Internal Control for Quality Assurance of Diagnostic RT-PCR


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Reverse transcription polymerase chain reaction (RT-PCR) is gaining major importance in the field of medical diagnostics. For example, RT-PCR assays are being tested to become standard methodology for detection of occult tumor cells in peripheral blood of cancer patients. However, there is heterogeneity in the frequency of tumor cell detection, e.g., in melanoma patients as reported in the literature (1,6,9). One of the main reasons for these discrepancies is differences in methodology, and a major problem is the limited availability of controls to assess the assay’s reliability. Controls for RT efficacy and RNA quality are usually accomplished indirectly by amplification of ubiquitously expressed housekeeping genes such as glyceraldehyde-3-phosphate dehydrogenase (GAPDH), β-actin and β2-microglobulin. However, the results should be interpreted with great caution for several reasons. First, the abundance of PCR targets for housekeeping genes can lead to satisfying PCR results for housekeeping genes in samples not suitable for reliable detection of tumor targets with low mRNA expression levels. Second, the increasing number of GAPDH and β-actin retropseudogenes (7,8), which can be amplified even if mRNA-specific primers were designed, can lead to an overestimation of the RT efficacy. Third, because the expression of housekeeping genes is regulated (3,4), the level of expression may be influenced by many factors. All of these reasons limit the usefulness of housekeeping gene PCR as a reliable control of RNA quality and RT efficacy, whereas application of an internal control would provide a tool for reliable evaluation of the entire RT-PCR process on a per sample basis.

We developed a model of a simple and universally applicable internal control for the RT-PCR assay system for detection of tumor cells in peripheral blood of cancer patients, in which a small defined number of control cells is added to the blood samples as soon as the sample reaches the laboratory. Because the control cells are carried throughout the whole assay procedure, they are a potent indicator of the efficacy of the RT-PCR assay system. We have evaluated the feasibility of this internal control for an RT-PCR assay to detect melanoma cells using the tissue-specific tyrosinase gene (tyr) as target gene. The Jurkat T-cell line was used as a control cell, and the unique Jurkat T-cell receptor β-variable gene (TCRBV) was used as a control gene to monitor sample processing efficacy.

We added 103 Jurkat cells to blood samples from melanoma patients and to a dilution series of SK-Mel 28 cells (101-104 were added to 10 mL of healthy donor blood) before processing. Processing involved RNA extraction performed with a modified guanidinium isothiocyanate (GITC) phenol–chloroform method, and the RT performed with random hexamers and avian myeloblastosis virus (AMV) reverse transcriptase (Promega, Madison, WI, USA) as described elsewhere in detail (11). PCR was carried out as co-amplification with two primer sets. One published primer pair (HTYR3 and HTYR4) specific for the tyr cDNA (10) and a second primer pair specific for the unique Jurkat TCRBV cDNA were used. The Jurkat’s specific 5′ primer (5′-TGACAGAGATGGGACAAGAA-G-3′) was devised from the TCRBV region, whereas the specific 3′ primer (5′-TAACCTGTCCCGAACCIG-3′) and the hybridization oligonucleotide probe (5′-TAGCGAACAGTGCAGAA-GA-3′) were designed according to the T cell’s unique complementary determining region (CDR) 3.

Two microliters of first-strand cDNA/RNA heteroduplex were diluted in 50 µL containing a final concentration of 50 pmol of each primer, 1.5 mM MgCl2, 0.2 mM dNTP, 1× PCR buffer (50 mM KCl, 10 mM Tris-HCl, pH 9.0, 0.1% Triton®-X-100) and 2 U Taq DNA Polymerase (Promega). The samples were overlaid with 70 µL mineral oil, and 45 cycles of amplification were carried out on a thermal cycler (Perkin-Elmer, Norwalk, CT, USA) with a hot start. Each cycle consisted of a 1-min denaturation step at 94°C, a 1-min annealing step at 55°C and a 1-min extension step at 72°C. The PCR fragments were 207 and 307 bp for the tyr and TCRBV genes, respectively. Eight microliters of PCR product of the tyr and TCRBV co-amplification were separated in a 2% agarose gel (SeaKem® LE agarose; FMC BioProducts, Rockland, ME, USA) and blotted onto a pos-
itively charged nylon membrane (Pall-Gelman Sciences, Dreieich, Germany). For hybridization, 20 pmol of each of the two digoxigenin (Dig)-labeled oligonucleotide probes, one specific for the tyr gene (5′-GCCTGAGTTGACC-GAATAT-3′) and a second specific for the TCRBV gene (5′-TAGCCGAACAGTGGTCGAGAA-3′) were used, using conditions described elsewhere (11). Chemiluminescence-based detection was performed with a DIG Luminescent Detection Kit (Boehringer Mannheim GmbH, Mannheim, Germany). After scanning the X-ray film with a Macintosh® Color One Scanner, (Apple Computer, Cupertino, CA, USA), the signal strength of the PCR product was determined using Image 1.41 software (National Cancer Institute, Bethesda, MD, USA; http://rsb.info.nih.gov/nih-image). We first tested the internal control for the tyr RT-PCR assay for a possible impact of the co-amplification on test sensitivity. PCR was performed on the serial dilution of SK-Mel 28 cells (10⁴–10¹ cells) with either the two primer pairs or with one primer pair, respectively (Figure 1A). The tyr signal intensities of the PCR products of the dilution series obtained after co-amplification remained almost linear from 10³ to 10¹ SK-Mel 28 cells, whereas with 10⁴ SK-Mel 28 cells, amplification of the tyr mRNA reached a plateau (Figure 1C). The PCR signal strengths of the co-amplification were comparable co-amplification were comparable with the signal strength obtained when PCR was performed either for tyr or TCRBV cDNA alone, indicating that co-amplification does not grossly influence the test sensitivity (Figure 1B).

