Dual-color real-time telomeric repeat amplification protocol

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Telomerase, a ribonucleoprotein that performs the maintenance of chromosome ends by a reverse-transcription mechanism, is an essential immortalizing factor in mammalian cells. While telomerase is repressed in most human somatic cells, its reactivation is strongly associated with 80%–90% of the advanced tumors of over 30 types studied (see Reference 1 for review). This makes telomerase the most universal tumor marker in oncology diagnostics, as well as a useful molecular probe of proliferative potential of cells in biomedical research. The telomeric repeat amplification protocol (TRAP) is the primary technique for the sensitive detection and semiquantitative evaluation of telomerase activity (2). However, it requires a relatively time-consuming analytical phase, typically involving polyacrylamide gel electrophoresis and densitometry. Alternatively, it is possible to evaluate telomerase activity by ELISA (Telo*TAGGG*™ Telomerase PCR ELISA; Roche Diagnostics GmbH, Mannheim, Germany) or by direct fluorimetric measurement of end products (TRAPeze® XL Telomerase Detection Kit; Chemicon International, Temecula, CA, USA), but with these the risk of false-positive results is relatively high when analyzing end products without subsequent electrophoretic analysis.

Here we have adapted the TRAPeze® XL kit for use in real-time quantitative TRAP (RQ-TRAP). The kit applies Amplifluor™ primers for detection of both amplified telomerase products and an internal PCR control labeled with fluorescein and sulforhodamine, respectively. Amplifluor primers consist of a 3′ end sequence complementary to the target sequence and a 5′ end hairpin structure. The fluorophore (energy donor) and the quencher dabsyl (dimethylaminobenzenesulfonyl, 4-dimethyl-aminobenzene-4′sulfonyl) are in close proximity within the 5′ hairpin. As the primer is incorporated into a double-stranded PCR product, the hairpin is unfolded through the activity of the polymerase. In this extended conformation, the distance between the fluorophore and quencher is increased and a fluorescence signal is generated. Inclusion of an internal control labeled with a second fluorophore serves to monitor the PCR amplifications and aids in the quantitation of telomerase activity. Such a control is missing in the recently described SYBR® Green RQ-TRAP (3), which increases the risk of false-negative results.

We demonstrate here that the kit can also be used for RQ-TRAP assays to monitor amplification of telomerase products and the control simultaneously using the fluorescein (FAM) and rhodamine (ROX) channels of the Rotor-Gene™ 3000 instrument (Corbett Research, Mortlake, Australia). The low cross-talk between channels (<1%) of this instrument is advantageous. The RQ-mode gives an opportunity to evaluate telomerase activity and to recognize false-positive results directly from reaction kinetics, thus avoiding time-consuming postamplification analysis. In particular, two kinds of false-positive results were observed with the dual color RQ-TRAP during its optimization that would have been missed using only end point fluorescence measurements. These are (*i*) a rapid increase of signal from the outset of the PCR in both channels arising from the partial degradation of Amplifluor primers by inappropriate (3′→5′ exonuclease-positive) DNA polymerase; (*ii*) primer-dimer formation occurring during the later PCR cycles (after cycle 35). The general advantage of real-time technique is that the basic value used for evaluation is not a total product generated by PCR, but rather a threshold cycle, which always occurs in the exponential phase of the amplification, and thus the quantification is not affected by any reaction component becoming limited in a plateau phase. The latter results in a systematic bias against the more abundant template and makes any quantification based on measurements of overall product yield intrinsically unreliable. Using TRAPezeXL kit in a real-time mode thus provides a possibility of avoiding this bias.

