# DISSERTATION

## INNOVATIVE APPROACHES OF NUCLEIC ACID QUANTIFICATION BY POLYMERASE-CHAIN-REACTION

## (INNOVATIVE ANSÄTZE ZUR NUKLEINSÄURE-QUANTIFIZIERUNG MITTELS POLYMERASE-KETTENREAKTION)

ausgeführt zum Zwecke der Erlangung des akademischen Grades eines Doktors der Naturwissenschaften unter der Leitung von

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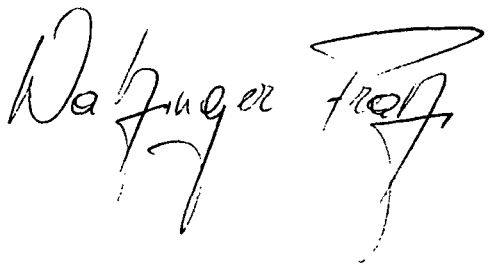

# *SUMMARY*

Over the past years, I was involved in a number of projects dealing with different aspects of quantitative PCR. This synopsis highlights some of the essential findings, which resulted from my work.

With the development of quantitative real-time PCR assays and instruments, the equipment of both routine and research labs has changed. Nowadays, real-time instruments are often regarded as basic equipment of PCR laboratories. With respect to this recent progress in nucleic acid quantification, some of the early projects described, like the development of a competitive PCR approach no longer seem to be up-to-date. It is therefore necessary to consider that at the time when the project was carried out, the establishment of such techniques represented an important technical step forward. For example, the *shifted restriction site* competitive PCR approach (SRS-cPCR) permits reliable and accurate quantification of nucleic acid targets without the need of expensive and sophisticated equipment. Despite new technical developments, competitive PCR assays continue to play an important role as the basic principle of some commercially available quantitative PCR systems (Cobas Amplicor System, Roche).

The incentive for the establishment of a multiplex PCR assay for quality control of RT-PCR tests was the limited ability of approaches available at that time to provide reliable information on the integrity of RNA targets. For the detection of minimal residual disease (MRD) in patients with malignant disorders, a more comprehensive approach was required to assess variations in RNA/cDNA quality and to permit correct interpretation of PCR results. The multiplex RT-PCR assay facilitated the control of four different mRNA species in the sample analyzed, thus providing more reliable information on the integrity of the RNA target of interest. Due to the selection of differentially expressed control genes, this assay allowed to assess gradual differences in the quality of RNA/cDNA preparations, providing a very useful tool for determining the expected sensitivity of RT-PCR assays.

Despite this technical improvement, the selection of the most appropriate control genes for RNA-based PCR assays continued to be a matter of debate. The problem was therefore addressed by a European multi-center laboratory network (Europe Against Cancer, EAC), in which we participated. This concerted action was initiated primarily to establish and standardize methods for the detection and monitoring of leukemia-associated fusion gene transcripts. A large number of candidate genes, selected on the basis of consensus criteria, such as the absence of pseudogenes, the level of expression and transcript stability were carefully evaluated. Based on our long-standing experience, two of the three genes selected by the European collaborative group as optimal control systems, the Abelson (*ABL*), and the beta-2-microglobulin (*B2M*), already had been included in the multiplex assay developed earlier in our laboratory. The data emanating from this collaborative study are currently in press (Leukemia 2003) and will in future serve as an international standard for quantitative analysis of RNA targets.

The establishment and implementation of twenty three RQ-PCR assays for the detection and monitoring of pathogenic human viruses in immunosuppressed pediatric patients resulted from a rather recently initiated project. The assays developed in our laboratory allow precise quantification of viral load in a variety of clinical specimens. The test systems were demonstrated to have a broad dynamic range and to display high sensitivity, reproducibility and specificity. The ability to use uniform PCR conditions for all assays permits simultaneous processing and detection of many different viruses, thus economizing the diagnostic work. Our observations based on more than 50.000 assays reveal the potential of the real-time PCR tests to facilitate early diagnosis of infection, to monitor the kinetics of viral proliferation and the response to treatment. In viral infections, for which specific treatment is available, the quantitative PCR assays established provide reliable diagnostic tools for timely initiation of appropriate therapy and for rapid assessment of the efficacy of antiviral treatment strategies.

A special focus of interest was the detection and monitoring of adenovirus (AdV) infection in patients receiving allogeneic stem cell transplantation (SCT). Patients with invasive AdV infection have very high transplant-related morbidity and mortality. With the development of RQ-PCR assays permitting sensitive detection of all 51 currently known human AdV serotypes, we intended to facilitate early detection and identification of patients carrying a high risk of disseminated disease. In a series of 132 consecutive pediatric SCT patients, more than 5000 samples derived from peripheral blood (PB), stool, urine, and throat were screened for adenovirus infection by PCR during the post-transplant period. We have demonstrated with high statistical significance that patients with the virus detectable in peripheral blood, but not at other sites, have an extremely high risk of developing fatal disseminated disease. More importantly, monitoring of PB specimens by real-time PCR permitted early diagnosis of invasive AdV infection in all instances. In patients who developed disseminated AdV disease, detection of the virus in PB preceded onset of clinical symptoms by a median of over three weeks. Based on this work, the detection of AdV in peripheral blood now serves as a basis for early initiation of preemptive antiviral treatment.

# ZUSAMMENFASSUNG

Während der vergangenen Jahre war ich in einer Reihe von Projekten involviert, die unterschiedliche Aspekte der quantitativen PCR beleuchteten. In dem folgenden Abschnitt werden die wichtigsten Erkenntnisse dieser Projekte zusammengefasst.

Mit der Entwicklung der quantitativen real-time PCR Methoden änderte sich auch die Ausstattung der Routine- und Forschungslabors. Heutzutage werden real-time Geräte oft als Grundausstattung von PCR Labors angesehen. In Anbetracht dieser Fortschritte bei der Quantifizierung von Nukleinsäuren scheinen manche der in dieser Arbeit beschriebenen Projekte, w. z. B. die Entwicklung einer kompetitiven PCR nicht mehr aktuell. Es erscheint daher notwendig noch einmal darauf hinzuweisen, dass zu jener Zeit, als die Methode etabliert wurde, die Entwicklung solcher Techniken einen wichtigen Schritt vorwärts darstellte. Die „Shifted Restriction Site Competitive PCR (SRS-cPCR)" ist beispielsweise eine zuverlässige und exakte Methode zum Quantifizieren von Nukleinsäuren, die in jedem Labor auch ohne teure und hoch entwickelte Technologie angewandt werden kann. Trotz neuer technischer Entwicklungen ist das Prinzip der kompetitiven PCR heute noch aktuell und findet sogar in manchen kommerziellen Systemen erfolgreich Anwendung (Cobas Amplicor System, Roche).

Eine wichtige Aufgabenstellung für unser Labor war der Nachweis einer minimalen Resterkrankung (MRD) bei Patienten mit malignen Neoplasien. Um die PCR Ergebnisse korrekt interpretieren zu können war es notwendig mögliche Unterschiede in der RNA/cDNA Qualität deutlich zu machen. Bislang verfügbare Methoden erlaubten nur sehr eingeschränkt Rückschlüsse auf die Intaktheit der RNA, deshalb war es wichtig eine Methode zur Qualitätskontrolle von RT-PCR Reaktionen zu entwickeln. Die im Rahmen eines eigenen Projektes entwickelte Multiplex RT-PCR Methode ermöglicht innerhalb einer Probe die Kontrolle von vier unterschiedlichen mRNA Spezies und liefert dadurch zuverlässigere Informationen über die Integrität der RNA als andere, herkömmliche Methoden. Aufgrund der Selektion von unterschiedlich exprimierten Kontrollgenen ist es mit dieser neuen Methode möglich graduelle Unterschiede in der Qualität der RNA/cDNA Präparation zu erkennen und damit die erwartete Sensitivität der RT-PCR Untersuchung besser abschätzen zu können.

Trotz der Fortschritte bei der Etablierung von geeigneten Kontrollsystemen wird in wissenschaftlichen Kreisen nach wie vor die Auswahl des am besten geeigneten Kontrollgens für RNA-PCR Methoden diskutiert. Dieses Problem war deshalb auch Gegenstand einer multizentrischen europäischen Studie (Europe Against Cancer, EAC), an der wir teilnahmen. Diese konzertierte Aktion wurde ursprünglich ins Leben gerufen, um Methoden zu etablieren und zu standardisieren, die für den Nachweis von Leukämie-assoziierten Fusionsgenen verwendet werden können. Als mögliche Kandidaten für das optimale Kontrollgen wurde, abhängig vom Vorhandensein von Pseudogenen, von der Expressionshöhe und von der Transkript-Stabilität, eine Reihe von Genen ausgewählt. Basierend auf unserer langjährigen Erfahrung hatten wir zwei der drei im Rahmen der Studie als optimale Kontrollsysteme favorisierten Gene, das Abelson (*ABL*) und das Beta-2-Mikroglobulin (*B2M*), bereits in der oben erwähnten Multiplex-PCR inkludiert. Die Daten dieser europäischen Studie sind gegenwärtig in Druck (Leukemia, 2003) und werden in Zukunft als internationaler Standard für die quantitative Analyse von RNA Targets herangezogen.

Das Etablieren und Implementieren von 23 RQ-PCR Assays für den Nachweis von humanpathogenen Viren bei immunsupprimierten Patienten war Gegenstand eines erst kürzlich initiierten Projekts. Die in unserem Labor entwickelten Tests erlauben ein präzises Quantifizieren der Virusmenge aus unterschiedlichsten klinischen Ausgangsmaterialien. Die Testsysteme zeigen einen breiten dynamischen Bereich, hohe Sensitivität, Spezifität und Reproduzierbarkeit. Alle Virus-Zielsequenzen können unter den gleichen Bedingungen amplifiziert werden, wodurch mit den beschriebenen Testsystemen ein hoher Durchsatz bei maximaler Flexibilität erreicht werden kann. Die Daten von mehr als 50.000 Untersuchungen haben gezeigt, dass es mit diesen Assays möglich ist, Infektionen frühzeitig zu diagnostizieren, und die Kinetik einer Virusinfektion sowie das Ansprechen auf die Behandlung zu überwachen. Für virale Infektionen, die gezielt behandelt werden können, stellen die etablierten quantitativen PCR Testsysteme diagnostische Verfahren dar, mit deren Hilfe der Zeitpunkt zur frühzeitigen Therapie und die Effizienz der antiviralen Behandlung besser abgeschätzt werden können.

Bei Patienten, die eine allogene Stammzell-Transplantation (SCT) erhalten haben, ist es von besonderem klinischem Interesse, Adenovirus (AdV) Infektionen nachzuweisen und zu überwachen. Patienten mit einer invasiven AdV Infektion zeigen eine sehr hohe transplantations-assoziierte Morbidität und Mortalität. Um AdV Infektionen spezifisch nachweisen und jene Patienten frühzeitig identifizieren zu können, die ein hohes Risiko einer disseminierten Erkrankung aufweisen, wurden AdV-spezifische RQ-PCR Testsysteme eingesetzt, die den Nachweis aller 51 gegenwärtig bekannten, humanen Adenoviren erlauben. Bei 132 konsekutiven, pädiatrischen Patienten nach SCT wurden mehr als 5000 Proben aus peripherem Blut (PB), Stuhl, Urin, und Rachen auf AdV Infektionen untersucht. Die Ergebnisse lassen mit hoher statistischer Signifikanz darauf schliessen, dass ausschließlich jene Patienten mit nachweisbarer AdV Infektion im PB ein aussergewöhnlich hohes Risiko aufweisen, eine schwerwiegende, disseminierte Erkrankung zu entwickeln. Das routinemässige Untersuchen von PB-Proben ermöglichte in allen Fällen das frühzeitige Erkennen einer invasiven Infektion. Bei jenen Patienten, die eine disseminierte Erkrankung entwickelten, war es mittels PCR möglich, die Viren im Schnitt drei Wochen vor dem ersten Auftreten klinischer Symptome nachzuweisen. Auf der Grundlage dieser Erfahrungen wird der Nachweis von AdV Infektionen im PB zukünftig als Entscheidungshilfe für den Beginn einer präemptiven antiviralen Therapie dienen.

# ACKNOWLEDGEMENTS

After finishing my graduate studies in the laboratory for Molecular Genetics of Childhood Leukemia at the Children's Cancer Research Institute, a permanent position as a scientist was offered to me, with the option to use the output of scientific projects for my Ph.D. thesis. I have decided to stay in order to gain more experience in the field of pediatric tumor biology, and to help developing new strategies for the diagnostics and monitoring of malignant disorders.

Although I would have had the chance to finish my Ph.D. thesis earlier, I never felt a strong wish to do this. Over the past years, I was involved in a number of challenging projects. I was able to publish the results in peer-reviewed journals and had the chance to participate in international studies. Moreover, I found a beautiful woman, married and produced three children. All the time, I was busy with other things and had no impetus to finish my thesis. Due to the relentless insistence of numerous people, I have finally decided to complete my thesis. To some of these people, I would like to express particular appreciation:

First of all, I wish to acknowledge with gratitude and affection the work of Prof. DDr. Lion. Dear Thomas, thanks a lot for your strong support - you have been an invaluable source of help in all matters. As a Ph.D.student, I will always remember the hours spent with brainstorming, analyzing of data and inspiring discussions. You never complained, but I guess it was sometimes rather laborious and time consuming to review my manuscripts. For your countless hints and suggestions, I will be eternally grateful - thank you! As a senior scientist, I appreciated the way you organized the lab. You always tried to avoid unnecessary bureaucracy and you always took the time for discussion if somebody was not happy - despite other obligations. In my opinion, these are essential points which make life in a lab much easier!

I also would like to express my gratitude to Prof. Kubicek, who accepted years ago to supervise me during the Ph.D. thesis at the Technical University. He has been one of the most reliable persons I ever met - to any query I had, he sent immediately a reply. Thank's a lot for your help!

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# *PREFACE*

A number of widely used procedures exist for the detection and quantitation of specific mRNAs in total or poly(A) RNA samples, such as Northern blot, slot blot and dot blot analyses, nuclease protection assays (NPAs), in-situ hybridization (ISH) and reverse transcriptase polymerase chain reaction (RT–PCR) techniques. All of these methodologic approaches allow relative and absolute quantitation of mRNA, but have some inherent limitations which are important to be considered. Northern blot, slot blot and dot blot analyses require considerable amounts of nucleic acids and have the disadvantage of relatively low sensitivity, with lower limits of detection in the range of $10^5$ to $10^7$ target molecules. The NPAs (including RNase protection and S1-nuclease assays) are 10- to 100-fold more sensitive than the techniques indicated above, but are time-consuming and technically difficult. The ISH technique offers higher sensitivity allowing the quantitation of as little as 10 to 100 molecules per cell. However, this method is also technically demanding and laborious, and requires special equipment for the preparation of samples and visualization of results (hybridization signals). For these reasons, ISH does not seem to be readily applicable to processing of large numbers of samples.

In contrast to the above techniques, RT–PCR is a powerful tool for the detection of minute amounts of nucleic acids. In RT-PCR, an RNA template is converted into complementary DNA (cDNA) using reverse transcriptase. The cDNA is then amplified exponentially by PCR. Due to the exponential amplification of the target sequence, the method reveals an impressive sensitivity, with a detection limit of less than 10 copies of any specific nucleic acid molecule against a background of hundreds of millions other molecules. Because of the high sensitivity combined with high specifity and versatility, PCR is increasingly used for routine diagnostic purposes, such as the detection and quantitation of viral, bacterial or fungal pathogens, as well as in the diagnosis of inherited genetic disorders and malignant tumors. The wide use of this powerful technology facilitates further development in the exploitation of potential applications, but at the same time requires great efforts in identifying the limitations and controlling possible problems and pitfalls.

This thesis provides the background and the theoretical basis for the application of the quantitative RT-PCR (QRT-PCR) technology in research and diagnosis. Moreover, the thesis addresses special problems and recent developments in RNA and DNA quantification. The formal structure of the thesis includes four parts divided into a total of six chapters. A synopsis of individual sections of the thesis is indicated below:

## PART 1 RNA QUANTIFICATION: A REVIEW

**Chapter I** **Approaches to quantification of RNA targets by PCR based techniques** In this chapter, different approaches to PCR-based quantification of gene expression including comparative, competitive, and real-time PCR are reviewed. Different formats of fluorescence-based real-time PCR and their applications in clinical diagnosis and research are presented.

## PART 2 CONTROL GENES

**Chapter II** **Multiplex PCR for quality control of template RNA/cDNA in RT-PCR assays**

In this chapter, the problems in the selection of appropriate control genes for PCR-based RNA quantification are addressed. The issues of different expression levels or discordant stability between the target and the control gene transcripts sre covered, and a technical approach to more efficient control of template RNA/cDNA is presented.

**Chapter III** **Control genes for diagnosis and MRD detection in malignant hematologic disorders by RQ-PCR**

This chapter focuses on the selection of the most appropriate control genes for the detection and the monitoring of leukemia-associated fusion gene transcripts. The data presented are derived from an international multi-center laboratory network ("Europe Against Cancer") supported by the European Union, with our participation as representatives of Austria. The control genes selected by this international consortium are presented and the implications for the establishment of international standards for quantitative analysis of RNA in leukemia diagnosis are discussed.

## PART 3 COMPETITIVE PCR ASSAYS

**Chapter IV** **Quantification of mRNA expression by competitive PCR using non-homologous competitors containing a shifted restriction site**

In this chapter, a novel competitive PCR assay permitting reliable and accurate quantification of nucleic acid targets is presented. The advantages of this system over other techniques, including real-time PCR methods, are discussed.

## PART 4 REAL-TIME QUANTITATIVE PCR ASSAYS

**Chapter V** **Real-time quantitative PCR assays for the detection and monitoring of pathogenic human viruses in immunosuppressed pediatric patients**

In this his chapter, the development of a panel of 23 RQ-PCR assays for the detection and monitoring of sixteen different pathogenic viruses and virus families is presented. The application of these assays to early diagnosis of infection, monitoring of virus proliferation kinetics and the monitoring of response to treatment is demonstrated. The potential of the technique to facilitate timely clinical decision making and assessment of the efficacy of antiviral treatment is discussed.

**Chapter VI** **Molecular monitoring of adenovirus load in peripheral blood after allogeneic bone marrow transplantation permits early diagnosis of disseminated disease,**

In this chapter, the clinical relevance of RQ-PCR monitoring of a life-thratening virus infection is demonstrated. Virus detection and monitoring of viral load are shown to be of great importance for timely treatment decisions. The impact on the clinical outcome of patients carrying this virus is discussed.

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# RNA QUANTIFICATION: A REVIEW

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# I

# RNA QUANTIFICATION BY PCR BASED TECHNIQUES

# Approaches to Quantification of RNA targets by PCR based techniques

## A. Introduction

The RT–PCR technique is a powerful tool for detection of minute amounts of gene transcripts. Due to the exponential amplification of the target sequence, it has exquisite sensitivity. Less than ten copies of any transcript can be readily detected, even against a high background of nucleic acids. The essential prerequisites for the acquisition of reliable quantitative data are the detection and accurate measurement of the PCR products during or at the end of amplification, and the ability to deduce from this information the number of target molecules present at the beginning of the reaction. Quantitative PCR analysis can be used either for the assessment of relative differences between different specimens or for absolute quantification of transcript copies in individual samples. The quality of RT-PCR assays is greatly affected by template integrity and purity, and by the efficiency of both reverse transcription (RT) and amplification.

Numerous PCR-based approaches to quantification of mRNA targets have been described:

## B. Quantitative RT-PCR methods

### 1. Quantification without standards.

The calculation of initial target molecules is performed using a mathematical model referred to as linear regression analysis. A series of identical PCR reactions are set up, and reaction tubes are sequentially removed from the block after an increasing number of amplification cycles. The measurement of PCR product in individual reactions of the series reflects the kinetics of product accumulation during PCR cycling, and provides a basis for quantitative analysis of the transcript investigated (*kinetic analysis*) (see below page 6). Tube-to-tube variations are controlled, and if necessary, corrected by analyzing each sample in several replicates.

The accumulation of PCR product ($P$) can be described by **Equation 1**:

$$P = P_0 (1+E)^n \qquad (Eq.\ 1)$$

$P_0$ represents the initial amount of target molcules, $n$ the number of cycles and $E$ the amplification efficiency.

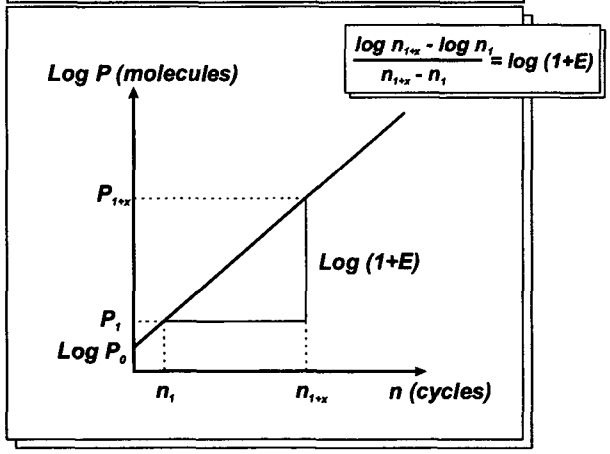
This equation can also be converted to:

$$\log P = [\log (1+E)]\, n + \log P_0 \qquad (Eq.\ 2)$$

Using this formula, a linear relationship between the PCR product ($P$) on the x-axis and the number of cycles ($n$) on the y-axis can be drawn [1].

The initial target concentration ($P_0$) can be determined as the y-intercept which can be extrapolated from the slope $\log (1+E)$ as shown in Fig. (1).

**Fig.1: Linear regression analysis**




**Legend:**
Graphical determination of the initial target concentration ($P_0$) by measurement of product amounts ($P_1$ and $P_{1+x}$) synthesized after cycle $n_1$ and $n_{1+x}$ .

In practice, the sample to be analyzed is split into several identical PCR reactions. After a given number of cycles during which the reaction can be expected to be in the exponential phase of amplification, PCR reactions are removed from the thermocycler at 3-4 cycle intervals. The amount of amplified product is determined by spectrophotometry or, in reactions using radioactively labelled primers, by scintillation counting. As indicated in Fig. (1), the results are plotted on a logarithmic scale against the cycle numbers.

An advantage of linear regression analysis is the assessment of amplification efficiency in each reaction. In other approaches, amplification efficiency of the respective target must first be determined by a series of experiments, and potential variations during acquisition of data are compensated for by parallel amplification of appropriate controls. Due to the need to analyze multiple sample aliquots, this method is time-consuming and labor-intensive. Moreover, the lack of controls for most other important variables of quantitative PCR may provide a serious impediment to accurate quantitative analysis. Hence, techniques that do not include any standards can only be applied in very well defined systems showing no variability of parameters affecting quantification. The use of this approach has therefore been restricted to few applications [2].

In all other quantitative PCR approaches, endogenous or exogenous standards were used. Recent advances in the detection of amplification products have led to the development of automated systems based on kinetic acquisition of data and calculation of results using the principle of linear regression analysis (see section 4 "real-time PCR").

## 2. ***Quantification with endogenous standards.***

This approach relies on the amplification of a control sequence either in a separate reaction or in the same test tube together with the target of interest, using an additional primer pair. The endogenous control is generally another mRNA expressed by the cells investigated. The PCR product of the endogenous standard is used for normalization of the target-specific signal. The corrected values are used to estimate ***relative*** differences in quantity between target and standard RNA. Because the target transcript is evaluated in comparison to an endogenous control, the method has been termed comparative RT-PCR [3-5].

Target and standard mRNAs are processed in parallel during the entire course of the assay. This approach allows controlling the yield and integrity of RNA as well as the enzymatic reactions of reverse transcription and PCR. However, for reliable relative quantification it is essential to ensure that the efficiency of the RT step is similar for both transcripts, and that the amplification efficiencies are nearly identical, at least until the PCR cycle at which the synthesized products are compared. This implies that both targets must be within the exponential phase of amplification at the time of analysis. It is necessary therefore to determine the optimum time point for PCR termination and data collection to meet the above requirements.

An advantage of this method is its simplicity, because the generation of endogenous standards only requires the design and synthesis of an additional primer pair. The endogenous standards are gene transcripts that are normally present in the samples and provide a means of controlling the integrity of RNA and the efficiency of RT and PCR. In most cases, mRNAs of ubiquitously expressed housekeeping genes have been used.

The requirements for the selection of adequate endogenous standards for quantitative expression studies are similar to the criteria described in the last section of this review dedicated to the controls recommended for RT-PCR tests.
Some investigators use comparative PCR assays as a tool for comparing expression levels of different mRNA species. The comparative PCR approach can also be used for the assessment of integrity of different transcripts. In our laboratory, an assay permitting the co-amplification of up to four differentially expressed endogenous standard genes has been established [6]. This multiplex assay reveals the ratio of relative transcript quantities of four housekeeping control genes which display a typical pattern depending on the quality of RNA/cDNA preparation. The test permits the assessment of gradual differences in the integrity of an RNA sample, thus enabling assessment of the expected sensitivity of RT-PCR assays targeting a transcript of interest.

For diagnostic laboratories involved in the analysis of extracellular RNA (e.g. detection of RNA viruses), it is important to consider that the use of endogenous standards is limited to the quantitation of RNA in cells and that it is not applicable to the quantitation of viral RNA in plasma, serum, or other cell-free body fluids.

### 3. ***Quantification with exogenous standards.***

This approach is based on the inclusion of synthetic RNA or DNA fragments, which are added to the reaction containing the target of interest either prior to RT (RNA) or before amplification (DNA). In contrast to endogenous standards, this type of control is not subject to temporal variation in expression, and can therefore also be used for the quantification of RNA targets in cell-free body fluids. The amount of exogenous standard added is well defined. If the amplification efficiencies of the standard and target fragments are equivalent, the ratio of the respective PCR products can be used to calculate the initial copy number of the target transcript of interest.

The consideration DNA or RNA standards depends on the question addressed. In principle, DNA standards are more convenient in terms of handling, and are not prone to degradation during storage. However, they provide no control for RT-efficiency. A frequently reported problem associated with the use of DNA standards is underestimation of the target transcript copy numbers. This error is most likely attributable to incomplete conversion of target RNA molecules to cDNA, a step, which is not controlled if a DNA standard, is used.

If the aim of the quantitative RT-PCR assay is calculation of the ***absolute*** number of target RNA molecules, it is advisable to use RNA standards. These are reverse transcribed together with the target transcript, thus eliminating the problem of variable efficiency of the RT step for subsequent quantification of the PCR products. The main disadvantage of RNA standards in quantitative RT-PCR assays is the danger of degradation, which would invariably lead to incorrect calculation of target transcripts.

A way to overcome the susceptibility of RNA to nuclease-induced degradation is the incorporation of modified nucleotides (RT-PCR Competitor Construction Kit, Ambion, Austin, TX, USA). These nucleotides confer nuclease resistance to the RNA molecules, and render the standards stable in the presence of most nucleases.

The requirements for an adequate standard may be less stringent if the quantitative RT-PCR assay is performed for detection of ***relative*** **differences** in the number of RNA molecules, e.g. changes in RNA expression over time, determined by serial follow-up samples. Provided that the assay is well established and the RT-step is known to have a low variability, the use of DNA standards will permit reliable quantification.

Depending on the design of primer-recognition sequences of the exogenous standard, different types of quantitative PCR reactions can be performed:

**a) Non-competitive PCR**

In this experimental set-up, standards lacking any sequence homology to the target of interest are used. Similar to the PCR approach using endogenous standards, two different primer pairs are necessary to amplify both the target and the standard fragments. The technique is referred to as "non-competitive" because the standard and the target sequence show no competition for the primers driving the amplification.

Due to the known quantity of standard molecules added to the reaction, it is possible to estimate ***absolute*** **copy numbers** of the target transcript. For the establishment of quantitative assays relying on the use of non-competitive exogenous standards, the same principles apply as described above for tests using endogenous standards.

**b) Competitive PCR**

If the exogenous standard is designed to share at least partially the primer binding sites with the target sequence of interest, the two sequences compete for primers, nucleotides and polymerase during each PCR cycle. Hence, the term "competitive PCR" has been coined for quantitative assays using a single primer pair for amplification of both the standard and the target sequence [7].

With a single set of primers it is more readily achievable that both fragments are amplified with identical efficiency. If target and competitor sequences are indeed amplified with equivalent efficiency, the synthesis of both products reaches the plateau phase at the same time, and the ratio between the two products remains constant even beyond the exponential phase of amplification. The initial quantity of target molecules in the sample can be calculated from the ratio of competitor- and target-derived amplicons generated during PCR [8].

Since equivalent amplification efficiency is an essential prerequisite for precise quantification by competitive PCR, great emphasis needs to be put on the design of appropriate competitors. Many investigators have used highly homologous competitors differing from the target sequence only by the presence or absence of single nucleotides or by short insertions or deletions. The great similarity between target and competitor facilitates identical amplification efficiencies, but mostly leads to the formation of heteroduplexes (i.e. double-stranded molecules composed of a competitor-derived strand and a target transcript-derived strand) during the annealing phase. This phenomenon has been described in several publications [9,10,11,12]. According to these reports, the formation of heteroduplexes occurs during the late phase of amplification, and thus, does not affect the target/competitor ratio in a relevant manner. In contrast to these reports, our investigations revealed the formation of heteroduplex fragments already during early cycles of PCR which compromised the results of competitive PCR [13].

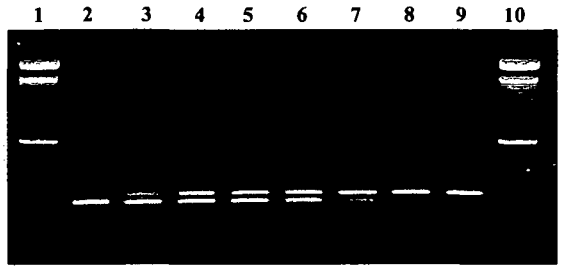
Mathematical models have been described to permit quantification outside the equivalence point

(EQP, see page 7) despite the generation of heteroduplexes [9,14]. However, particularly in situations in which target and competitor differ by a single nucleotide, theoretical predictions based on mathematical models may be problematic [15].

In order to avoid the pitfalls of heteroduplex formation, non-homologous competitors, also referred to as heterologous competitors or "mimics", were designed. These competitors display equal size and identical primer binding regions, but contain an internal nucleotide sequence which is different from the target sequence. It was demonstrated for different targets that the lack of extensive homology between the nucleotide sequences of the competing fragments did not affect the PCR kinetics. Non-homologous competitor molecules of a size identical to the target fragment were amplified with the same efficiency, and prevented the formation of heteroduplexes [10,16,17]. The versatility of non-homologous competitors has also been demonstrated by different commercially available quantitative PCR (Q-PCR) systems. For example, the automated *Cobas Amplicor Analyzer* (Roche) is used in routine screening of human blood products for the presence and amount of different RNA and DNA viruses. The system is based on a PCR-ELISA format controlling the RT- and amplification efficiency by the co-amplification of a non-homologous competitor fragment.

In most competitive assays, the standard (=competitor) is serially diluted into multiple reactions containing a constant amount of the target RNA transcript. The quantity of target RNA/cDNA can be most conveniently assessed at the so-called equivalence point (EQP). At this point the target- and competitor-derived amplification products display the same signal intensity, indicating identical amounts of target and competitor at the beginning of the PCR reaction (Fig.(2)).

**Fig. 2: Competitive PCR**




**Legend:**
Lanes 2 to 9 contain $10^6$ molecules of an in vitro transcribed cloned human wild type target fragment (284 bp) and a semilogarithmic dilution of the competitor RNA (246 bp) ranging from $10^4$ to $3x10^7$ molecules. The equivalence point (EQP) is indicated by an arrow (lane 5) and reveal $10^6$ copies of the target in the sample investigated. It was determined by comparison of signal intensities of the 246bp and 284bp fragments. Lanes 1 and 10 show a size marker (100bp ladder).

To approach the EQP as closely as possible, several PCR reactions covering a range of competitor concentrations must be set up for quantification of individual targets, thus rendering the procedure rather laborious.

This disadvantage can be overcome. We have recently developed a modified Q-PCR approach for quantification outside the EQP requiring a significantly reduced number of competitor dilutions for quantitative analysis of the target [13]. The assay is less laborious and costly, and therefore more broadly applicable. Essentially, it is based on the design of competitors that do not form heteroduplexes with the target sequence, permitting the quantification of target copies across a range of three logs using only three competitor concentrations.

In summary, well-standardized competitive PCR assays yield parallel amplification curves for both target and competitor throughout the logarithmic phase of PCR. The PCR product accumulation of both fragments reaches the plateau at the same time. Since the ratio of target to competitor molecules remains constant throughout

the entire amplification process, it is possible to perform a high number of cycles without compromising the result of quantitative analysis. The possibility of collecting data outside the exponential range of amplification greatly increases the effective dynamic range and the sensitivity of the method.

