The Usefulness of Quantitative Real-Time PCR in Immunogenetics

Maurizio Provenzano, MD,[1] Carlo Riccardo Rossi, MD,[2] Simone Mocellin, MD[1,2]

[1] Department of Transfusion Medicine, Clinical Center, National Institutes of Health, Bethesda, MD and
[2] Department of Oncological and Surgical Sciences, Istituto di Clinica Chirurgica II,
University of Padova, Padova, Italy. E-mail: mocellins@hotmail.com

The polymerase chain reaction (PCR) has revolutionized the detection of DNA and RNA, allowing the detection of as little as a single copy of a given sequence. PCR is largely used in immunogenetics studies for a variety of purposes, such as the detection of viral load in infectious diseases, identification of markers that can predict survival of patients undergoing organ transplantation[1] and, in general, to assess the expression of immune related genes.

Theoretically, there is a quantitative relationship between the amount of starting material and the PCR product at any cycle. However, it is a common experience for replicate reactions to yield different amounts of PCR product. Furthermore, at the end of a PCR reaction in which the number of cycles are empirically pre-determined, different amounts of starting material can yield similar amounts of amplification product due to the consumption of the reagents. As a consequence, conventional PCR cannot be considered a quantitative assay and the results are usually expressed in terms of positivity/negativity. A semi-quantitative evaluation is possible on the basis of the appearance of the electrophoretic band (weak/strong positivity), but this approach is highly subjective and the sensitivity is very low. To address the issue of PCR product quantitation, methods such as competitive PCR,[2] sequence analysis gene expression (SAGE)[3] and high-throughput microarray[4] have been proposed. These techniques are cumbersome, time consuming, and require multiple manipulations of the samples, thus increasing the risk of carry-over contamination.

Quantitative real-time PCR (qr-PCR) allows a highly sensitive quantification of transcriptional levels of the gene of interest in a few hours with minimal handling of the samples.[5,6] Here we describe the principles and the technical aspects of this technique, and review its main applications in the field of immunogenetics.

Principles and Techniques

Higuchi and colleagues pioneered the analysis of PCR kinetics by constructing a system that detects PCR products as they accumulate.[7] This “real time” system utilized the intercalator ethidium bromide that binds to the increasing amounts of amplified double stranded DNA, resulting in an increase of fluorescence. This method has been improved by the introduction of fluorescent gene specific probes in the PCR reaction.

TaqMan methodology is based on the 5' nuclease assay first described by Holland et al.,[8] which uses the 5'-3' exonuclease activity of *Taq* DNA polymerase to cleave a dual-labeled probe annealed to a target sequence during PCR amplification. Briefly, after total RNA extraction and cDNA synthesis, the cDNA is added to a PCR reaction mixture containing standard PCR components plus a probe that anneals to the template between the two primers (Figure 1). This probe contains both a fluorescent reporter dye at the 5'-end (6-carboxyfluorescein, 6-FAM; emission λ_{max} = 518 nm) and a quencher dye at the 3'-end (6-carboxytetramethylrhodamine, TAMRA; emission λ_{max} = 582 nm). The quencher can only quench the reporter fluorescence when the two dyes are close to each other. This is only in the case of an intact probe. Once amplification occurs, the probe is degraded by the 5'-3' exonuclease activity of the Taq DNA polymerase, and the fluorescence will be detected by means of a laser integrated in the sequence detector (TaqMan ABI Prism 7700 Sequence Detection System, Perkin Elmer, Foster City, CA). The PCR cycle number at which fluorescence reaches a threshold value of 10 times the standard deviation of baseline emission is used for quantitative measurement. This cycle number is called the cycle threshold (Ct) and it is inversely proportional to the starting amount of target cDNA.

The number of target gene copies is extrapolated from a standard curve equation. For each gene, cDNA from a positive control is first generated by the reverse transcription reaction. Using 1 µl of this cDNA, the gene under investigation is amplified using Taqman primers by means of a standard PCR reaction. The amount of amplicon obtained is then quantified by spectrophotometry and the number of copies calculated on the basis of the molecular weight of each individual gene amplicon. Serial dilutions of this amplicon are tested with qr-PCR assay to generate the gene specific standard curve. Optimal standard curves are based on PCR amplification efficiency from 90 to 100% (100% meaning that the amount of template is doubled after each cycle), as demonstrated by the slope of the standard curve equation. Linear regression analysis of all standard curves should show a high correlation (R^2 coefficient ≥ 0.98).