Each reaction (final volume 25 µL) for the RQ-TRAP assay contained 5 µL of 5× TRAPezeXL reaction mix, 1 µL of 25 mM $MgCl_2$, 16.6 µL of H_2O, 0.4 µL of Thermo-Start® *Taq* DNA polymerase 5 U/µL (ABgene, Surrey, UK) and 2 µL of sample extract. From a number of tested hot start *Taq* DNA polymerases, the Thermo-Start *Taq* DNA polymerase had the best compatibility with the reaction mixture. The reaction conditions used were: 30°C for 30 min (telomerase extension step), 95°C for 15 min (heat inactivation of telomerase and activation of hot start *Taq* DNA polymerase), 45 cycles of denaturation at 94°C for 30 s, annealing at 59°C for 30 s (fluorescence is acquired in FAM and ROX channels in this step), and extension at 72°C for 60 s. Cycling was followed by the final extension step at 72°C for 3 min.

It is possible to perform either comparative or absolute quantification of fluorescence curves using the take-off or threshold cycle values (C_t) evaluated by Rotor-Gene 3000 software version 4.6. While C_t data in the FAM channel are used for quantification, C_t data from the ROX channel (reflecting amplification of the internal control) should be invariable in the absence of PCR inhibition and need not be included in the calculation. However, it is necessary to check for amplification in the ROX channel of all samples, including the nontemplate control, and that the end point fluorescence values in both channels display an inverse relationship arising from semicompetitive amplification of the telomerase products and

control template. Results can then be calculated to relative telomerase units (TU) using a standard curve obtained from serial dilutions of control HeLa cell extracts included in the kit. A value of 100 TU corresponds to activity of 1000 control positive cells. To calculate TU, the simplified formula: $TU = (2^{-\Delta Ct})*100$ may be used, where ΔCt is the difference between C_t in a given sample and C_t in a reaction containing extract of 1000 control positive cells. Results expressed as dependence of C_t on logarithm of cell number (Figure 1A) or as TU_{versus} cell number (Figure1B) are shown. Both graphs are linear throughout the whole tested range of cell numbers. Moreover, it is also possible to use the end point fluorescence data from the fluorescence curves acquired to evaluate the results following the manufacturer's instructions. Figure 1C shows such an evaluation using the ratio of ΔFAM (end point fluorescence value of sample in FAM channel minus the corresponding value in a telomerase-negative control) and ΔROX (the end point fluorescence value of sample in ROX channel minus the corresponding value in a control without *Taq* DNA polymerase). A bilogarithmic plot of ΔFAM/ΔROX versus TU (calculated from the number of control positive cells) also shows linear fitting throughout the whole range of concentrations tested. In the case of any remaining doubts about the specificity of reaction products, these may still be analyzed by conventional polyacrylamide gel electrophoresis.