Nevertheless, shortcomings of methods relying on the use of exogenous standards include the lack of controls for RNA yield and integrity, and for RT-efficiency. It is necessary therefore to include additional controls for these variables in each assay.

### 4. *Quantification by real-time PCR.*

Real-time quantitative PCR (RQ-PCR), the most recent technical development in PCR quantification, is based on the release of fluorescence signals during amplification corresponding to the number of amplicons generated during PCR. Continuous measurement of the fluorescence emitted during the reaction permits the monitoring of amplification kinetics. By registering the increase of fluorescence, accumulation of PCR product can be documented in real-time. RQ-PCR can be used for assessment of relative levels of gene expression or for absolute quantification of transcript copies, if the system is appropriately calibrated.

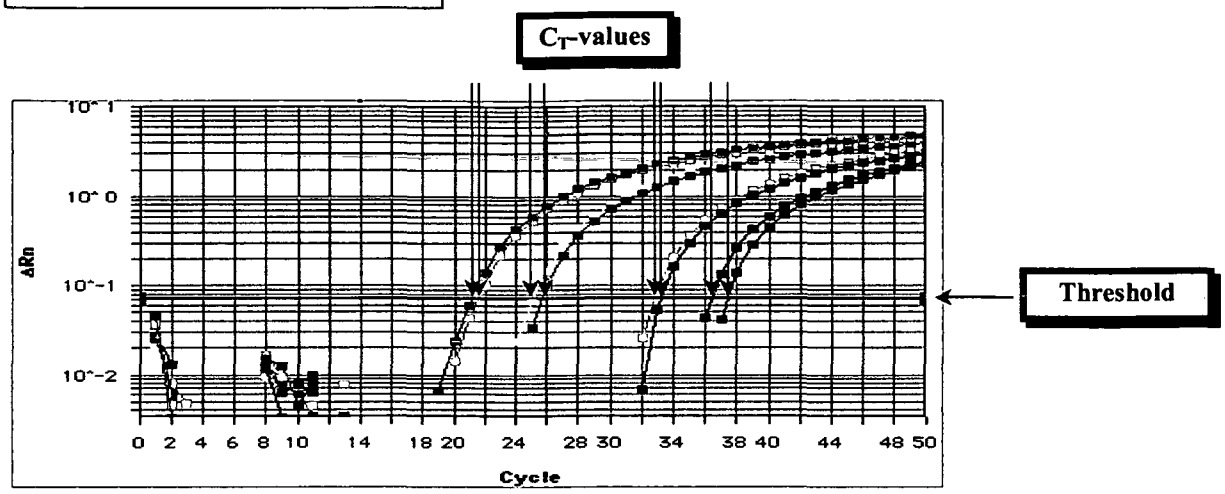
In contrast to quantitative PCR approaches based on the analysis of data at the end of amplification (end-point quantitative PCR), the real-time PCR technology permits continuous collection of data during PCR cycling (kinetic quantitative PCR).

One of the first attempts at kinetic PCR quantification was the linear regression analysis, described above. Initially, the use of radioactive isotopes for the detection and quantification of PCR products was common.

The introduction of fluorescence-based product detection in quantitative PCR analysis has been an important step forward. Higuchi et al. performed pioneering work in this field by establishing a PCR assay in which product accumulation was indicated by increasing fluorescence signals emitted by intercalating ethidium bromide molecules [18]. By coupling the thermal cycler with fiber-optic cables and a spectrofluorometer [18], and later with a charged-coupled device (CCD camera) [19], Higuchi et al. were able to continuously monitor the fluorescence released during PCR. This technique has eliminated the need of post-amplification handling of the samples, and has paved the way towards automated RQ-PCR. Nevertheless, like in other approaches, controls for variables such as the integrity and purity of template and the efficiency of RT and amplification must be included.

The results are typically displayed as amplification plots resulting from a series of fluorescence measurements taken at defined time points during the amplification process (Fig. (3)). One of the important features of the real-time technology is the ability to monitor the increasing amount of product during the PCR reaction. This facilitates quantification of the target at an early point in the exponential phase of PCR, when the amplification product first becomes detectable. Quantification by real-time PCR is therefore not adversely influenced by limiting concentrations of reagents, nor by other variables such as cycling conditions affecting quantification in endpoint analysis-based PCR assays.

**Fig. 3: Real-time amplification plot**




**Legend:**

Duplicates of plasmid dilutions ($10^6$, $10^5$, $10^3$, $10^2$) were amplified by the ABI 7700 real-time PCR system. Quantitative data are derived from determination of the cycle at which the amplification signal crosses a preset threshold (termed "Cycle above threshold" or $C_T$). The results of all plasmid dilutions are highly reproducible, only the highest dilution containing $10^2$ plasmid molecules (last duplicate) shows minimal differences in the amplification signal.

The x-axis shows the number of PCR cycles, the y-axis the amount of synthesized product.

**a) Detection formats and chemistries**

Currently, four alternative detection formats are available for the acquisition of RQ-PCR data. The simplest method relies on the use of intercalating dyes such as ethidium bromide (EtBr) or SYBR® Green I (FMC Bioproducts, Rockland, ME, U.S.A.) that preferentially bind to double-stranded DNA fragments in a sequence-independent manner. All other detection formats are based on specific hybridization of one or two fluorescence-labelled oligonucleotide probes to the target sequence during amplification. The 3'-ends of these probes are chemically modified to avoid their extension by the polymerase during PCR, i.e. to prevent them from serving as primers. In contrast to the use of intercalating agents, which bind also to primer dimers and other spurious PCR products, employment of specifically binding fluorogenic probes leads to higher specificity of target detection.

The inclusion of a hybridization step in the PCR reaction also increases the sensitivity of detection, which is comparable to that achieved by nested PCR or Southern blot hybridization of PCR products.

***(1) Intercalating dyes***

DNA binding dyes like EtBr or SYBR® Green I intercalate in a non-specific manner into double-stranded (ds) DNA molecules, and in the bound state emit fluorescence when excited by an appropriate light source. For real-time PCR approaches, SYBR® Green I is used preferentially because it is more sensitive [20]. During the annealing and extension steps, an increasing amount of dye binds to the newly-synthesized DNA leading to maximum fluorescence emission at the end of the elongation phase. As soon as the DNA is denatured again during PCR cycling, intercalated dye molecules are released into the solution resulting in a drop of fluorescence. The fluorescence is recorded after each cycle at the end of the elongation phase, and reflects the number of PCR products generated during the amplification process [21] (Fig. (4)).

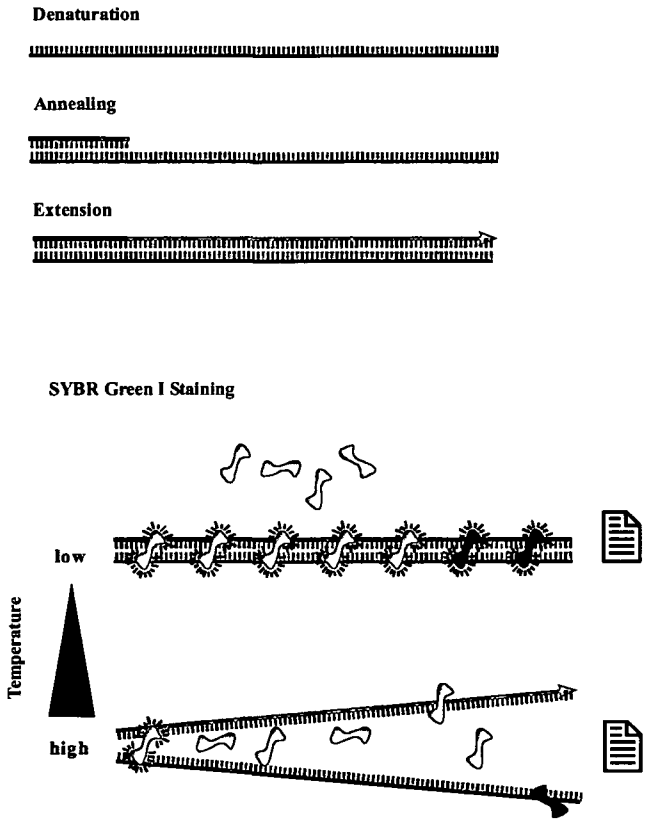
It is important to consider that the number of intercalating dye molecules, and thus, the amount of fluorescence signal emitted is greatly influenced by the length of the PCR amplicons. If the absolute amount of initial target copies is to be determined, a correction factor must be used to correctly assess the number of target molecules [22].

Compared to other real-time detection formats, systems based on intercalating dyes are easier to establish and less expensive because no target-specific fluorogenic probes are required.

However, the lack of probes in the assay results in lower sensitivity and specificity. Amplification and detection of specific PCR products are determined solely by the amplification primers. Dye molecules binding to non-specific PCR products or primer dimers contribute to overall fluorescence signal intensity, and may therefore lead to wrong quantification of target transcripts.

Specificity of amplified fragments can be controlled by melting curve analysis at the end of the PCR: measurements of fluorescence are performed while the temperature in the reaction tubes is gradually raised, until complete denaturation of the amplicons occurs. During denaturation of the amplicons, intercalated dye is released into solution leading to decreasing fluorescence signals. The analysis is done by plotting the intensity of fluorescence against the temperature gradient on a logarithmic scale. Because each double-stranded DNA fragment has its characteristic melting profile defined by its length and nucleotide composition, it is possible to differentiate specific from non-specific products, and to determine the quantity of specific amplicons as a basis for the calculation of initial target copy numbers [23].

**Fig.4: Melting curve analysis using SYBR Green I**




**Legend:**
At the end of PCR, the products are gradually heated. The fluorescence signals are measured at frequent intervals, e.g. at 0.5°C increments. As soon as the dsDNA starts denaturing, the intercalating dye (SYBR® Green I, ) is released, resulting in a decrease in fluorescence.
Fluorescence data are collected continuously.

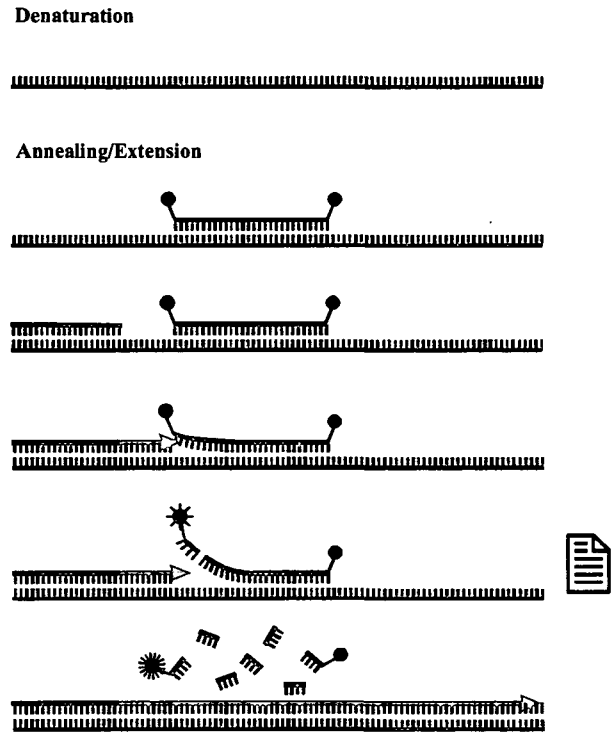
### *(2) Hydrolysis probes*

This method is based on the use of three oligonucleotides for amplification and detection of the target including the forward and reverse primers, and a non-extendible target-specific oligonucleotide probe that binds to the target strand between the PCR primers (Fig. (5)).

Hydrolysis probes, also referred to as TaqMan® probes (Applied Biosystems, Foster City, CA, USA), are dually labelled with a fluorescent reporter dye (e.g. FAM or VIC) covalently attached to the 5′-end, and a quencher dye (e.g. TAMRA), covalently attached to the 3′-end. When the reporter molecule on the TaqMan

probe is stimulated by an appropriate light source to emit fluorescence, the energy is transferred to the quencher, thereby suppressing the emission of fluorescence by the reporter. This physical principle is known as the *fluorescence resonance energy transfer (FRET)* [24].

**Fig.5: Quantitation using hydrolysis probes**




**Legend:**
The dually labelled hydrolysis probe binds to the template between the denaturation- and annealing-steps, when the temperature is decreased. During elongation, the probe is displaced and subsequently cleaved by the Taq DNA polymerase. Due to the fragmentation, quencher ( )- and reporter ( )-molecules are separated, leading to a measurable increase of reporter fluorescence ( ). The time point of data collection is indicated by an arrow.

The transfer of energy works efficiently only across very short distances, and decreases rapidly when the reporter and quencher molecules move apart. During PCR, when the DNA polymerase extends the primers, the hybridized probes are cleaved by the 5′exonuclease activity of the enzyme and the corresponding quencher and reporter molecules are separated. The energy transfer to the quencher molecule is thus abrogated, and the reporter starts emitting fluorescence which can be measured at the end of each extension step [25,26].

To ensure that the enzyme reveals its maximum 5′-3′ exonuclease activity, the amplification is usually performed by combining the annealing and extension steps at a temperature between 60-62°C. To prevent dissociation of the hybridized probes from the template before or during the extension step it is essential to design probes with a considerably higher melting temperature ($T_m$) than that used for the annealing/extension steps. It is recommended to design TaqMan probes displaying a $T_m$ of 68-70°C.

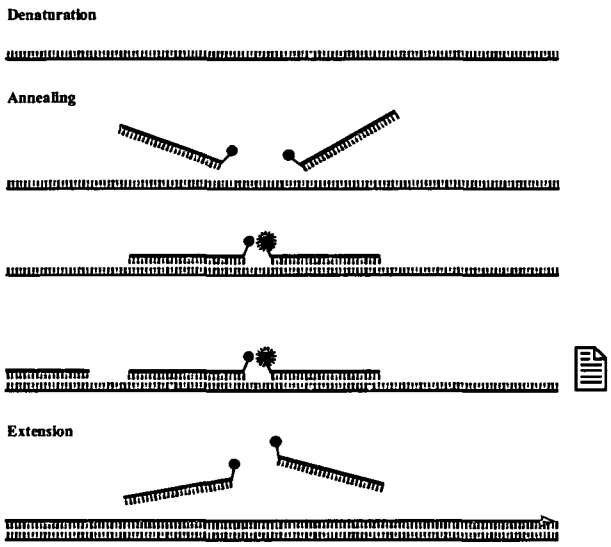
In well-established assays, there is a linear correlation between the number of released reporter molecules and the number of amplicons synthesized during each PCR cycle. This correlation serves as a basis for calculation of initial copy numbers of the target transcript.

### *(3) Hybridization probes*

In addition to the amplification primers this method requires the use of two additional single-labelled oligonucleotide probes hybridizing to a sequence located between the primers (Fig. (6)). One of these oligonucleotides is labelled with a *donor* dye at the 3′end (Fluorescein, emitting green light), the other is labelled with an *acceptor* dye at the 5′end (LC Red 640 or LC Red 705, emitting red light). The probes are designed to hybridize during the annealing step to the same strand in a head-to-tail arrangement, at a distance of 1 to 5 nucleotides to bring the two dyes in close proximity. The donor dye is stimulated by an appropriate light source to emit fluorescence. If both probes are bound to the target, the fluorescence energy is transferred to the acceptor molecules (FRET), and the excited LC Red 640 fluorophore emits a red fluorescent signal, which is detected and measured at the end of each annealing step. After the annealing step, the temperature in the cycler is raised for strand

extension, and the hybridization probes detach from the target. In solution, the hybridization probes are not close enough to permit relevant energy transfer. The amount of red fluorescence emitted during each annealing step reflects the number of target copies present in the PCR reaction. Measurement of the fluorescence kinetics during PCR permits calculation of the initial target copies [27].

**Fig.6 Quantitation using hybridization probes**




**Legend:**

The two hybridization probes bind to the template between denaturation- and annealing-steps, when the temperature is decreased. Due to the close proximity of donor ( ) and acceptor ( ) dyes, FRET occurs stimulating the acceptor dye to emit red fluorescent light ( ). After the annealing step, the temperature is raised, leading to dissociation of the hybridization probes.

The time point of data collection is indicated by an arrow.

An advantage of this method is that the hybridization probes are not hydrolyzed, facilitating the generation of melting curves (see below). A potential disadvantage could be the need to design two oligonucleotide-probes capable of hybridizing between the amplification primers in close proximity to each other. For example, when investigating fusion gene transcripts or single nucleotide polymorphism (SNP)-loci, it may be difficult to identify appropriate probes hybridizing with equal efficiency near the fusion point or at the polymorphism, particularly if the flanking sequences display a major difference in GC content.

### *(4) Molecular Beacons*

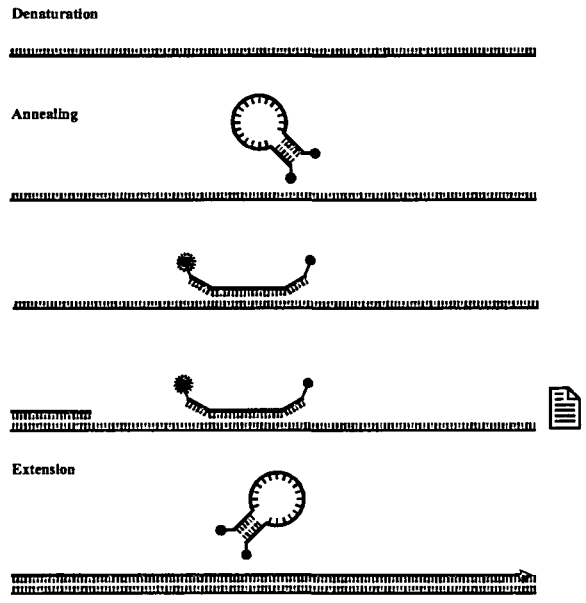
Molecular beacons are hybridization probes with flanking sequences of 5 to 7 nucleotides designed to be complementary to each other, and an intervening sequence complementary to the target of interest (Fig. (7)). The ends of the probe are labelled with a fluorescent and a non-fluorescent quenching dye, respectively. The term molecular beacon is derived from the fact that in solution the complementary sequences of the probe anneal to each other forming a stem-like structure, whereas the intervening sequence remains single-stranded and loops out. The result is a hairpin structure that brings the fluorescent dye and the quencher in close proximity, leading to efficient quenching of the fluorophore (FRET). In presence of the specific template, the intervening loop sequence of the molecular beacon binds to the target which leads to a conformational transition from the hairpin structure to a linear structure, resulting in the separation of fluorophore and quencher. Energy transfer no longer occurs, and the fluorescence emitted can be detected at the end of each annealing step.

According to earlier reports, well-designed molecular beacons are able to discriminate single base-pair mismatches more accurately than hydrolysis probes [28]. The reason for the higher specifity is the high thermal stability of the hairpin structure. This conformation is favored over the linear structure if the target sequence is not perfectly matched to the complementary region of the probe. If the probe is not linearized by hybridizing to its target, no fluorescence signal is generated.

The real challenge of working with molecular beacons is the design of the stem and the loop. If the stability of the stem is too high, the hairpin structure is retained even in the presence of

complementary target. Is the stability too low, the molecular beacon may fold into alternate conformations that do not place the fluorophore in the vicinity of the quencher, leading to an increase in background signals. It is also essential to select an intervening loop sequence, which is not complementary to other genomic regions or to the sequence of the primers used for amplification. Cross-hybridizations with other sequences would also result in high background fluorescence.

**Fig.7: Quantitation using molecular beacons**




**Legend:**

Molecular beacons in solution form a hairpin structure. After the denaturation step, the molecular beacons bind with its loop sequence to the complementary region within the template DNA. This hybridization step leads to unfolding of the beacon, separating the reporter ( )- and the quencher ( )-dyes, and leads to the emission of fluorescence ( ). After the annealing step, the temperature is raised, leading to dissociation of the probe which folds into a hairpin structure again.

The time point of data collection is indicated by an arrow.

Under appropriate conditions, molecular beacons and hybridization probes are not hydrolyzed during the amplification process, and can be used for the generation of melting curves after the final extension step at the end of PCR. The $T_m$ is not only dependent on the length and the GC-content, as discussed previously, but also on the degree of homology between the target and the hybridizing probe. The melting curve therefore permits identification of the PCR products synthesized, and can reveal even simple nucleotide differences.

### b) Hardware

In Table 1, important parameters of instruments from different manufacturers are compared. Our own experience is limited to the ABI7700 Sequence Detection System (AB) and the Light Cycler (Roche, Basel, Switzerland) instruments, all other data presented are based on information provided by the respective manufacturers.

#### *(1) Amplification performance (see Table 1a)*

The amplification is performed either in conventional thermoblock cyclers (AB, BIO-RAD, Stratagene, Cepheid, MJ Research) or in airstream cyclers (Roche, Corbett Res.) using a moved rotor-like carousel as sample rack. With thermoblock cyclers, it is possible to perform high-throughput analysis by processing 96 or even 384 samples per run. The airstream cyclers have a lower capacity in a range between 32 and 72 samples per run, but the strength of this technology is the rapid heating and cooling rate. In comparison to the thermoblock cyclers, the time required can be reduced by at least 50%, resulting in total cycling times in the range of 30 minutes for amplification of a 100 bp fragment.

The RQ-PCR system recently launched by BIO-RAD (iCycler) offers an additional intriguing feature. This instrument has an additional temperature gradient function that facilitates testing of optimal annealing temperature and PCR set-up by processing up to 12 samples at 8 different temperature profiles in a single experiment. This function is an attractive feature permitting rapid establishment of optimal assay conditions.

When comparing the design and specifications of sample processing the Smart Cycler (Cepheid) is constructed according to a concept different from all others. This system is composed of 16 modules equipped with a solid-state heater and forced air-cooling. Each of these modules is independently programmable, providing the highest flexibility in simultaneous amplification of targets using different detection formats or time/temperature profiles.

***(2) Detection performance (see Table 1b)***

For excitation of reporter dyes to emit fluorescence, the instruments are either equipped with a laser (AB), a light-emitting diode (LED) (Roche, Corbett Res., Cepheid, MJ Research) or a halogen lamp (Stratagene, BIO-RAD). The fluorescence emission is detected either by a CCD camera (AB, BIO-RAD), a photodiode (Cepheid), photo multipliers (Corbett Res., MJ Research, Stratagene) or an optical unit (Roche).

All currently available systems support all detection formats discussed previously.
If hybridization probes are used, it is essential to select suitable fluorophores matching with the excitation and detection characteristics of the individual system. Selection of fluorophores is determined mainly by the light source and, if present, by the filters used in the instrument.

Halogen lamps display an excitation range of 350 to 750nm, which is adequate for most of the fluorophores available. By contrast, lasers produce light of a single wavelength, thus limiting the number of eligible fluorophores to those efficiently excited by this wavelength. An advantage of laser-generated beams is the high-intensity permitting the detection of minute fluorophore amounts, and thus providing very high sensitivity.

Nearly all of the instruments use emission filters, some in combination with additional excitation filters. The only exceptions are the ABI Prism 7700 and 7900 instruments (AB) that allow detection of emission spectra within a range of 500-660nm. The advantage of unfiltered signal detection is greater flexibility in the selection of fluorophores displaying different emission wavelengths. All other systems are restricted to the use of fluorophores with excitation and emission wavelengths matching with the filters installed. A disadvantage of unfiltered systems becomes apparent in experiments aiming at quantification of more than one fluorophore signal in one reaction. If the wavelengths of the emission spectra show overlaps, a portion of the light emitted by each fluorophore is detected in the wrong channel, thus increasing the measured signal intensities. This phenomenon is known as cross talk, and with the currently available software it is hardly possible to distinguish specific signals of low intensity from cross talk signals. To overcome the problem of cross talk signals and to permit dual-fluorescence analysis in systems using unfiltered signal detection (AB), fluorophores whose emission spectra show minimal overlap have been introduced (e.g. FAM in combination with the recently launched fluorophore NED). In contrast, the use of filters or lens (Roche, BIO-RAD, Corbett Res., Stratagene, Cepheid) minimizes the cross talk signals by selective measurement of defined wavelengths.

For melting curve analysis, the smallest possible increment of temperature per second (minimal ramp rate) is an essential parameter. The lower the minimal ramp rate, the better the resolution of melting curves. The Light Cycler instrument (Roche) displays a particularly low temperature ramp rate, and permits easier identification of specific amplicons and discrimination from spurious PCR products.

c) **Applications and new developments**

Due to the high sensitivity, reproducibility, and broad dynamic range, RQ-PCR instruments are being increasingly used for routine diagnostic purposes. In the case of RNA targets, the spectrum of common clinical applications includes detection and quantification of various RNA viruses [29,30], diagnosis of tumor-associated genetic alterations [31,32], and the validation of array-based gene expression profiling results [33,34].

Recently, different formats of real-time PCR have also been used for the detection and quantification of point mutations at the DNA and RNA level. A new type of probes carrying a chemical modification at the 3'-end, which facilitates highly specific binding to the minor groove of DNA, has been introduced (TaqMan Minor Groove Binding (MGB) probes, AB). The tight binding increases the $T_m$ of the hybridized probe. Moreover, the difference in $T_m$-values between completely and incompletely matched probes is pronounced, permitting accurate discrimination between different alleles. Additionally, these probes contain a new quencher dye (DARK) that does not emit fluorescence within the detectable range of wavelength. This improvement eliminates spectral overlaps with fluorescence emitted by the reporter dye, and results in greater accuracy in the measurement of reporter-specific signals.

For allelic discrimination, two differently labelled (e.g. FAM and HEX) TaqMan MGB probes, each specific for one allele, can be used in a PCR reaction driven by a single set of primers. Our experience with the use of MGB probes on the ABI Prism 7700 instrument revealed an excellent ability of this system to differentiate between homo- and heterozygous samples (unpublished observations). However, precise quantification of the two alleles was hampered by spectral interference (cross talk) of the FAM and VIC fluorescence signals in the detection channels.

Melting curve analysis of PCR products in the presence of SYBR® Green I is an alternative approach for discrimination and quantification of different alleles. The main advantage of this format is its simplicity. The absence of differently labelled fluorescence probes eliminates the problem of cross talk signals. Theoretically, a single base pair difference is sufficient to change the $T_m$ of the amplicon, and to allow discrimination between the different alleles by melting curve analysis: homozygous samples result in single peaks. A problem we encountered in analyses of heterozygous samples was the observation of one or two peaks in addition to the specific allelelic signals, which resulted from heteroduplex formation between the non-complementary single-stranded DNA molecules (data not shown). This phenomenon is an impediment to accurate quantitative analysis of alleles. For this application therefore, the use of intercalating dyes may not be an ideal approach. Alternatively, formats based on hybridization probes or molecular beacons may be used for detection and quantification of different alleles. Due to the thermodynamic characteristics of the hairpin structure of molecular beacons, the specificity of these probes is expected to be superior.

Another possibility to increase the specificity of amplicon-specific probes directed against individual alleles is the use of the recently introduced peptide nucleic acids (PNAs) [35]. PNAs are nucleic acid analogs in which the phosphate/sugar backbone is replaced by an uncharged polyamine backbone. The side groups consist of nitrogenous purine and pyrimidine bases, like in biological nucleic acids. Owing to the

neutral backbone, the binding to complementary base sequences is stronger and more specific than that achieved with DNA or RNA probes [36]. Thus, PNA/DNA mismatches are less stable than DNA/DNA or RNA/DNA mismatches. Moreover, PNA probes show excellent chemical and biological stability, and are only slightly more expensive than normal TaqMan probes. Due to these intriguing properties, it might be possible to improve the specificity of quantification by substituting nucleic acid probes by PNA oligomers.

**d) *Comparison of* real-time PCR instruments**

The ABI Prism 7700 Sequence Detection System and the Light Cycler were the first real-time instruments on the market and, according to the number of published papers, are apparently still the most widely used systems. The ABI Prism 7700 has the capacity of processing 96 samples per run, and has therefore been the instrument of choice for high throughput applications. By contrast, the Light Cycler with its smaller sample capacity, but rapid cycling profile (Table 1), has been the preferred instrument for research applications, with a focus on processing rather small sample numbers, but a greater variety of different targets.

The properties of the other thermal block-based instruments (iQ-Cycler, BioRad; MX4000, Stratagene; ABI7900, AB) appear to be comparable to the ABI Prism 7700 apparatus, with an emphasis on large scale applications. In particular, the ABI7900 instrument facilitates processing of up-to 384 samples per run at a higher cycling speed (Table 1). Due to its enormous capacity, it may become the apparatus of choice for high throughput tasks.

The iCycler (BIO-RAD) and the Rotor-Gene instrument (Corbett Res.) are based on airstream heating and display properties similar to the first apparatus relying on this principle, the Light Cycler.

To assess the compatibility of real-time PCR assays performed on different types of instruments, we compared a number of different protocols on the ABI7700 and on the Light Cycler. For both instruments the set-up of PCR reactions was identical including the same reaction volumes, TaqMan® probes, primers, DNA or cDNA templates, and master mixes containing Uracil-DNA Glycosylase (UNG) (see below). The targets tested included 17 different RNA and DNA viruses and 10 leukemia-associated fusion gene transcripts. The qualitative and quantitative results of both systems were highly concordant. The amplification efficiency of individual PCR reactions and the sensitivity achieved on both instruments were very similar under identical assay conditions (unpublished data). Due to the different properties of the ABI Prism 7700 and the Light Cycler, characterized by high capacity versus versatility and speed, the main areas of application are different. In this regard, the instruments are not likely to be competitors on the market for the same applications. On the contrary, the different features may render the instruments complementary in the clinical diagnostic setting. The speed of the Light Cycler could be utilized for the establishment and optimization of assays, and for rapid analysis of urgent clinical samples. Once established and optimized, the tests could be transferred to the ABI Prism 7700 or another high throughput instrument for large scale analysis.

**Table 1a: Comparison of various real-time instruments**

| Manufacturer | Instrument | Amplification Performance | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | Sample rack | Sample Format | Capacity (max) | Sample Volume | Heating/ Cooling system | Time/ run[1] | Heating ramp rate (max) | Cooling ramp rate (max) | Temperature Accuracy |
| AB | ABI Prism 7000 Sequence Detection System | Sample block | 0.2ml tubes or microtiter plate | 96 | 25 to 100µl | Peltier-based thermal cycler | 100 min | 5.0°C/sec from 4°C to 99.9°C | 5.0°C/sec from 99.9°C to 4°C | ± 0.25°C from 35°C to 99.9°C |
| | ABI Prism 7700 Sequence Detection System | Sample block | 0.2ml tubes or microtiter plate | 96 | 25 to 100µl | Compressor-based thermal cycler | 115 min | 3.5°C/sec from 4°C to 99.9°C | 3.5°C/sec from 99.9°C to 4°C | ± 0.75°C from 35°C to 99.9°C |
| | ABI Prism 7900HT Sequence Detection System | Sample block | 0.2ml tubes[2] or microtiter plate[2,3] | 96 or 384 | 25 to 100µl[2] 5 to 20µl[3] | Peltier-based thermal cycler | 100 min | 5.0°C/sec from 4°C to 99.9°C | 5.0°C/sec from 99.9°C to 4°C | ± 0.25°C from 35°C to 99.9°C |
| Roche | Light Cycler | Rotor-like carousel | glass capillaries | 32 | 10 to 20µl | Airstream cycler | 50 min | 20°C/sec from 50°C to 95°C | 20°C/sec from 95°C to 50°C | ± 0.3°C from 40°C to 98°C |
| BIO-RAD | ICycler iQ | Sample block | microtiter plate or 0.2ml tubes | 96 | 15 to 100µl | Peltier- and Joule-based thermal cycler | 95 min | 3.3°C/sec from 40°C to 99°C | 2°C/sec from 99°C to 40°C | ± 0.4°C from 40°C to 99°C |
| Corbett Research | Rotor-Gene 2 channel | Rotor-like carousel | 0.1ml[4] or 0.2ml tubes[5] | 36 or 72 | 10 to 50µl | Airstream cycler | 68 min | 5°C/sec from 50°C to 95°C | 5°C/sec from 50°C to 95°C | ± 0.5°C from 40°C to 98°C |
| | Rotor-Gene 4 channel | Rotor-like carousel | 0.1ml[4] or 0.2ml tubes[5] | 36 or 72 | 10 to 50µl | Airstream cycler | 68 min | 5°C/sec from 50°C to 95°C | 5°C/sec from 50°C to 95°C | ± 0.5°C from 40°C to 98°C |
| Stratagene | MX4000 | Sample block | 0.2ml tube strips or microtiter plate | 96 | 10 to 50µl | Thermal cycler | 90 min | 1.7°C/sec from 50°C to 95°C | 1.7°C/sec from 95°C to 50°C | ± 0.25°C from 40°C to 98°C |
| Cepheid | Smart Cycler | 16 Sample blocks | 0.2ml tubes | 16 | 25 to 100µl | Thermal cycler with air cooling | 72 min | 10°C/sec from 50°C to 95°C | 2.5°C/sec from 95°C to 50°C | ± 0.5°C from 60°C to 95°C |
| MJ Research | DNA Engine opticon | Sample block | 0.2ml tubes or microtiter plate | 96 | 10 to 50µl | Peltier-based thermal cycler | 100 min | 3°C/sec from 4°C to 99.9°C | 3°C/sec from 99.9°C to 4°C | ± 0.3°C from 35°C to 99.9° |

**Legend:**

1 Amplification profile for the amplification of a 100bp fragment. The profile was adapted to the use of hydrolysis probes in the presence of hot start polymerase and UNG. Thermal block cycler amplification profile [50°C/2min, 95°C/10min, 40x (95°C/15sec, 60°C/1min)]. Airstream cycler amplification profile [37°C/2min, 95°C/10min, 40x (95°C/10sec, 60°C/20sec)].

