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Review

Quantitative Real-Time PCR in Cancer Research

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Abstract. In the era of the Human Genome Project, quantitation of gene expression by tumor/host cells is of paramount importance to investigate gene patterns responsible for cancer development, progression and response/resistance to treatment. Quantitative real-time PCR (qrt-PCR) technology has recently reached a level of sensitivity, accuracy and practical ease that support its use as a routine bioinstrumentation for gene level measurement. Several applications have been already implemented in the field of cancer research, and others are being validated, showing that this molecular biology tool can provide both researchers and clinicians with precious information concerning the behaviour of tumors. The knowledge of the biochemical principles underlying this biotechnology can be of great value to correctly interpret qrt-PCR data.

Key words: quantitative PCR; cancer research.

Introduction

Polymerase chain reaction (PCR)-based techniques allow us to obtain genetic information through the specific amplification of nucleic acid sequences starting with a very low number of target copies. These reactions are characterized by a logarithmic amplification of the target sequences, i.e. an increase of PCR copies, followed by a plateau phase showing a rapid decrease to zero of copy number increment per cycle. Accordingly, the amount of specific DNA product at the end of the PCR run bears no correlation to the number of target copies present in the original specimen. However, many applications in medicine or research require quantification of the number of specific targets in the specimen, both to study the reaction of the cell or cell population to a stimulus and to compare the gene profile of different samples. The fundamental importance of gene expression quantification methods in basic research, pharmaco-genomics and molecular diagnostics[91] continues to direct efforts aimed at improving current methodologies as well as the development of novel technologies. Not all are based on target amplification: the "Invader" assay is a development of the invasive signal amplification assay that combines two signal amplification reactions in series to generate and amplify a fluorescent signal in the presence of the correct target sequence[21]. The assay is sensitive down to sub-attomole levels and may well become a method of choice in the future. For now, however, reverse transcription (RT)-PCR-based assays are the most common method for characterizing or confirming gene expression patterns and comparing messenger RNA (mRNA) levels in different sample populations[77]. Despite the wide variability of results characteristic of conventional RT-PCR assays[86] and their unreliability as a clinical

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diagnostic tool[15], considerable attention is still focused on conventional, competitive protocols[32] with steady improvements to internal standards, references for data normalization as well as the introduction of new mathematical models for data analysis[39].

Serial analysis of gene expression, originally developed by VELCULESCU et al.[109], allows for high-throughput gene profiling. However, this technique is cumbersome, time consuming and requires multiple manipulations of the samples, increasing the risk of carry-over contamination. Furthermore, like Northern and Southern blot, it requires large amounts of input mRNA, making impossible the analysis of hypocellular specimens.

Among the most promising innovations applied to conventional RT-PCR protocols is the development of quantitative RT-PCR such as competitive standardized RT-PCR and quantitative real-time PCR (qrt-PCR). These technologies address some of the major problems associated with conventional RT-PCR protocols: 1) the use of standardized competitor templates or standard curves allows comparison between experiments and 2) the use of internal standards solves the problem of variation in template starting amounts and operator loading errors. Competitive RT-PCR is a time-consuming system which is limited to sets of primers available from one supplier. Furthermore, it does not eliminate the errors associated with individuals carrying out the reactions.

Conceptual simplicity, practical ease[112] and the promise of high throughput[19] have made the homogeneous real-time fluorescence detection assay[41] the most widely used mRNA quantification method for research application. Its potential as a clinical diagnostic assay[12] is also being realized, and its use has been reported for the detection of bacterial[36] and viral[16, 24, 89, 98] DNA/ /RNA in clinical samples, the analysis of cellular immune responses in the peripheral blood[42], the evaluation of tissue-specific gene expression[11] and for cytokine mRNA profiling[100].

In the oncology field, qrt-PCR is experiencing a rapid diffusion among investigators because of its potential applicability to a wide range of research. Qrt--PCR allows a highly sensitive quantification of DNA and transcriptional gene levels in a few hours with minimal handling of the samples. The recent flood of reports using qrt-PCR in cancer research testifies the transformation of this technology from an experimental tool into the scientific mainstream. The range of applications of qrt-PCR in the oncology field is immense and has been fuelled in part by the proliferation of low-cost instrumentation and reagents. Many of the applications of qrt-PCR include measuring mRNA expression levels[70, 84, 117], DNA copy number[34, 52, 74], allelic discrimination[76], confirmation of microarray data[68, 88, 115], and measuring titers of (tumor) oncology-related viruses[16, 106]. Some of the most recent applications, which demonstrate the sensitivity of this technology when applied to expression analysis of hypocellular samples, include expression analysis of fine-needle aspirate material[50, 69, 75], laser capture of microdissected material[35], paraffin-embedded tissues[97], and linearly amplified RNA[115].