Since both the amount of total RNA added to each reverse transcription reaction tube (based on wavelength absorbance) and its quality (i.e., degradation) are not reliable parameters to measure the starting material, transcripts of a housekeeping gene are quantified as an endogenous control. Beta-actin is one of the most used nonspecific housekeeping genes, but according to the experimental conditions, other internal references can be adopted (e.g., leukocyte markers). For each experimental sample the value of both the target and the housekeeping gene are extrapolated from the respective standard curve. The target value is then divided by the endogenous reference value to obtain a normalized target value independent of the amount of starting material.

Quantitative PCR of both specimen cDNA and standard amplified cDNA are conducted in a 25 µl final volume mixture containing Taqman Master Mix (Perkin Elmer), primers and probe at optimized concentrations (probe: 200 nM, primers: 400 nM). Thermal cycler parameters include 2 min at 50°C, 10 min at 95°C and 40 cycles of denaturation at 95°C for 15 s and annealing/extension at 60°C. The TaqMan ABI Prism 7700 Sequence Detection System allows 96 samples to be run simultaneously.

The Applications

Quantitative real-time PCR (qr-PCR) has been utilized in humans to measure viral load for diagnosis of different dis-

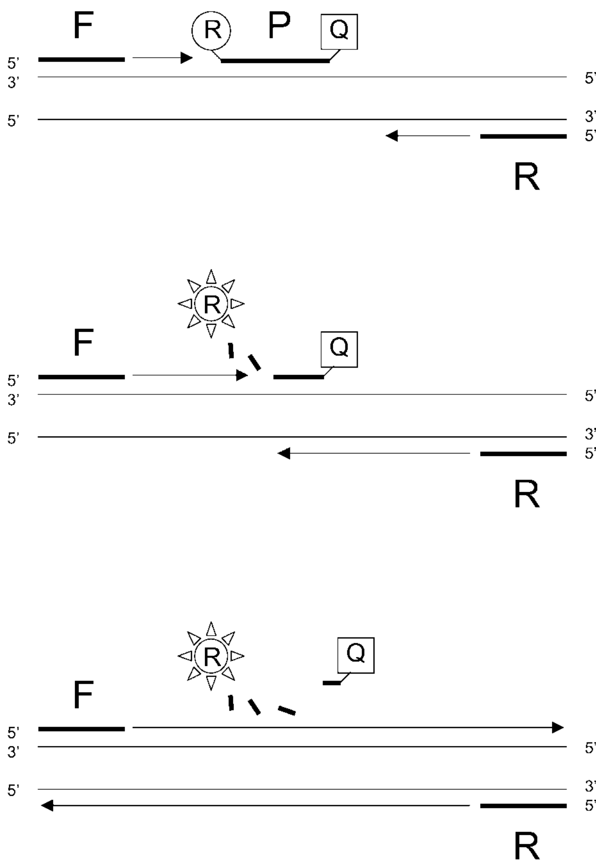

Figure 1. During quantitative real time PCR, in addition to a forward (Fp) and a reverse (Rp) primer, a fluorescent probe (P) is utilized. Two fluorescent dyes, a reporter (R) and a quencher (Q) are attached to the 5' and 3' ends of the Taqman probe, respectively. When both dyes are attached to the probe, reporter dye fluorescent emission is quenched (top panel). During each extension cycle, the *Taq* DNA polymerase cleaves the reporter dye from the probe (middle panel). Once separated from the quencher, the reporter emits its characteristic fluorescence throughout the DNA polymerization (bottom panel).

eases caused by DNA viruses such as CMV, EBV and HBV. Investigators have adopted this system to titer viral genome density in clinical specimens as an alternative to antigenemia assays. Tanaka and colleagues[9] demonstrated that the CMV load in peripheral blood leukocytes was positive in 100% of immunocompromised symptomatic patients and in 24% of asymptomatic patients, as well as in the plasma of 76% of symptomatic patients studied, paralleling and enhancing the results of the antigenemia assay in all cases. Moreover, the monitoring of patients with CMV infection demonstrated that the CMV DNA copy number changed proportionally with anti-CMV therapy, confirming that this technique is quite helpful for diagnosing the onset of CMV-related diseases and monitoring virus reactivation in patients with latent CMV infections. Other investigators quantified the serum load of CMV and other viruses after anti-viral treatments or post-transplantation using this technique.[10,11] Although discrepancies in the correlation between the qr-PCR technique and the antigenemia assay were observed in samples with a low viral load,[12] the results obtained from the follow-up of bone marrow transplant patients for CMV and EBV reactivation confirmed that this new procedure should be widely used for monitoring viral infections in transplanted patients.[13,14]