2 for 96-wells block 3 for 384-wells block 4 for 72-wells carousel 5 for 36-wells carousel

**Table 1b: Comparison of various real-time instruments**

| Manufacturer | Instrument | Detection Performance | | | | | Minimal Ramp rate | On-line monitoring | Price (EUR) |
|---|---|---|---|---|---|---|---|---|---|
| | | Emission filters or lens | Detection channels (in nm) | No of fluorophores/ sample | Mode of data acquisition | Time required for data acquisition | | | |
| AB | ABI Prism 7000 | Yes | 530, 540, 580, 610 | 4 | | 1 sec for 96 wells[7] | 0.023°C/sec | Yes | 56.690.- |
| | ABI Prism 7700 | No | measured from 500-660 | no limit | sequentially | 7 sec for 96 wells | 0.03°C/sec | No | 88.960.- |
| | ABI Prism 7900 | No | measured from 500-660 | no limit | sequentially | 7 sec for 96 wells | 0.023°C/sec | No | 106.830.- |
| Roche | Light Cycler | Yes | 530, 640, 710 | 3 | sequentially | 5 sec for 32 capillaries | 0.05°C/sec | Yes | 50.150.- |
| BIO-RAD | iCycler | Yes | 530, 575, 595, 620, 660,710[6] | 4 | parallel | 1 sec for 96 wells[7]/ 8 sec for 96 wells[8] | 0.1°C/sec | Yes | 47.170.- |
| Corbett Research | Rotor-Gene 2 channel | Yes | 470, 530, 585, 625 | 2 | sequentially | 8 sec for 72 wells | 0.5°C/sec | Yes | 42.510.- |
| | Rotor-Gene 4 channel | Yes | 510, 555, 610, 660, 580, 610 high pass | 4 | sequentially | 8 sec for 72 wells | 0.5°C/sec | Yes | 47.600.- |
| Stratagene | MX4000 | Yes | 4 detection channels with different filter sets ranging from 350-830 nm, custom made filter are available | 4 | sequentially | 7 sec for 96 wells[8] | 0.5°C/sec | Yes | 98.000.- |
| Cepheid | Smart Cycler | Yes | 4 detection channels with different filter sets ranging from 505-800 nm | 4 | each module seperately | 6 sec/module | 0.1°C/sec | Yes | 47.000.- |
| MJ Research | DNA Engine opticon | Yes | 530 | 1 | sequentially | 7 sec for 96wells | 0.1°C/sec | Yes | 43.000.- |

**Legend:**

**6** filter change done by the user

**7** if a single fluorophore has to be detected

**8** if four fluorophores have to be detected

### e) Cost of quantitative PCR assays

Approaches to quantitative analysis by PCR that do not require the use of costly instruments, such as gel electrophoresis-based competitive or comparative assays, are also inexpensive in terms of consumables. However, these assays are more labor-intensive, and, in contrast to real-time PCR approaches, cannot be readily automated. It may be expected therefore that their use will be restricted to instances in which real-time instruments are not accessible.

Among the real-time PCR apparatuses available, the differences in price are quite considerable. The current range is between 43K EURO (Rotor-Gene 2 channel; Corbett) and 107K EURO (ABI Prism 7900 Sequence Detection System; AB) (Table 1)*. As an alternative to the investment, some companies offer the possibility of leasing the instruments.

Another important consideration is the cost of reagents and other consumables. In tests not relying on the use of SYBR Green chemistry, it is necessary to purchase target-specific fluorescent probes. The prices vary greatly depending on the supplier (Table 2), ranging between 175 and 600 EURO for a synthesis at a scale of about 25nmol*.

**Table 2: Prices for hydrolysis probes**

| Supplier | Price [Euro] | Yield [nmol] |
|---|---|---|
| Applied Biosystems | 595.- | 15 - 25 |
| Sanova Eurogentec | 334.- | 10 - 50 |
| Genset | 600.- | ~25 |
| Metabion | 245.- | 15 - 25 |
| IBA | 175.- | 25 |
| Microsynth | 222.- | 10 - 25 |
| TIB-MOLBIOL | 450.- | ~30 |

**Legend:**
The prices reflect the costs for dually labeled hydrolysis probes, synthesized at a scale of about 25 nmol*. The costs for purification are included.

**Footnote:**
* ... The indicated prices are based on information obtained between October, and December 2001.

It is important to emphasize that the quality of probes may vary between suppliers. In our experience, low-price probes sometimes display an increased hydrolysis rate, leading to higher background signals. This may impede the detection and quantification of low copy numbers of specific target.

The factor most strongly affecting the cost of real-time assays is the price of PCR reagents. It is nevertheless not advisable to use homemade preparations. Commercially available kits are more expensive, but permit better standardization and inter-laboratory comparison. Two of the companies producing RQ-PCR instruments (AB and Roche) provide their own reagent sets for real-time amplification. The current cost of reagents calculated for a 20µl reaction vary between 0.93 EURO (AB) and 2 EURO (Roche)*. The reagent mix ("Master Mix") supplied by AB contains hot-start DNA polymerase, buffer, dNTPs, a reference dye, and UNG (see below). The latter two reagents are not part of the Light Cycler amplification protocol and are therefore not included in the reagent kit supplied by Roche. The glass capillaries used by the Light Cycler are rather expensive (0.5 EURO each) in comparison to the reaction tubes or plates used by the AB instrument. Hence, the overall cost of consumables other than liquid reagents per 20 µl reaction for the Light Cycler is about 1 EURO versus 0.5 EURO for the AB instruments. The cost of PCR assays on other instruments for which reagents are not provided by this manufacturer depends on the selected supplier of reagents.

### f) Controls and necessary precautions

Accurate quantification of mRNA transcripts by RT-PCR not only requires judicious selection of the most appropriate methodology and instrumentation, but also careful control of potential problems inherent in the technique.

*(1)* ***False negative results***

In view of the great sensitivity of PCR, the occurrence of false negative results is a highly underestimated problem. The absence of positive signals in samples containing the target transcript of interest may result from several factors including 1) inhibitory substances present in the clinical specimen, 2) degradation of the targeted nucleic acid, 3) low efficiency or failure of extraction, reverse transcription or amplification. To discriminate between true and false negative samples, these variables need to be carefully assessed by appropriate controls. The co-amplification of endogenous control genes, such as ubiquitously expressed constitutive mRNAs of housekeeping genes, is used for this purpose.

**The requirements for the selection of adequate endogenous control genes are the following:**

**1.** Endogenous RNA standards have to be carefully evaluated for the presence of pseudogenes. Genes known to have processed pseudogenes, such as Histone H3.3 (H3.3) [37], Glyceraldehyde-3-phosphate dehydrogenase (G3PDH) [38], or Beta-Actin (β-Actin, M55014) [39] have to be excluded to avoid the risk of false results in case of contamination with genomic DNA (gDNA). In these instances, PCR amplification of the control gene would be positive even in samples containing completely degraded RNA. This could lead to false negativity of the target transcript amplification. We have recently published a list of legitimate endogenous control genes known to lack pseudogenes in the human genome [6,40].

**2.** Adequate endogenous controls should not be expressed at considerably higher levels than the target gene of interest. If very low amounts of test material are available or if the RNA sample tested is partially degraded, a highly expressed endogenous gene may still test positive, and absence of the specific target signal could lead to false negative results.

The use of endogenous controls is not restricted to the surveillance of critical steps of the assay. Due to the constant expression level of appropriate control genes, the copy number of target transcripts is commonly indicated in relation to the endogenous control gene (e.g. target mRNA copies /10E6 control gene copies) [41]. For this application, it is necessary that:

**a.)** control genes reveal expression levels that are constant during the cell cycle and in the cell types investigated, and their expression is not affected by the disease in question nor by the treatment applied [40];

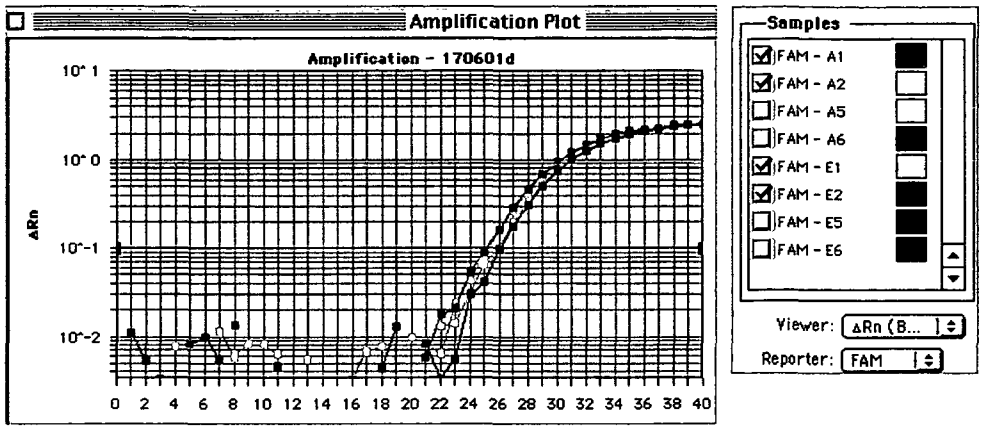
**b.)** the stability, i.e. the degradation rate, of the control and the target transcripts should be similar [40].

The problem of different sensitivity to nucleases becomes apparent in partially degraded samples, in which preferential decay of the target transcript has occurred. Greater stability of the control gene transcript would lead to false negative analysis of the target mRNA. Moreover, differences in stability may lead to incorrect quantitative analysis if the results are expressed as the ratio between target and control transcript copies. The problem of RNA degradation is particularly relevant in central referral laboratories receiving specimens from distant locations for qualitative and quantitative RT-PCR analysis. In these instances, the target/control gene ratio may be adversely affected by exposure of the sample to ambient temperature during transportation.

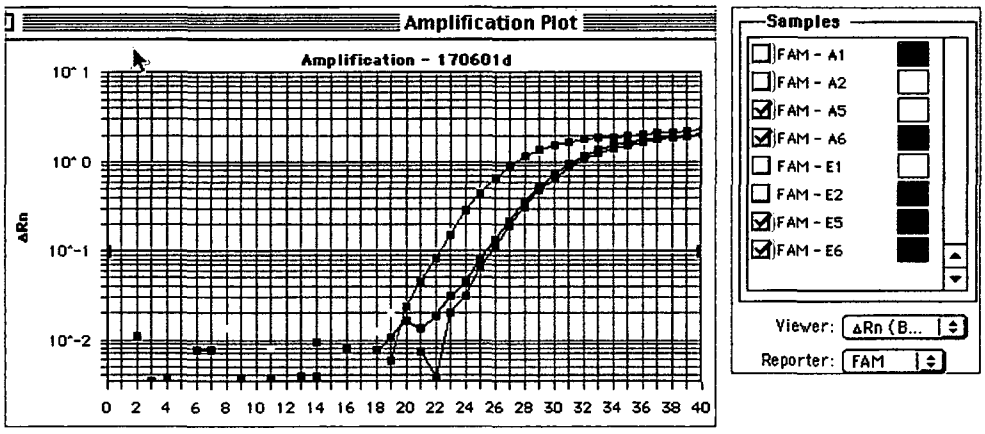
Little information is available in the literature on the stability of different mRNA transcripts, demonstrating that this problem may be underestimated. Our experience indicates that the degradation rate of various control genes may differ significantly (Fig. (8), unpublished data).

**Fig. 8: Stability of control genes**

A)




B)




**Legend:**

Fresh peripheral blood samples were split into 5 aliquots. Mononuclear cells were isolated by density gradient centrifugation from the first aliquot upon receipt in the laboratory (day 0), from the second aliquot after 1 day-, from the third aliquot after 2 days-, from the fourth aliquot after 3 days- and from the last aliquot after 4 days of storage on the bench at room temperature. A total of 2 x $10^6$ cells from each of these isolation steps were stored in lysis buffer at -20°C. Finally, all samples were subjected simultaneously to RNA extraction, reverse transcription and real-time amplification.

**Panel A** shows the amplification of the ABL (Abelson) control gene transcript in duplicates on day 0 (A1, red and A2, green) and on day 4 (E1, pink and E2, blue). The amplification curves of both duplicates are nearly identical (average $C_T$-values of 25.2 vs. 26.0), indicating a high stability of this control gene over the time period investigated.

**Panel B** shows the amplification of the GUS (β-Glucronidase) control gene transcript in duplicates on day 0 (A5, yellow and A6, blue) and on day 4 (E5, green and E6, red). The amplification curves of both duplicates differ significantly (average $C_T$-values of 22.2 vs. 25.6), indicating a decrease in amplifiable template amount in the range of one log. Thus, the GUS transcript displayed a lower stability than the ABL control mRNA over the time period investigated

It is important to keep in mind that the use of a single control gene only allows to draw conclusions about the integrity of the target gene if the degradation rate of both transcripts is known to be similar. If no data on the stability of the control and target genes are available, parallel processing and amplification of the control gene may not permit reliable assessment of the integrity of the target sequence. To avoid selection of an inappropriate control gene, it is recommended to assess the degradation rate of both transcripts in over-time stability studies.

Depending on the system studied, a number of commonly used endogenous standards were shown to display expression levels that are either variable or too high to provide a relevant control for

most transcripts of interest [42-44]. These studies demonstrate the apparent difficulties in selecting suitable endogenous standards. Because individual housekeeping genes reveal different degradation rates and display tissue- and developmental stage-specific differences in the expression level, it is not likely that a universal control gene will be identified. Rather, it will be necessary to carefully select an appropriate control gene for each specific application.

An alternative control for RT-PCR assays is the implementation of the so–called "armored RNA" technology, in which the samples investigated are spiked with a known concentration of a synthetic RNA standard protected from degradation by packaging in pseudoviral particles [45]. Such external standards do not provide information on the integrity of the RNA sample under investigation, but facilitate control of each individual step of RT-PCR. Moreover, variations in the efficiency of each of the processing steps can be assessed by adding different types of RNA standards at known concentrations prior to nucleic acid isolation, and RT.

*(2)* ***False positive results***

The problem of false positivity in PCR assays is generally well appreciated. Most commonly, the problem is caused by carryover contamination of PCR reactions with amplification products or genomic DNA (gDNA), and occasionally, by homology of the primers to irrelevant sequences.

Although in real-time PCR assays using fluorescence-based instruments, the absence of post-amplification handling greatly reduces the risk of contamination by PCR products, it should be emphasized that this problem is not entirely eliminated. Contamination may occur by leakage from tubes or microtiter plates with lids not tightly closed or by breakage of glass capillaries leading to spillage of the amplification mixture. The contamination with PCR product can be easily reduced by a digestion step with Uracil-DNA-glycosylase (UNG) prior to each amplification if the nucleotide dTTP was substituted by dUTP in all PCR reactions. In this instance, all PCR products differ from gDNA by the presence of dUTP. The greater problem is a contamination with template gDNA/cDNA strands lacking dUTP thus being not affected by the enzymatic digestion. When using UNG, it is essential to prevent digestion of newly synthesized PCR product. The enzyme is therefore inactivated by heat denaturation before the start of amplification.

Although the routine use of UNG is highly effective, it does not completely eradicate the problem of contamination. The inclusion of control reactions lacking any template, the so-called "no-template controls", and reactions including non-specific template, the "no-amplification controls", are also in real-time PCR assays necessary as a means of preventing false positive results.

## C. Future Prospects

The rapidly growing number of publications on quantification of RNA targets demonstrates the increasing importance of quantitative RNA detection technologies in different areas of research and in clinical diagnosis. The expression level of diagnostically important genes or the load of infectious pathogens, such as RNA viruses, have become clinically important parameters. The level or the kinetics of gene expression over time, and the dynamics of infection have prognostic relevance in a number of clinical situations, and may serve as a basis for guiding therapeutic interventions [46-49].

The variety of methods used for quantification of RNA targets renders the comparison of data generated by different laboratories rather difficult. It is desirable therefore to establish standardized technical approaches to quantitative RNA analysis. Automation of the entire process, from RNA isolation to quantification, would provide the most convenient way of eliminating inter-laboratory variation. The instruments required for automated sample processing are available, but the relatively high cost of the equipment and the necessary consumables have been an impediment to broad implementation of automation. In can be expected, however, that the increasing competition on the market will inevitably lead to reduced instrument costs in the near future, making automation of RNA analysis more readily affordable.

To achieve the goal of establishing international standards in molecular diagnosis, it is necessary to organize multi-center laboratory networks focusing on the development and standardization of diagnostic protocols. Examples of such international collaborative efforts include the *Concerted Actions* supported by grants from the European Union, aiming at the standardization and clinical implementation of different diagnostic approaches. The data emanating from these collaborative studies are available to the scientific community. One of the recent international studies, the DG V Concerted Action supported by the Europe Against Cancer Program, focused on the quantification of leukemia-associated fusion gene transcripts [50]. The study, which is still ongoing, has provided detailed information on adequate endogenous control genes, and has resulted in the establishment of standardized protocols for RNA isolation, RT and real-time PCR quantification of fusion gene transcripts. This international collaboration may serve as a good example for future efforts aiming at the development of widely applicable concepts for quantitative analysis of RNA.

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# PART 2

# CONTROL GENES

# CHAPTER

# II

# MULTIPLEX PCR FOR QUALITY CONTROL OF TEMPLATE RNA/cDNA IN RT-PCR ASSAYS

# Multiplex PCR for quality control of template RNA/cDNA in RT-PCR assays

## A. Introduction

Reverse transcription-polymerase chain reaction (RT-PCR) is a powerful tool for the detection and quantification of target mRNAs in basic research and routine clinical diagnosis. To assess the presence of amplifiable template RNA/cDNA, amplification of a control gene is used as an external standard. Commonly, housekeeping genes with minimal tissue- and developmental stage-specific variations are employed [1,2]. In assays directed at the detection of tumor-cell specific fusion gene transcripts, a segment of one of the genes involved in the translocation is frequently used as a control [3,4]. In most instances, a sequence from a single control gene is amplified to assess the availability of intact and amplifiable RNA. In view of the differences in stability of various mRNA species [5], this approach may not provide a reliable control for integrity of the target sequence in all instances.

In a diagnostic setting, central referral laboratories receiving specimens from distant locations are often faced with the problem of partially degraded RNA-samples, because the cell material may be exposed to ambient temperature for several days during transportation. In such samples, the use of a highly expressed control gene may yield a positive signal while the actual target transcript may no longer be detectable by the PCR assay. In patients with leukemia carrying a characteristic marker amenable to investigation by RT-PCR, which most commonly is a fusion gene transcript, one-step PCR is generally sufficient to permit its detection at the time of diagnosis. We nevertheless perform two-step nested PCR analysis of the target sequence as a means of confirming specificity of the amplified product, while the control gene is only submitted to one-step amplification. In largely degraded RNA preparations from diagnostic blood or bone marrow samples, a two-step amplification of the fusion transcript targeted is sometimes necessary to reveal its presence, while a one-step amplification of a moderately expressed control gene may be negative, and that of a highly expressed control gene positive. This scenario emphasizes the problem of different expression levels or discordant levels of stability between the target and the control gene transcripts. On the other hand, it indicates that the employment of a control gene expressed at a higher level may be useful in some instances, because it may indicate the presence of small amounts of amplifiable RNA/cDNA which could be sufficient to permit detection of the target sequence after two-step PCR-amplification.

While in many diagnostic RT-PCR assays successful amplification of a specific target may be possible even in largely degraded RNA samples, investigation of the same targets for detection of minimal residual disease (MRD) requires RNA preparations of very good quality to achieve adequate sensitivity. Therefore, control gene analysis in RT-PCR assays for detection of residual tumor cells should help assess variations in RNA/cDNA quality to permit correct interpretation of the results. Integrity of the target sequence which is one of the essential factors influencing sensitivity of PCR tests, can only be assessed by indirect evidence provided e.g. by control gene

amplification. The employment of a single control gene may not be an ideal approach to this task.

We have therefore established a multiplex PCR assay for co-amplification of sequences from four transcripts expressed at different levels. Selection of the control genes and the target sequences was based on extensive testing of more than ten genes commonly used as controls in RT-PCR assays including Aldolase, Histone H3.3 (H3.3), Beta-2-microglobulin ($\beta_2$-MG), Glucose 6-phosphate dehydrogenase (G6PDH), Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), β-actin, Hypoxanthine phosphoribosyltransferase (HPRT), Porphobilinogen deaminase (PBGD), Dihydrofolate reductase (DHFR), Abelson (ABL) and BCR.

Genes known to have processed pseudogenes (Aldolase [6], H3.3 [7], G3PDH [8], β-actin [9] (M55014), HPRT (U10112), and DHFR [10]) were excluded to avoid the risk of false positive results in case of contamination with genomic DNA [5,11]. Primers located in different exons were designed to facilitate the discrimination of PCR products derived from gDNA and cDNA. Concentrations of PCR components and cycling parameters were optimized for each of these genes, and finally, primers suitable for multiplex PCR were selected on the basis of compatible reaction conditions and product sizes. Based on these selection criteria, we established a multiplex PCR assay permitting co-amplification of mRNA segments derived from four different genes including BCR, ABL, $\beta_2$–MG, and PBGD. The former two are expressed at moderate levels, whereas the latter two show relatively high levels of expression in most cell types. Co-amplification of the four target sequences selected yields PCR products ranging from 128 to 377 bp. The minimum difference in length between individual products of 65-94bp permits easy evaluation upon agarose gel electrophoresis. Primer sequences, genomic location and predicted size of amplified cDNA and gDNA fragments are indicated in Table 1.

Contemporaneous amplification of differentially expressed control genes by the multiplex PCR assay permits assessment of the extent of RNA degradation.

## B. Materials and Methods

PCR was carried out in a Perkin-Elmer Cetus DNA thermal cycler (model 2400). Individual reactions contained 10 mM Tris (pH 8.3), 50 mM KCl, 2.5 mM MgCl2, 150 µM of each of the deoxynucleotide triphosphates, 0.5 units of AmpliTaq Gold (all reagents were from Perkin Elmer), approx. 50 ng template, 5.4 pmol of the BCR and $\beta_2$–MG primers, 4.8 pmol of the ABL primers, and 4.3 pmol of the PBGD primers in a total volume of 30µl. Reactions were initiated by heating the samples at 95°C for 8 min. Subsequently, thirty-five cycles were performed with denaturation at 96°C for 30 sec, annealing at 55°C for 30 sec, and extension at 72°C for 40 sec. A final extension step for 7 min at 72°C was included. Fifteen microliters of the PCR product were electrophoresed on 2% agarose gels and visualized with ethidium bromide. It is important to note that the use of other reagents or another thermal cycler will likely require new optimization of the protocol.

**Table 1: Primer and product details**

| Primer Sequence | Genomic Location | Accesion Number | Product Size | |
|---|---|---|---|---|
| | | | gDNA | cDNA |
| **PBGD 3/2-S**<br>5′-TGA GAG TGA TTC GCG TGG GTA C-3′ | 264 - 285 | D 12722 | ≈ 890bp◆ | 128 bp |
| **PBGD 4-5/1-AS**<br>5′-CCC TGT GGT GGA CAT AGC AAT G-3′ | 189 - 210* | X 04217 | | |
| **ABL I2/1-S**<br>5′-AGC ATC TGA CTT TGA GCC-3′ | 463 - 480 | M 14752 | 793 bp | 193 bp |
| **ABL 3/4-AS**<br>5′-CCC ATT GTG ATT ATA GCC TAA GAC-3′ | 632 - 655 | M 14752 | | |
| **$\beta_2$-MG 2/2-S**<br>5′-ATT TCC TGA ATT GCT ATG TG-3′ | 177 - 196 | M 17987 | 2147 bp | 287 bp |
| **$\beta_2$-MG 4/2-AS**<br>5′-GAA TTC ACT CAA TCC AAA TG-3′ | 2304 - 2323 | M 17987 | | |
| **BCR 10-11/7-S**<br>5′-GAG AAG AGG GCG AAC AAG-3′ | 119274-119279*<br>121237-121248*<br>(2889 - 2906) | U 07000<br><br>(M 24603) | 5212 bp | 377 bp |
| **BCR 14/1-AS**<br>5′-CTC TGC TTA AAT CCA GTG GC-3′ | 124467-124486<br>(3246 - 3265) | U 07000<br>(M 24603) | | |

**Legend:**

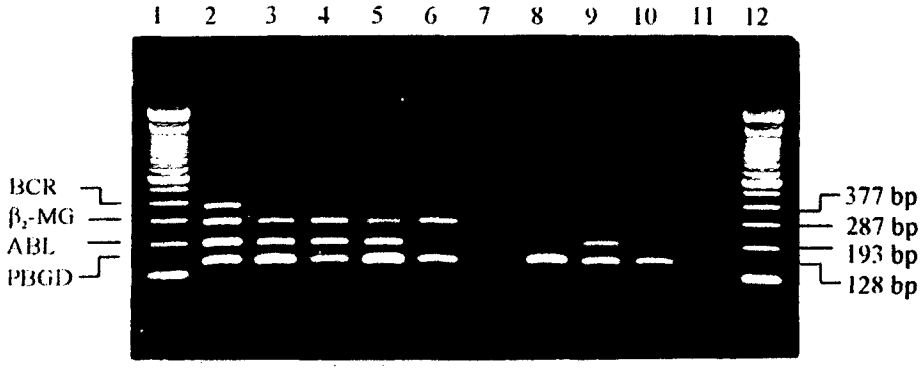
* Primers spanning exon-exon junctions.
◆ No information on the location of introns available. The size given is based on estimation of PCR products upon electrophoresis through agarose gels.

## C. *Results and Discussion*

The reliability of this method was evaluated by screening of more than hundred tumor specimens that had been previously studied for the expression of ABL and $\beta_2$–MG mRNAs by conventional RT-PCR analysis. In all samples displaying a strong amplification signal of the moderately expressed ABL gene in single-target PCR analyses, all four control gene fragments were amplified in the multiplex assay, indicating adequate mRNA/cDNA quality. RNA/cDNA preparations of suboptimal quality were characterized by loss of the BCR signal, while further deterioration of sample quality resulted in an additional loss of either the ABL or the $\beta_2$–MG signal. In single-target PCR assays, such samples were negative for ABL, but positive for the highly expressed $\beta_2$–MG gene. In samples of very poor mRNA/cDNA quality displaying a weak $\beta_2$–MG signal in the single-target PCR assay, the multiplex amplification revealed a PBGD signal only. The inability to amplify a $\beta_2$–MG fragment in a sample by single-target PCR corresponded to complete lack

of signals in the multiplex assay. Examples of multiplex PCR amplification of good, intermediate, and poor RNA/cDNA quality preparations are shown in Figure 1. The discrepancy between single-target and multiplex PCR regarding the presence or absence of the $\beta_2$–MG signal might be attributable to competition for available substrates between $\beta_2$–MG and other targets in the multiplex assay. This notion is supported by the observation that samples negative for $\beta_2$–MG, and positive for PBGD in the multiplex PCR, generally show successful $\beta_2$–MG amplification when tested with the same reagents and under the same cycling conditions in a conventional single-target PCR assay.

**Fig 1: Quality assessment of RNA/cDNA preparations by multiplex PCR.**



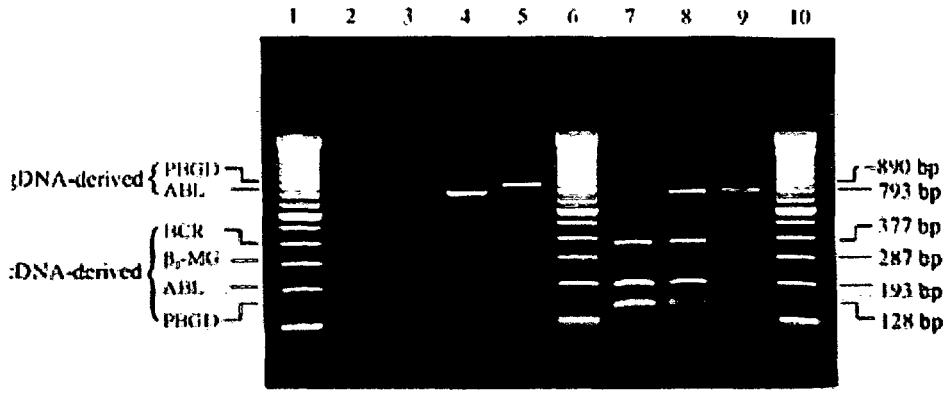

**Legend:**

Transcript segments of four genes (BCR, ABL, $\beta_2$–MG, and PBGD) were co-amplified by one-step PCR. In samples with high quality RNA/cDNA (lanes 2-4), all four fragments were successfully amplified. Loss of the BCR signal (lanes 5,6) indicated suboptimal quality, and additional loss of the $\beta_2$–MG signal (lane 8) or the ABL signal (lane 9) was indicative of relatively poor RNA/cDNA quality. Very poor quality templates yielded a PBGD signal only (lane 10).

In this multiplex assay, PBGD has been the most stable target, present in over 99% of the samples investigated. In a series of over 100 specimens with amplifiable cDNA tested, the only sample lacking the PBGD-derived band exhibited $\beta_2$–MG and ABL signals (lane 7). Lanes 1 and 10: molecular size marker (100 bp ladder; GIBCO/BRL); lane 11: negative control.

The primer combinations used in the multiplex PCR reaction were tested for their ability to amplify genomic DNA fragments of the respective genes. To address this question, all primer pairs were tested in single-target PCR reactions using pure gDNA as template. Due to the relatively small introns spanned by the primers for ABL and PBGD, specific PCR products of about 0,9 and 0,8 kb were obtained (Table 1), while no amplification products of BCR and $\beta_2$–MG were seen under the assay conditions employed. To test the effect of gDNA contamination of RNA preparations on the multiplex PCR assay, cDNA samples were mixed with gDNA at various proportions. Under these artificial assay conditions, contaminating gDNA down to a 10% level was sufficient to produce a 0,8 kb amplification signal of the ABL gene. In contrast to the results of single-target PCR assays, no gDNA signal of the PBGD gene was amplified under the multiplex PCR conditions.

**Fig 2: Amplification of genomic DNA fragments in single-target PCR, and multiplex PCR of cDNA samples with gDNA contaminants**




**Legend:**

Each of the four primer pairs was tested in single-target PCR assays containing gDNA as template.

The reaction conditions and cycling parameters of the single-target PCR were as indicated in "Materials and Methods", the only modification being an adaptation of the primer concentration to 10 pmol. With BCR primers (lane 2) and $\beta_2$-MG primers (lane 3) which span large introns, no genomic DNA product was amplifiable, but with ABL primers (lane 4) and PBGD primers (lane 5), genomic fragments of the expected size could be amplified.

To test the effect of contaminating gDNA on the multiplex PCR assay, RNA/cDNA templates derived from cell lines were mixed with different amounts of gDNA. In samples containing a cDNA/gDNA ratio of 1:1(lane 8), the ABL gene-derived DNA fragment of 0,8 kb was detected in addition to the c-DNA-derived amplification products. At a cDNA/gDNA ratio of 9:1, the ABL-derived band was still visible (lane 7). Lane 9 shows multiplex PCR amplification of a clinical sample containing an unknown amount of contaminating gDNA. As shown in lanes 7-9, the presence of contaminating gDNA compromises the efficiency of cDNA target amplification. Multiplex PCR analysis of the above RNA/cDNA samples derived from cell lines revealed products of all four cDNA targets when no gDNA was admixed (not shown). Lanes 1, 6, and 10: molecular size marker (100bp ladder, GIBCO/BRL).

The results of amplification using different cDNA/gDNA mixtures in the multiplex PCR assay are shown in Figure 2.

The multiplex PCR assay presented cannot supplant other approaches to assessment of RNA integrity, such as evaluation of ribosomal RNA bands on (non)-denaturing agarose gels. However, in comparison to single-target control PCR tests, this assay provides a more comprehensive control of different mRNA species in the sample analyzed, which may yield relevant information on the integrity of the target sequence. The ability to assess gradual differences in the quality of RNA/cDNA preparations provides an additional tool for determining the expected sensitivity of RT-PCR assays.