This review was not meant to report comprehensively on the use of qrt-PCR in cancer research. Instead, we described the principles underlying qrt-PCR, illustrated its technique and discussed its main limits. Furthermore, we reported on the main utilizations of qrt--PCR in the field of oncology. In particular, we focused on two emerging applications, which are tumor immunology and minimal residual disease assessment, showing that this laboratory tool can provide both researchers and clinicians with precious information regarding the behavior of tumors.

Principles

HIGUCHI et al.[43] pioneered the analysis of PCR product amounts by studying PCR kinetics. The concept of "real-time" PCR consists of the detection of PCR products as they accumulate. The first system constructed to this aim included the DNA intercalator ethidium bromide in each amplification reaction and an adapted thermal cycler to irradiate the samples with ultraviolet light. The reaction produces increasing amounts of double-stranded DNA, which binds ethidium bromide, resulting in an increase in fluorescence emission. The main drawback of intercalator-based detection of PCR product accumulation is that both specific and non-specific products generate signal. Real--time systems for PCR were then improved by probe-based, rather than intercalator-based PCR product detection. The 5' nuclease assay, first described by HOLLAND et al.[44], is based on the cleavage of a target probe during PCR by the 5' nuclease activity of *Thermophylus aquaticus* (*Taq*) DNA polymerase. During amplification, annealing of the probe to its target sequence generates a substrate that is cleaved by the *Taq* polymerase when the enzyme extends from an upstream primer into the region of the probe. This dependence on polymerization ensures that probe cleavage occurs only if the target sequence is being amplified. With this first generation qrt-PCR, after the PCR reaction the

amount of cleaved probe is measured by thin layer chromatography to separate cleavage fragments from intact probe. The development of fluorogenic probes by LEE et al.[57] allowed the elimination of post-PCR processing for the analysis of probe degradation. Two main systems have been developed, which exploit the extension or the annealing phase, respectively, to generate fluorescence emission (Fig. 1 and 2). In both cases, the fluorescence signal increases with each PCR amplification cycle. The PCR cycle number at which fluorescence reaches a threshold value of ten times the standard deviation of baseline fluorescence emission is used for quantitative measurement (Fig. 3). This cycle number is called the cycle threshold (Ct) and it is inversely proportional to the starting amount of target cDNA (Fig. 4).

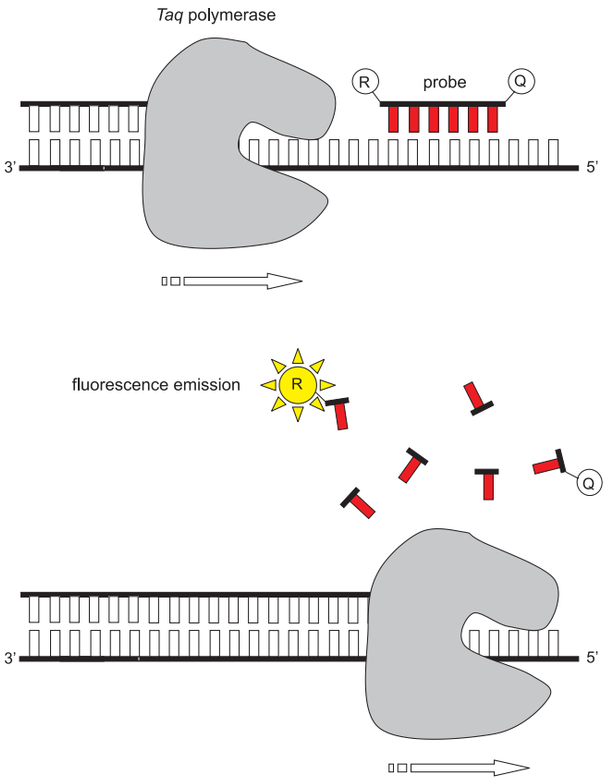

Fig. 1. Principles of qrt-PCR using fluorogenic probes: scheme of the extension phase method. In addition to the reaction components used for conventional PCR, the probe is an oligonucleotide with both a reporter fluorescent dye and a quencher dye attached at its 5'- and 3'-end, respectively. During the extension phase, the quencher can only quench the reporter fluorescence when the two dyes are close to each other. This is only the case of an intact probe. In fact, 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. This reaction is measured by the ABI 7700, or more recently by ABI 5700 ABI Prism machines, from Perkin Elmer