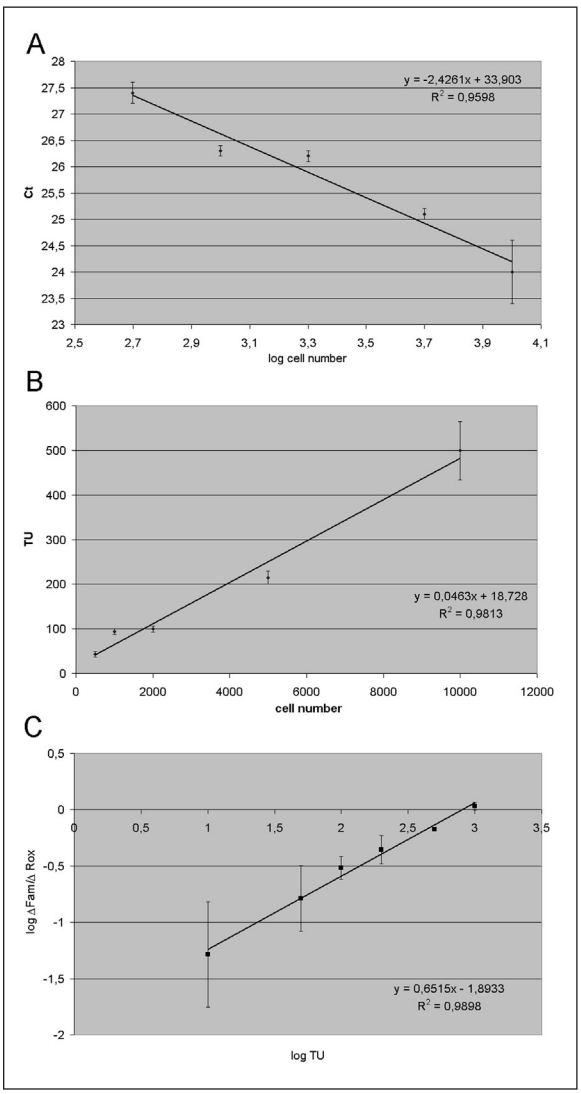

Figure 1. **Evaluation of dual-color real-time TRAP.** The result of the regression analysis is shown at the top of the graph. Each data point was measured in two replicates and reproducibility has been checked in three experiments. (A) Relationship between take-off values (C_t) obtained from comparative analysis of the FAM channel (Rotor-Gene 3000, version 4.6) and the log number of the control positive cells (HeLa) used in the assay. (B) Telomerase activity [expressed in telomerase units (TU)] of serial dilutions of extract from control positive cells. (C) Alternative evaluation of telomerase activity using end point fluorescence data. ΔFAM, end point fluorescence value of sample in the FAM channel minus the corresponding value in telomerase-negative control; ΔROX, end point fluorescence value of sample in the ROX channel minus the corresponding value in a control without *Taq* DNA polymerase. Telomerase activity (TU) was calculated from the number of control positive cells (100 TU corresponds to the activity extracted from 1000 control positive HeLa cells).

Figure 2 demonstrates that dual-color RQ-TRAP, using the TRAPeze XL kit, can be used to analyze telomerase activity in clinical samples. Telomerase activity was measured in $CD138^+$ multiple myeloma cells obtained from a patient's bone marrow by immunomagnetic separation (4). Activity was assessed in parallel using the polyacrylamide gel electrophoresis (PAGE) pattern of TRAP products, end point fluorescence and C_t data, and three replicates (each of 500 cells) tested. They demonstrate that dual-color RQ-TRAP meets the requirements for high sensitivity and reliability in quantifying telomerase activity for diagnostic and research applications. It also saves the considerable amount of time and consumables necessary for postamplification procedures.

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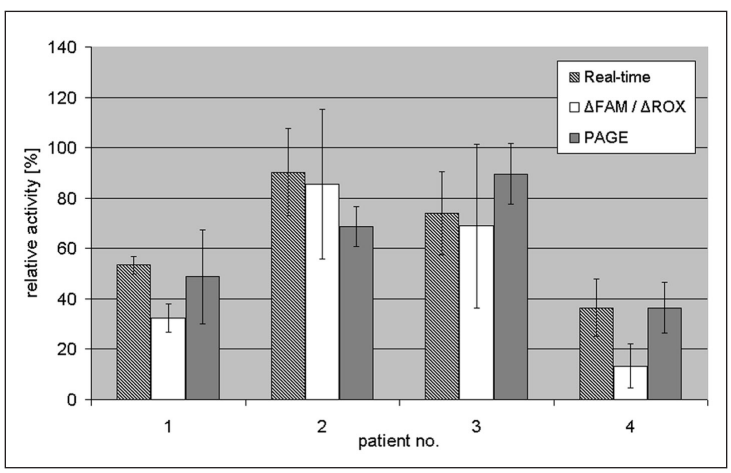

Figure 2. Comparison of results of telomerase activity in $CD138^+$ cells immunoseparated from bone marrow of four multiple myeloma patients. Data obtained using real-time TRAP, end point fluorescence measurement, and conventional evaluation from polyacrylamide gel electrophoresis are shown in parallel. Results are expressed as a percentage of activity in the standard ARH77 multiple myeloma cell line. The values are mean ±SEM of three reactions.

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