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# CHAPTER

# III

# CONTROL GENES FOR DIAGNOSIS AND MRD DETECTION BY PQ-PCR

# Evaluation of candidate control genes for diagnosis and residual disease detection in leukemic patients using "real-time" quantitative reverse-transcriptase polymerase chain reaction (RQ-PCR) - A Europe Against Cancer Program

## A. Introduction

The diagnosis and follow-up of leukemia patients has evolved from being solely based on morphological criteria in the past to increasingly based upon molecular methodologies [1-8]. The development of PCR methods has resulted in a more accurate diagnosis of balanced translocations, particularly in cases of cryptic translocations, which before were only detectable by molecular methods [9-11]. Although appropriate in the diagnostic setting, standard reverse transcriptase-polymerase chain reaction (RT-PCR) methods are less suitable for the evaluation of the disease status during clinical remission, i.e. detection of minimal residual disease (MRD). Qualitative RT-PCR determinations were therefore only of limited value in the longitudinal follow-up of acute leukemia patients, since many of these patients became PCR negative.

Real-time quantitative PCR (RQ-PCR) has recently been introduced and allows rapid and sensitive detection of the fusion gene (FG) transcript in follow-up samples [12]. Compared to standard RT-PCR, real time RQ-PCR enables accurate quantification of gene expression. An attractive feature of this technique is that crucial parameters such as the RNA quality and quantity can be evaluated. This is accomplished by parallel amplification of the target gene and one or more control genes (CG), also called house-keeping or endogenous reference genes.

A suitable CG in any application of RQ-PCR analysis can be defined as a gene with a stable expression in all nucleated cells among different analyzed samples and which is unaffected by any experimental treatment [13,14]. Furthermore, the selected gene should not present any pseudogenes, in order to avoid any genomic DNA amplification [14,15]. An impaired amplification of CGs should be accompanied by a corresponding reduction in the quantity of target gene transcript(s).Variation in amplification would reflect variations in RNA quality, quantity and/or cDNA synthesis efficiency [14]. Thus, quantification of CG expression could be used for detecting poor quality samples, based on reference values observed in a large number of fresh samples. RQ-PCR would allow to assess an experimental sensitivity per sample, which is particularly important for PCR negative follow-up samples.

Although the literature on RQ-PCR assays is rapidly expanding, no concerted effort related to the selection of appropriate CGs has been published so far. The choice of a CG remains a crucial issue and a consensus has still to be found [14]. To date, numerous CGs for MRD detection by RQ-PCR are still used: *ABL* [16-20], *ACTB* [21,22], *B2M* [16,23,24], *GAPDH* [16,22,25], *PBGD* [26], *TBP* [27], and 18s rRNA [28]. Inclusion of CG analysis should significantly enhance the reliability of MRD detection in leukemic patients. However, this is critically dependent upon optimization and standardization of CG and FG assays. For these reasons, a collaborative action within the Europe Against Cancer (EAC) program was initiated

involving 26 laboratories in 10 member countries. The aim of this network was to define and standardize RQ-PCR protocols suitable for measuring the levels of FG transcripts in leukemia with special reference to the selection and optimization of CGs. In this study, we focused on the selection and validation of suitable CGs for RQ-PCR assays from a large panel of normal and leukemic samples, which can be eligible not only for the quantification of fusion gene transcripts, but also for the quantification of aberrantly expressed genes. The design and optimization of the FG RQ-PCR analysis are described in the EAC manuscript [29].

## B. Materials and Methods

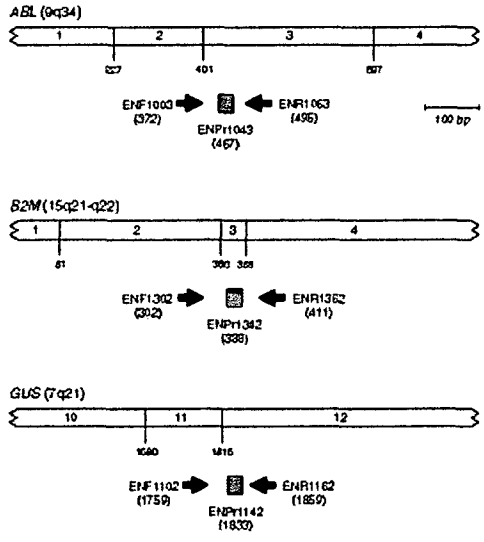
### 1. Organization of the CG network

Our concerted action, initiated in March 1999, involved twenty-six laboratories from ten European Community member countries [29]. All members of the network used the ABI PRISM 7700 sequence detection system (Applied Biosystems, ABI, Foster City, CA). For the identification and validation of suitable CGs in the MRD setting, a subgroup consisting of six laboratories was formed. Test material was prepared centrally (Aarhüs, Denmark) and distributed to network members unless otherwise indicated. Details about the organization of the EAC program, optimization and standardization of the RQ-PCR protocol for the detection of the FG transcripts can be found in the EAC manuscript [29] and the indicated web site: http://meidia.nord.univ-mrs.fr/EAC/publications.html.

### 2. Primers and probes

In the initial CG screening, a pre-developed human endogenous control plate (kindly provided by ABI) containing primer and probe sets for 11 CGs was used as recommended by the manufacturer (Table 1). Six additional CG primer and probe sets, designed by EAC laboratories, were analyzed: one EAC and one in-house [16] set for Abelson (*ABL*), one in-house [16] set for beta-2-microglobulin (*B2M*), two EAC sets for Porphobilinogen deaminase (*PBGD* and *PBGD2*) and one in-house set for Transcription Factor IID (*TBP*) (Table 1). After selection, the number of CGs was ultimately reduced to three, which were subjected to further analysis (Figure 1 and Table 2).

**Figure 1: Schematic diagram of EAC primer/probe sets for *ABL*, *B2M*, and *GUS***




**Legend:**
*ABL* EAC set: ENF1003, ENPr1043, ENR1063
*B2M* EAC set: ENF1302, ENPr1342, ENR1362 set
*GUS* EAC set: ENF1102, ENPr1142, ENR1162.
The number below the primers and probes refers to their 5' nucleotide position in the gene transcript (see Table 2).
ENF = forward primer, ENPr = TaqMan reverse probe, ENR = reverse primer.

Oligonucleotide primers and probes were designed employing the Primer Express 1.0 program (ABI) and the Oligo 6 program (Molecular Biology Insights Inc., Cascade, CO, USA). Primer and probe sets were also tested using 100 ng genomic DNA per PCR to confirm that assays were cDNA specific.

**Table 1: List of the 14 housekeeping genes analyzed in the first round**

**Candidates CGs**

| Code | Name | Chromosome | $Ct_{median}$ value | Reason(s) for exclusion |
|---|---|---|---|---|
| 18S rRNA [a] | 18S rRNA | 12p12 | 14.5 | Very high expression |
| *ABL* [b,c] | **Abelson** | **9q34** | 29.0 [b]<br>**27.7** [c] | |
| *PO* [a] | Acidic ribosomal protein | 11p15 | 22.3 | Pseudogenes (n=8) [d] |
| *B2M* [a,b] | **Beta-2-microglobulin** | **15q21** | 20.7 [a]<br>**21.6** [b] | |
| *ACTB* [a] | Beta-actin | 7p15 | 20.5 | Pseudogenes (n=18) [d] |
| *GUS* [a] | **Beta-glucuronidase** | **7q21** | **25.9** | |
| *CYC* [a] | Cyclophilin | 7p13 | 27.6 | Pseudogenes (n=63) [d] |
| *GAPDH* [a] | Glyceraldehyde 3 phosphate dehydrogenase | 12q13 | 21.1 | Pseudogenes (n=52) [d] |
| *HPRT* [a,b,d] | Hypoxanthine phosphoribosyltransferase | Xq26 | 26.8 | Ch(X) |
| *PGK* [a] | Phosphoglycerokinase | Xq13 | 24.2 | Ch(X) |
| *PBGD* [b] | Porphobilinogen deaminase | 11q23 | 27.1 | alternative transcriptional start sites |
| *PBGD2* [b] | Porphobilinogen deaminase 2 | 11q23 | 27.0 | |
| *TBP* [a,b,d] | Transcription Factor IID | 6q27 | 30.0 [a]<br>25.7 [b] | Low expression [a]<br>Variable in PBMNC [b] |
| *TFRC* [a] | Transferrin Receptor | 3q26 | 26.6 | Variable expression in hematopoietic cells |

**Legend:**
[a] Pre-developed human endogenous control plate from ABI (65 samples tested).
[b] In-house primer/probe set tested (36 samples tested).
[c] EAC primer/probe set tested (20 samples tested).
[d] Number of pseudogenes identified on the following web site http://www.pseudogene.org/. Pseudogenes were also found for *HPRT* (n=1) and *TBP* (n=1) genes.
Samples were tested before optimizing RT and RQ-PCR conditions. Thus median Ct values per CG might differ from the subsequent extensive analysis. In bold, selected genes for the final validation.

### 3. *Plasmids calibrators*

PCR products of *ABL*, *B2M* and *GUS* gene transcripts for preparation of plasmids were amplified with respectively ABL-F (5'-CCT TCA GCG GCC AGT AGC-3') & ABL-R (5'-GGA CAC AGG CCC ATG GTA C-3'), B2M-F (5'-CCT TGA GGC TAT CCA GCG T-3') & B2M-R (5'-CCT GCT CAG ATA CAT CAA ACA TG-3') and GUS-F (5'-CCT GTG ACC TTT GTG AGC AA-3') & GUS-R (5'-GTC TGC CGT GAA CAG TCC A-3'). PCR conditions of the BIOMED-1 Concerted Action were used [30].

The protocol for cloning and preparation of plasmid dilutions is detailed in the EAC manuscript [29]. For *ABL* and *GUS* plasmids, three dilutions ($10^5$, $10^4$, and $10^3$ copies in 5 µl) were used to calculate the standard curve. For the *B2M* plasmid, three different dilutions ($10^7$, $10^6$, and $10^5$ copies in 5 µl) were used. Corresponding coefficients of variation for Ct values were below 4% (*ABL*), 5% (*B2M*) or 3% (*GUS*) for all dilutions (data from all phases). Plasmid dilutions were centrally prepared initially in J. Gabert's laboratory and subsequently kindly provided by Ipsogen (Marseille, France) for phase IV (see EAC manuscript [29]).

**Table 2: Sequences of the three finally EAC selected CG RQ-PCR sets**

| Gene Acc No | EAC code[a] | Nt Sequence (5'- 3') | 5' Position (bp-size) |
|---|---|---|---|
| **Abelson** | ENF1003 | TGGAGATAACACTCTAAGCATAACTAAAGGT | 372 (31) |
| (*ABL*) | ENPr1043 | Fam-CCATTTTTGGTTTGGGCTTCACACCATT-Tamra | 467 (28) |
| X16416 | ENR1063 | GATGTAGTTGCTTGGGACCCA | 495 (21) |
| **Beta-2-Microglobulin** | ENF1302 | GAGTATGCCTGCCGTGTG | 302 (18) |
| (*B2M*) | ENPr1342 | Fam-CCTCCATGATGCTGCTTACATGTCTC-Tamra | 388 (26) |
| NM_004048 | ENR1362 | AATCCAAATGCGGCATCT | 411 (18) |
| **Beta-Glucuronidase** | ENF1102 | GAAAATATGTGGTTGGAGAGCTCATT | 1759 (26) |
| (*GUS*) | ENPr1142 | Fam-CCAGCACTCTCGTCGGTGACTGTTCA-Tamra | 1833 (26) |
| M15182 | ENR1162 | CCGAGTGAAGATCCCCTTTTTA | 1859 (22) |

**Legend:**
[a] ENF = forward primer, ENPr = TaqMan reverse probe (chosen in the anti-sens strand in order to select the C-rich strand), ENR = reverse primer

## 4. *Biological material and preparation of RNA*

From heparinized peripheral blood (PB), bone marrow (BM), and PB stem cells (PBSC) mononuclear cells (MNC) were obtained by Ficoll-Hypaque density centrifugation. All patient sampling was performed according to protocols approved by the local ethical committees of the given institutions and/or geographical areas. The RS4;11 cell line was grown in medium RPMI1640 with 10% fetal calf serum and harvested in the exponential growth phase. RNA was extracted by routine methods in the participating laboratories employing either a TRIzol reagent (Invitrogen, Cergy, France), a RNAzol reagent (Biotech Italia, Rome, Italy) or a column based system (Qiagen, Hilden, Germany) according to the manufacturer's recommendations. After extraction and isolation, the RNA concentration was determined by measurement of the optical density at 260 nm and the RNA was stored at -80°C until use.

## 5. *RT and RQ-PCR protocols*

Different cDNA protocols were initially used by the participating laboratories. During the first phases of the program, the cDNA protocol for the

RQ-PCR was optimized. As a starting point, the BIOMED-1 protocol [30] was used and modified as described in the results section. For the detailed cDNA protocol, see the accompanying EAC manuscript [29]. The TaqMan 7700 machine was operated using the default RQ-PCR protocol as recommended by the manufacturer, except that the number of PCR cycles were increased from 40 to 50 and the PCR reaction volume was decreased from 50 µl to 25 µl. For analysis, a threshold value of 0.1 was used and a baseline was set to 3-15 for *ABL* and *GUS* and 3-10 for *B2M*. For a more thorough description of the TaqMan PCR protocol, see the EAC manuscript [29].

### 6. *Expression of the results*

Results are relative to 100 ng of total RNA, corresponding to 5 µl ($1/10^{th}$) of the RT reaction. The mean Ct value and the mean value of the $\log_{10}$ of the Copy Number (CN) for each CG were used for the statistical analysis, since CN did not show a normal distribution. Log-transformed data were subsequently converted into decimal values for presentation.

### 7. *Collection of the data*

The data from material distributed by the Aarhus laboratory, data from the first selection process of suitable CG and data from the multicenter prospective study were collected and analyzed in Aarhus, Denmark.

For testing of the 14 candidate CGs (17 primer and probe sets), data were obtained from i) ABI endogenous control plate, ii) in-house and iii) newly designed EAC primer and probe sets. For the ABI endogenous control plate, five laboratories tested a total of 65 different archived samples: 22 normal PBMNC, 15 ALL (4 PB / 11 BM), 15 CML (10 PB / 5 BM), and 13 AML (5 PB / 8 BM) samples obtained at diagnosis. This number was reduced to 53 for *TFRC* (data partially missing for one laboratory) and 21 for 18S rRNA (data available from one laboratory only). A minimum of four normal PB and four leukemic samples was expected per laboratory. For in-house *ABL*, *B2M* and *TBP* primer and probe sets, 20 samples were tested in one laboratory: 4 normal PBMNC, 3 ALL, 7 AML and 6 CML. For EAC *ABL*, *PBGD* and *PBGD2* sets, 36 samples were tested: 4 PBMNC, 11 ALL, 7 AML and 14 CML in two laboratories. Paired results obtained with two different sets of primer for the same CG (one laboratory only) were available for three CGs: *ABL* (n=20), *B2M* (n=12) and *TBP* (n=12). All these experiments were performed before having optimized the RT step and before establishing a common threshold for data analysis.

In the prospective study, fresh normal (PB, BM or PBSC), ALL, AML or CML (either PB or BM) samples were tested in individual laboratories for the three EAC selected CGs (Table 2). MNC were isolated and cells were lysed (initial step of the RNA extraction procedure) on the same day as the samples were obtained. Initially, 316 samples were collected (Table 3). Six leukemia samples were excluded because CG analysis was impossible due to a poor *B2M* plasmid amplification. Consequently, 310 samples were analyzed to define reference values on the same set of samples. Information on the presence of a putative FG transcript was not available.

**Table 3: Origin and type of fresh normal donor and leukemia patient samples**

| | Laboratory | Normal Donor | | | Diagnostic Leukemic Samples | | | | | | Nr. of Samples per lab |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | PB | BM | PBSC | AML PB | AML BM | ALL PB | ALL BM | CML PB | CML BM | |
| Members of the EAC CG group | Aarhus | 11 | 5 | 7 | 6 | 9 | 6 | 7 | 8 | 6 | 65 |
| | Amsterdam | 11 | | 4 | | 3 | | | 1 | 1 | 20 |
| | Paris Necker | 11 | 1 | 1 | | 3 | 9 | 4 | 5 | 3 | 37 |
| | Rotterdam | 11 | | | 3 | 5 | 2 | 2 | | | 23 |
| | Turin | 18 | 9 | | | | | | | | 27 |
| | Vienna | 11 | 2 | 4 | 2 | 3 | 5 | 3 | | | 30 |
| Contributors to the EAC CG group | Salamanca | | 9 | 9 | 4 | 2 | | 2 | 1 | 2 | 29 |
| | Marseille Nord | | | | | | 10 | 10 | | | 20 |
| | Uppsala | | | | | 1 | | | 14 | 4 | 19 |
| | Napoli | | | | | 11 | | 1 | | 6 | 18 |
| | Paris R. Debré | 1 | | | | | | 7 | | | 8 |
| | Leuven | | | | 1 | 1 | | 1 | 3 | 1 | 7 |
| | Marseille IPC | | | 1 | | | | | 5 | 1 | 7 |
| | Stockholm | | | | 2 | 1 | 1 | | 2 | | 6 |
| | **Sample-number in total** | **74** | **26** | **26** | **18** | **39** | **33** | **37** | **39** | **24** | **316** |

**Legend:**
Among 316 included samples, six were excluded due to a poor *B2M* plasmid amplification (see Materials and Methods section).

For the retrospective study, 311 archived ALL, AML or CML samples (either PB or BM) with an identified FG transcript were tested in individual laboratories for the three EAC selected CGs. Results were collected and tabulated by the FG network leaders. Only samples with a CG Ct value within the reference range defined below were selected for the analysis. Contributors appear in Table 3 for the prospective study or in each particular section of the EAC manuscript for the retrospective study [29].

### 8. *Statistical analysis*

The statistical analysis was performed in Marseille, France, using the SPSS 10 Software (SPSS Inc., Chicago, USA). Ct values were analyzed using non parametric tests (Mann-Whitney test for unpaired samples or Wilcoxon test for paired samples) for the first step of CG selection since the number of tested samples per group could be low and heterogeneous.

Subsequently, Ct and CN values obtained with the three EAC selected CGs on a large number of samples were analyzed using a global linear

model in order to compare each group with the others. For example, three groups (BM, PB and PBSC tissues) in normal samples were compared. A post-hoc analysis (Tukey method) was used to define sub-groups with no statistically significant differences between each other (p value at least 0.05) if more than two groups were concerned (see results on *GUS* CG expression in normal samples below). In addition, the CG expression was compared in fresh or archived samples according to various parameters:

* tissue origin (BM, PB, PBSC),
* leukemia type (ALL, AML or CML)
* sample type (normal or leukemia).

For example, six groups (2x3) were compared by combining the tissue (PB or BM) and the leukemic type in fresh leukemic samples (see results below). The 95% range of expression, also called range of reference values, refers to the range between the 3$^{rd}$ and the 97$^{th}$ percentile for the selected gene and cell subtype according to the results of the post-hoc analysis. The level of significance was set at a p value below 0.05.

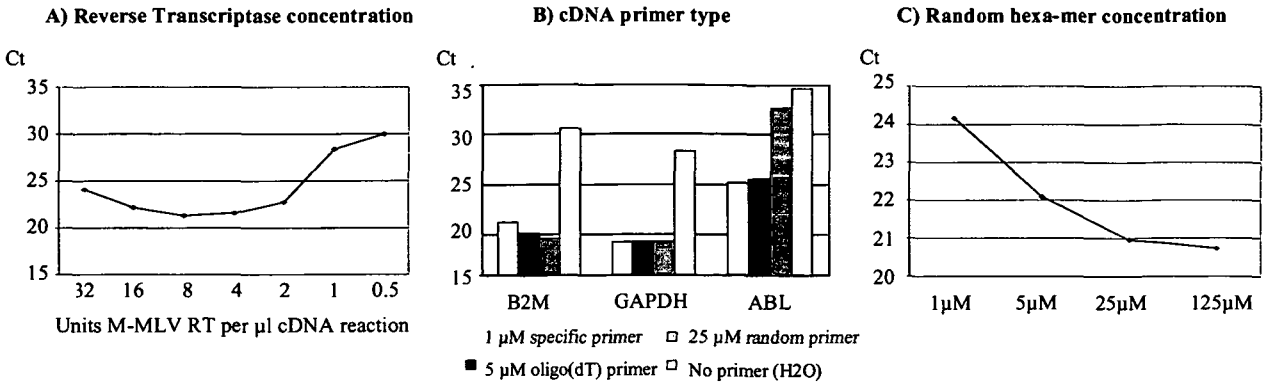
All raw data and statistical results are available on the following EAC web site: http://meidia.nord.univ-mrs.fr/EAC/publications.html

## C. Results

### 1. cDNA synthesis protocol

The efficiency of the RT step is a critical determinant of the sensitivity of RQ-PCR assays. Therefore, the CG network aimed to optimize this part of the RQ-PCR assay. The optimal concentration of M-MLV reverse transcriptase was found to be between 4-8 U per µL reaction and a concentration of 5 U per µL reaction was subsequently used (Figure 2a). In addition, comparison of three different types of cDNA primers showed that random hexamers and target specific primers gave the most efficient cDNA synthesis (Figure 2b). Since specific primers must be designed for each gene target, generally applicable random hexamer primers were selected. Finally, increment of primer concentration was found to increase the cDNA yield. Concentration up to 125 µM was tested but a concentration of 25 µM was retained since Ct values reached the plateau phase (Figure 2c). No differences between hexamers and nonamers were observed (data not shown). These conditions for performing the RT step were adopted by the EAC network in all further experiments.

**Figure 2: Optimization of cDNA reaction for RQ-PCR**

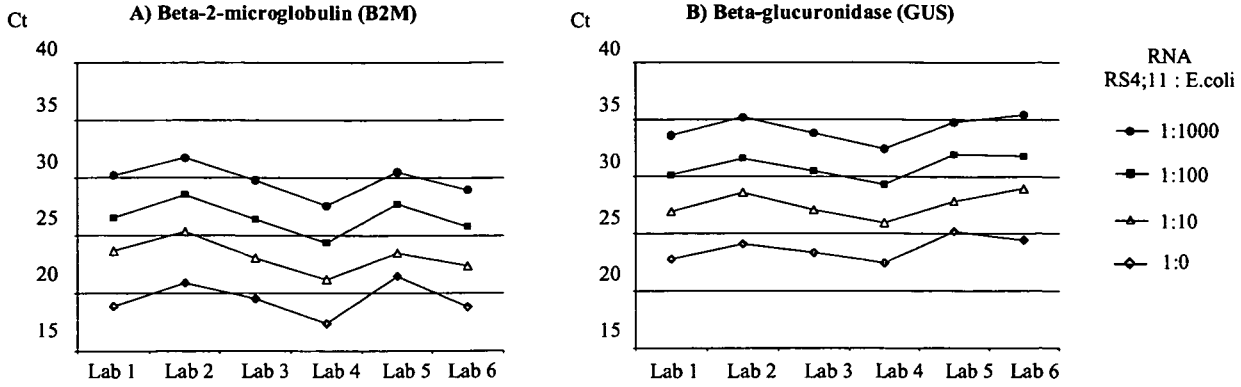



**Legend:**
**Panel A)** Effect of reverse transcriptase (RT) concentration on RT-RQ-PCR. Seven aliquots of K562 cell line RNA were reverse transcribed to cDNA using 25 µM random primer and two-fold dilutions from 0.5-32 U of MMLV RT per microliter of cDNA reaction. TaqMan PCR was performed using primer sets for *B2M, GAPDH, ABL* and *BCR-ABL* on each cDNA and the average Ct values of the four target genes in each cDNA plotted against the RT concentration.
**Panel B)** Comparison of different cDNA primer types in K562 RNA: Mixture of target gene specific primers (1 mM each), random hexa-mer primers (25 mM), an oligo(dT) primer (5 mM) and no primer.
**Panel C)** Effect of primer concentration on cDNA yield. RNA aliquots from the cell lines K562 and REH were reverse transcribed using 5 U of MMLV RT per microliter of cDNA reaction and 1-125 µM random hexa-mer primers.

The cDNA yields were monitored by TaqMan PCR using *B2M, GAPDH* and *ABL*. The median Cts of the three target genes in each cDNA were plotted against primer concentration

To evaluate the inter- and intra-laboratory variations, cDNA synthesis and subsequent RQ-PCR were performed on centrally prepared and distributed RNA dilutions of RS4;11 cell line into *E. coli*. As indicated in Figure 3 six laboratories participated on this study. After quantification of *B2M* and *GUS* gene transcripts the results varied between 2 to nearly four Ct-values (four Cts are equivalent to a 16-fold difference). Comparable results were observed for *ABL* gene transcripts (data not shown). The differences in cDNA synthesis efficiencies were highly reproducible throughout the RNA dilutions and - most importantly - between genes as suggested by the parallel lines (Figure 3). This indicates that although the efficiency of cDNA-synthesis varies between different laboratories, it is rather constant within each of the laboratories participating. Thus, CGs could be used for normalization of FG expression.

**Figure 3: Inter-laboratory reproducibility of cDNA synthesis assessed by *B2M* and *GUS* gene transcript amplification**




**Legend:**
Ten-fold dilutions of RS4;11 cell line RNA into *E.coli* RNA were prepared centrally by lab1 and aliquots were distributed on dry-ice to the participating laboratories. Each laboratory performed cDNA synthesis and TaqMan analysis.
Similar results were observed for *ABL* gene transcripts.

## 2. *Selection of control genes*

### a) Criteria for selection

The control gene group started with a screening of candidate CGs on normal PB and diagnostic leukemic samples using:

i) pre-developed human endogenous control plates (ABI) containing primer/probe sets for 11 commonly used CGs and

ii) six primer/probe sets available or developed during the study (Table 1).

Of the 17 primer/probe sets covering 14 CGs, the network aimed to identify at least one with a high median expression (Ct between 16.4 and 23.0) and at least one with a medium median expression (Ct between 23.0 and 29.6). These arbitrarily defined limits covered a range of two logs, since a 10 fold difference in cDNA amount is equivalent to 3.3 PCR cycles (Cts) if amplification efficiency is 100%. The lower limit was selected in order to obtain an adequate number of records for the baseline calculations and the higher limit to achieve sufficient sensitivity for sample evaluation.

The selected CGs should fulfill the following **major criteria**:

**i)** absence of pseudogenes (known or identified by comparing mRNA sequences with the human genome sequence by BLAT search on http://genome.ucsc.edu),

**ii)** high or medium expression, excluding very high or low expression,

**iii)** no significantly different CG expression between normal PB samples and leukemic samples,

**iv)** and no significantly different CG expression between PB and BM.

**Minor criteria** for exclusion were also identified:

**i)** variability within one leukemic type (AML, ALL or CML) at diagnosis or normal PB,

**ii)** cell cycle dependent expression,

**iii)** and X-chromosomal location.

**Selection of CGs out of pre-developed human endogenous control plates (ABI) (see Table 1):**

After the first experiments the *18SrRNA* CG was excluded due to the exorbitant high expression level ($Ct_{median}$ = 14.5), and the TBP CG due to the very low expression level ($Ct_{median}$ = 30). Among the four high expressed CGs (*PO*, *ACTB*, *GAPD* and *B2M*), *B2M* was the only one that did not reveal a pseudogene [15,31,32]. However, if the ABI primer/probe set was used for amplification the expression level of *B2M* varied significantly between normal and leukemic samples and between PB and BM ($p<0.001$ in both cases, n=65, Mann-Whitney test). Therefore the ABI *B2M* set was discarded. Among the five CGs with medium expression, three were discarded due to minor reasons: *HPRT* and *PGK* (X-chromosomal location) and *TFRC* (variable expression in hematopoietic cells [33,34]). CYC and GUS revealed median expression levels ($Ct_{median}$ = 27.6 vs. 25.9) and were therefore selected for further analyses.

**Selection of CGs out of in-house (available) or EAC (developed) primer/probe sets (see Table 1):**

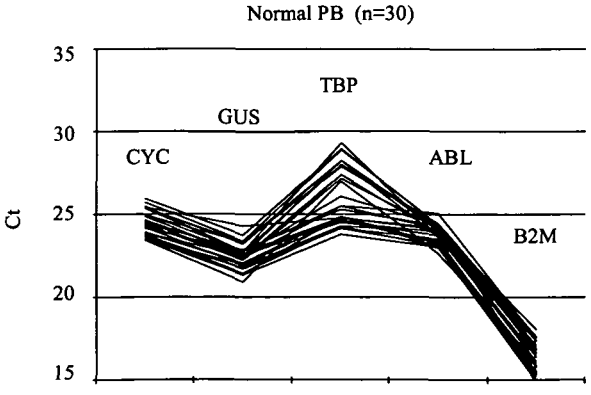
Among the six additional primer and probe sets, covering five CGs, three were excluded. *PBGD* and *PBGD2* sets exhibited nearly identical median Ct values (27.0 vs. 27.1) but due to the presence of alternative transcriptional start sites, these *PBGD* sets were finally discarded. Comparing the two *ABL* sets, the EAC *ABL* set was preferred since the median Ct value was lower. The *B2M* primer/probe set appeared to be suitable since no statistically significant difference between PB and BM was observed. Comparison between normal and leukemic samples was not possible since only four normal samples were tested (see Material and Methods section). The amplification of *TBP* transcript with the EAC revealed a median expression level ($Ct_{median}$ = 25.7) and was therefore also selected for further analyses.

After the first selection process, the number of genes was reduced to five: *ABL* (EAC), *B2M* (EAC), *CYC* (ABI), *GUS* (ABI) and *TBP* (EAC) (Table 1), taking into account that *ABL* primer and probe sets could amplify both normal and translocated allele of *BCR-ABL* positive leukemias (Figure 1).

**b) Variability of expression in normal PBMNC**

These five initially selected CGs were subjected to further analysis using five locally prepared PB samples from normal donors in each of the six CG laboratories. RQ-PCR analysis was performed using optimized EAC RT and PCR protocols. The variations in Ct values of *ABL*, *B2M*, *CYC* and *GUS* were comparable and within three Ct values (Figure 4). The connected lines of these four CGs were nearly parallel. In contrast, the *TBP* gene transcript revealed a variation of five Ct values (corresponds to 32-fold amplification) and non-parallel sample lines, indicating larger variation in the *TBP* gene expression (Figure 4). The *TBP* gene was therefore excluded from further analysis.

**Figure 4: Variation in CG expression in normal PB samples**




**Legend:**
PBMNC from 30 normal donors were analyzed with the *CYC*, *GUS*, *TBP*, *ABL* and *B2M* gene specific RQ-PCR sets. Each line represents data from one sample.

### 3. *cDNA specificity of primers/probe sets*

Since primer and probe sequences used for the human endogenous control plate were not available, new primer and probe sets (EAC sets) for the *CYC* and *GUS* genes had to be designed and tested. To evaluate the risk of false positive results due to pseudogenes or fortuitous genomic homologies, the selected EAC primer/probe sets were tested in different laboratories for the amplification of genomic DNA.

Three different *CYC* primer/probe sets were evaluated separately in two laboratories. Each set was found to amplify genomic DNA very efficiently, revealing Ct values between 25 and 30 (data not shown) [35]. Thus, *CYC* was excluded from further analysis.

*ABL*, *B2M* and *GUS* EAC primer/probe sets were tested in five different laboratories on 150 genomic DNA samples (30 per laboratory) obtained from normal donors and leukemia patients.

All RQ-PCR results for *B2M* and *GUS* were negative (Ct=50), whereas 7% (10/150) of *ABL* PCR-data were positive (Ct<50) in three out of five testing laboratories (n=2, 3 and 5 positive results, respectively). However, the *ABL* Ct values ranged from 35 to 45 (data not shown). Such Ct values are far away from the Ct values obtained using good quality RNA samples. Even if some remaining gDNA was present in the RNA sample, this would not significantly affect the *ABL* Ct values. Based on the low level amplification of gDNA, it was decided not to exclude the *ABL* primer set.

In conclusion, *ABL*, *B2M* and *GUS* primer/probe sets shown in Table 2 were selected for further testing as potential candidate CGs in the EAC program.

### 4. *CG expression in normal and leukemic samples*

After establishing the optimal conditions for cDNA synthesis and RQ-PCR protocols and after defining the most suitable CGs in the initial studies, the EAC network proceeded to establish the biological variation in the expression of the selected CGs in normal and leukemia samples.

#### a) Expression of the selected CGs in fresh samples

This prospective study was performed on normal donors (n=126) as well as on leukemia patients at diagnosis (n=184). Normal PBSC (n=26) were tested since MRD can also be studied in this particular harvest. Contributing laboratories as well as sample type and numbers are indicated in Table 3.

