and the integrity of the RNA preparation. Relative telomerase hTERT and hTR expression levels were obtained by comparing the levels of hTERT and hTR RNA with the expression levels of the PBGD housekeeping gene in the same sample.

The primers for hTERT and PBGD were selected to span exon-intron boundaries, thus preventing the co-amplification of the corresponding genomic DNA targets. As there is no intron-exon structure of the hTR gene, an hTR-specific primer cannot be made to differentiate between RNA-specific PCR products and those derived from contaminating DNA in the RNA preparations. Therefore, when analyzing hTR expression levels, it is essential to check possible DNA contamination in the sample by comparing the results of the RT-PCR analysis with those of the corresponding minus-RT control. DNA contaminations of less than 0.1% have typically been observed with the preparation methods used here.

Amplification efficiency and kit concept

Initial quantitative approaches for detecting hTERT RNA suggest that telomerase mRNA hTERT is expressed at very low levels, indicating the need for a highly sensitive RT-PCR approach. Besides primer specificity, the strongest impact on the sensitivity of the assay is the amplification efficiency of the PCR step. In an ideal PCR, the number of target molecules is doubled during
In order to reach maximal sensitivity of the assays, the conditions for the amplification of hTERT and hTR RNA, as well as for the housekeeping gene PBGD, have been optimized to give an optimal amplification efficiency of E = 2. As shown in Figure 1, analysis of serial dilutions of sample RNAs for hTERT and PBGD expression reveals amplification efficiencies E = 2 for all three targets, as indicated by parallel slopes of the corresponding standard curves.

As a consequence of hTERT, hTR, and PBGD RT-PCR having identical amplification efficiencies, the same standard curve for the quantification of different targets, i.e., hTERT and PBGD, can be used for the accurate quantitation of relative expression levels.

Additionally, as shown in Figure 2, amplification efficiency is independent of any nucleic acid background in the reaction, allowing the use of external standards for accurate quantification.

### Linearity and sensitivity of the assays

In order to determine the linearity of the assay, samples containing increasing concentrations of RNA transcripts were analyzed. There is a linear correlation between the input RNA and the amount of transcripts detected in the range of $10^2$ to $10^5$ copies for all three targets (Figure 3). The 95% positive cut-off point, which defines the copy number per reaction at which 95% of repetitive determinations are found as positive signals, was statistically identified by determining the fraction of positive results when analyzing increasing concentrations of target RNA. As depicted in Figure 4, the 95% positive cut-off value for the detection of hTERT transcripts is 20 copies per reaction.

### Precision of the assays

Using repetitive determinations of increasing concentrations of RNA targets in the same LightCycler run and in different LightCycler runs, we statistically determined the intra-run and inter-run variances of the assays. As indicated by the overlaying amplification curves at the different concentrations, coefficients of variation for intra-assay and inter-assay variances are typically less than 10% (copy numbers) and 0.8% (crossing points) when analyzing > $10^3$ copies per reaction (Figure 5, table part). Analysis of concentrations of $\geq 10^3$ copies/reaction produces increased variation coefficients as a result of the influence of Poisson statistics on the PCR process when analyzing very low concentrations of a target molecule.
Conclusions

With its TeloTAGGG hTERT Quantification Kit and the TeloTAGGG hTR Quantification Kit presented here, Roche Molecular Biochemicals is providing additional research tools for the analysis of telomeres and telomerase. These LightCycler assays complement the Roche Molecular Biochemicals’ TeloTAGGG product line for the analysis of telomerase activity and telomere length.

The real-time kinetic RT-PCR assays presented here enable fast, highly sensitive, and accurate quantitative analysis of the expression of the telomerase catalytic subunit hTERT mRNA and the telomerase-associated RNA hTR. Utilizing fluorescent hybridization probes for the on-line detection of the PCR products, these assays allow detection of as few as 100 copies of hTERT and hTR over a five-log dynamic range. In addition, the closed-tube homogenous format eliminates the necessity for post-PCR analysis and the risk of carry-over contamination.

Quantitative results can be obtained in less than 45 min with the LightCycler On-line Detection System, allowing the screening of large numbers of specimens when trying to elucidate the role of telomerase in the diagnosis and prognosis of cancer.

References