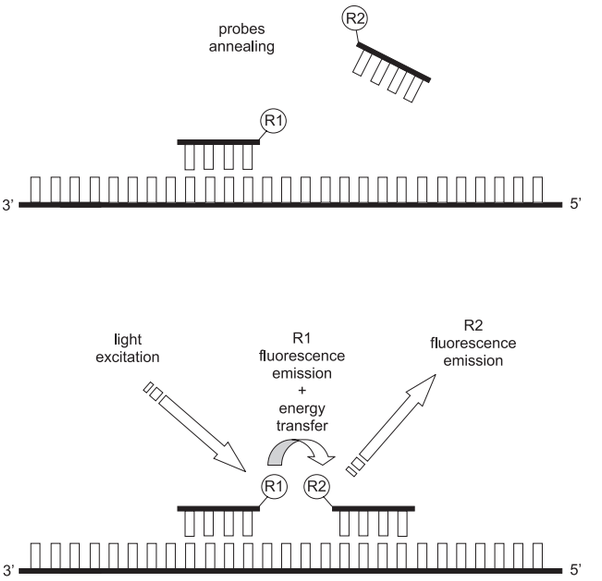

Fig. 2. Principles of qrt-PCR using fluorogenic probes: scheme of the annealing phase method. In this case, two different probes are used, one carrying a fluorescein label at its 3'-end, whereas the other carries another label (LC Red 640) at its 5'-end. The sequences of these two oligonucleotides are selected such that they hybridize to the amplified DNA fragment in a head to tail arrangement. When the oligonucleotides hybridize in this orientation, the two fluorescence dyes are positioned in close proximity to each other. The first dye (fluorescein) is excited by the filtered light source and emits green fluorescent light at a slightly longer wavelength. When the two dyes are in close proximity, the emitted energy excites the LC Red 640 attached to the second hybridization probe that subsequently emits red fluorescent light at an even longer wavelength. This energy transfer, referred to as fluorescence resonance energy transfer is highly dependent on the spacing between the two dye molecules. Only if the molecules are in close proximity (a distance between 1–5 nucleotides) is the energy transferred at high efficiency. Choosing the appropriate detection channel, the intensity of the light emitted by the Red 640 is filtered and measured. Since LC Red 640 only emits a signal when both oligonucleotides are hybridized, the fluorescence measurement is performed after the annealing step. This reaction is measured by the Light Cycler System, from Roche

Experimental Design

There are primarily two types of qrt-PCR analysis: relative quantitation and absolute quantitation[11]. However, the latter is a misleading name. In fact, no matter what the source or how carefully it is measured, there is no way to know exactly how much or how many copies of a known template truly exists in a given well

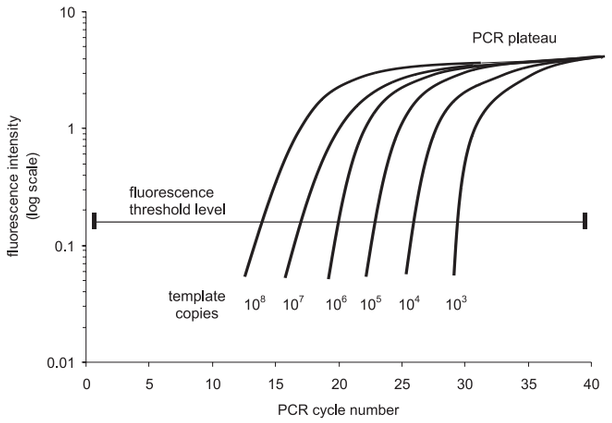

Fig. 3. β-actin amplification plot illustrating the nomenclature typically used in qrt-PCR experiments. The amplification plot is the plot of fluorescence signal *vs* PCR cycle number. The signal measured during these PCR cycles is used to plot the threshold. The threshold is calculated as 10 times the standard deviation of the average signal of the baseline fluorescent signal. A fluorescent signal that is detected above the threshold is considered a real signal that can be used to define the cycle threshold (Ct) for a sample. The Ct is defined as the fractional PCR cycle number at which the fluorescent signal is greater than the minimal detection level. The Ct values of different β-actin concentrations are used to generate the standard curve and then calculate the relative equation (Fig. 4)