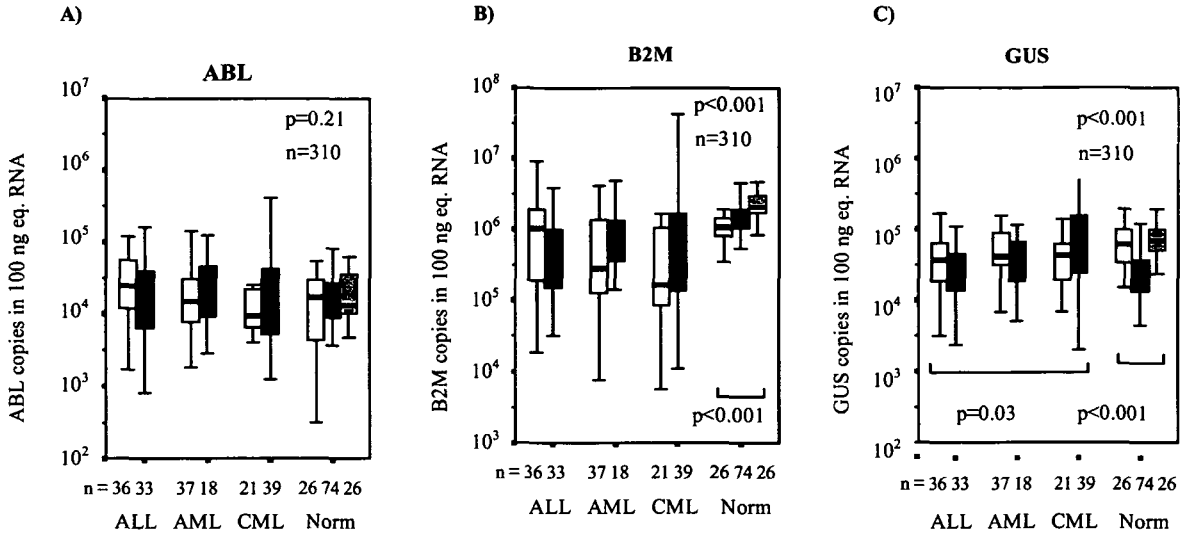
For analyzing the following data a global linear model was used. In normal samples, *ABL* gene expression did not differ significantly between BM, PB and PBSC (Figure 5A). On the contrary, *B2M* gene expression differed significantly between the three different sample types ($p<0.001$, n=126). However, post-hoc analysis revealed that *B2M* expression level in PB or PBSC samples was comparable (Figure 5B). *GUS* gene expression varied significantly between the three sample types investigated ($p<0.001$, n=126). Post-hoc analysis showed that *GUS* expression was significantly lower in PB but rather similar between BM and PBSC (Figure 5C).

In leukemia samples, CG expression was not significantly different between the BM and PB or between ALL, AML and CML, except for *GUS* expression ($p=0.03$, n=184) (Figure 5A-5C).

Comparison between normal and leukemia samples showed that only *ABL* expression did not differ significantly ($p=0.21$, n=310, Figure 5A). In contrast, significant differences were found for *B2M* and *GUS* expression ($p<0.001$, n=310) (Figures 5B and C).

When Ct values were analyzed instead of CN on the same samples (n=310), comparable results were obtained. Figures showing the CG Ct values in normal BM, PB and PBSC samples and leukemia BM and PB samples are available on the EAC web site.

**Figure 5: Comparison of the three CG transcripts expression in 310 fresh normal and leukemic samples**




**Legend:**

**Panel A) Expression level of *ABL*, panel B) Expression level of *B2M* and panel C) Expression level of *GUS* per 100ng RNA/cDNA.**

For normal samples, analysis performed according to the tissue (BM ☐ , PB ■ or PBSC ▩ ). After post-hoc analysis comparable expression levels were found for *B2M*/PB and *B2M*/PBSC and for *GUS*/BM and *GUS*/PBSC.

The indicated *p-values* are the result of the analyses by the global linear model. The *p-value* indicated in the upper right corner corresponds to the overall comparison between the 9 groups (BM, PB, and if available PBSC for ALL, AML, CML and normal individuals) whereas the *p-values* indicated below a specified subgroup refer to the value for only this subgroup. The black bold line indicates the median value. The box refers to the range defined by the 25$^{th}$ and the 75$^{th}$ percentile.

## b) Reference values for CG expression in fresh normal and leukemic samples

We decided to establish reference values on fresh samples in order to evaluate the range of CG expression and subsequently to identify poor quality samples (see Material and Method section). Reference values were defined by a target (median value) and two limits (3$^{rd}$ and 97$^{th}$ percentiles). Samples (6% of the fresh samples) outside this range were considered as unexpected results. Samples with a too high Ct value and consequently a too low CN were presumably degraded samples or samples containing an inhibitor, whereas samples with a too low Ct value and thus a too high CN could be related to an overestimated RNA quantification. The reference values, based on 126 normal samples and 184 leukemia samples at diagnosis are shown in Table 4.

In normal samples (n=126), an approximately 50-fold difference in *ABL* and *GUS* expression and up to 70-fold difference in *B2M* expression was found (Table 4). In leukemia samples (n=184), an approximately 100-fold difference in *ABL* and *GUS* gene expression and up to 500-fold difference in *B2M* expression was observed (Table 4). A parametric approach using the mean value (and not the median) and two standard deviations gave similar results to the methodology based on percentiles (data not shown).

**Table 4: Reference values of transcript expression level in normal and leukemia samples for the three selected CGs**

| Genes | Samples | n | Ct [a] median [3rd-97th perc.] | CN [a] median [3rd-97th perc.] |
|---|---|---|---|---|
| *ABL* | Leukemia and Normal [a] | 310 | 24.9 [21.9-29.3] | 18 000 [$1.3x10^3$-$1.3x10^5$] |
| *B2M* | Leukemia | 184 | 18.7 [15.6-24.9] | 550 000 [$9.6x10^3$-$4.3x10^6$] |
| | Normal PB and PBSC [a] | 100 | 17.2 [15.7-20.0] | 1 600 000 [$2.5x10^5$-$6.8x10^6$] |
| | Normal BM | 26 | 17.8 [16.7-22.9] | 1 100 000 [$2.9x10^4$-$2.0x10^6$] |
| *GUS* | Leukemia | 184 | 23.8 [20.8-28.0] | 41 000 [$2.8x10^3$-$3.2x10^5$] |
| | Normal BM and PBSC [a] | 52 | 23.0 [21.1-26.3] | 66 000 [$7.6x10^3$-$2.0x10^5$] |
| | Normal PB | 74 | 24.5 [22.6-27.7] | 22 000 [$2.6x10^3$-$7.1x10^4$] |

**Legend:**
[a] Data have been merged according to the results of the post-hoc analysis.

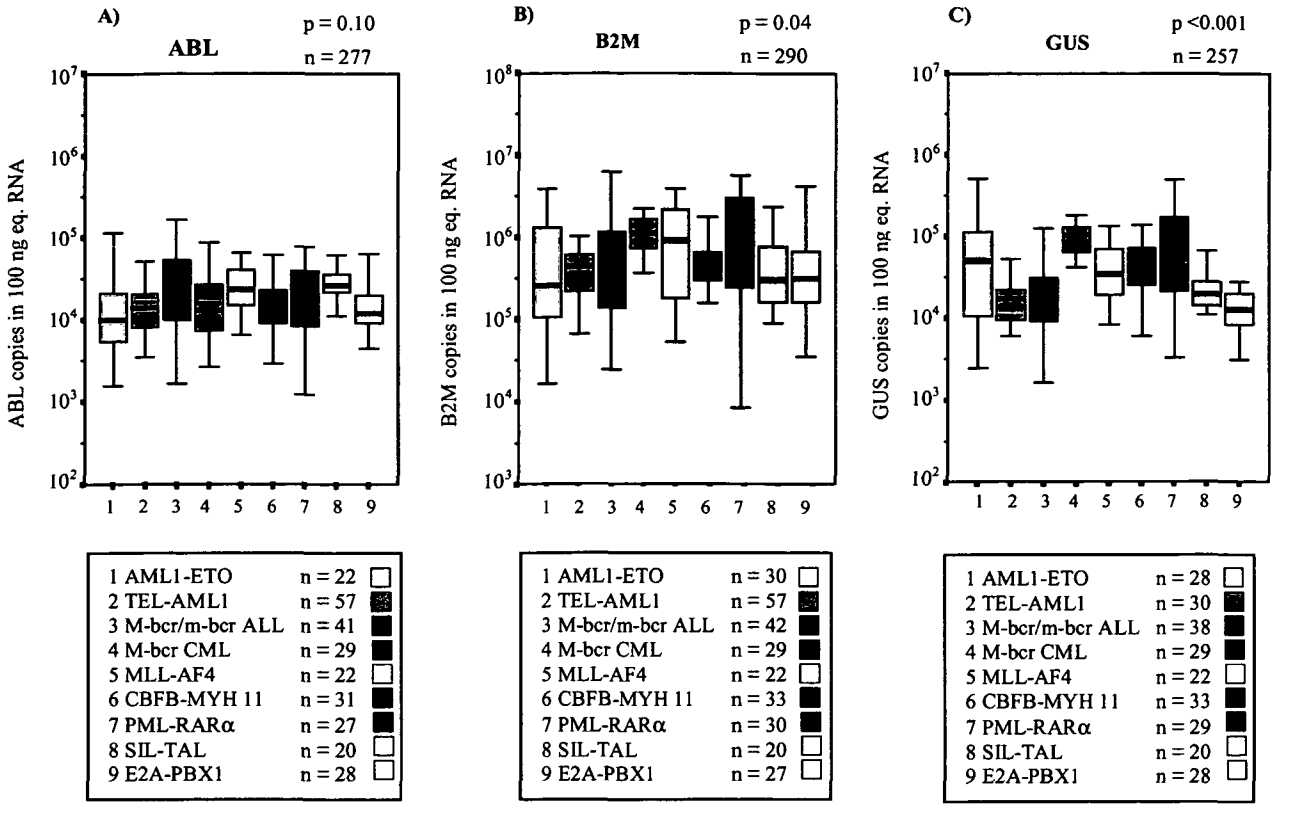
c) **CG expression in archived samples**

Data on archived leukemic samples at diagnosis (n=311) with an identified FG transcript were obtained from the FG groups (see accompanying manuscript [29]). Good quality samples were selected according to the reference values defined above, thus excluding cases with poor or no amplification of CG due to degradation or presence of inhibitors. *ABL* was a more restrictive CG for including samples compared to *GUS* or *B2M* (proportion of excluded samples, respectively 11%, 9% and 6%) resulting in a lower number of samples analyzed for this CG.

An analysis similar to the previous one performed on fresh leukemia samples (see Fig.5) was performed. Only *ABL* gene transcript CN did not differ significantly between tissues and leukemia type (p=0.50, n=277) or between FG transcript groups, when BM and PB samples were merged (p=0.10, n=277, Figure 6a). In contrast, the *B2M* gene transcript CN was significantly different between tissues and leukemia types (p=0.03, n=290) and between FG transcript groups (p=0.04, n=290, Figure 6b). Also *GUS* gene expression differed significantly between tissues and leukemia types (p<0.001, n=257) and between FG transcript groups (p<0.001, n=257, Figure 6c).

Additional statistical results and additional figures using Ct values instead of CN are available on the web site.

**Figure 6: Comparison of the three CG transcripts expression in archived leukemia samples at diagnosis according to the FG**




**Legend:**

**Panel A) expression level of *ABL*, panel B) expression level of *B2M*, panel C) expression level of *GUS*, per 100ng *RNA/cDNA***

Data from BM and PB samples were merged per FG transcript.

The indicated *p-values* are the result of the analyses by the global linear model. The *p-value* indicated in the upper right corner corresponds to the overall comparison between the 9 groups (BM, PB, and if available PBSC for ALL, AML, CML and normal individuals) whereas the *p-values* indicated below a specified subgroup refer to the value for only this subgroup. The black bold line indicates the median value. The box refers to the range defined by the 25$^{th}$ and the 75$^{th}$ percentile.

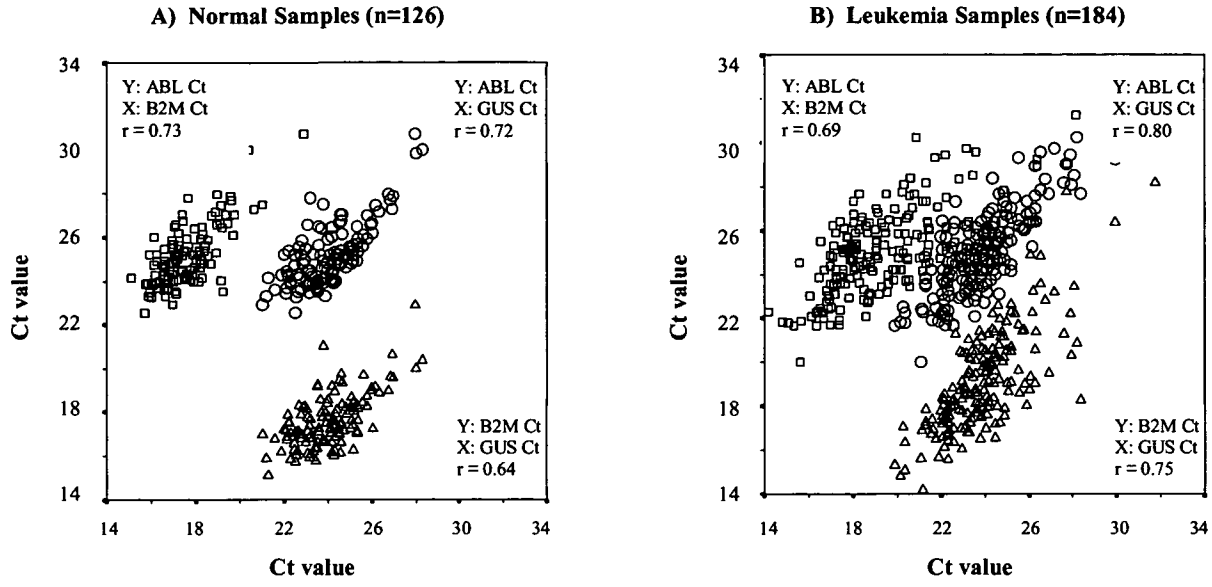
### d) Correlation between control genes and fusion genes

The expression level of the three CGs was always correlated ($p<0.01$), whatever Ct or CN values were used. The highest correlation was found between *ABL* and *B2M* Ct values ($r=0.73$) and *ABL* and *GUS* Ct values ($r=0.72$) in fresh normal samples (Figure 7a) and between *ABL* and *GUS* Ct values ($r=0.80$) in fresh leukemia samples (Figure 7b).

In archived leukemia samples, the highest correlation was found between *B2M* and *GUS* Ct values ($r=0.67$), whereas the lowest correlation was observed between *ABL* and *GUS* Ct values in the same samples ($r=0.54$), suggesting a differential degradation kinetic of these two genes.

Finally, the correlation between the FG transcript expression and the *ABL* gene transcript expression was higher or identical to the two other EAC selected CGs (see EAC manuscript [29] and EAC web site).

**Figure 7: Correlation between the three EAC selected CGs**




**Legend:**

**Panel A) FACS analyses of ABL, B2M, and GUS in fresh normal,**

**Panel B) FACS analyses of ABL, B2M, and GUS in leukemic samples.**

Results appeared globally more widespread in leukemia samples compared to normal samples. This observation could be related to the increased number of samples (184 versus 126) and laboratories (14 versus 6) included, and to an effective heterogeneity of CG expression. All *p-values* are below 0.01 ($p<0.01$).

## D. Discussion

### 1. *EAC Data*

This multi-center study, which included fourteen laboratories for the prospective study (Table 3) and the entire EAC group for the retrospective study (twenty-six laboratories), aimed at selecting CGs that were applicable for RQ-PCR-based analysis of leukemia patients, both at diagnosis and during follow-up. The choice of suitable control genes has been the subject of discussion for a long time, at least in the onco-hematology field [14,36].

The use of a CG for RQ-PCR detection of an FG transcript in leukemia patients appeared mandatory for different reasons: i) to evaluate the RNA quantity and quality (degradation or presence of an inhibitor); ii) to correct for such variations; iii) to exclude poor quality samples and iv) to calculate theoretical sensitivity of FG transcript detection.

In this study we present the most reliable control gene, selected out of 14 candidate control genes and we propose guidelines for interpreting the results of FG detection. These guidelines are based on statistically significant data assembled on CG amplification in fresh normal and diagnostic samples, easing the daily routine work with FG

diagnostics. The data presented in this study are eligible not only for quantification of fusion gene transcripts, but also for the quantification of aberrantly expressed genes.

During the evaluation of 14 potential CGs, genes with known or encountered pseudogenes, with X-chromosomal location, alternative transcriptional start sites, and with too high or low expression level were rejected. For these reasons, we excluded *ACTB*, CYC, *GAPDH, PO, HPRT, PGK, PBGD, 18SrRNA* genes and a single primer/probe combination of *TBP* (ABI).

We did not test the real impact of gender on gene expression but the literature suggests that this criterion may not be relevant.[39]

Finally, we ultimately selected *ABL*, *B2M* and *GUS* genes for extensive analysis of expression levels in normal samples and leukemia samples at diagnosis. Out of these three genes the *ABL* gene transcript was found to be the most reliable CG to compare diagnostic and MRD samples[29]. Firstly, the expression levels of *ABL* gene transcripts were similar between normal and diagnostic samples as well as within normal samples. Secondly, the correlation between *ABL* gene expression and the different FGs expression was the highest observed among the three extensively tested CGs (*ABL*, *B2M* and *GUS*) [29]. Based on these observations, *ABL* transcript quantification offers two advantages: it allows to correct for variations in RNA quality or quantity since variation of *ABL* and FG transcript expression are correlated, and it offers the possibility to assess the sensitivity level of the RQ-PCR experiment for the detection of a FG transcript- either at diagnosis or during MRD follow-up (see also EAC manuscript [29]).

The *ABL* gene was already introduced a decade ago as a control gene for competitive PCR in *BCR-ABL* positive leukemias [7,38] and was used as a control gene for RQ-PCR analysis in other investigations [18,37].

Two objections can be raised concerning the *ABL* primer/probe combination used in this study. Firstly, due to the relatively short size of *ABL* intron 2, we were able to amplify genomic DNA in 7% of pure genomic DNA. Secondly, according to the location of the *ABL* primer/probe set on the gene between *ABL* exon 2 and 3, we can amplify both the wild-type *ABL* and the *BCR-ABL* gene transcripts. Indeed, it appears that these two points are negligible:

The contamination of RNA samples with genomic DNA will never be a major problem if Ct-value is within the reference range, since contribution of DNA will be very minor (see results).

The co-amplification of *ABL* and *BCR-ABL* transcripts with *ABL* EAC RQ-PCR set may indeed lead to a limited inaccuracy for cells expressing a high level of *BCR-ABL* transcripts [29] but the calculation will be correct if the quantification of *BCR-ABL* expression is regarded as a proportion of rearranged *ABL* alleles to the total number of *ABL* alleles (rearranged and normal). A similar primer/probe combination amplifying the *ABL* housekeeping gene and the *BCR/ABL* fusion gene transcripts was used in another recent study on CML patients for the comparison of cytogenetic techniques (chromosome banding, interphasic or hypermetaphasic FISH) and RQ-PCR data. The authors found a highly significant correlation between these data, clearly indicating that such a primer/probe set is eligible for diagnosis and follow-up of residual disease.

For the clinical use, accurate quantification during treatment follow-up is probably more important than precise quantification of FG transcript at diagnosis. Thus, the co-amplification of normal and rearranged *ABL* alleles should not be considered as a major limitation for using *ABL* as a control gene in RQ-PCR analysis for *BCR-ABL* positive samples.

To circumvent the co-amplification of both alleles, alternative *ABL* primer/probe sets can be designed and synthesized, spanning the region between *ABL* exon 1b and between 2 or exon 2 and 4 [18,37]. We kept the RQ-PCR set on *ABL* exons 2 and 3 since a longer template size could reduce the efficiency of the TaqMan PCR.

In the EAC concerted action we optimized and standardized the process of cDNA-synthesis and RQ-PCR, but still there are many other variables that seem to influence the result of quantification, representing a potential source for inter-laboratory variations: i.e. the timing of the sampling, the various treatment protocols and the different treatment steps of leukemia patients and BM regeneration (such as induction, consolidation, and intensification).

The influence of the treatment protocol on the expression level of various control genes was investigated in an in-vitro study focusing on human T helper cells [41]. Conclusions were that *ACTB* and *GAPDH* gene expression levels could change up to 17 fold (for *GAPDH*) according to the conditions and time of culture. In another monocentric study performed on a limited number of hematopoietic samples (four PBMNC and eight malignancies) the investigators focused on the expression of *ACTB*, *B2M* and *PBGD* [40]. According to this paper, the *B2M* gene was the most suitable endogenous reference gene, but the authors also suggest that the expression levels of the three selected CGs might be affected by the various therapeutic protocols and during the recovery phase after aplasia. This assumption needs to be further assessed in another project.

In this study, some control genes were not studied, such as glucose-6-phosphate dehydrogenase (G6PD) because all potential CGs could not be tested. The use of the pre-developed human endogenous control plate in the first selection process was a starting point and few additional genes were added according to the experience of participating EAC laboratories on control gene amplification.

We tried to estimate the variations of CG expression observed in routine conditions on fresh samples. We found that samples with an *ABL* value within the reference range, respectively $1.3x10^3$-$1.3x10^5$ copies or Ct between 21.9 and 29.3, should be considered as an amplifiable sample and could be qualified for subsequent analysis (Table 4). However, such limits might differ according to the RQ-PCR protocol and to the technology of PCR machines. Samples in our study with a higher CG CN (or a lower Ct value) than expected could be qualified for subsequent analysis, although only 3% of normal samples are supposed to exhibit such a result, which could probably be related to an analytical problem. On the other hand, samples with a lower CG CN (or higher Ct value) than expected are supposed to be poor quality samples and should be rejected, since only 3% of normal samples would present such values. Nevertheless, if those samples are kept for subsequent analysis, precautions should be taken for interpreting negative MRD results in these poor quality samples. Furthermore, in these samples the kinetics of degradation may be different between the FG and the CG transcripts, resulting in an over or

under-estimation of MRD levels. The stability of CG and FG transcripts is currently under investigation within our EAC network (van der Velden et al., manuscript in preparation).

Quantification of CG and FG transcripts in archived diagnostic samples showed that the FG expression correlated with the expression of the three CGs [29]. In such samples, we could correct variations in RNA quality/quantity using a ratio FG CN / CG CN, also called normalized copy number (NCN), or using ΔCt method by amplifying in parallel a suitable CG [29]. Furthermore, we showed that amplification of CGs greatly improved the quantification of FG transcripts in diluted samples. We observed that variations in CG expression within the reference range could reflect variations in experimental sensitivity. We will therefore propose two formulas to calculate the sensitivity of the RQ-PCR experiments both at diagnosis and during follow-up, based on CG quantification (see below).

## 2. *Proposals for calculation and presentation of MRD results*

The use of CGs as internal reference in a MRD setting has the potential to detect poor quality samples as well as to correct for cDNA synthesis efficiency, thus allowing reproducible quantification of FG transcript in diagnostic and follow-up samples. But it also offers the possibility to quantify the sensitivity of experiments, which is a crucial point for FG negative results. An important aim of the EAC network was to develop algorithms to express data in a fashion that is intuitively understandable but which also highlights the differences in day-to-day sensitivity encountered when a patient is followed longitudinally during cyto-reductive treatment. Based on the quantification of CG expression in a patient's follow-up samples and FG and CG expression in the patient's diagnostic sample, we propose two methodologies for calculation of MRD results and experimental sensitivity: the normalized copy number (NCN) and ΔΔCt method. Results can be calculated either using slopes and intercepts from previously generated plasmid standard curves (ΔΔCt method) or by employing normalized copy numbers (NCN method), where plasmid standards are always tested on each PCR plate.

### a) The ΔΔCt method

In the ΔΔCt method, the Ct of the FG ($Ct_{FG}$) obtained during follow-up is first normalized by subtracting the Ct of the CG ($Ct_{CG}$) and subsequently normalized by the $\Delta Ct_{DX}$, i.e. ($Ct_{FG} - Ct_{CG}$), of the patient at diagnosis. The resulting MRD value (MRDv) and the day-to-day sensitivity value (SENSv) of the assay can then be determined from the equations appearing in table 5. The ΔΔCt method is based on the assumption that intercepts and slopes are identical between FG and CG. No difference was found between FGs and CGs plasmid standard curves in the accompanying manuscript.[29] Thus average intercept and slope values of 40 and –3.4, respectively, can be used for calculation (Table 5). However, if differences were to be seen, the intercept and slope of the FG should be used, since the CG Ct should be within the window of the reference values and therefore only have a minor impact on the calculation. The ΔΔCt method, which employs Ct values, does not include any plasmid or RNA standard curve, reducing the risk of contamination, degradation and batch variation from these.

### b) The NCN method

In the NCN method, the MRD value is a ratio between the CG normalized expression of the FG in follow-up $(FG_{CN}/CG_{CN})_{FUP}$ and diagnostic samples $(FG_{CN}/CG_{CN})_{DX}$ (Table 5). SENSv is calculated according to the relative expression of

the FG at diagnosis $(FG_{CN}/CG_{CN})_{DX}$ and CG expression $(CG_{CN,FUP})$ in follow-up sample (Table 5). Since the NCN method makes use of standard curves, the SDS software may perform the calculation of CN. In this manuscript, we reported the use of plasmid based standard curves but other standard curves (e.g. cell line dilutions) might be used (see EAC manuscript [29]). At least three plasmid standard dilutions should be employed for each gene to ensure proper standard curves. This methodology has the advantage that degradation of probes can be compensated for and that data generated on different types of RQ-PCR machines can be compared. However, this methodology suffers an increased cost per sample.

**Table 5: EAC Formula for calculation of MRD value and theoretical sensitivity based on EAC selected FG and CG RQ-PCR sets**

| Parameters | ΔΔCt method<br>*No standard curve needed* | NCN method<br>*FG and CG standard curves* |
|---|---|---|
| **MRD value (MRDv)** | $10^{((\Delta CtFUP-\Delta CtDX)/-3.4)}$ | $\frac{(FG_{CN} / CG_{CN})_{FUP}}{(FG_{CN} / CG_{CN})_{DX}}$ |
| **Sensitivity (SENSv)** | $10^{(40-CtCG,FUP-\Delta CtDX)/-3.4)}$ | $\frac{CG_{CN,DX}}{CG_{CN,FUP} \times FG_{CN,DX}}$ |

**Legend:**
**DX:** diagnostic; **FUP:** follow-up; **NCN:** normalized copy number; $\mathbf{Ct_{CG}}$: Ct value of the CG; $\mathbf{Ct_{FG}}$: Ct value of the FG; $\mathbf{FG_{CN}}$: fusion gene copy number, $\mathbf{CG_{CN}}$: control gene copy number. $\mathbf{\Delta Ct_{FUP}}$: $(Ct_{FG} - Ct_{CG})$ during follow-up, $\mathbf{\Delta Ct_{DX}}$: $(Ct_{FG} - Ct_{CG})$ at diagnosis.
Values 40 and –3.4 are respectively experimental results for the intercept value and mean slope observed in our EAC program for plasmid standard curves. If CG and FG values for the patient are not available at diagnosis, data of the EAC manuscript can be used[29].

For calculation of MRD value, EAC data for the corresponding FG at diagnosis can be used if patient value at diagnosis is not available. Sensitivity (SENS) of the experiment can be expressed as $10^{SENS}$ (ex: $10^{-5}$) and should be calculated as follow: $SENS = \log_{10}(SENSv)$ (see also accompanying manuscript [29]).

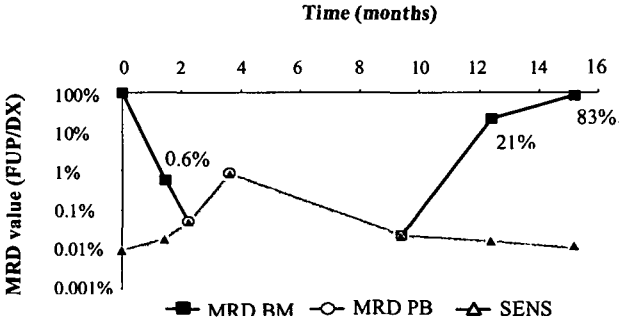
### c) Data presentation

With NCN or ΔΔCt method, MRDv and SENSv values can be plotted on a graph for presentation of MRD data (Figure 8). Both lead to a common presentation, for which results of follow-up samples are expressed relative to the patient's sample at diagnosis. Results can be depicted graphically with a time-scale on the x-axis whereas day-to-day sensitivity calculated from CG values and MRD results are plotted on the y-axis. The diagnostic value is set at 1 (or 100%), independently of the FG expression at diagnosis which is nevertheless taken into account to calculate sensitivity in follow-up samples.

The advantages of this scale are that calculations are simpler, since only one equation is needed per FG setup. More importantly the FG expression in different patients may be compared during follow-up, which would be highly relevant for CML patients whom may show differences in the FG level at diagnosis [37]. On the other hand, when using this scale, information concerning variability of FG expression per patient at diagnosis is lost. A more exhaustive discussion concerning the expression of results and the choice of a standard curve can be found in the accompanying manuscript [29].

Since MRD results are expressed relative to the diagnostic value, MRDv should be below 1 in follow-up samples. Each time MRD is undetectable, only the point corresponding to the sensitivity appears. An example for longitudinal monitoring of an *MLL-AF4* positive patient is shown in figure 8. Both methods of calculation lead to similar results either for MRDv or SENSv results, therefore only one graph is shown.

**Figure 8: Longitudinal MRD monitoring of a *MLL-AF4* positive patient using EAC *MLL-AF4* and *ABL* RQ-PCR sets**




**Legend:**

Time 0 was diagnosis, Time 12 was cytological relapse. Value at diagnosis is 1 (100%) and subsequent FG positive results are expressed relatively to diagnostic sample. Four months after diagnosis, the PB sample was clearly degraded (*ABL* Ct value: 32.3, *ABL* CN: 257). Although result appears in the figure, care should be taken for interpreting this negative result. The gray area defines a zone in which the minimal residual disease cannot theoretically be detected by RQ-PCR according to the value of the CG amplification at the time point. The lower the frontier between the white and the gray zone, the better the sensitivity is. Calculation was performed according to ΔΔCt and NCN methods found in table 5. Raw data corresponding to the amplification of the CG and the FG can be found on the EAC web site. Both methods of calculation lead to similar results either for MRD value or for sensitivity, consequently only one graphic is shown.

## E. Conclusions

Our EAC program selected the most suitable CGs for RQ-PCR experiments in assessing MRD in leukemic patients and defined reference values for CG expression. To our knowledge this is the first multi-center study of this scale (number of laboratories, number of tested samples) attempting to evaluate candidate control genes for RQ-PCR experiments. These EAC data should serve as gold standard for laboratories using the EAC protocol for the clinical implementation of RQ-PCR delineated in this and the accompanying report [29]. Furthermore, these data are eligible not only for quantification of fusion gene transcripts, but also for the quantification of aberrantly expressed genes. However, each laboratory should be able to define its own normal range and compare it to these reference values.

We conclude that the initial promise of revolutionizing MRD detection by this method seems to be within reach due to the exceedingly high reproducibility and robustness of the RQ-PCR method. The inclusion of CGs to correct for sample variations seems to make the method applicable for gene transcript detection, thus creating a platform for future studies, where RQ-PCR assays should be crucial to assess therapeutic efficiency of classical and innovative treatment approaches, notably for inhibitors of protein tyrosine-kinases, phosphatidyl-inositol-3-kinase or farnesyl-transferase proteins.

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PART 3

# COMPETITIVE PCR ASSAYS

# CHAPTER

# IV

# QUANTIFICATION BY SHIFTED RESTRICTION-SITE COMPETITIVE PCR

# Quantification of mRNA expression by competitive PCR using non-homologous competitors containing a shifted restriction site

## A. Introduction

Competitive PCR is a powerful tool for accurate quantification of DNA or RNA. The procedure relies on the co-amplification of the sequence of interest with a serially diluted synthetic DNA fragment of known concentration (competitor) using a single set of primers [1,2]. The initial quantity of target molecules in the sample can be calculated from the ratio of competitor- and target-derived amplicons generated during PCR, provided that the target and competitor sequences are amplified with equivalent efficiency [3]. The quantity of target DNA (cDNA) can be most conveniently assessed at the so-called equivalence point (EQP), at which the target- and competitor-derived amplification products display the same signal intensity, indicating identical amounts of target and competitor at the beginning of the PCR reaction. To approach the EQP as closely as possible, several PCR reactions covering a range of competitor concentrations must be set up for quantification of individual targets, thus rendering the procedure rather laborious. In principle, it is feasible to use a small number of reactions with competitor concentrations covering the range of interest, and to quantify the target outside of the EQP. For quantification beyond the EQP it is essential that the initial target/competitor ratio is not affected by the amplification process.