Although qr-PCR has been applied mostly to virus related investigations, some authors have reported on the utilization of this technique to study the immunological phenomena occurring during HLA class I peptide-based immunotherapy for metastatic melanoma. Kammula et al. identified T cell reactivity to melanoma associated antigens used for the vaccination of patients by measuring interferon-gamma (IFN-γ) transcript levels in PBMC before and after treatment.[15] We recently applied qr-PCR to the identification of CTL reactivity to CMV-derived peptides in order to set up a sensitive screening for candidate immunodominant CMV peptides and create a large library of HLA class I restricted CMV peptides. PBMC from serologically CMV positive donors were directly exposed *ex vivo* to candidate CMV epitopes and, after three hour incubation, IFN-γ mRNA levels were measured by qr-PCR. With this method we could quickly identify the *ex vivo* immunogenicity of a panel of peptides on the basis of CTL reactivity (Provenzano et al., in preparation). With this technique, a new HLA-A24 restricted peptide was found to be the most

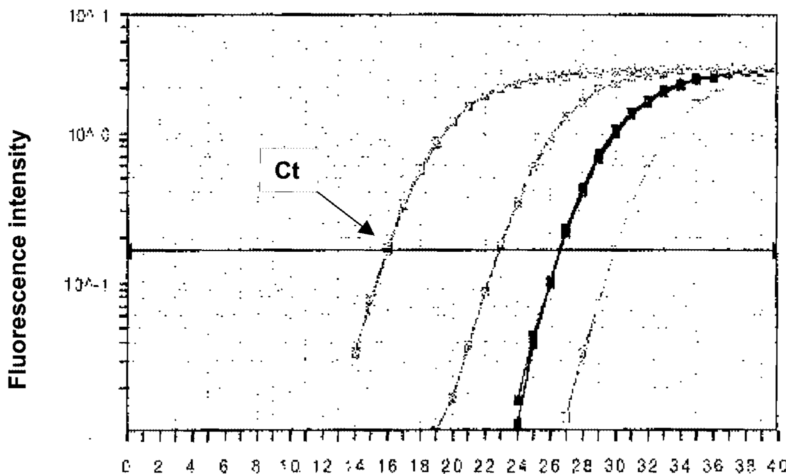

Figure 2. The copy number of each gene is extrapolated on the basis of a standard curve. Serial dilutions of known number of gene copies are run in triplicate. In this diagram, PCR cycles (X axis) are plotted against the fluorescence intensity (Y axis). The cycle at which the fluorescence reaches a threshold value (ten times the standard deviation of baseline emission) is called threshold cycle (Ct) and is inversely proportional to the starting amount of target DNA.

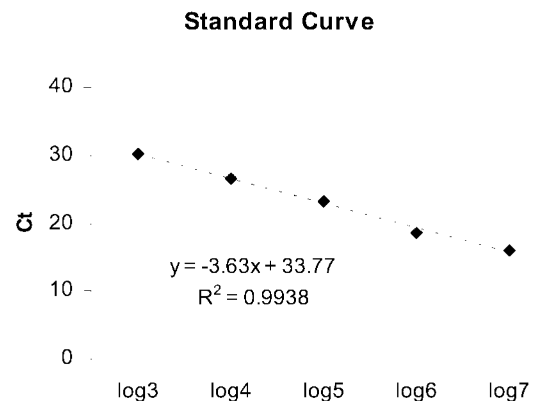

Figure 3. When the known concentrations (expressed in logarithmic form, X axis) of target gene are plotted against the corresponding cycle threshold (Ct, Y axis) obtained by qr-PCR, the result is a line representing the linear correlation between the two parameters. The equation describing this relationship is used to extrapolate the gene copy number in experimental samples.

immunogenic peptide among those screened in all HLA-A24 donors studied, demonstrating that qr-PCR can increase the rate at which immunogenic peptides are identified.

Recurrence of hematological cancers has also been evaluated using qr-PCR.[16] To measure the risk of relapse of T-lineage acute lymphoblastic leukemia (ALL) in children, these researchers investigated the detection and quantification of residual leukemic cells that harbor the TAL-1 deletion. Seventeen out of 23 patients enrolled in the study were TAL-1 positive, the assay having a sensitivity of detection of one leukemic cell among $1x10^5$ normal cells. Thus, TAL-1 qr-PCR is a sensitive and accurate method for assessment of minimal residual disease (MRD) in T-lineage ALL. In the wake of these results, a Japanese group utilized qr-PCR to monitor the MRD in leukemia and lymphoma patients by assessing PRAME (preferentially expressed antigen of melanoma) expression in peripheral blood samples,[17] demonstrating in 8 out of 8 patients monitored a significant reduction of PRAME transcription levels after chemotherapy and an increased expression in two patients who relapsed. Moreover, PRAME positive cell lines were found to be susceptible to lysis by specific cytotoxic T lymphocytes, suggesting that this tumor associated antigen can represent a target for a cell mediated immune response.