Applicability of the internal control for tyr RT-PCR was demonstrated when the RNA of serial SK-Mel 28 dilutions (10³–10¹ cells) and from peripheral blood of five melanoma patients were analyzed for tyr gene expression (Figure 2). PCR of patients’ blood samples was carried out in duplicate. The PCR signal strength of TCRBV gene expression indicates efficacy of individual sample processing, while the serial dilution of the SK-Mel 28 cells proves the individual assay’s sensitivity. The results of tyr gene expression of samples A, B and D are reliable because the internal control could be

**Figure 1.** Southern blot analysis of the amplified tyr gene and the control target TCRBV gene of a serial dilution of SK-Mel 28 (10¹–10⁴) cells after co-amplification and amplification. 10⁴ Jurkat cells were added to 10 mL of peripheral blood from healthy donors spiked with 10³–10⁴ SK-Mel 28 cells. After RNA extraction and RT, 45 cycles of co-amplification were carried out using two primer pairs, one pair specific for the tyr gene and the second pair specific for the internal control TCRBV gene resulting in PCR products of 207 and 307 bp, respectively. Separate tube amplification was performed for 45 cycles with either one of the specific primer pairs. PCR products were run on a 2% agarose gel and visualized after Southern blot with chemiluminescence-based detection (A). Optical densities (OD) of the dilution series after co-amplification (B) and amplification (C) were determined using Image 1.4 software.
detected. Sample C was processed with low efficiency according to the weak TCRBV signal strength. The negative result for tyr gene expression of sample E is questionable because the internal control is not detected even after duplicate amplification. In this case, negativity of the internal control indicates poor sample-processing efficacy. Therefore, sample E must be reanalyzed for tyr gene expression. Blood samples of 9 healthy donors tested negative for tyr mRNA expression, and because no Jurkat cells were added to these 9 samples, no internal control gene expression was detected (data not shown).

The internal control we described combines easy applicability with reliable evaluation of the RT-PCR assay. The principle of the internal control used here is of general applicability. Although we selected human Jurkat cells, any other cell (e.g., of nonhuman or even plant origin) can be used as an internal control given that: (i) the control gene is not expressed in any of the cells present in the clinical specimen to be analyzed; (ii) before routine investigations, primer competition between control gene and target gene with influence on the test sensitivity is ruled out; and (iii) the signal strength of the control gene is adapted to the level of the target gene expression to be investigated. This is accomplished by choosing the respective number of Jurkat cells per sample. Once the number of control cells is determined, aliquots of a stock of Jurkat cells diluted in GITC buffer can be cryopreserved at -70°C and added to the blood sample upon arrival in the laboratory.

In our laboratory, we perform tyr and TCRBV RT-PCR on whole-blood samples. In other laboratories where density gradient separations are performed, viable control cells can be added alternatively to the blood samples, facilitating control of cell recovery. However, because natural tumor cells and Jurkat T cells might not have the same density, the use of a density gradient separation can alter overall cell recovery and generate sample-to-sample variation.

Application of the internal control using gradient separation with specific density of 1.077 g/mL, separating the mononuclear cell fraction, assures good recovery of the Jurkat T-cell line but not necessarily of circulating melanoma cells, in which a proportion of cells are lost in the erythrocyte fraction (5). Therefore, application of the internal control for sample processing with specific density of 1.077 g/mL is questionable reliability. However, when the specific density of the separation medium is increased to 1.09 g/mL, separating all peripheral blood leukocytes from erythrocytes, recovery of circulating melanoma cells from peripheral blood is remarkably improved, and split-sample analysis revealed that the number of positive samples corresponds to the results obtained after whole-blood processing (5). In the case in which gradient separation with a specific density of 1.09 g/mL is performed, our internal control allows an estimation of the efficacy of sample processing.

A further advantage of the internal control is that it can also be used for semiquantitative analysis of the PCR assay. For this purpose, the signal strength of the internal control can be used for normalization of the target gene expression, which can subsequently be compared, e.g., in our tyr RT-PCR assay to a standard curve of SK-Mel 28 cells run in the same assay (2).

Taken together our results show that the internal control circumvents a number of inherent problems of alternative controls to assess pre-PCR procedures. The overall RT-PCR assay sensitivity can be reliably evaluated on a per sam-

![Figure 2](image-url)
ple basis, and the sensitivity limit of the RT-PCR assay can be assessed for every sample. This type of reliability can improve homogeneity of results from clinical investigations in the future.

REFERENCES


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