Since equal amplification efficiency of competitor and target sequences is a necessary prerequisite for quantitative PCR-assays, competitors are usually designed to resemble the target sequence as closely as possible. Many investigators have used highly homologous competitors differing only by the presence or absence of a unique restriction enzyme site. The strategies commonly used to separate and quantitate the PCR products include cutting either target or competitor with a single restriction endonuclease [4,5,6,7] or digesting both target and competitor with two different enzymes [8,9]. The latter strategy may result in false quantification if the restriction enzymes do not cut with equivalent efficiency. In the former approach, in which a restriction site is present in either the competitor or the target fragment, incomplete digestion leads to false quantification because undigested products co-migrate with the fragments lacking the restriction site.

In both approaches digestion-resistant heteroduplexes are generated during PCR [4,10,11,12] that co-migrate upon electrophoretic separation with the non-digested homoduplex products. This may lead to a change in the ratio between target and competitor fragments, resulting in wrong or inaccurate quantification. To prevent errors in quantification by competitive PCR assays, different measures were taken to minimize or eliminate the formation of heteroduplexes. Some investigators resolved heteroduplexes by adding fresh PCR components prior to the last amplification cycle [13,11], others introduced an additional denaturation/renaturation step after PCR, resulting in a binomial distribution of homo- and heteroduplexes [14]. Mathematical models have been described to permit quantification outside the EQP despite the generation of heteroduplexes [15,12].

However, particularly in situations in which target and competitor differ by a single nucleotide, theoretical predictions based on mathematical models may be problematic [16].

In order to avoid the pitfalls of heteroduplex formation, we have designed non-homologous competitors that display equal size and identical primer binding regions, but contain an internal nucleotide sequence which is different from the target sequence. It was demonstrated that non-homologous competitor molecules with a size identical to the target fragment are amplified with the same efficiency and prevent the formation of heteroduplexes [17,11,18].

In this report, we present a novel modification of non-homologous competitor molecules permitting precise quantification outside the EQP, that require a single restriction endonuclease digest for distinction between target and competitor. The technique is presented using mRNA quantification of the human multidrug resistance gene *MDR1* as a model.

## B. Materials and Methods

### 1. Generation of DNA and RNA competitors

The non-homologous competitor was designed to contain *MDR1*-specific primer binding sequences of ~20bp at the ends encompassing a bacterial sequence lacking homology to human DNA. It was generated by amplification of the bacterial cloning vector pBluescript (pBS) II $KS^+$ with hybrid primers. The 5′ ends of these primers contained an *MDR1*-specific sequence, and the 3′ ends were homologous to the pBS sequence: MDR*hyb* 22a/pBS S 5′-AGT TTG CAG GTA CCA TAC AGT TGC CTA ATG AGT GAG CTA AC-3′, MDR*hyb* 24a/pBS AS 5′-CTG TAG CTG TCA ATC AAA GGA TGT TCT TTC CTG CGT TAT C- 3′ (pBS sequence location 899-918 and 1137-1155, respectively, according to GenBank data AccNo X52331). The plasmid sequence was selected to contain a natural recognition site for the same restriction endonuclease as the target, but the position within the competitor molecule was shifted. PCR was performed in 10mM Tris-HCl, 50mM KCl, 2mM $MgCl_2$, 200µM of each dNTPs, 0.1µM of forward and reverse primers, 5 ng pBS template, and 1.25 units of AmpliTaq DNA polymerase (Applied Biosystems, Foster City, CA, USA) in a total volume of 50µl. The amplification profile consisted of thirty-five cycles of 40 s at 95°C, 30 s at 54°C, and 40 s at 72°C. The cycles were preceded by an initial denaturation step at 95°C for 3 min, and followed by a final extension step at 72°C for 7 min. The competitor fragment generated by PCR was cloned into the EcoRV site within the polylinker sequence of Bluescript phagemid according to the manufacturer's recommendations (USB, Editorial Comments Vol.19. No1. pp 4-6). Identity and orientation of the cloned insert were determined by DNA sequencing using T3/T7 primers.

For the synthesis of DNA competitors, the plasmid was cut with BamHI and HindIII, (Roche, Basel, Switzerland) the insert-containing fragments were purified, and the competitor concentration measured by spectrophotometry and by comparison with a DNA Mass Ladder (Life Technologies, Paisley, UK) upon electrophoresis in an agarose gel. The competitor fragments were aliquoted and stored as a stock solution at a final concentration of $10^9$ molecules per 5µl at –20°C.

For the synthesis of RNA competitors, the plasmid was linearized and transcribed in vitro using the MAXIscript kit (Ambion, Austin, TX,

USA). The full length transcripts were purified from prematurely terminated transcription products by separation on polyacrylamide gels (5% acrylamide, 8M urea), eluted overnight at 45°C in DEPC-treated water, extracted with acid-equilibrated saturated phenol/chloroform (pH 4.5) (Amresco, Solon, OH, U.S.A.), and precipitated with 2.5 volumes of 100% ethanol. The concentration of the synthesized RNA was measured by spectrophotometry and stored as a stock solution at a final concentration of $10^9$ molecules per 5µl at –70°C.

**a) cDNA synthesis and PCR template preparation**

When using RNA competitors, a semi-logarithmic serial dilution of *MDR1* RNA competitor molecules was added covering a range of 3–4 logs, depending on the range of expression of the target transcript. Total cellular RNA (1 µg) was mixed with 1 mM of each of the dNTPs, 25 µM $pd(N)_6$, 4 µl of nuclease-free water and incubated at 72°C for 5 min. The mixture was placed on ice for 1 min before the addition of 4µl reaction buffer (50mM Tris/HCl (ph 8.3), 75 mM KCl, 5 mM $MgCl_2$), 10 mM DTT, 1 U/µl RNasin (Promega, Mannheim, FRG), and 5 U/µl Moloney murine leukemia virus reverse transcriptase (Life Technologies). The reaction was incubated at 37°C for 45 min, and finally, the enzymes were inactivated by heating at 98°C for 3 min.

When using DNA competitors, 1 µg of total RNA extracted from the cells of interest was converted to cDNA as described above. Two microliters of the reverse transcription reaction corresponding to 100 ng total RNA were added to a semi-logarithmic serial dilution of the DNA competitor stock solution.

**b) Competitive PCR**

The following primers were used for specific amplification of competitor and target: MDR 22a S 5′-AGT TTG CAG GTA CCA TAC AG- 3′ (location 100-119, according to GenBank data AccNo M29441) and MDR 24a AS 5′-CTG TAG CTG TCA ATC AAA GG- 3′ (location 140-159, according to GenBank data AccNo M29443). The PCR mixture and the amplification profile were as described in generation of DNA and RNA competitors. An example of *MDR1* mRNA expression analysis by competitive PCR is shown in Fig. 1A.

To assess the kinetics of amplification, target and competitor cDNA fragments were mixed at different ratios and co-amplified in the presence of $P^{32}$ end-labeled primer ($10^6$ cpm per reaction) over 12 to 40 cycles. Aliquots of each sample were separated on a 2% agarose gel, the bands were excised and the radioactivity determined by liquid scintillation counting (Fig. 2).

**c) Restriction endonuclease digestion**

All products were designed to show small, yet clearly detectable differences in length following digestion. After the restriction digest, only products smaller than 300bp, with a difference in length between 15-30bp were obtained.

The competitor- and target-derived *MDR1* amplicons were digested in the PCR buffer using the restriction enzyme Pvu II (Roche). To test the applicability of this approach to a broad spectrum of target sequences, we have investigated a number of restriction endonucleases for their ability to cut efficiently in PCR buffer. PCR products of different target genes including the human multidrug resistance gene *MDR1*, the multidrug resistance associated protein (*MRP*), the gene for the pi form of glutathione S-transferase (*GST-pi*), and the thymidylate synthase gene (*TS*) were tested by a panel of restriction endonucleases. We have

identified 20 different enzymes providing adequate results under the conditions indicated in Table 1. Most of the restriction endonucleases revealed excellent enzymatic activity in PCR buffer. Only two of the enzymes (Mae I and Mvn I, Roche) required the addition of 50% (vol/vol) specific enzyme buffer (supplied by the manufacturer) to the PCR reaction to permit efficient digestion of the amplicons.

### 2. *Quantitative analysis:*

Twenty microliters of each PCR mixture were electrophoresed in 2% TBE agarose gels containing ethidium bromide. Gels were photographed and the EQP was determined using a Kodak Digital Science™ DC120 Zoom Digital Camera and the 1D Image Analysis Software (Kodak, Rochester, NY, USA). To account for the fact that ethidium bromide signal intensity obtained under UV light is dependent on the size of the respective DNA fragment, it may be necessary to consider the length of the products when determining the EQP [19,20]. Our system, however, did not require the application of a correction factor to compensate for the influence of size on signal intensity, because the effect on the results was negligible: due to the small differences in size between target and competitor, application of the correction factor, e.g. 1.27 for the 100bp signal, for comparison with the 127 bp band, and 1.16 for the 172bp fragment, for comparison with the 199 bp band [19,21], would only result in minor differences not exceeding the intrinsic variability of the methodology used (0.5 log).

Fig. 1B shows the quantitation of MDR1 transcripts in a model test system containing a known amount of cloned target ($1x10^6$ per reaction), and a serial dilution of competitor molecules ranging from $10^4$ to $3x10^7$. The result of competitive analysis showed the EQP at the expected position, i.e. in the reaction containing $10^6$ competitor molecules (lane5). In a parallel competitive PCR experiment (not shown), a control gene transcript of *b2-microglobulin* (*β2-MG*) was quantified using the identical amount of template cDNA from the same preparation. In accordance with other publications, the result of quantification was expressed as the ratio between the target and the control gene [22]. For the purpose of *MDR1* monitoring, the data were expressed as the number of transcripts per $10^6$ transcript molecules of the control gene.

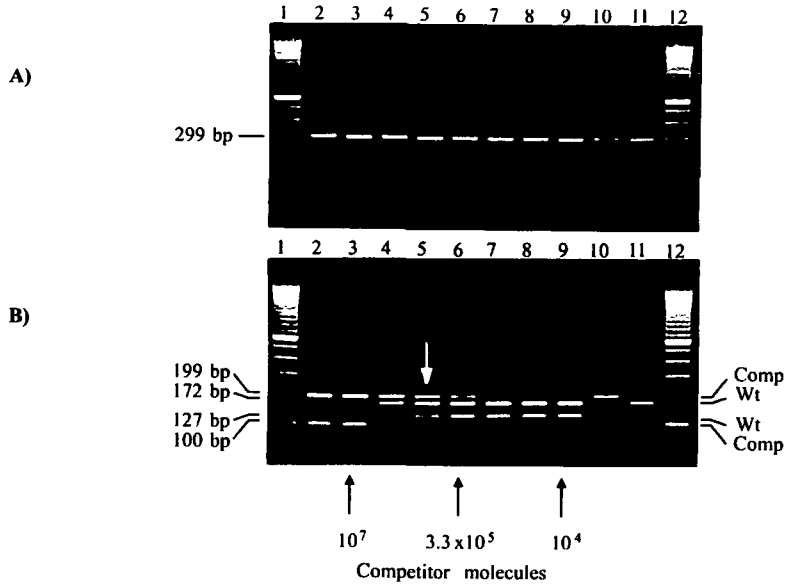
Amplification of *β2-MG* transcripts is a common approach to controlling factors such as RNA degradation and RT efficiency, permitting quantitative comparison between samples [23,24]. When using constant starting amounts of RNA, highly consistent *β2-MG* signal intensities were observed indicating stable RNA quality and low variability of the RT step in our experimental setting. An additional indication of the low RT variability was provided by the analysis of multiple replicates of RNA samples which revealed high reproducibility of the results.

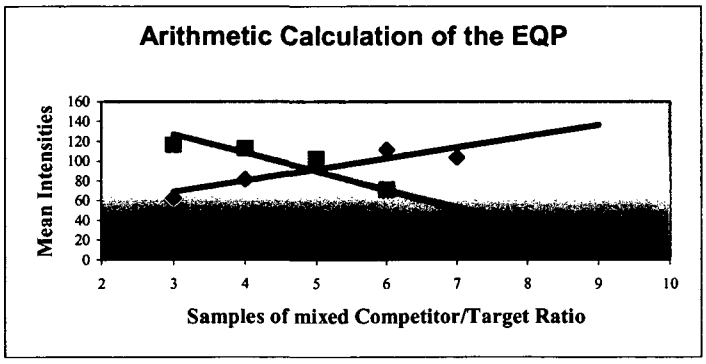
## C. Results and Discussion

The competitive PCR approach presented is based on the use of a heterologous competitor, sharing only the primer binding sites with the target sequence. Due to the low homology between the competing amplicons, this method has the advantage of avoiding the formation of heteroduplexes. This prevents incorrect quantification caused by co-migration of the heteroduplex product with the undigested fragments. Moreover, elimination of heteroduplex formation in competitive PCR assays permits the calculation of target copy numbers without the need

to visualize the equivalence point, thus greatly reducing the number of PCR reactions per assay (Fig. 1C). The linear range of this competitive PCR approach is around three logs, i.e. 1.5 logs above and below the EQP.

**Fig.1 *MDR1* quantification using SRS-cPCR**



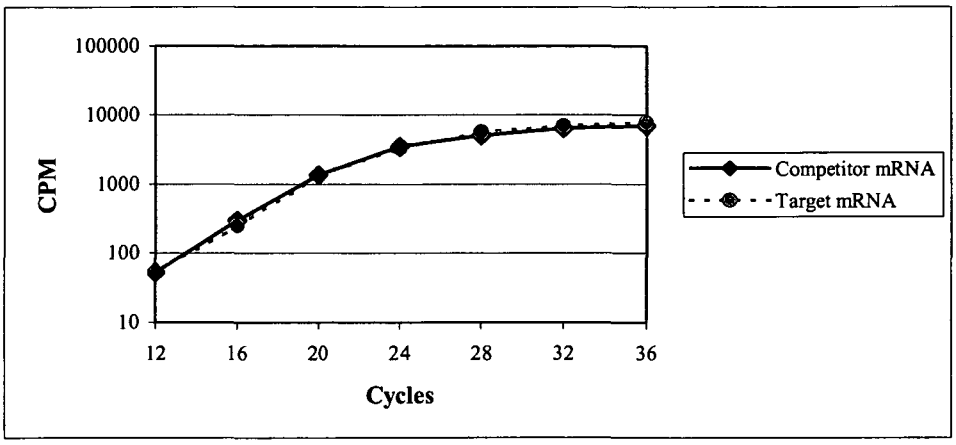




**Legend:**

Competitive PCR and restriction digest were carried out as described in Materials and Methods. Lanes 1 and 12 in each panel: size marker (100bp ladder). Lanes 10 and 11 represent amplification products of only competitor and target mRNA, respectively. **(A)** 20 µl PCR product were loaded on a 2% agarose gel. The target and the competitor products were identical in size [299bp]. Lanes 2 to 9 contain $10^6$ molecules of an in vitro transcribed cloned human wild type *MDR1* fragment and a semilogarithmic dilution of the competitor RNA ranging from $10^4$ to $3x10^7$ molecules. **(B)** After digestion with the restriction endonuclease Pvu II, the wild-type PCR products were cleaved into 127- and 172 bp fragments, and the competitor PCR products into 100- and 199 bp fragments. The equivalence point (EQP) is indicated by an arrow (lane 5). It was determined by comparison of signal intensities of the 100 bp and 127 bp fragments, or of the 172 bp and 199 bp fragments, respectively. The competitive amplification of an external control gene, beta-2- microglobulin, permitting correction of target quantification (29) is not displayed. In competitive PCR reactions, the subdominant template yields visible products if it represents at least 1% of the total. Three competitor dilutions covering a range of three logs (lanes 3, 6, and 9, indicated by arrows) usually permit the visualization of target- and competitor-derived products in at least one of the reactions (lanes 3 and 6), thus permitting calculation of the target molecules. **(C)** The band intensities of competitor and target derived fragments were digitalized and measured by using the 1D Image Analysis Software. In the graph the mean intensities from the larger competitor (line B) and the target (line A) fragments were used for the performance of a regression analysis. The EQP is indicated by the intersection of the two lines. The numbers on the abscissa correspond to the lane numbers on the agarose gel. Elimination of any data points between the extreme values has no effect on the EQP. This demonstrates that it is possible to reduce the number of competitor dilutions needed for quantitative analysis of target transcripts, thus rendering the assay less laborious.

A key to successful and reliable competitive PCR is the identical amplification efficiency of competitor and target fragments. Under ideal conditions, the competitor/target ratio should remain constant throughout the amplification process. The kinetic analysis shown in Fig. 2 revealed two identical curves within the exponential phase of PCR, indicating equivalent amplification efficiencies for both fragments co-amplified in the same reaction. The PCR kinetics was not affected by the lack of homology between the nucleotide sequences of the competing fragments. These observations were also made in a number of other quantitative PCR assays using the type of competitor described (not shown).

**Fig.2 Amplification kinetics of endogenous human *MDR1* mRNA and competitor RNA in presence of $\alpha$-$^{32}$P-dCTP**




**Legend:**
Competitor (solid, blue line) and target (broken, red line) mRNA were mixed at different ratios, and amplified for 12, 16, 20, 24, 28, 32, 36 and 40 cycles. The data are plotted as a function of cpm vs. cycle number. The amplification kinetics of mixtures were identical regardless of the target/competitor ratio. In the example displayed, the ratio was 1:1.

The use of either RNA and DNA competitors showed that both types permit measurement of changes in the relative amount of a specific target RNA with reasonable accuracy. DNA competitors are more convenient in terms of handling, and are not prone to degradation during storage. A problem associated with the use of DNA competitors is underestimation of the concentration of mRNA target molecules. The error is most likely attributable to incomplete conversion of target RNA molecules to cDNA. This problem can be largely eliminated by using RNA competitors which are reverse transcribed in the same reaction as the target RNA. In our hands, however, the differences in quantitative results depending on the use of RNA or DNA competitors were not significant for a variety of target and competitor combinations including *MDR1*, *MRP* and *BCR/ABL* (data not shown). These observations indicate a low variability of the RT step under well-standardized experimental conditions. If the aim of the quantitative RT-PCR assay is calculation of the absolute number of target molecules, it is advantageous to use RNA competitors. If the assay is performed for detection of relative differences in the number of RNA molecules, e.g. changes in RNA expression over time assessed by serial follow-up samples, the use of DNA competitors will be adequate, provided that the RT-step has a low variability.

The only requirements for construction of the competitor type described herein are two hybrid primers. They permit generation of a competitor displaying the following features: 1) identical length to the target sequence, 2) lack of homology to the target sequence, except of the primer binding sites, and 3) a recognition site for a restriction endonuclease which is present at a different position within the target sequence. The application of restriction endonucleases for discrimination between target- and competitor-derived PCR products is greatly facilitated by the finding that many common enzymes cut efficiently in PCR buffer (Table 1). This renders the entire procedure less time- and labor-intensive and permits selection of a suitable restriction endonuclease in virtually most sequence of interest, thus enabling quantitative analysis by the SRS-cPCR assay in any experimental settings.

**Table 1 Optimum conditions for digestion of unpurified PCR fragments in PCR reaction buffer**

| *Enzyme* | *Recognition sequence* | *Units/Time* | *Application* | *Manufacturer* |
|---|---|---|---|---|
| Acc II | CG/CG | 10U/1hr | 1 x PCR buffer | Amersham Pharmacia Biotech (Uppsala, Sweden) |
| Cfo I | GCG/C | 5U/1hr | 1 x PCR buffer | Roche |
| Csp 6I | G/TAC | 5U/1hr | 1 x PCR buffer | MBI Fermentas (Buffalo, NY, USA) |
| Hpa II | C/CGG | 5U/1hr | 1 x PCR buffer | Roche |
| Mae I | C/TAG | 10U/1hr | 0.5 x PCR buffer<br>0.5 x Enzyme buffer | Roche |
| Msp I | C/CGG | 5U/1hr | 1 x PCR buffer | Roche |
| Mvn I | CG/CG | 5U/1hr | 0.5 x PCR buffer<br>0.5 x Enzyme buffer | Roche |
| Nde II | /GATC | 10U/2hr | 1 x PCR buffer | Roche |
| Rsa I | GT/AC | 5U/1hr | 1 x PCR buffer | Roche |
| Sau3A | /GATC | 5U/1hr | 1 x PCR buffer | Roche |
| Apa I | GGGCC/C | 10U/3hr | 1 x PCR buffer | Roche |
| BamH I | G/GATCC | 10U/3hr | 1 x PCR buffer | Roche |
| Bgl II | A/GATCT | 10U/1hr | 1 x PCR buffer | Roche |
| EcoR I | G/AATTC | 5U/1hr | 1 x PCR buffer | Roche |
| Hind III | A/AGCTT | 5U/1hr | 1 x PCR buffer | Roche |
| Kpn I | GGTAC/C | 5U/1hr | 1 x PCR buffer | Roche |
| Pst I | CTGCA/G | 10U/1hr | 1 x PCR buffer | Roche |
| Pvu II | CAG/CTG | 10U/1hr | 1 x PCR buffer | Roche |
| Sma I | CCC/GGG | 5U/1hr | 1 x PCR buffer | Roche |
| Xho I | C/TCGAG | 10U/1hr | 1 x PCR buffer | Roche |

**Legend:**

The panel of endonucleases displayed includes ten enzymes with hexanucleotide and ten with tetranucleotide recognition sequences. The conditions indicated permit complete digestion of 1 µg PCR-product at the appropriate temperature (as recommended for each restriction endonuclease by the respective manufacturer).

The approach described has the advantage of being independent from the efficiency of the restriction enzymes used. Both target and competitor are digested concurrently in the same tube by the same enzyme, but at different positions. If incomplete digestion occurs it affects both target and competitor to the same extent, and has no impact on the target/competitor ratio. Hence, the origin of molecules within the undigested band is not relevant for quantitative analysis.

In comparison with recently introduced real-time Q-PCR approaches [25,26,27,28], competitive PCR analysis is more laborious, but permits equally sensitive detection and precise target quantification at substantially lower cost. Competitive PCR approaches may therefore continue to play an important role in PCR-based quantification of DNA and RNA targets as long as the cost of RQ-PCR prevents many researchers from adopting this technique. The use of competitor molecules containing a shifted restriction site contributes to increased reliability and precision of competitive PCR assays.

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# PART 4

# RQ-PCR ASSAYS

# CHAPTER

# V

# RQ-PCR ASSAYS FOR THE DETECTION AND MONITORING OF PATHOGENIC HUMAN VIRUSES IN IMMUNOSUPPRESSED PEDIATRIC PATIENTS

# Real-time quantitative PCR assays for the detection and monitoring of pathogenic human viruses in immunosuppressed pediatric patients

## A. Introduction:

The employment of polymerase chain reaction (PCR) techniques for virus detection and quantification offers the advantages of high sensitivity and reproducibility, combined with an extremely broad dynamic range. A plethora of qualitative and quantitative PCR virus assays have been described, and commercial PCR kits are available for quantitative analysis of a number of clinically important viruses such as HIV [1,2], Hep B and C [3,4], and CMV [5]. In addition to permitting the assessment of viral load at a given time point, quantitative PCR tests offer the possibility of determining the dynamics of virus proliferation, monitoring of the response to treatment and, in viruses displaying persistence in defined cell types, distinction between latent and active infection. Moreover, from a technical point of view, the employment of sequencial quantitative PCR assays in virus monitoring helps identifying false positive results as caused by inadvertent contamination with traces of viral nucleic acids or PCR products [6].

We have established quantitative virus detection assays based on the real-time PCR (RQ-PCR) technology for 16 different viruses or virus families, which play an important role in the clinical surveillance of immunosuppressed children. All assays were designed to run under identical PCR conditions, in an attempt to render the diagnostic work as economic as possible.

The RQ-PCR assays are presented in a ready-to-use format, and clinical applications of quantitative virus analysis in immunosuppressed patients are discussed.

## B. Materials and Methods

### 1. Sample preparation

#### a) Nucleic Acid Extraction

For the isolation of DNA and RNA, commercially available kits were used, essentially as recommended by the manufacturer. DNA extraction from largely cell-free liquids, except urine, and from peripheral blood leukocytes for the detection of intracellular virus particles was performed using the QIAamp DNA Mini Kit (QIAGEN GmbH, Hilden, Germany). Isolation of virus DNA from urine was done using the QIAamp Viral RNA Mini Kit, and from stool by the QIAamp DNA Stool Mini Kit (QIAGEN). For the isolation of RNA from each of these sources, the QIAamp Viral RNA Mini Kit was used. The only modifications performed included the adjustment of the input and the elution volumes: for nucleic acid extraction, the input volume for all samples was 200 µl for DNA and 140 µl for RNA, the elution volume was 240 µl for DNA and 120 µl for RNA.

#### b) Reverse transcription

For reverse transcription of the viral RNA, a total of 30 µl viral RNA eluate and 5 µl of nuclease-free water were mixed with 1 mM of each of the dNTPs, and 25 µM $pd(N)_6$, and this mixture was incubated at 72°C for 5 min. The denatured RNA was placed on ice for 1 min before the addition of 12 µl reaction buffer (50mM Tris/HCl (ph 8.3), 75 mM KCl, 5 mM $MgCl_2$), 10 mM DTT, 1.5 µl RNasin (40U/µl; Promega, Mannheim, FRG), and 1.5 µl Moloney murine leukemia virus reverse transcriptase (200U/µl; Invitrogen, Carlsbad, CA, U.S.A.). The reaction was incubated at 37°C for 45 min, and finally, the enzymes were inactivated by heating at 98°C for 3 min.

## 2. *Target sequence selection and primer/probe design*

Specific primers and probes were selected and designed using the "Primer Express, Vers.2.0" software (Applied Biosystems (AB), Foster City, CA, USA). The oligonucleotide sequences and their location, the amplicon length and the GenBank accession number of the corresponding target genes are indicated in **Table 1**. As indicated in this table, some of the primers reveal a degenerated code. This was a prerequisite necessary for the detection of viral subspecies differing from each other by single nucleotides.

For the experiments described below, hydrolysis probes labeled with FAM (6-carboxyfluorescein)-reporter molecules at the 5′-end and TAMRA (6-carboxy-tetramethylrhodamine)-quencher molecules at the 3′-end (AB) were used. The optimal concentration of primers was assessed by performing serial PCR reactions across a concentration range from 50 to 900nM.

## 3. *Real-time PCR*

The reactions were set up in a total volume of 25µl containing 12.5µl Universal Master Mix, [2x conc., incl. ROX-reference dye and uracil N′-glycosylase (UNG), AB], 50-900nM of primers, 200nM of TaqMan probe (see table 1), and 6 µl of genomic (gDNA) or complementary DNA (cDNA) template. The mixtures were prepared in 96-well optical microtiter plates (AB), centrifuged 1 minute at 1,200 rpm and amplified on the ABI 7700 or 7900 Sequence Detection System using the following, uniform cycling parameters: 2 minutes at 50°C (degradation of potentially present contaminating dUTP-containing amplicons by UNG), 10 minutes at 95°C (inactivation of UNG and activation of AmpliTaq Gold DNA polymerase), and 50 cycles of 15 seconds at 95°C and 60 seconds at 60°C (amplification of the specific target sequence).

During the amplification process, fluorescence signal emission is measured every 7 seconds within each well and plotted in an amplification curve by relating the normalized fluorescence signal intensities (ΔRn) to the cycle numbers. The crossing point of these amplification curves with the threshold, which is set at 0.05 U, is defined as threshold cycle (**Ct**). The Ct values are inversely related to the initial amount of template molecules: the lower the initial number of virus copies at the beginning of PCR, the higher the Ct values generated. The theoretical detection limit of the reaction is represented by the Y-intercept, the point at which the standard curve intersects with the ordinate. It indicates the theoretical Ct value obtained in the presence of a single template molecule at the start of PCR. Samples revealing threshold cycles greater than the calculated Y-intercept were by definition regarded as negative for the respective target.

## 4. *Specificity*

All primer/probe combinations were tested for potential cross-reactivity with unrelated viral and other microbial sequences based on the available data by alignment software (BLAST). None of the selected primer/probe combinations displayed significant homologies to any other sequences. Moreover, the theoretically conceivable cross-reactivity with human DNA/RNA sequences has been excluded by testing the primer/probe combinations against preparations of human nucleic acids.

## 5. *Standardization*

For standardization of quantitative virus detection assays, commercially available, quantified DNA control panels (ABI, Advanced Biotechnologies Inc., Columbia, Maryland, U.S.A.), in-house cloned plasmid standards or high-titer virus preparations derived from culture supernatants were used. The

calculation of virus particle numbers was based on spectrophotometric or fluorometric measurement of purified viral DNA or RNA. For the establishment of standard curves, serial logarithmic dilutions covering a range between $10^1$ and $10^5$ virus particles were employed, as described in more detail in the Results section.

### 6. *Controls*

#### a) Negative controls

A number of precautions were undertaken to prevent and control the occurrence of false positive virus tests. Every clinical RQ-PCR test performed included control reactions lacking template [no-template controls (NTCs)] and reactions including non-homologous template [no-amplification controls (NACs)] to test for the presence of contamination or the generation of non-specific amplification products under the assay conditions used. Moreover, to further reduce the risk of false positive tests resulting from contamination with PCR products, all PCR reactions were performed by substituting the nucleotide dTTP by dUTP. Prior to each amplification, a digestion step with Uracil-DNA-glycosylase (UNG) was carried to digest any contaminating PCR product, if present.

#### b) Positive controls

In addition to the DNA/cDNA of the respective control virus strain, the following controls were used in each assay to document efficient nucleic acid extraction and absence of enzyme inhibitors in the template preparation:

i) In largely cell-free clinical samples, such as plasma, serum, cerebrospinal fluid, urine, sputum, bronchoalveolar lavage, or stool a defined quantity of a non-human control virus [Phocid herpesvirus 1 (PHHV)– kindly provided by Dr. H.Niesters, University of Rotterdam, The Netherlands] was spiked into each sample prior to DNA/RNA extraction. Since constant DNA quantities of the control virus are co-extracted even when RNA isolation kits are used (personal communication by H.Niesters and own unpublished observations), the virus can also serve as a control in RNA virus detection assays. Under the standardized assay conditions used, constant levels of the seal virus were detected, provided that the nucleic acid extraction was efficient and no inhibitors of reverse transcription or PCR amplification were present (see Results).

ii) In clinical samples containing cells, such as peripheral blood, buccal swabs or biopsy material, a human single-copy housekeeping gene [beta2-microglobulin (B2-MG)-see Table 1 [7] was co-amplified in parallel to the virus sequence of interest.

In instances, in which the Ct values of the above controls were off scale (below the expected reading), an appropriate correction factor was applied to the calculation of virus copy number in the corresponding clinical samples, to compensate for impaired nucleic acid extraction or amplification efficiencies. Negative virus test results in the presence of low-positive (>1 log below normal) or negative PHHV/B2-MG controls were regarded as not interpretable.