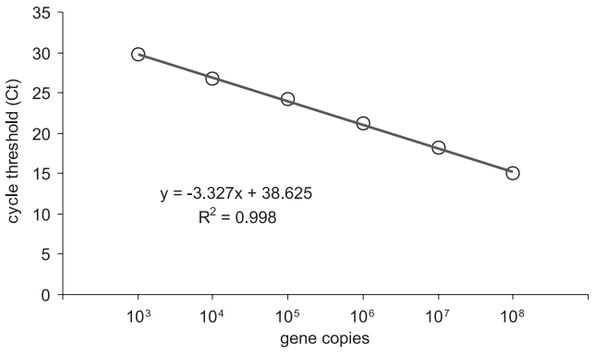

Fig. 4. β-Actin standard-curve plot for calculation of PCR efficiency and quantitation. A 10-fold serial dilution of a positive control template is used to generate the standard curve. The resulting Ct values for each input amount of template are plotted as a function of the log concentration of input amounts and a linear trend-line is fit to the data. This is done both for optimizing a PCR reaction as measured by the PCR efficiency and for quantitation of unknown samples. Determining PCR efficiency is especially important when using the relative quantitation method. The resulting slope of the line fit to the data is used to determine the PCR efficiency as shown in the formula. An ideal slope should be 3.32 for 100% PCR efficiency; in this example it is 97.6%. 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 ≥ 0.99) to be considered suitable for gene-level quantitative analysis. The function that defines this slope is also used to calculate the amount of unknown samples. Most real-time PCR instruments have software that can automatically compute the amount of template of an unknown sample from a standard curve. However, it can be done manually by putting the observed Ct value for an unknown sample into the formula: (observed Ct – y intercept)/slope

of a known sample[33]. A more appropriate term for this method is standard curve-based quantitation, as a standard curve (5- or 10-fold serial dilution) of "known" amount of a given gene is used to quantify the gene abundance in a sample of interest.

For relative quantitation, a "within sample" comparison is made between the gene of interest and that of a control gene. In this case, quantitation is done by subtracting the Ct of the control gene from the Ct of the gene of interest. The resulting difference in cycle number (ΔCt) is the exponent of the base 2 (due to the doubling function of PCR), representing the fold difference of template for these two genes. The assumption must be made that the chosen control gene does not vary in copy number or expression level amongst the samples of study. Only if this assumption holds true can a lot more samples can be run on any given plate or multiple plates that will be completely comparable.

Technique

Here we briefly describe the qrt-PCR protocol we routinely adopt when using the TaqMan ABI Prism 7700 Sequence Detection System (Perkin Elmer, CA). Using the dedicated program (Primer Express) included in this system, probes and primers are designed to obtain amplicons less then 100 bp in length in order to enhance PCR efficiency. Probes are labeled with 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). Furthermore, they are designed so to cross an intron-exon junction in order to avoid genomic DNA amplification. To ensure that the sets of probes and primers in use do not amplify genomic DNA, an agarose gel electrophoresis can be run. Given the small size of the amplicons, we adopt the FluorImager 595 scanning system (Molecular Dynamics, CA) to detect the fluorescence of the Vistra Green intercalator (Amersham Biosciences, UK). An argon laser for excitation at 488 nm and an emission band-pass filter 530 DF30 are recommended.

Using total RNA extracted from cells known to express the gene/s of interest, cDNA synthesis is performed. For each gene considered, cDNA from a posi-

tive control is first generated. Then, the gene under investigation is amplified using TaqMan primers by means of a standard PCR reaction. To this aim, 1 µl cDNA is added to the following reagents: 1 µl TaqMan primers (10 µM, Perkin Elmer, CA), 5 µl 10 × PCR buffer (Perkin Elmer, CA), 11 ml $MgCl_2$ (25 mM), 1 ml dNTP (10 mM) and 0.5 µl AmpliTaq Gold (Perkin Elmer, CA) in a final volume of 50 µl. The thermal cycler conditions for this PCR reaction are set as follows: after 10 min at 95°C, 15 s at 95°C followed by 1 min at 60°C are repeated 40 times. 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 qrt-PCR assay to generate the gene-specific standard curve.

Qrt-PCR of both sample cDNA and standard amplified cDNA is conducted in a 25 µl final volume mixture containing primers, probe and 1 × TaqMan Master Mix (Perkin Elmer, CA) at optimized concentrations (probe: 200 nM, primers: 400 nM). The TaqMan ABI Prism 7700 Sequence Detection System allows running 96 samples at a time. Thermal cycler parameters include 2 min at 50°C, 10 min at 95°C and 40 cycles involving denaturation at 95°C for 15 s and annealing/extension at 60°C.