Since reverse transcriptase PCR is often used for detection of micrometastasis in blood, lymph nodes and bone marrow, some investigators analyzed the expression level of cytokeratin-18 (CK18) mRNA by qr-PCR in gastrointestinal carcinoma cell lines.[18] As high CK18 expression levels were detected in carcinoma cell lines using this technique, compared to levels in non-epithelial cells, it was concluded that not only qualitative but also quantitative analysis of target mRNA is important for detection of micrometastasis in cancer.

Conclusions

Techniques used to analyze the presence of target genes by DNA replication, or their activity by protein production, are less accurate and require more time to be evaluated. Quantitative real-time PCR is a method to rapidly and precisely quantify gene activity by detecting mRNA levels of the gene of interest. In this review, we reported the broad applicability of this technique in microbiology, virology, oncology and immunology. The rapid identification and quantification of the target studied make this method useful for diagnosis of different diseases and for monitoring after treatment. Moreover, the *ex vivo* identification of specific CTL activity to viruses and/or tumor antigens will improve the understanding of the mechanisms responsible for anti-viral and anti-tumor immune responses. Thus, application of qr-PCR should allow the rapid identification of new diagnostic targets as well as assist in the development and improvement of treatments.

References

1. Shulzhenko N, Morgun A, Rampim GF, et al. Monitoring of intragraft and peripheral blood TIRC7 expression as a diagnostic tool for acute cardiac rejection in humans. *Hum Immunology*; 62:342-347.
2. Freeman W, Walker S. 1999. Quantitative RT-PCR: pitfalls and potential. *Biotechniques*; 26:112-122.
3. Velculescu V, Zhang L. 1995. Serial analysis of gene expression. *Science*; 270:484-487.
4. Wang E, Miller L, Ohnmacht GA, Liu E, Marincola FM. 2000. High fidelity mRNA amplification for gene profiling using cDNA microarrays. *Nature Biotech*; 17:457-459.
5. Kammula US, Marincola FM, Rosenberg SA. 2000. Real-time quantitative polymerase chain reaction assessment of immune reactivity in melanoma patients after tumor peptide vaccination. *J Natl Cancer Inst*; 92:1336-1344.
6. Mandigers C, Meijerink J, Raemaekers J. 1998. Graft versus lymphoma effect of donor leucocyte infusion shown by real time quantitative PCR analysis of t(14;18). *Lancet*; 352:1522-1523.
7. Higuchi R, Dollinger G, Walsh P. 1992. Simultaneous amplification and dectection of specific DNA sequances. *Biotechnology*; 10:413-417.
8. Holland P, Abramson R. 1991. Detection of specific polymerase chain reaction product by utilizing the 5' to 3' exonuclease activity of Thermus Aquaticus DNA polymerase. *Proc Natl Acad*. Sci U.S.A.; 88:7276-7280.
9. Tanaka N, Kimura H. 2000. Quantitative analysis of cytomegalovirus load using a real-time PCR assay. *J Med Virol*; 60:455-462.
10. Gault E, Michel Y. 2001. Quantification of human cytomegalovirus DNA by real time PCR. *J Clin Microbiol*; 39:772-775.
11. Brechtuehl K, Whalley S. 2001. A rapid real time quantitative polymerase chain reaction for hepatitis B virus. *J Virol Methods*; 93:105-113.
12. Najioullah F, Thouvenot D, Lina B. 2001. Development of a real time PCR procedure including an internal control for the measurement of HCMV viral load. *J Virol Methods*; 92:55-64.
13. Ohga S, Kubo ENA. 2001. Quantitative monitoring of circulating Epstein-Barr virus DNA for predicting the development of post-transplantation lymphoproliferative disease. *Int J Hematol*; 73:323-326.
14. Limaye A, Huang MLW. 2001. Cytomegalovirus (CMV) DNA load in plasma for the diagnosis of CMV disease before engraftment in hematopoietic stem cell transplant recipients. *J Inf Disease*; 183:377-382.
15. Kammula US, Lee K-H, Riker A, et al. 1999. Functional analysis of antigen-specific T lymphocytes by serial measurement of gene expression in peripheral blood mononuclear cells and tumor specimens. *J Immunol*; 163:6867-6879.
16. Chen XPQ, Stow P. 2001. Quantification of minimal residual disease in T-lineage acute lymphoblastic leukemia with the TAL-1 deletion using a standardized real time PCR assay. *Leukemia*; 15:166-170.
17. Matsushita M, Ikeda H, Kizaki M. 2001. Quantitative monitoring of the PRAME gene for the detection of minimal residual disease in leukemia. *Br J Haematol*; 112:916-926.
18. Tokunaga E, Maehara Y. 2000. Application of quantitative RT-CR using Taqman technology to evaluate the expression of CK 18 mRNA in various cell lines. *J Exp Clin Cancer Res*; 19:375-381.