**Table 1: Primers and probes for specific virus detection by real-time PCR**

| Virus type | Target | Amplicon length | Oligonucleotide Sequences (5'-3') Forward / Probe / Reverse | Conc. (nM) | Nucleotide position | GenBank Acc. No. |
|---|---|---|---|---|---|---|
| **Human Herpes Viruses (DNA):** | | | | | | |
| CMV | MIE protein | 76 bp | AAC TCA GCC TTC CCT AAG ACC A | 300 | 2414 – 2435 | M21295 |
| | | | CAA TGG CTG CAG TCA GGC CAT GG | 200 | 2437 – 2459 | |
| | | | GGG AGC ACT GAG GCA AGT TC | 300 | 2470 - 2489 | |
| EBV | BNT p143 | 74 bp | GGA ACC TGG TCA TCC TTT GC | 300 | 4679 - 4698 | NC_001345 |
| | | | CGC AGG CAC TCG TAC TGC TCG CT (AS) | 200 | 4700 - 4722 | |
| | | | ACG TGC ATG GAC CGG TTA AT | 300 | 4733 - 4752 | |
| HHV 6 | DNA polymerase gene | 74 bp | GAA GCA GCA ATC GCA ACA CA | 300 | 57517 - 57536 | NC_001664 |
| | | | AAC CCG TGC GCC GCT CCC | 200 | 57544 - 57561 | |
| | | | ACA ACA TGT AAC TCG GTG TAC GGT | 900 | 57568 - 57590 | |
| HHV 7 | Major capsid protein | 124 bp | CCC AAC TAT TTA CAG TAG GGT TGG TG | 300 | 84230 - 84255 | U43400 |
| | | | CTA TTT TCG GTC TTT CCA ATG CAC GCA (AS) | 200 | 84258 - 84284 | |
| | | | TTT AGT TCC AGC ACT GCA ATC G | 900 | 84332 - 84353 | |
| HHV 8 | ORF 26 | 111 bp | GTG CTC GAA TCC AAC GGA TT | 300 | 47308 - 47327 | U75698 |
| | | | TGT TCC CCA TGG TCG TGC C | 200 | 47336 - 47354 | |
| | | | CGA TAT TTT GGA GTA GAT GTG GTA CAC | 300 | 47392 - 47418 | |
| HSV 1 | US 4 gene | 166 bp | TTC TCG TTC CTC ACT GCC TCC C | 900 | 137279 - ..301 | NC_001806 |
| | | | CGT CTG GAC CAA CCG CCA CAC AGG T (AS) | 200 | 137379 - ..404 | |
| | | | GCA GGC ACA CGT AAC GCA CGC T | 50 | 137423 - ..445 | |
| HSV 2 | Glycoprotein D gene | 71 bp | CGC CAA ATA CGC CTT AGC A | 300 | 99 - 117 | AF021342 |
| | | | CTC GCT TAA GAT GGC CGA TCC CAA TC | 200 | 123 - 148 | |
| | | | GAA GGT TCT TCC CGC GAA AT | 300 | 150 - 169 | |
| VZV | ORF 38 | 82 bp | AAG TTC CCC CCG TTC GC | 300 | 69313 - 69329 | X04370 |
| | | | CCG CAA CAA CTG CAG TAT ATA TCG TCT CA | 200 | 69336 - 69364 | |
| | | | TGG ACT TGA AGA TGA ACT TAA TGA AGC | 300 | 69368 - 69394 | |
| **Human Adenoviruses (DNA):** | | | | | | |
| AdV A | Hexon gene | 135 bp | GGK CTG GTG CAA TTC GCC | 300 | 17818 - 17835 | X73487 |
| | | | CCA CGG ACA CCT ACT TCA CCC TGG G | 200 | 17840 - 17864 | |
| | | | CAC GGG CAC AAA ACG CA | 300 | 17936 - 17952 | |
| AdV B | Hexon gene | 138 bp | CGC CGG ACA GGA TGC TT | 900 | 45 - 61 | X76549 |
| | | | AGT CCG GGT CTG GTG CAG TTC GCC | 200 | 73 - 96 | |
| | | | CTA CGG TCG GTG GTC AC | 900 | 166 - 182 | |
| AdV C | Hexon gene | 138 bp | ACC TGG GCC AAA ACC TTC TC | 300 | 2884 – 2903 | J01966 |
| | | | AAC TCC GCC CAC GCG CTA GA | 200 | 2910 – 2929 | |
| | | | CGT CCA TGG GAT CCA CCT C | 900 | 2940 - 2958 | |

| Virus type | Target | Amplicon length | Oligonucleotide Sequences (5'-3') { Forward / Probe / Reverse } | Conc. (nM) | Nucleotide position | GenBank Acc. No. |
|---|---|---|---|---|---|---|
| AdV D | VA RNA gene | 143 bp | AAA AAC GAA AGC GGT TGA GC | 300 | 2 – 21 | U10675 |
| | | | CCA ATA CCA CGT TAG TCG CGG CT | 200 | 104 – 126 | |
| | | | CGG GTC GAG ACG GGA GT | 50 | 128 - 144 | |
| AdV E | Hexon gene | 75 bp | CAA CAC CTA CTC GTA CAA AGT GCG | 900 | 225 – 248 | X84646 |
| | | | CGC CCA CGG CCA GCG TGT | 200 | 251 – 268 | |
| | | | TAG GTG CTG GCC ATG TCC A | 300 | 281 - 299 | |
| AdV F | L5 IV-2 gene | 113 bp | CCC GTG TTT GAC AAC GAA GG | 300 | 31277 - 31296 | L19443 |
| | | | ATC GAC AAG GAC AGT CTG CCA ACA CTA ACG | 200 | 31326 - 31355 | |
| | | | TTA GAG CTA GGC ATA AAT TCT ACA GCA | 300 | 31363 - 31389 | |
| **Human Polyomaviruses (DNA):** | | | | | | |
| BKV | VP3 gene | 116 bp | TGT ACG GGA CTG TAA CAC CTG C | 300 | 1525 - 1546 | V01108 |
| | | | TGA AGC ATA TGA AGA TGG CCC CAA C | 200 | 1550 - 1574 | |
| | | | TTT GGM ACT TGC ACG GG | 300 | 1624 - 1640 | |
| JCV | Late mRNA gene | 123 bp | TGA ACC AAA AGC TAC ATA GGT AAG TAA TG | 900 | 474 – 502 | NC_001699 |
| | | | TTC ATG GGT GCC GCA CTT GCA | 200 | 523 – 543 | |
| | | | AAT CCT GTG GCA GCA G | 900 | 581 - 596 | |
| **Parvovirus (DNA):** | | | | | | |
| PVB 19 | VP2 gene | 75 bp | AAG CCG TGT GCA CCC ATT | 300 | 4947 - 4964 | AF162273 |
| | | | TAA ACA CTC CCC ACC GTG CCC TCA | 200 | 4966 – 4989 | |
| | | | GTA CTG GTG GGC GTT TAG TTA CG | 300 | 4999 - 5021 | |
| **Enterovirus (RNA):** | | | | | | |
| EV | 5' UTR gene [1] | 148 bp | CCC TGA ATG CGG CTA ATC C | 900 | 455 - 473 | D00820 |
| | | | CGG AAC CGA CTA CTT TGG GTG TCC GTG TTT C | 200 | 535 - 565 | |
| | | | ARA TTG TCA CCA TAA GCA GCC A | 900 | 581 - 602 | |
| **Paramyxoviruses (RNA):** | | | | | | |
| Parainfl 1 | HN Gene [4] | 109 bp | GTT GTC AAT GTC TTA ATT CGT ATC AAT AAT T | 900 | 1191 - 1220 | U70948 |
| | | | TAG GCC AAA GAT TGT TGT CGA GAC TAT TCC AA | 200 | 1232 - 1263 | |
| | | | GTA GCC TMC CTT CGG CAC CTA A | 900 | 1278 - 1299 | |
| Parainfl 2 | HN Gene | 90bp | GCA TTT CCA ATC TTC AGG ACT ATG A | 900 | 767 - 791 | D00865 |
| | | | CCA TTT ACC TAA GTG ATG GAA TCA ATC GCA AA | 200 | 795 - 826 | |
| | | | ACC TCC TGG TAT AGC AGT GAC TGA AC | 900 | 831 - 856 | |
| Parainfl 3 | HN Gene | 136 bp | AGT CAT GTT CTC TAG CAC TCC TAA ATA CA | 900 | 779 - 807 | L25350 |
| | | | AAC TCC CAA AGT TGA TGA AAG ATC AGA TTA TGC A | 200 | 828 - 861 | |
| | | | ATT GAG CCA TCA TAA TTG ACA ATA TCA A | 900 | 887 - 914 | |
| RSV | N gene | 149 bp | GGC AGT AGA GTT GAA GG | 900 | 1801 - 1817 | M11486 |
| | | | ACT TGC CCT GCA CCA TAG GCA TTC ATA AAC AAT | 200 | 1830 - 1862 | |
| | | | ACA ACT TGT TCC ATT TCT GC | 300 | 1930 - 1949 | |

| Virus type | Target | Amplicon length | Oligonucleotide Sequences (5'-3') | | Conc. (nM) | Nucleotide position | GenBank Acc. No. |
|---|---|---|---|---|---|---|---|
| | | | | Forward / Probe / Reverse | | | |
| **Orthomyxoviruses (RNA):** | | | | | | | |
| Infl A | M gene [2] | 132 bp | CAT GGA ATG GCT AAA GAC AAG ACC | | 900 | 126 - 149 | U49116 |
| | | | TTT GTG TTY ACG CTC ACC GTG CCC A | | 200 | 184 - 208 | |
| | | | CCA TTT AGG GCA TTT TGG ACA | | 900 | 237 - 257 | |
| Infl B | HA gene [3] | 137 bp | AGA CCA GAG GGA AAC TAT GCC C | | 300 | 134 - 155 | AB036449 |
| | | | ACC TTC GGC AAA AGC TTC AAT ACT CCA | | 200 | 219 - 245 | |
| | | | TCC GGA TGT AAC AGG TCT GAC TT | | 900 | 248 - 270 | |
| **Positive Controls:** | | | | | | | |
| PHHV | gB gene [5] | 89 bp | GGG CGA ATC ACA GAT TGA ATC | | 900 | 267 - 287 | Z68147 |
| | | | TTT TTA TGT GTC CGC CAC CAT CTG GAT C | | 200 | 305 - 332 | |
| | | | GCG GTT CCA AAC GTA CCA A | | 900 | 337 - 355 | |
| B2 MG | DNA | 105 bp | TGA GTA TGC CTG CCG TGT GA (ex 2) | | 300 | 343 - 362 | M17987 |
| | | | CCA TGT GAC TTT GTC ACA GCC CAA GAT AGT T (ex 2) | | 200 | 364 – 394 | |
| | | | ACT CAT ACA CAA CTT TCA GCA GCT TAC (intr 2) | | 300 | 421 - 447 | |
| B2 MG | RNA | 82 bp | TGA GTA TGC CTG CCG TGT GA (ex 2) | | 300 | 343 - 362 | M17987 |
| | | | CCA TGT GAC TTT GTC ACA GCC CAA GAT AGT T (ex 2) | | 200 | 364 – 394 | |
| | | | TGA TGC TGC TTA CAT GTC TCG AT (ex 3) | | 300 | 1018 - 1040 | |

## C. Results

The sequence information of primers and probes for 23 real-time PCR virus detection assays is displayed in Table 1, together with an indication of their precise positions within the targeted genes. Moreover, the optimal primer and probe concentrations are indicated for each detection assay. All virus PCR tests presented were designed to be conducted under identical cycling conditions, as outlined in the Methods section, in order to facilitate the molecular diagnostic work.

### 1. *Efficiency, and sensitivity of the RQ-PCR virus assays*

These parameters were assessed by repeated testing of serial logarithmic dilutions of the standard reference virus strains covering a range of 5 logs. The number of virus copies used to prepare the serial dilutions had been determined by spectrophotometric or fluorometric measurement of the g/cDNA concentration of individual virus strain preparations.

After PCR amplification, the Ct-values of individual dilutions steps were plotted against the initial virus copy number, leading to typical standard curves. The standard curves provided information on the amplification efficiency, the consistency of replicate reactions, and the theoretical and actual detection limits of the assay. The amplification efficiencies, defined by the standard curve slopes, were generally at or around 3.5. The consistency of replicates was measured by the correlation coefficient ($R^2$), which indicates the linearity of the Ct values plotted in the standard curves. The $R^2$ indices were higher than 0.990 in all measurements.

The actual sensitivity of the assays was determined by the lowest standard dilution consistently detectable in replicate reactions. In all assays presented, 1E+02 virus particles were reproducibly detected.

### 2. *Reproducibility of the RQ-PCR virus assays*

To evaluate the intra-assay variation of the virus tests, control samples across a wide range of virus copy numbers were analyzed concomitantly, in triplicate reactions. The inter-assay variation was assessed by investigating a minimum of three different DNA/cDNA aliquots of individual virus samples in independent assays. The results obtained document the high precision and low variation within and between individual virus tests.

### 3. *Quantification of virus copy numbers in clinical samples*

The virus loads in individual patient specimens were investigated by testing the most recent and, if available, a previously quantified patient sample in duplicate reactions, together with the appropriate external viral standard preparations. The efficiency of virus DNA/RNA isolation from clinical samples and the possible presence of reverse transcriptase or polymerase inhibitors were monitored by using internal controls, as described in the Methods section. These controls permitted appropriate correction in the calculation of virus copy numbers in the specimen investigated. For the calculation of virus particles in the sample tested, the slope and the Y-intercept values of the corresponding standard curve, and the Ct-value of the target virus amplification were used, according to the following equation:

$$\mathbf{P_0 = Inv\ log\ (Ct - Y/s)^*.}$$

***Footnote:** $P_0$= number of virus copy equivalents in the PCR reaction prior to amplification; Y=Y-intercept; s=slope)*

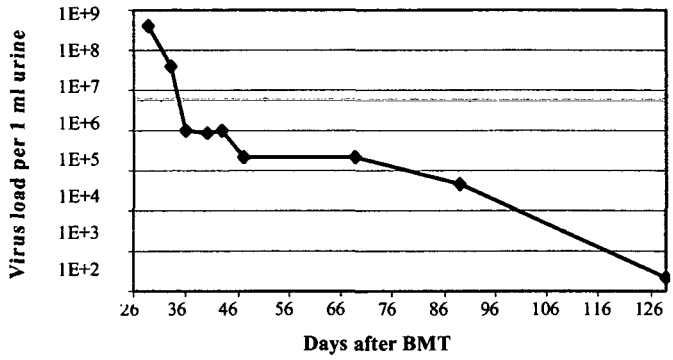
## 4. *Examples of clinical application in immunosuppressed patients*

The panel of virus tests presented covers viral pathogens of well established or supposed importance in pediatric patients with severe immunosupression. The following examples were derived from virus monitoring in immunocompromised children after allogeneic stem cell transplantation, and illustrate the clinical utility of the RQ-PCR virus detection assays in this particular clinical setting.

### a) Rapid diagnosis of viral cause of disease symptoms

Severe inflammation of the urinary bladder in immunocompromised patients may occur as a result of infection with a variety of bacterial and viral pathogens. The polyoma virus BKV is a relatively common cause of hemorrhagic cystitis in children undergoing allogeneic stem cell transplantation. Although no specific antiviral treatment is currently available for this type of infection, detection and monitoring of the virus during the course of disease is of clinical relevance with regard to idenfication of the cause of the symptoms observed and with regard to differential diagnosis from other pathogens requiring specific treatment. **Figure 1** illustrates the detection and surveillance of BKV virus load in serial urine samples of a patient displaying hemorrhagic cystitis after allogeneic SCT. All tests for other pathogens in urine were negative. The causative agent and the course of infection under symptomatic therapy could be documented by RQ-PCR monitoring.

**Fig. 1: Kinetics of BKV load during hemorrhagic cystitis**




**Legend:**

Documentation of BKV infection of the urinary bladder and clearance of the virus by serial RQ-PCR analysis of urine samples during the post-transplant period. The virus load (Y-axis) is plotted against the time after transplantation (X-axis).

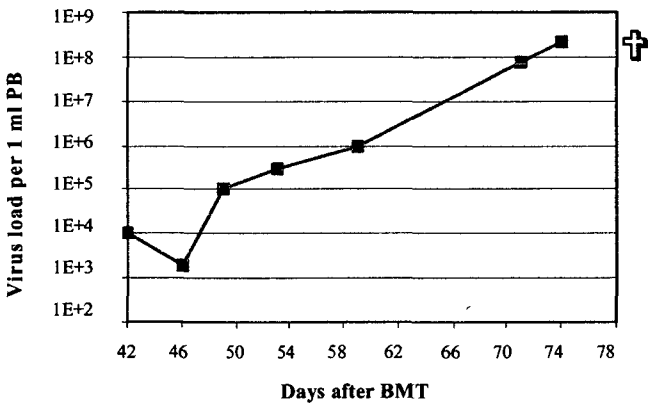
### b) Latent virus infection and reactivation

Following primary exposure, a number of viruses, including members of the herpes virus family and adenoviruses, may persist as latent infections in peripheral blood (PB) leukocytes.

In view of the high prevalence of infections with these viruses, low viral copy numbers documenting persistence are detectable in B cells of the peripheral blood in a large proportion of healthy individuals. In immunosuppressed patients, reactivation of latent virus infection, associated with serious clinical symptoms, represent a frequent finding [8]. In these instances, the viruses proliferate within the affected cells and are released into the extracellular compartment. Since viral reactivation may lead to life-threatening complications in patients with impaired immune response, early diagnosis is of major clinical importance, in order to permit timely initiation of appropriate antiviral treatment.

The Epstein-Barr virus (EBV) is commonly detectable at low copy numbers in peripheral blood lymphocytes, when present in latent state. Reactivation of the virus in immunocompromised patients may lead to a lymphoproliferative disease (LPD), which may result in the occurrence of fatal malignant lymphoma [9,10]. In these instances, increasing copy numbers of the virus are detectable within PB lymphocytes and rising levels of free virus are detectable in plasma. Early detection of EBV proliferation kinetics provides a basis for timely initiation of preemptive treatment [11,12]. In the example presented, a retrospective analysis in a child who died from EBV-associated lymphoma is shown (**Figure 2**). Increasing levels of the virus in peripheral blood were documented by RQ-PCR over a period of several weeks, before the lymphoma was diagnosed clinically.

**Fig. 2 Kinetics of EBV load in peripheral blood in a posttransplant lymphoproliferative disorder**



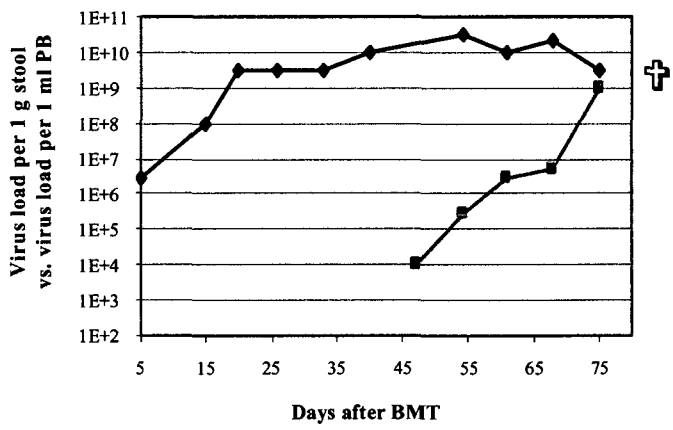

**Legend:**

Serial RQ-PCR analysis documents the reactivation of a latent EBV infection by revealing constantly increasing virus copy numbers. This retrospective analysis of virus proliferation kinetics, which heralded the development of EBV-associated malignant lymphoma, underlines the potential of molecular detection and monitoring of EBV load to provide a basis for early initiation of preemptive antiviral treatment.

## c) Early detection of viral dissemination

Intestinal virus infections are commonly observed in immunosuppressed children during the post-transplant period [13]. The clinical symptoms, if present, do not require antiviral treatment in most instances. Earlier observations made in our laboratory indicate that invasive adenovirus (AdV) infections require very early onset of antiviral treatment [6]. In a number of instances, an example of which is shown in **Figure 3**, intestinal AdV infections with rapid proliferation kinetics can be detected in serial stool samples by RQ-PCR, heralding fatal dissemination of the virus.

**Fig. 3 Kinetics of AdV load in stool preceding fatal invasive infection**



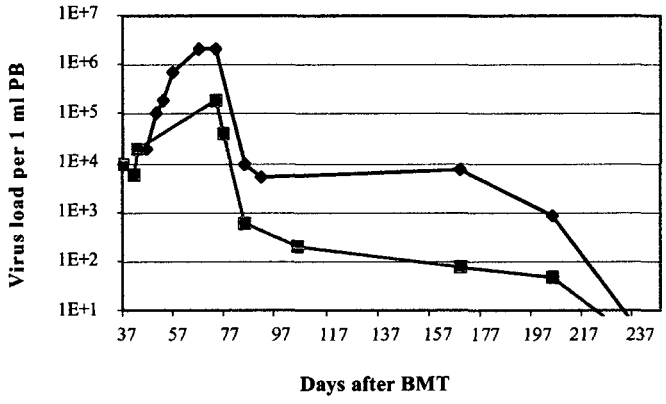

**Legend:**

Serial RQ-PCR analysis in stool samples (blue line) indicates rapid virus proliferation in the intestinal tract to an extremely high AdV load. This kinetics preceded dissemination of the virus by several weeks as documented by the appearance and expansion of the pathogen in peripheral blood (red line). The monitoring of AdV in stool may therefore permit timely onset of appropriate antiviral treatment, in attempts to prevent disseminated disease.

**d) Documentation of the response to antiviral treatment**

In immunocompromised patients suffering from potentially life-threatening virus infections, the surveillance of the success of treatment is of paramount importance for appropriate clinical management, including the choice and duration of appropriate antiviral therapy [14,15,16]. The example displayed in **Figure 4** shows the documentation of decreasing cytomegalovirus levels both in plasma and peripheral blood leukocytes, followed by elimination of the virus below the limit of detection, in response to antiviral treatment with gancyclovir.

**Fig. 4 Kinetics of CMV load in peripheral blood in response to antiviral treatment**




**Legend:**

Monitoring of CMV by RQ-PCR in serial plasma (blue line) and peripheral blood leukocyte samples (red line) during the post-transplant period reveals viral reactivation by rising levels of CMV-DNAemia and subsequent clearance of the virus following antiviral therapy.

## D. Discussion

In this paper, we present real-time PCR assays for qualitative and quantitative analysis of sixteen viruses or virus families, which appear to be of particular importance in the clinical management of immunosuppressed children undergoing high-dose chemotherapy or allogeneic stem cell transplantation. Qualitative virus detection revealing merely the presence or absence of a viral pathogen is not sufficient in a variety of clinical situations. In many instances, the rate of disease development was shown to be related directly to the viral DNA or RNA levels detected in plasma, serum or peripheral blood lymphocytes. The association between disease progression and viral load is well established for infections with a number of viruses including particularly HIV, HBV, and HCV [17,3,18].

In immunosuppressed patients, particularly after allogeneic SCT, the need to quantitatively monitor infections with CMV and EBV has been long appreciated [19,5,20]. The clinical relevance of a number of other viral infections in this setting is less well established, but there is a growing body of evidence indicating that viruses such as AdV [6,21], HHV6 [22], VZV [23], PVB19 [24] and others [25,26,27] merit careful monitoring in these patients. Molecular diagnostic assays, such as the tests presented herein are therefore of increasing clinical importance.

To render the screening for multiple viruses more practicable, all tests presented were conceived to permit target amplification and quantification under identical PCR conditions. The examples shown represent important paradigms of clinical application of quantitative virus detection assays in immunocompromised patients. As demonstrated, quantitative virus tests are useful when assessing a

clinically suspected viral cause of infection, because the documentation of rising virus copy numbers, in absence of other detectable pathogens, provides support for a role of the virus as a causative agent. Moreover, the observation of increasing viral load in sequential assays virtually excludes the possibility of false interpretation of positive PCR results resulting from contamination with amplification products or traces of viral nucleic acids harboring the amplifiable sequence.

Another clinically important application of quantitative virus tests is the possibility of differentiating between latent infection and reactivation. Persisting viruses may occur after primary infection in healthy immunocompetent individuals as well as in asymptomatic patients [28], and cause universally positive results in qualitative PCR assays. Mere detection of viral pathogens by qualitative PCR might be not relevant for the clinical outcome in these individuals, but consecutive assessment of the virus load seems to play an important role for the diagnosis and prognosis in patients with viral reactivation, by providing a basis for timely initiation of appropriate treatment [5,6,29,30,31,32]. Similarly, the observation of virus proliferation in compartments such as the intestinal tract, which per se may not require antiviral therapy, could permit early prediction of impending invasive infection. Timely onset of appropriate therapy could help preventing progression to disseminated viral disease, at least in a proportion of patients.

Finally, the ability of quantitative virus tests to facilitate monitoring of the response to antiviral treatment is an invaluable tool in clinical care of imunocompromised patients, providing control of the appropriate choice and the necessary duration of therapy. Quantitative virus testing has therefore become an indispensable diagnostic instrument in many different clinical situations. The real-time PCR tests presented provide a contribution to the rapidly growing field of molecular investigation of viral infections, as a basis for improved patient care.

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# CHAPTER

# VI

# RQ-PCR FOR DETECTION AND QUANTIFICATION OF ADENOVIRUS

# MOLECULAR MONITORING OF ADENOVIRUS IN PERIPHERAL BLOOD AFTER ALLOGENEIC BONE MARROW TRANSPLANTATION PERMITS EARLY DIAGNOSIS OF DISSEMINATED DISEASE

## A. Introduction

Adenoviruses are pathogens causing serious infections in allogeneic bone marrow transplant (allo-BMT) recipients [1-3] . To date, 51 different human AdV serotypes have been identified [4,5]. They are divided into six major subgroups (subgenera or species A-F) on the basis of their oncogenic, hemagglutinating, morphological, and DNA sequence properties [6-8]. In immunosuppressed patients, any adenovirus species may cause life-threatening infections [2,3,8,9]. In most clinical situations involving adenovirus infection, species identification of an AdV isolate is as informative as a finer identification by serotype [6].

In different studies, adenoviruses have been found to infect up to 20% of patients receiving allo-BMT, with a particularly high incidence of infection and virus disease in children [8,10,11]. Infections can be asymptomatic or cause localized disease such as enteritis, upper respiratory tract infection or cystitis [12]. However, AdV infections in allo-BMT recipients tend to become invasive, and disseminated disease is associated with very high mortality [8,10-17].

Earlier diagnostic approaches to AdV detection relied mainly on serological tests and cell culture [18-23]. In immunocompromised patients, however, the use of serological tests is limited due to the impaired immune response, and evaluation of positive cultures is a relatively slow method [24]. The recent introduction of PCR-based assays has opened new ways to rapid, specific and highly sensitive AdV detection [4,12-15,17,25].

In view of the fact that many of the published diagnostic approaches do not effectively cover all AdV types, we have established species-specific real-time PCR assays permitting reliable detection and quantification of all 51 currently known human AdV serotypes. In the current study in pediatric patients after allogeneic stem cell transplantation, we have addressed the clinical significance of molecular AdV detection, and investigated the potential of serial real-time PCR analysis to facilitate diagnosis of invasive infection early in its pre-clinical stage.

## B. Patients and Methods

### 1. Patients

All pediatric patients who underwent allogeneic stem cell transplantation (allo-SCT) at St.Anna Children's Hospital, Vienna, Austria, between June 1996 and May 2002 were included in the study (n=132). Written informed consent was obtained from each patient and/or the parents, and the transplant protocol has been approved by the institutional review board of St. Anna Children's Hospital. Patient characteristics and transplant modalities are summarized in Tab.1. Ex vivo T-cell depletion by CD34+ selection was performed in case of 1-AG mismatched unrelated or haploidentical family donors. Patients with severe combined immunodeficiency, patients with severe aplastic anemia and patients with mismatched or unrelated donors received ATG from day -3 to -1. GvHD prophylaxis consisted of cyclosporine A and, in recipients of non-T-cell depleted grafts from unrelated donors, of an additional short MTX course.

All patients were nursed in laminar air flow units. Antiviral prophylaxis consisted of acyclovir 30 mg/kg/day i.v. from day -7 until day + 28 and immunoglobulin substitution every three weeks, until day +100.

Patients with a positive PCR test in peripheral blood for CMV or AdV were eligible for preemptive antiviral treatment, regardless of the viral load. Treatment was initiated upon availability of PCR results, generally within 48-72 hours after sampling. Patients with CMV-DNAemia received primary preemptive therapy with gancyclovir and, in case of persistent DNAemia, secondary preemptive treatment with foscarnet, until two consecutive negative results were obtained. Patients with adenovirus-DNAemia transplanted between October 1998 and May 2002 (n=7) received preemptive treatment with cidofovir (5mg/kg/week; according to a protocol requiring continuation of treatment until attainment of two negative PCR results).

**Tab. 1: Patient characteristics, transplant modalities and acute GvHD**

**N = 132**

| | | | |
|---|---|---|---|
| **Observation time:** | median 23 months (range: 2-71) | | |
| **Age at SCT:** | median 8 years (range: 0.1-20) | | |
| **Diagnosis:** | Acute leukemia | | n=63 |
| | Chronic leukemia/Myelodysplasia/Solid tumor | | n=23 |
| | Severe combined immunodeficiency | | n= 8 |
| | Inborn errors/Severe aplastic anemia | | n=38 |
| **Conditioning:** | Chemoconditioning | | n= 80 |
| | Irradiation (TBI/TLI)-containing conditioning | | n= 52 |
| | Additional anti-thymocyte globulin (ATG) | | n=103 |
| **Donor:** | HLA-matched sibling donor | | n= 35 |
| | HLA-matched unrelated donor | | n= 68 |
| | HLA-mismatched family donor | | n= 29 |
| **Graft:** | Bone marrow without CD34+ positive selection | | n= 62 |
| | Peripheral blood stem cells without CD34+ positive selection | | n= 12 |
| | Peripheral blood stem cells with CD34+ positive selection | | n= 58 |
| **Acute GvHD:** | All patients (pts) (n=132): | GvHD, any grade | n= 72 |
| | | GvHD, grade III-IV | n= 27 |
| | AdV negative pts (n=96): | GvHD, any grade | n= 51 |
| | | GvHD, grade III-IV | n= 18 |
| | AdV positive pts at any site (n=36) | GvHD, any grade | n= 21 |
| | | GvHD, grade III-IV | n= 9 |
| | AdV positive pts in PB (n=11) | GvHD, any grade | n= 5 |
| | | GvHD, grade III-IV | n= 3 |

## 2. *Sample collection*

Whenever possible, patients were tested prior to transplantation, and prospective virus screening in peripheral blood (PB), stool, urine and throat for CMV, EBV, AdV, HHV6, and HHV7 was performed at three- to seven-day intervals until day +28.

Subsequently, the intervals in patients who tested negative were extended to one to two weeks, until day +100 post-transplant. In patients who tested positive for any virus until day + 28, the screening was continued at three- to seven-day intervals, until two consecutive negative results were obtained.

After day +100, tests were carried out only in case of clinically suspected viral infection. More than 5000 samples derived from the sites indicated above, and occasionally from cerebrospinal fluid, organ biopsies, and bone marrow, were screened by PCR-

based DNA analysis. The average number of samples investigated in each patient was 50 (range: 11-75).

### 3. *Isolation of viral DNA*

DNA extraction from largely cell-free liquids except urine, and from peripheral blood leukocytes for detection of intracellular virus particles was performed using QIAamp DNA Mini Kit. Isolation of virus DNA from urine was done using the QIAamp Viral RNA Mini Kit, and from stool by the QIAamp DNA Stool Mini Kit (QIAGEN GmbH, Hilden, Germany) according to the manufacturer's recommendations.

### 4. *Quantitative PCR analysis of AdV*

Six different primer/probe systems were designed on the basis of the available AdV DNA sequence information (NCBI Database) to permit detection of the individual AdV species (subgenera) A-F. The AdV DNA sequences targeted by the primer/probe systems were derived from the hexon, and VA RNA genes. The characteristics of the primers/probes used are indicated in Tab.2. Reference strains of all 51 human adenovirus serotypes (kindly provided by H. Niesters, Dept Virology, University of Rotterdam, The Netherlands) were used as positive controls for the establishment of AdV-specific real-time PCR assays. Each of the six primer/probe systems were shown to permit sensitive and specific detection of all AdV serotypes belonging to the respective species. The only cross reactivity observed, involved the detection system for AdV species B which recognized also species E (including only serotype 4). By contrast, the detection system for species E detected exclusively this genotype under the conditions used.

PCR reactions were set up in a total volume of 25 µl including 6 µl template DNA, and 12.5 µl TaqMan Universal Master Mix. The concentration of primers and FAM-labelled probe for each detection system are displayed in Tab.2. Amplifications were carried out using the ABI Prism 7700 or 7900 Sequence Detectors (Applied Biosystems, Foster City, Ca, USA) for a total of 50 cycles. After an initial denaturation step for 10 min at 95°C, each cycle consisted of denaturation for 15 sec at 95°C, and annealing and primer extension for 60 sec at 60°C.
Strict precautions were undertaken to prevent contamination of PCR reactions with exogenous products as described [26]. A dUTP glycosylase step was performed prior to each PCR reaction. Each DNA sample was analyzed in duplicate, and multiple negative controls were included in each assay.

For quantitative analysis of virus load, real-time TaqMan PCR was performed using the equipment indicated above. External standard curves for each AdV species were established by making serial dilutions of quantified virus preparations derived from reference strains. For assessment of virus copies per cell, a single copy gene (beta2 microglobulin) was quantified in parallel by real-time PCR [27]. When investigating cell-free liquids, quantitative results were expressed as the number of virus copies per ml. In stool samples, the virus copies were calculated per gram material, and intracellular virus copies were indicated per $10^6$ cells.

The sensitivity of the PCR assays permitted reliable detection of $2x10^2$ virus particles per ml of the medium investigated. For reproducible quantification of virus load, however, the presence of $\geq 10^3$ particles per ml was necessary. When analyzing cell material, sensitivity of the assays permitted detection and quantification of virus copies at a level of 10 particles per $10^6$ cells.

### 5. *Statistical analysis*

The risk factors for AdV infection analyzed in the univariate analysis included T cell depletion in- and ex-vivo, underlying disease, type of donor, graft versus host disease of all grades, and presence of other virus infections.