Since both the amount of total RNA added to each reverse transcription reaction tube (based on wave length absorbance) and its quality (i.e. degradation) are not reliable parameters in order to measure the starting material, we also quantify the sample transcripts of a housekeeping gene as an endogenous control: β-actin is chosen as a common housekeeping gene (Fig. 3), while leukocyte markers (e.g. CD8) are used as cell-specific internal reference[51].

For each experimental sample the value of both the target and the housekeeping gene are extrapolated from the respective standard curve equation (Fig. 4). The target value is then divided by the endogenous reference value to obtain a normalized target value independent of the starting material amount.

Limits of the technique

Results variability. The variability of RT-PCR results obtained from identical samples assayed in different laboratories continues to be a problem[7]. In qrt-PCR, the single most likely source of data variation is the variability introduced by the person carrying out the experiment. Since there are several steps involved in going from a tissue sample to a quantitative result, it is not surprising that this is so.

The recent introduction of robotics into molecular biology should reduce the variability and contamination observed when different operators prepare multiple templates[65]. Fully automated robotic workstations, already made commercially available by Applied Biosystems, Genovision, Roche and Qiagen, use an integrated vacuum system or magnetic bead system to purify RNA or DNA. They are designed to dispense reagents for the RT-PCR assay, and in the case of the Applied Biosystems 6700 present the operator with a sealed plate that is ready to load on the thermal cycler.

Result normalization. The identification of a valid reference for data normalization remains the most stubborn of problems and none of the solutions proposed appear to be ideal. It is especially difficult when dealing with *in vivo* samples and comparing gene expression patterns between different individuals. Surprisingly, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) continues to be utilized as a normalizer despite continuing reports that emphasize the problems associated with its use[53, 105]. In fact, it is now well documented that GAPDH mRNA levels are not constant[120], particularly under some pathological conditions[37]. Ribosomal RNA (rRNA), which makes up the bulk of a total RNA sample, is another normalizer that has been proposed[6, 119], despite reservations concerning its expression levels, transcription by a different RNA polymerase, and possible imbalances in rRNA and mRNA fractions between different samples[95]. We and other authors routinely use β-actin as housekeeping gene[10, 27, 38, 90, 94, 99, 116]. Even though the issues of β-actin gene regulation and pseudogene existence has been raised[82, 93], we believe that the consistency of results yielded over time in our experience support *ex adjuvantibus* the use of this reference gene.

Alternatively, some investigators have advocated normalization to total cellular RNA as the least unreliable method[11]. However, little is known about the total RNA content per cell of different tissues *in vivo,* or how this might vary between individuals or between normal and tumor tissue. Moreover, a major problem arises when RNA is prepared from extremely small samples, e.g. from fine-needle aspirates. Here it is not possible to quantitate the RNA sample, and it becomes necessary to normalize copy number to some internal RNA template.

The best solution to this basic issue might be to choose a set of reference genes in order to minimize the potential variability characteristic of each single gene. To this aim, an endogenous control plate avail-

able from Applied Biosystems evaluates the expression of eleven housekeeping genes.

mRNA cell source. When dealing with cell lines or *in vitro* purified cell populations, the issue of gene expression normalization is strictly about the best way to correctly measure gene copy number. *Ex vivo* samples constitute an even worse problem of qrt-PCR data interpretation. In fact, until recently, *in vivo* RNA extractions and subsequent analyses could be carried out from whole tissue biopsies with little regard for the different cell types contained within that sample. This inevitably results in the averaging of the expressions of different cell types and the expression profile of a specific cell type may be masked, lost or ascribed to and dismissed as illegitimate transcription[17] because of the bulk of the surrounding cells. Indeed, it is not surprising that significant differences have been detected in the gene expression profiles of microdissected and bulk tissue samples[30, 102]. This is particularly relevant when comparing gene expression profiles between normal and cancer tissue since normal cells adjacent to a tumor may be phenotypically normal, but genotypically abnormal or exhibit altered gene expression profiles due to their proximity to the tumor[23], and some tumors have significantly larger immune cell infiltrates than others[14]. Therefore, quantitative information *per se* is no longer sufficient. Again, technology developments might bring a solution to this important issue. The introduction of laser capture microdissection[29] represents a crucial step forward, allowing the extraction of a pure subpopulation of cells from heterogeneous *in vivo* cell samples for detailed molecular analysis[111].