**Tab. 2: Primers and probes for species-specific adenovirus detection by real-time PCR**

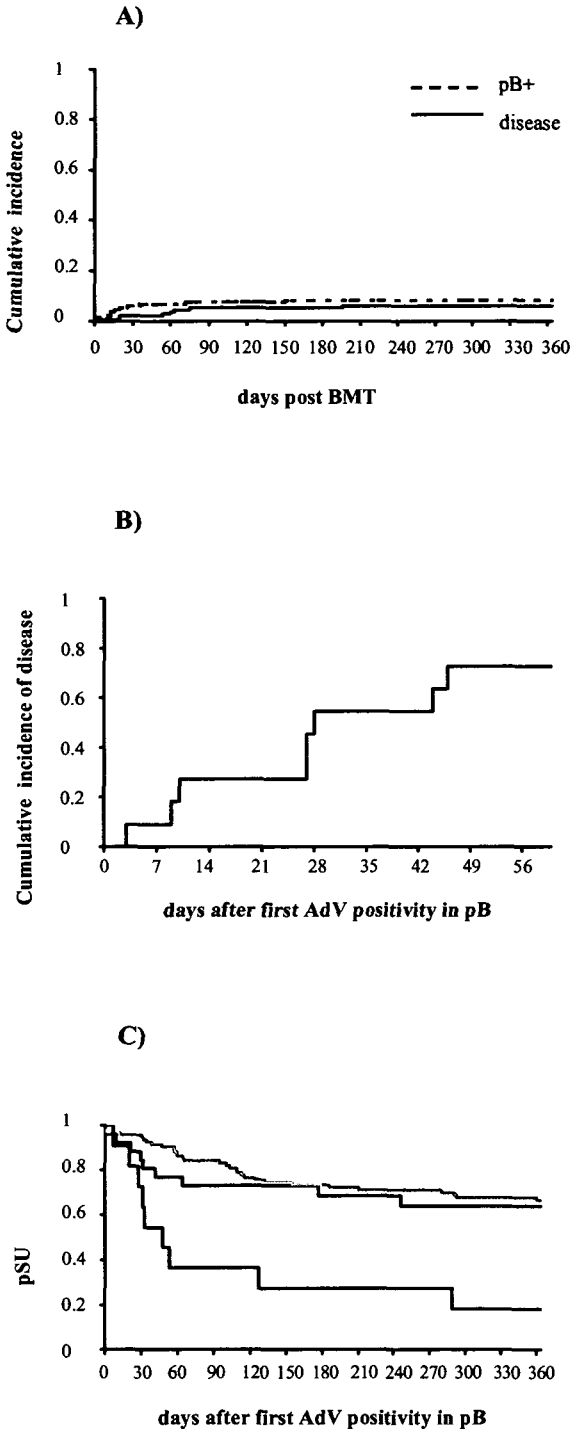
| Acronym/ Virus type | Target | Amplicon length | Oligonucleotide sequence (5'-3') Forward \| Probe \| Reverse | Conc. (nM) | Nucleotide position | GenBank Acc. No. |
|---|---|---|---|---|---|---|
| AdV A | Hexon gene | 135 bp | GGK CTG GTG CAA TTC GCC | 300 | 17818 - 17835 | X73487 |
| | | | CCA CGG ACA CCT ACT TCA CCC TGG G | 200 | 17840 - 17864 | |
| | | | CAC GGG CAC AAA ACG CA | 300 | 17936 - 17952 | |
| AdV B | Hexon gene | 138 bp | CGC CGG ACA GGA TGC TT | 900 | 45 - 61 | X76549 |
| (B + E) | | | AGT CCG GGT CTG GTG CAG TTC GCC | 200 | 73 - 96 | |
| | | | CTA CGG TCG GTG GTC AC | 900 | 166 - 182 | |
| AdV C | Hexon gene | 138 bp | ACC TGG GCC AAA ACC TTC TC | 300 | 2884 – 2903 | J01966 |
| | | | AAC TCC GCC CAC GCG CTA GA | 200 | 2910 – 2929 | |
| | | | CGT CCA TGG GAT CCA CCT C | 900 | 2940 - 2958 | |
| AdV D | VA RNA gene | 143 bp | AAA AAC GAA AGC GGT TGA GC | 300 | 2 – 21 | U10675 |
| | | | CCA ATA CCA CGT TAG TCG CGG CT | 200 | 104 – 126 | |
| | | | CGG GTC GAG ACG GGA GT | 50 | 128 - 144 | |
| AdV E | Hexon gene | 75 bp | CAA CAC CTA CTC GTA CAA AGT GCG | 900 | 225 – 248 | X84646 |
| | | | CGC CCA CGG CCA GCG TGT | 200 | 251 – 268 | |
| | | | TAG GTG CTG GCC ATG TCC A | 300 | 281 - 299 | |
| AdV F | Hexon gene | 128 bp | GCA GGA CGC CTC GGA GTA | 300 | 268 – 285 | D13781 |
| | | | TAC TTC AGC CTG GGG AAC AAG TTC AGA AA | 200 | 329 – 357 | |
| | | | TGT CTG TGG TTA CAT CGT GGG T | 900 | 374 - 395 | |

Differences between patient groups including 1) AdV negative patients, 2) patients positive for AdV at one or two sites, but negative in PB, and 3) patients positive for AdV in PB were analyzed using Chi-Square Test. For analysis of small cohorts, Fisher's Exact Test was employed. The

association between AdV infection and graft versus host disease (GvHD) of all grades was analyzed by Mantel-Haenszel Chi-Square Test.

The overall survival rate was estimated by the method of Kaplan-Meier. The cumulative incidence was estimated for the time to first AdV positivity in PB and time to onset of AdV disease. Depending on the subgroup analyzed, the intervals start at BMT, at first AdV positivity in PB, or at the time of AdV disease onset, respectively (Fig.1). For the comparison of patients who tested AdV positive in PB with patients who tested negative in PB, the interval for the latter group starts at the median time to AdV positivity (i.e. day +16), to account for the time-dependent nature of AdV positivity in PB (Fig.1c). The impact of CMV positivity, AdV positivity, and AdV disease on survival was studied by including the respective variables as time-dependent co-variates in a Cox regression model.

**Fig. 1: Incidence of invasive adenovirus infection, disseminated disease and overall survival**




**Legend:**

**Panel A. Probability of invasive AdV infection and disease**

In the cohort of patients investigated, the one-year cumulative incidence of invasive AdV infection post SCT (blue colored curve), documented by virus detection in peripheral blood, was 8% (±8%), that of AdV-associated disease (red colored curve) 6% (±6%).

**Panel B. Probability of disease after AdV detection in PB**

The cumulative incidence of disease was 73% (±10%) within 60 days after the first AdV positive PCR test in PB.

**Panel C. Probability of survival in patients with AdV positive/negative PCR tests**

The green colored (bottom) curve indicates the overall survival in patients with AdV positivity in PB. The one-year probability of survival (pSU) in this cohort was 18% (±9%). The two upper, nearly identical curves illustrate the overall survival in patients who tested AdV negative in PB, but positive at one or two other sites (blue colored curve; one-year pSU of 64% (±10 %).), and in AdV negative patients (orange colored curve; one-year pSU of 67% (±5 %). The difference between patients positive for AdV in PB and the other two cohorts illustrated is highly significant ($p<0.001$). By contrast, there was no statistically significant difference in pSU between AdV negative patients and patients who tested AdV positive at sites other than PB ($p=0.706$).

The impact of CMV positivity on the time to AdV positivity in PB was analyzed in the same fashion. Relative hazard rates (relative risk) are indicated in addition to p-values. All indicated p-values are two-sided. P values ≤0.05 were considered significant, p values ≤0.01 highly significant.

## 6. *Definitions*

Localized and disseminated adenovirus infection, definite and probable adenovirus disease were defined as described [11,28], with minor modifications reflecting the use of PCR detection assays: Patients with reproducibly positive adenovirus PCR screening assays in specimens derived from any site or positive tests from sources related to their clinical signs and symptoms were considered to have adenovirus infection. Disseminated (invasive) infection was defined as the presence of positive tests from two or more organ systems and/or positive tests from peripheral blood.

Definite disease was defined as the presence of typical adenovirus nuclear inclusions on routine histopathology, a positive culture from tissue (except from the gastrointestinal tract), or both. Probable adenovirus disease was defined as the presence of two or more positive PCR assays in samples from peripheral blood or from more than two different body sites, in association with compatible symptoms, without other identifiable causes. All patients with fever, unexplained symptoms, or laboratory test abnormalities were carefully tested for infections and for GvHD.

## C. Results

### 1. *Incidence and spectrum of adenovirus infections in children after allogeneic stem cell transplantation*

Patients who tested AdV positive are summarized in Tab.3. Thirty six patients (27%) showed positive results in samples derived from at least one site.

Among the AdV positive patients, twenty five (69%) had detectable virus at one or two sites, most commonly in stool and/or throat samples, but never tested positive in peripheral blood (PB) (Tab.3, upper panel). In these patients, AdV positivity has appeared at a median of 18 days post-transplant (range –7/> +100). Patients who tested AdV positive at more than two different sites also showed presence of the virus in PB. Adenoviruses were detected in PB samples of eleven children, i.e. 31% of the AdV positive cases, and 8% of all patients (Fig. 1a; Tab.3, bottom panel). First appearance of adenoviral DNA in PB specimens has been observed at a median of 16 days post-transplant (range –7/> +100).

In our series, adenoviruses of all but one species were detected: groups A, B, C, D, and F were represented, but no sample was positive for species E, the smallest subgroup including only one serotype (No 4). The most prevalent AdV species in the cohort investigated was group C (78% of all positive cases), while other types were observed less frequently: (A:8%; B:8%; D:8%, F:8%). Four patients were positive for two different AdV species: in two instances, the species C+B were present, two other patients displayed the species A+B, and D+F, respectively. Among patients who tested positive in PB, nine (82%) displayed species C, and two (18%) species A.

### 2. *Risk factors for AdV infection*

T-cell depletion in vivo by ATG combined with T-cell depletion ex vivo by CD34+ positive selection was associated with a significantly increased incidence of AdV infection (p=0.014). T-cell depletion by ATG alone showed no significant association (p=0.493). Children who were transplanted from matched sibling donors (MSD) had a significantly lower occurrence of AdV infection in comparison to the cohort of patients with matched unrelated (MUD) and mismatched family (MMFD) donors (p=0.014).

No statistically significant correlation with AdV detection has been found for other parameters including the presence of graft versus host disease (GvHD), regardless of the grade (p=0.725), and for the underlying disease (*non-malignant versus malignant)* (p=0.314).

### 3. *Association of AdV detection with presence of other viruses*

Of the viruses tested in addition to AdV including CMV, EBV, HHV6, and HHV7, only CMV was significantly correlated with detection of AdV in PB. Within the group of AdV PB positive patients, 8/11 (73%) had detectable CMV in PB versus 33/96 (34%) in AdV negative patients (p=0.020). By contrast, the difference in the detection of CMV in PB

between AdV negative cases and patients who tested AdV positive at sites other than PB, 33/96 (34%) versus 10/25 (40%), was not significant (p=0.601).

In the patients with positive PB tests for both AdV and CMV during the time of observation, all but one revealed CMV positivity before the first positive AdV test (Table 3). However, in contrast to the dynamics of AdV load observed in most of these patients (see below), there was no evidence of increasing CMV levels in PB. Cox regression analysis revealed that patients who tested CMV positive in PB had a 4.4-fold increased risk of invasive AdV infection.

However, detection of CMV in PB per se, in absence of expanding viral load, was not a significant factor for survival in the patients studied (relative risk: 1.3). Hence, despite a significant association with later occurrence invasive AdV infection, CMV was not an independent co-variable with regard to transplant related mortality (TRM).

### 4. *Adenovirus infection and transplant related mortality*

In children with AdV detectable at sites other than PB, the only signs of adenoviral disease included enteritis in seven patients with AdV positivity in stool specimens, and generalized rash in one patient with AdV positive skin biopsy (Tab.3). By contrast, eight of eleven patients who had detectable AdV in PB had clinical evidence of disseminated adenoviral disease (Fig. 1B; Tab.3).

In the group of AdV negative patients, 28 children (29%) died during the observation period. In eight instances (8%), the fatal outcome was attributable to disease relapse, and in 20 cases (21%) to TRM. In the cohort of adenovirus positive patients who had no detectable AdV in PB (Tab.3, pts 1-25), eight (32%) died. One patient (4%) died from the underlying disease, and seven (28%) from TRM (i.e. organ toxicity n=3, or infectious complications including CMV-pneumonia n=1, legionella n=1, and invasive aspergillosis n=2). The difference in TRM-related deaths between the two cohorts, 21% vs 28%, was not statistically significant (relative risk:1.2; p=0.706). In the group of patients who were AdV positive in PB (Tab.3, pts 26-36), ten (91%) died. One patient (9%) died from relapse, and nine (82%) from TRM. The association of AdV positivity in PB with TRM was highly significant (relative risk: 5.8; p<0.001)(Fig. 1C). One of these patients died from CMV pneumonia over three months after transient AdV positivity in PB, and eight children developed disseminated AdV disease with multiorgan (hepatic, pulmonary, renal) failure. The correlation between onset of AdV-related disease and mortality was highly significant (relative risk: 31.4; p<0.001).

Adenoviral DNA was detected in PB at a median of 29 days before death (range 7 to 112 days). The AdV types observed in PB of these patients belonged either to species C (n=6) or A (n=2).

### 5. *Quantification of virus load in patients with invasive AdV infection*

In patients who tested AdV positive at any site, virus load was monitored at three- to four-day intervals. The virus load was usually low in patients presenting with AdV positivity at sites other than PB ($<2x10^4$ virus copies per gram of tissue, e.g. stool, or per milliliter of liquid, e.g.urine), and there was mostly no evidence of rising virus copy numbers during the observation period. Only three patients (Tab.3; No 19, 35, and 36) showed dramatically increasing AdV levels in stool samples, reaching copy numbers of $>10^8$/g. During this period, one of the patients (No 19) had hemorrhagic diarrhea, which was treated with cidofovir, and resolved together with prompt reduction of AdV load in stool. Two other patients (No 35, 36) later showed AdV positivity in PB with rapidly rising virus load, and died shortly thereafter.

**Tab. 3: Characteristics of adenovirus-positive patients**

| Patient No. | Age | Diagnosis | Donor | Source | T-cell depletion | aGvHD during AdV infection / Grade | AdV-species | Site of detection | Day of CMV detection in PB pre/post BMT | Day of AdV detection pre/post BMT | AdV disease | Outcome | Day of death / post BMT | / Cause |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 9 | WG | MSD | BM | no | no | D | stool | +56 | +7 | no | a/w | | |
| 2 | 3 | AML | MUD | PBSC | 1+2 | no | C | stool | -13 | 0 | no | a/w | | |
| 3 | 20 | ALL | MUD | BM | 1 | no | F | stool, organ biopsy (postmortem) | n.d. | +59 | no | died | +101 | / CMV |
| 4 | 1 | HLH | MMFD | PBSC | 1+2 | n.e. | F | stool, throat | -10 | -7 | enteritis | died | +2 | / HLH |
| 5 | 8 | ALL | MMFD | PBSC | 1+2 | no | C | stool | + 1 | > +100 | enteritis | a/w | | |
| 6 | 3 | ALL | MUD | PBSC | 1+2 | no | A | stool | +17 | > +100 | enteritis | died | +131 | / toxicity |
| 7 | 8 | ALL | MUD | BM | 1 | no$^{+}$ | C | stool | +12 | +90 | no | a/w | | |
| 8 | 3 | FHL | MUD | PBSC | 1+2 | no | C+B | stool, throat (C) / stool (B) | -5 | -7 (C) / > +100 (B) | no | died | +153 | / aspergillus |
| 9 | 5 | FA | MMFD | PBSC | 1+2 | no | C | stool | n.d. | +49 | enteritis | died | +70 | / toxicity |
| 10 | 2 | FHL | MMFD | PBSC | 1+2 | no | C | throat | n.d. | > +100 | no | a/w | | |
| 11 | 10 | ALL | MMFD | PBSC | 1+2 | no | C | stool | n.d. | +91 | no | died | +120 | / legionella |
| 12 | 10 | CML | MSD | BM | no | no | C | skin | n.d. | > +100 | rash | a/w | | |
| 13 | 7 | SAA | MFD | PBSC | 1+2 | no | C | throat | n.d. | > +100 | no | a/w | | |
| 14 | 3 | AML | MUD | PBSC | 1 | no | C | organ biopsy (postmortem) | n.d. | +39 | no | died | +39 | / toxicity |
| 15 | 16 | SAA | MMFD | PBSC | 1+2 | n.e. | C | throat | n.d. | +10 | no | died | +74 | / aspergillus |
| 16 | 4 | XLP | MUD | PBSC | 1+2 | no | C | stool | n.d. | +8 | no | a/w | | |
| 17 | 11 | AML | MSD | BM | no | no | D | stool | +52 | +10 | no | a/w | | |
| 18* | 1 | MPS I | MUD | PBSC | 1+2 | yes/3 | C | stool | +6 | +53 | enteritis | a/w | | |
| 19* | 1 | MPS I | MUD | PBSC | 1+2 | yes/3 | C | stool | n.d. | +23 | enteritis | a/w | | |
| 20 | 7 | MDS | MSD | BM | no | no | C | stool | -12 | -7 | no | a/w | | |
| 21 | 0 | SCID | MMFD | PBSC | 1+2 | no | C | stool | n.d. | +18 | enteritis | died | +170 | /SID |
| 22 | 9 | ALL | MUD | PBSC | 1 | no | C | stool | n.d. | -7 | no | a/w | | |
| 23 | 1 | HLH | MUD | PBSC | 1+2 | no | C | stool | n.d. | -7 | no | a/w | | |
| 24 | 8 | AML | MUD | BM | 1 | no | D+F | stool | n.d. | -7 (D)/+19 (F) | no | a/w | | |
| 25 | 1 | SGD | MUD | BM | 1 | no | C | stool | n.d. | -7 | no | a/w | | |
| 26* | 5 | ALL | MMFD | PBSC | 1+2 | no | C | PB | +4 | +16 | disseminated | died | +69 | / AdV |
| 27 | 0 | JMML | MUD | BM | 1 | no | C | PB, stool, throat | -11 | +18[PB: +20] | no | died | +424 | / CMV |
| 28 | 5 | ALL | MMFD | PBSC | 1+2 | no$^{+}$ | C | PB, stool, organ biopsy (postmortem) | > +100 [+139] | > +100 [PB:+150] | disseminated | died | +198 | / AdV |
| 29 | 3 | AML | MMFD | PBSC | 1+2 | no | C | PB, stool, urine | +41 | -7 [PB:+13] | no | died | +302 | / relapse |
| 30 | 9 | FA | MUD | PBSC | 1+2 | no | C | PB, stool, urine, throat | +38 | +72 | disseminated | died | +79 | / AdV |
| 31* | 9 | CML | MUD | PBSC | 1+2 | no | C | PB, organ biopsy (postmortem) | -43 | -7 | disseminated | died | + 8 | / AdV |
| 32 | 2 | JMML | MUD | BM | 1 | yes/2 | C | PB | n.d. | +13 | no | a/w | | |
| 33 | 12 | AML | MUD | PBSC | 1 | no | C | PB, stool, throat, urine, organ biopsy (postmortem) | +14 | +36 | disseminated | died | +64 | / AdV |
| 34 | 3 | AML | MUD | PBSC | 1+2 | no | A | PB, throat, liver | -10 | -7 | disseminated | died | +24 | / AdV |
| 35 | 8 | ALL | MMFD | PBSC | 1+2 | no | A+B | PB, stool, throat, urine, CSF (A) / stool (B) | n.d. | +11 (A)/-7 (B) | disseminated | died | +31 | / AdV |
| 36* | 2 | MPS I | MUD | PBSC | 1+2 | yes/4 | B+C | PB, stool, throat, urine, CSF, eye (C)/ stool (B) | n.d. | -7(C) [PB:+26]/> +100 (B) | disseminated | died | +151 | / AdV |

**Legend:**
Upper panel (No 1-25): patients who tested RQ-PCR positive at one or two sites other than peripheral blood. Bottom panel (No 26-36): patients who tested RQ-PCR positive at sites including peripheral blood. Patients who had undergone a prior SCT (No 18, 19, 26, 31, 36) are marked by an asterisk (column *Patient No*). In the column *aGvHD*, the highest grade of acute graft versus host disease observed during the time of AdV infection is indicated. (For overall incidence of acute GvHD during the post-transplant period, see Table 1). Two patients (marked by $^{+}$) had chronic GvHD during the course of AdV infection. In both instances (No 7, 28) the GvHD was graded as extensive. In the column *Day of AdV detection*, the appearance of AdV in PB is indicated in brackets, if the virus was first detected at a site other than PB. In the column *Day of CMV detection*, patient No 31 is indicated as CMV positive on day –43. The availability of CMV data at this unusual time point is attributable to the fact that the patient had undergone his first SCT over 3 months earlier. In patients who had both AdV and CMV detectable in PB (No 26-31, 33, 34), the median time point of first PCR positivity was day +9 (range –43 to +139) for CMV and +14.5 (range –7 to +150) for AdV. In one instance (No 31), AdV detection in PB preceded that of CMV by 28 days, but in the remaining cases detection of CMV preceded first AdV positivity in PB by 3 to 36 days (median 17 days). None of the patients who tested CMV positive prior to transplantation had evidence of active infection, as revealed by RQ-PCR monitoring. Two patients (No 31 and 34) had AdV detectable in PB at the commencement of conditioning, but the transplantation could not be postponed due to the presence of leukemia in progression. In all other patients with AdV infection prior to transplantation, the virus was detectable in stool only.
**Abbreviations:** BMT=bone marrow transplantation; WG= Wegener's granulomatosis, AML=acute myeloid leukemia; ALL=acute lymphoblastic leukemia; HLH=hemophagocytic lymphohistiocytosis; FHL=familial hemophagocytic lymphohistiocytosis; FA=Fanconi anemia; CML=chronic myeloid leukemia; SAA=severe aplastic anemia; XLP=X-linked lymphoproliferative diseae; MPS 1= mucopolysaccharidosis type 1; JMML=juvenile myelomonocytic leukemia; SCID=severe combined immunodeficiency; SGD=chronic granulomatous disease; MSD=matched sibling donor; MUD=marrow unrelated donor; MMFD=mismatched family donor; BM=bone marrow; PBCS=peripheral stem cell transplantation; T-cell depletion: 1=anti-thymocyte globulin (ATG); 2=CD34+ positive selection; CSF=cerebrospinal fluid; a/w=alive and well; SID=sudden infant death; CMV=cytomegalovirus; AdV=adenovirus; n.d.=not detected, n.e.=not evaluable.

Among patients who tested AdV positive in PB (Tab.3, bottom panel), three showed only transient positivity with virus copy numbers ranging between $2x10^2$ and $2x10^3$ per ml PB or per $10^6$ leukocytes, without any dynamics indicative of virus proliferation. None of these patients had evidence of adenoviral disease during the observation period; one is alive and well 30 months post-transplant, one died from disease relapse, and one died from CMV pneumonia more than three months after AdV had become negative in PB (Tab.3, No 32, 29, and 27, respectively).

All other patients who had detectable AdV in PB developed disseminated disease (Fig. 1b). The maximum AdV load in PB detected in these patients prior to the onset of disease varied over more than four logs: three patients had very high maximum levels $\geq 10^7$ (Tab.3; No 26, 28, 34), two had intermediate levels of $10^5$ and $5x10^6$, respectively (Tab.3; No 35, 36), but in three patients the maximum virus copy numbers detected were low, ranging from $1\text{-}9x10^3$ (Tab.3; No 30, 31,33). With the exception of two children who died very shortly after first detection of the virus in PB (Tab.3; No 30, 31), the virus load in PB could be monitored by quantitative PCR analysis during the course of infection. In these patients (Tab.3; No 26, 28, 33-36), the kinetics of AdV proliferation revealed a tenfold increase in virus load at a median of 21 days (range 6 to 37 days) prior to the onset of clinical symptoms, and 26 days (range 12-118 days) prior to fatal outcome.

## D. Discussion

In the current study, pediatric patients undergoing allogeneic SCT were investigated for adenovirus infections by a pan-adenoviral PCR-assay system. The observation of AdV infections in 27% of the children investigated is rather high in comparison with earlier retrospective studies [11,13,20,24,29-31]. In the patients presented, detection of adenovirus at more than two sites was always associated with presence of the virus in peripheral blood (PB), indicating the presence of invasive virus infection [11,21,32]. In these patients, AdV was first detected at a median of 16 days post-transplant. In children with

non-invasive AdV infections, with detectable virus at one or two sites not including PB, the virus was detected at a median of 18 days post SCT. However, the time range of first AdV detection was rather large in both groups (Tab.3), as reported previously [11,29,31].

In the clinical setting, species identification of an AdV isolate has been reported to be as informative as the analysis of individual serotypes [6,25) . Specific targeting of AdV DNA sequences by PCR is hampered by major genetic differences between the six species, with an overall sequence homology of less than 25% [8,33,34] . Most of the published PCR assays for AdV detection are based on amplification of fragments derived from the hexon gene, the most highly conserved gene in the AdV genome [17,34-37]. Comparative analysis of the currently available sequence information on the hexon gene from different types of adenovirus revealed a nucleotide homology of about 50% (NCBI Database). This observation explains the apparent difficulty in establishing diagnostic assays that permit detection of the entire spectrum of human adenoviruses. Indeed, careful sequence analysis indicated that some of the published PCR tests targeting the hexon gene that were supposed to cover all AdV types are not likely to meet the expectations [4,38-43].

For the purpose of this study, we have therefore established species-specific real-time quantitative PCR assays covering the entire spectrum of known human AdV types. Fatal infections were observed in patients infected with the AdV species A and C. Other studies in patients after allogeneic SCT, after chemotherapy of malignant disorders or after organ transplantation have reported fatal outcome for infections with other AdV types including also the species B, D, E, and F [2; 3; 8; 11; 21; 24; 40; 44]. In our series, species C was by far the most prevalent type of adenovirus, and most of the fatal infections were associated with this AdV subgroup. However, the reported prevalence of individual AdV types associated with severe infections at various transplant centers was quite heterogeneous [2,3,8,11,21,24,40,44]. The data available to date indicate that adenoviruses of any species may cause life-threatening infections in allografted patients. In view of the fact that there is no clear evidence against the association of individual AdV types with severe infections, the screening methods employed in immunocompromised patients should be required to permit sensitive detection of all adenoviral serotypes.

Our experience indicating that detection of adenovirus at multiple (i.e. more than two) sites reflects presence of invasive infection, is in concert with other reports [21,31,32]. In our series, however, 73% of the patients with AdV detectable in PB, but none of the patients with AdV detectable at sites other than PB, developed fatal disseminated AdV disease. First detection of the virus in PB preceded fatal outcome of the infection by a median of 29 days. In agreement with a recent retrospective pilot study [13], our results demonstrate that detection of AdV in PB is highly predictive for TRM ($p<0.001$).

Some of the patients who developed disseminated disease had very high levels of AdV copies in PB ($>10^7$/ml or per $10^6$ cells), while others had relatively low peak levels, between $10^3$ and $10^4$. Regardless of the maximum AdV levels reached, quantitative monitoring of virus load by real-time PCR usually revealed rising virus copy numbers before the onset of clinical symptoms. It should be noted that the documentation of virus proliferation virtually eliminates a problem inherent in PCR diagnosis, the occurrence of false positive PCR tests due to inadvertent contamination with extraneous nucleic acids. Moreover, the assessment of virus proliferation kinetics permits clear distinction between latent and active adenovirus infection. We have shown

previously for quantitative PCR assays that a tenfold increase of the respective target copy number is well beyond the intrinsic variability of the method, and provides reliable evidence of an expanding process [45]. Tenfold increase in virus load preceded clinical signs of adenovirus disease by a median of three weeks. These observations indicate that monitoring of adenovirus proliferation kinetics is more informative than the assessment of absolute virus load at a given time point. Our results therefore suggest that repeated detection of AdV in peripheral blood, and documentation of rising virus copy numbers may provide the most reliable approach to diagnosis of invasive infection and prediction of adenovirus disease. It is necessary to keep in mind, however, that the options for effective therapy directed against adenoviral disease are still limited [12,30,32,46-50), and that delayed onset of treatment has been associated with a greater risk of treatment failure [49]. In view of the highly significant association of adenovirus detection in peripheral blood with the risk of fatal virus disease, a positive PCR test in PB should prompt the initiation of preemptive antiviral treatment. The availability of firm evidence for actively proliferating, invasive adenoviral infection, as revealed by rising virus numbers in PB, will gain clinical relevance when more effective anti-adenoviral treatment becomes available. Currently, the main use of quantitative monitoring of adenoviral load is the assessment of the efficacy of treatment. The surveillance of patients after hematopoietic stem cell transplantation by molecular techniques that permit rapid and sensitive detection of the entire spectrum of human adenoviruses can be expected to contribute to an improvement of clinical outcome in patients with invasive adenoviral infections.

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# PART 5

# APPENDIX

# ABBREVIATIONS

| | |
|---|---|
| ABL | Abelson |
| ABL | Abelson gene |
| ALL | acute lymphoblastic leukemia |
| B2M | beta-2-microglobulin |
| BM | bone marrow |
| CCD camera | charged-coupled device camera |
| cDNA | complementary DNA |
| CG | control gene |
| CML | chronic myeloid leukemia |
| CN | copy number |
| DNA | deoxyribonucleic acid |
| ds | double stranded |
| EAC | Europe Against Cancer |
| ENF | European network forward primer |
| ENPr | European network TaqMan reverse probe |
| ENR | European network reverse primer |
| EtBr | ethidium bromide |
| EQP | equivalence point |
| FG | fusion gene |
| FRET | fluorescence resonance energy transfer |
| Fw | forward |
| G3PDH | Glyceraldehyde-3-phosphate |
| gDNA | genomic DNA |
| GUS | beta-glucuronidase |
| H3.3 | Histone H3.3 |
| LED | light emitting diode |
| MNC | mononuclear cells |
| MRD | minimal residual disease |
| MRDv | minimal residual disease value |
| mRNA | messenger RNA |
| NCN | normalized copy number |
| PB | peripheral blood |
| PBL | peripheral blood lymphocyte |
| PBMNC | peripheral mononuclear cells |
| PBSC | peripheral blood stem cell |
| PCR | polymerase chain reaction |
| PNA | peptide nucleic acid |

| | |
|---|---|
| QRT-PCR | quantitative reverse transcription polymerase chain reaction |
| RNA | ribonucleic acid |
| RQ-PCR | real-time quantitative PCR |
| RT | reverse transcription |
| RT-PCR | reverse transcription polymerase chain reaction |
| Rv | reverse |
| SENSv | sensitivity value |
| SNP | single nucleotide polymorphism |
| ss | single stranded |
| $T_m$ | melting temperature |
| UNG | uracil-DNA glycosylase |

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*ABSENCE OF N-RAS MUTATIONS IN MYELOID AND LYMPHOID BLAST CRISIS OF CHRONIC MYELOID LEUKEMIA*, F. Watzinger, A. Gaiger, H. Karlic, R. Becher, and T. Lion. Cancer Research 54 (14): 3934-3938, July 15, 1994

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# CURRICULUM VITAE

## PERSONAL DATA:

| | |
|---|---|
| *Name:* | Franz Watzinger |
| *Date of Birth:* | 11th of August 1966 |
| *Place of Birth:* | Ried im Innkreis/ Upper Austria |
| *Nationality:* | Austrian |
| *Present Address:* | Obere Marktfeldstrasse 1<br>A-2563 Pottenstein |
| *Marital Status:* | Married |
| *Children:* | Three (born 1995, 1997, 1998) |

## EDUCATION:

| | |
|---|---|
| *1972 - 1980* | Elementary School |
| *1980 - 1984* | High School, Second Language: English |
| *1985 - 1986* | Major in agriculture, Universität f. Bodenkultur, University of Vienna |
| *1986 - 1993* | Major in Molecular Biology, Minor in Biochemistry<br>Institute of Biology, University of Vienna<br>Completion of undergraduate studies - Bachelor of Science degree<br>Graduate studies and research work at the Children's Cancer Research Institute (CCRI) at St. Anna Children's Hospital (Medical Director: Prof. Dr. H. Gadner,) under the supervision of Prof. Dr. D. Schweitzer (Dept. of Cytogenetics, University |

of Vienna) and Prof. DDr. T. Lion (Dept. of Medicine, University of Vienna).

Topic of my master's thesis: "**Incidence of N-ras Mutations in the Blast Crisis of Chronic Myeloid Leukemia**" (published in the Journal Cancer Research, Watzinger F. et al. 54:3934, 1994).

*1993/May* Graduation with the degree "Master of Science (MSc)".

*1993 - 2003* Scientist and Senior Scientist at the CCRI

Postgraduate studies and research under the supervision of Prof. DDr. T. Lion, and Prof. Dr. C. P. Kubicek, (Dept. of Techn. Microbiology, Techn. University of Vienna).

Topic of my Ph. D. thesis:

**" Innovative Approaches of Nucleic Acid Quantification by Polymerase-Chain-Reaction."**