Applications in cancer research

Tumor immunology. The major goal of therapeutic cancer vaccine trials is to mediate tumor regression. However, it is critically important to devise *in vitro* immunological assays that correlate with clinical outcome for use as surrogate markers of vaccine efficacy. Analysis of immune responses in circulating lymphocytes that address the presence of T cells bearing T cell receptors-specific for the epitope used for vaccination can accurately enumerate the number of T cells elicited by the vaccines[3] but does not yield information about their functional status[58]. Because of their low frequency, the study of cytotoxic T cell (CTC) cytofluorimetrically sorted using HLA/peptide/tetramer complexes is greatly limited. We recently reported on the use of qrt-PCR to assess directly the immune status of peripheral blood mononuclear cells (PBMC) from patients with melanoma undergoing peptide-based vaccination[78]. By testing PBMC for interferon γ (IFN-γ) mRNA expression, it was found that PBMC can respond to a vaccine-specific stimulus in a direct assay without requiring prolonged *in vitro* manipulations[50]. Comparative analysis of different monitoring methods demonstrated that vaccine-induced sensitization of circulating lymphocytes correlated with results obtained with classical *in vitro* sensitization methods[51] as well as T cell phenotyping with tetrameric HLA/epitope complexes and intracellular cytokine detection by FACS analysis[71, 73]. In addition to monitoring immune reactivity against individual tumor-associated peptides restricted by specific HLA molecules, qrt-PCR can also be applied to analyze immune reactivity against whole proteins, mixtures of proteins, or even whole tumor cells without knowledge of the relevant peptides or restriction elements. HOUSSEAU et al.[46] have applied qrt-PCR to monitor the effect of vaccination with a whole protein construct. In their case the immunogen consisted of poxvirus expression vectors encoding the tyrosinase melanoma antigen. The detection system consisted of autologous dendritic cells infected with an adenovirus expression vector encoding for tyrosinase. In this way, reactivity developed *in vivo* toward the poxvirus proteins could be excluded and only reactivity toward possible tyrosinase epitopes could be tested.

Human tumor immunology is at a standstill whereas implemented cancer vaccines have shown effectiveness in inducing immune responses detectable in circulating lymphocytes. In most circumstances, however, such immune responses are not sufficient to induce cancer regression. This paradoxical observation could be explained in several ways depending upon the immunological endpoint used for immune monitoring. In the assessment of the therapeutic efficacy of specific cancer treatment, analysis of immune responses in circulating lymphocytes (systemic response) may not be as relevant as the analysis of the same effector populations within the tumor microenvironment (peripheral response). A very promising application of qrt-PCR for the immune monitoring of cancer patients is its utilization for the documentation of immune/tumor cell interactions within the tumor microenvironment[113]. This can only be done using qrt-PCR, since other methods (e.g. immunohistochemistry, flow cytometry) require larger amounts of material. We applied qrt-PCR to the analysis of specimens obtained from fine needle aspirates (FNA). With this strategy we could follow dynamic changes in expression of tumor-associated antigens[75], cytokine expression and other immune cell-specific markers[50, 69] during immunization. Although some of these markers could have been followed by immuno-

histochemistry, for others there was no available antibody. In addition, the limited amount of material obtainable with FNA would not have allowed the preparation of a sufficient number of cytology slides to study more than few markers, while the RNA extracted from individual RNA and amplified to Wang's method[114] allowed the study of an unlimited number of genes[113]. Likewise, we followed the mRNA expression of some cytokines (IFN-γ, IL-10, TGF-β1, TGF-β2) before and during patients' vaccination[69]. Surprisingly, IL-10, which is generally considered an immunosuprressive molecule, was overexpressed in pre-treatment samples that responded to vaccination. This finding led us to revisit IL-10 functions and further study IL-10 properties, showing that this cytokine might have an important role as a bridge between innate and adaptive immune response. The identification of HLA-restricted immune dominant CTC epitopes is a limiting step when designing peptide-based vaccines. In a viral model, we set up a rapid and precise method to identify new HLA-restricted epitopes from the cytomegalovirus (CMV) pp65 protein[81]. Candidate peptides identified with a computer algorithm were screened by direct *ex vivo* stimulation of PBMC from CMV-seropositive HLA-A*2402 individuals. Qrt-PCR was used to evaluate CTC responses to peptides by measuring IFN-γ transcripts. These authors demonstrated that the measurement of IFN-γ mRNA by qrt-PCR can be used to detect CTC responses 3 h after peptide stimulation of a small quantity of PBMC without requiring time consuming *in vitro* sensitisation of CTC.

Detection of minimal residual disease. A high percentage of patients with leukemia, lymphoma, and solid tumors achieve a complete clinical remission after initial treatment. However, many of these patients will eventually relapse from residual tumor cells undetected by the common staging procedures. The focus of the study of minimal residual disease (MRD) is to redefine the concept of remission by using more sensitive molecular techniques to detect levels of disease burden below the resolution threshold of conventional pathology.

Specific genetic translocations characterizing some hematological malignancies can be used as tumor markers while monitoring the response to therapy. Treatment options often involve chemotherapy, radiotherapy, and/or bone marrow transplants. Over recent decades these advances have greatly improved the chances for survival for these patients. However, the risk of recurrence remains a significant obstacle for complete remission. Thus, the detection of MRD is a crucial step towards further refining treatment regimens.

The qualitative (yes vs no) detection of MRD is associated with a relative increase in relapse rate[26, 54]. The quantification of disease burden by qrt-PCR can greatly strengthen the relationship of MRD and subsequent relapse, allowing a truly "tailored" individual therapy. For instance, the reciprocal translocation t(8;21)(q22; q22), in which the gene for acute myeloblastic leukemia (AML)-1 transcription factor is fused with a gene (MTG8) on chromosome 8, is perhaps the most frequent chromosomal aberration resulting in AML[54]. The disruption of normal transcriptional regulation by AML-1 is likely to result in leukemogenesis. Despite the positive outcome of treatment for many AML patients, a significant proportion will relapse. Moreover, even without relapse many patients will still test positive for an AML-1/MTG8 fusion transcript using standard PCR techniques[118]. Perhaps limited by the detection of rare pre-leukemic cells, standard PCR techniques are not capable of stratifying patients, as the amount of fusion product may be a better prognostic indicator. Therefore, recent studies[55, 61] have demonstrated the use of qrt-PCR for detecting fusion products, which is helpful in identifying those patients with quantifiable minimal residual disease and may prove valuable as a prognostic indicator or for the assessment of treatment options. Similar studies have been conducted to quantify other translocation fusion transcripts, such as BCR-ABL in chronic myeloid leukemia (CML)[4, 80] before and after allogeneic transplant[28], or to determine the response to treatment with IFN-α in CML patients[5]. Quantification of leukemia-specific TEL-AML-1 fusion transcript levels in the prediction of relapse of childhood acute lymphoblastic leukemia (ALL)[79, 92] or to measure minimal residual disease of ALL[110] is possible using qrt-PCR. Furthermore, qrt-PCR has been used to quantify the chromosomal translocation t(14;18)(q32; q21)[59, 60] and bcl-2 rearrangement in patients with follicular lymphoma[45] or stem-cell harvests used to treat patients with non-Hodgkin's lymphoma[56]. It was also used to measure the prevalence of bcl-2 rearrangements in the peripheral blood of normal individuals in order to determine how background levels of this rearrangement might impact the measurement of disease in patients with follicular lymphoma[103]. Many of these studies have benefited greatly by the application of qrt-PCR methods. In fact, for the molecular detection of rare tumor cells in clinical samples, qrt--PCR offers two important advantages over conventional RT-PCR assays: the results are quantitative and, perhaps more importantly, it facilitates exact sensitivity controls on a per sample basis as well as exact comparison of different assay protocols. With highly sensi-

tive and quantitative RT-PCR assays available, stronger clinical correlations to molecular events may now be possible. In particular, the use of qrt-PCR is becoming a necessary research tool for detecting the molecular events underlying disease recurrence and may guide therapeutic decisions based on how individual patients respond at the molecular level. Thus, quantitative measurements can be used to define correlations between the amount of fusion products and clinical outcome.

While the clinical utility of PCR-based MRD evaluation for hematological malignancies is well established, the experience with solid tumors is still limited and it is not clear whether the detection of disease at molecular level is of prognostic significance[107].

The assessment of CTC by means of qrt-PCR has been evaluated for different solid tumor types such as melanoma as well as colon, breast and prostate carcinoma[2, 8, 13, 25, 31, 40, 48, 49, 62, 63, 101]. Unlike hematological malignancies, solid tumors are rarely characterized by specific chromosomal translocations and tumor-specific markers are expressed only by some tumor types and in a low percentage of cases. To obtain maximum specificity, some authors isolated epithelial cancer cells from blood samples by means of immunomagnetic beads[22]. However, this technique cannot be applied to non-epithelial cancer such as melanoma. To overcome this limitation, other investigators developed a nested qrt-PCR using tyrosinase as a melanocyte/melanoma-specific gene[64]. Unfortunately, qrt-PCR cannot be currently recommended for routine clinical use in patients with solid tumors, since no study has definitively demonstrated a significant correlation between the detection of CTC and a patient's clinical outcome.

A novel application of qrt-PCR is the detection and quantification of MRD in sentinel lymph nodes. After the introduction of lymphatic mapping by MORTON et al.[72], the use of sentinel node biopsy has rapidly spread around the world as a sensitive technique for the early diagnosis of lymph node involvement by tumor metastasis. Histopathological examination of sentinel nodes is time consuming and might miss some microscopic metastases made of few malignant cells. Qrt-PCR has been proposed as a rapid method to identify sentinel node micrometastases[20, 67, 83, 96, 108]. No data are currently available on either the reliability of the technique or the biological significance of such hypocellular metastases. However, based on quantitative PCR data, molecular remission and molecular relapse could be exactly defined and validated in prospective clinical trials to assess the biological and clinical significance of MRD in various types of solid tumors.

Other applications. DNA copy number measurements are important in determining the extent of genomic imbalance that underlies most malignancies. There are numerous techniques available for measuring DNA copy number in tumors; each method has specific advantages and disadvantages. Comparative genomic hybridization can detect imbalances across the entire genome, but at relatively low resolution. Fluorescent *in situ* hybridization can provide copy number measurements in a cell-specific manner, but it is difficult to perform in high throughput and is difficult to count 25 or more DNA copies. PCR has been used for determining allelic imbalance (or loss of heterozygosity) using polymorphic simple-sequence repeats; however, if it is performed as an end-point PCR assay, quantitative conclusions can be misleading.

Qrt-PCR has been used in several studies[18, 34, 74, 104] in which allelic imbalance is determined. Loss of heterozygosity in a critical region of chromosome 22q13 has been correlated with prognosis of patients with head and neck tumors[87]. In order to evaluate the clinical relevance of SMAD4 deletion, BOULAY et al.[9] determined gene copy alterations by using qrt-PCR in colorectal tumor biopsies. Patients with normal SMAD4 diploidy turned out to have a 3-fold higher benefit of 5-fluorouracil-based adjuvant chemotherapy, thereby suggesting SMAD4 as a predictive marker of chemosensitivity in colorectal cancer. A secreted member of the tumor necrosis factor receptor superfamily, the decoy receptor 3 (DcR3), was reported to be amplified in colorectal cancer as a negative regulator of Fas-mediated apoptosis. MILD et al.[66] analyzed DcR3 gene copy number in a large series of colorectal cancers from a randomized multicenter trial of 5-fluorouracil/ /mitomycin-C adjuvant chemotherapy using qrt-PCR. They observed that adjuvant chemotherapy was significantly more beneficial in patients with normal DcR3 gene copy number than in patients with amplification.

Identification of BRCA1/BRCA2 mutations allows molecular diagnosis for breast cancer susceptibility. A high-throughput automated allelic-discrimination assay has been proposed to detect the prevalent mutations in these genes[1]. Two allele-specific oligonucleotides are directly used in the PCR reaction, in both of which the fluorescent reporter and quencher dyes are attached to the 5'- and 3'-ends, respectively. During PCR, fluorescence is generated after cleavage of the annealed primer by the 5' nuclease activity of *Taq* polymerase. The wild-type BRCA sequence can be distinguished from the mutant sequence by the differential fluorescence emission of two different reporter dyes. The sensitivity of allelic-discrimination assay is at the level of a single cell following a nested PCR without

the need for radioactivity, gel electrophoresis, or membrane blotting/hybridization. The same technique has been recently utilized for the identification of single nucleotide polymorphisms (SNP) in the human genome to determine the relative percentages of donor and recipient cells present in the recipient after allogeneic bone marrow transplant engraftment. Compared to Southern hybridization analysis, qrt-PCR results highly correlated. Additional development will be necessary to produce a panel of highly informative SNP for clinical use. A qrt-PCR-based SNP assay may ultimately provide more accurate quantification and shortened turnaround time compared with current post-engraftment assays[76].

Besides DNA amplification, cancer cells can increase the expression of an oncogene by augmenting its transcription rate. Some authors[47] found that survivin levels are higher in hepatocellular carcinoma than in normal liver tissue. Moreover, in that study patients who had tumors with higher survivin levels were found to have a higher incidence of disease recurrence.

To examine the prognostic and predictive significance of the apoptosis-related marker Fas ligand (FasL): Fas ratio in breast cancer, tumor biopsies from primary invasive breast cancer patients were examined for the expression of FasL and Fas mRNA transcripts by qrt-RT-PCR. Using a cutoff value of 1, a FasL: Fas ratio greater than 1 was found to have significant prognostic value for disease-free survival. Moreover, hormone receptor-positive women exclusively treated with tamoxifen and with a FasL: Fas ratio greater than 1 had a significantly decreased disease-free and overall survival[85].

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