Establishment and Study of Different Real-Time Polymerase Chain Reaction Assays for the Quantification of Cells with Deletions of Chromosome 7

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The evaluation of residual disease, which has prognostic value in the treatment of hematological malignancies, is currently assessed by scoring a limited number of cells by karyotyping and molecular cytogenetics. Real-time polymerase chain reaction (PCR) is an easier and more sensitive technique, enables analysis of a larger number of cells, and decreases sampling error. However, real-time PCR has been applied only to target transcripts of fusion genes. Here, we considered two real-time PCR strategies to quantify a number of cells carrying a partial deletion of chromosome 7 mixed with normal disomic cells. The first strategy was based on the amplification of two sequences, one on chromosome 7 and the other on chromosome 14. In the second strategy residual disease was assessed by the ratio between the two alleles of a bi-allelic marker, mapped on chromosome 7, measured with allele-specific assays. Precision and accuracy of the two approaches were tested by reference samples with nominal values of residual disease ranging from 2 to 95%. As expected the second strategy resulted in more precise and accurate monitoring within the range from 5 to 95%. Furthermore, this method may be applied to assess the number of dysplastic or neoplastic clones carrying any unbalanced chromosome changes. (*J Mol Diagn* 2006, 8:218–224; DOI: 10.2353/jmoldx.2006.050111)

The evaluation of residual disease is essential in the clinical management of patients with myeloproliferative and lymphoproliferative disorders in relation to chemotherapy and bone marrow transplantation procedures.¹ A significant proportion of hematological neoplastic and dysplastic disorders are associated with clonal chromosome changes that are usually monitored by conventional cytogenetics and fluorescent *in situ* hybridization (FISH). The reverse transcription of chimeric transcripts

and their amplification by polymerase chain reaction (PCR) may be applied only if a structural rearrangement has been detected. It is important to remember, however, that the most frequent anomalies found in acute myeloid leukemia and myelodysplastic syndrome are numerical.² In this case monitoring by conventional cytogenetics is feasible but on a limited number of cells in mitosis. A quantitatively more important result may be reached by performing FISH on interphase nuclei with chromosome-specific probes, usually those recognizing centromeric alphoid DNA.^{3,4} Several hundred nuclei may so be scored to detect the presence of cells with a number of signals that reveal the normal disomy or abnormal monosomy or trisomy. In this kind of assay, healthy individuals are used as controls to set a cutoff threshold to establish the lower level of detection of aneuploid cells.⁵ A definite evaluation of interphase-FISH precision and accuracy is not feasible because of a large number of technical variables that influence the data acquisition. Kibbelaar and colleagues⁶ made an intriguing effort to quantify small subpopulations of aneuploid cells by interphase-FISH and concluded that monosomy could be detected at a threshold level of 5% and trisomy at 1% when 400 nuclei are scored. In their study, Dreyling and colleagues⁷ accounted for a threshold of 7.5% to detect monosomic cells. This value is comparable to previous studies that described a threshold of 10 to 12%.^{8,9}

The application of molecular biological techniques in malignant hematological diseases, particularly leukemias, has led to a rapid increase in knowledge and deeper insight into the diagnosis and monitoring of the pathogenesis of these diseases. At the end of the 1990s, many studies reported successful retro-transcription and quantification by reverse transcriptase-quantitative PCR (RT-QPCR) of leukemia-specific fusion transcripts.^{10,11} This application of RT-QPCR proved to be reliable and sensitive: Mensink and colleagues¹¹ assessed the sensitivity of their assay and showed that as few as 10 copies

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of the BCR/ABL cDNA were still detectable. Furthermore, RT-QPCR is an easy and time-saving procedure, enabling frequent assessments of the minimal residual disease (MRD) by monitoring not only bone marrow specimens but also peripheral blood.^{12,13} An important limit is that RT-QPCR is applied only to cases characterized by fusion gene transcripts as consequence of translocations or other structural rearrangements.^{14–16} Boehm and colleagues¹⁷ reported the possibility to identify constitutive deletions or duplications of subtelomeric regions by real-time quantitative PCR (RQ-PCR) on genomic DNA, but their approach was not intended to quantify the number of cells carrying the anomalies because no normal cells were present in their samples. Here, we report two distinct strategies to use RQ-PCR on genomic DNA to quantify subpopulations of cells with a deletion of the long arm of chromosome 7. Monosomy of chromosome 7 or deletions of 7q are associated with many hematological disorders^{18,19} and have been used here as the experimental model with which to study the applicability of RQ-PCR to assess the number of dysplastic or neoplastic clones carrying any unbalanced chromosome changes.

Materials and Methods

Materials and Marker Selection

Human fibroblasts carrying a terminal deletion of chromosome 7, with karyotype 46,XY,del(7)(pter→q34), were purchased from the Human Coriell Cell Repository, Camden, NJ (repository number GM03240). They were originally obtained from a 7-day-old male with microcephaly and other congenital anomalies. Genomic DNA was isolated from the fibroblasts with the 7q deletion and from the peripheral blood of 10 healthy patients. A bi-allelic polymorphism mapping on 7q35 was selected from the Marshfield Center for Medical Genetics (Marshfield, WI) database of short insertion/deletion (marker name MID1064). The two alleles differ from each other in the insertion/deletion of four nucleotides and have frequencies of 0.55 and 0.44, respectively, within the European population. Therefore, the rate of heterozygosity is 50%. A homozygote for each of the two alleles of MID1064 and a heterozygote were selected from the healthy volunteers by direct sequencing. DNA extractions were performed by FlexiGene DNA kit (Qiagen Science, Hilden, Germany). Genomic DNA samples were accurately quantified using TaqMan RNase P control reagents (Applied Biosystems, Foster City, CA) according to the manufacturer's protocol and then diluted to 20 ng/μl with 10 mmol/L Tris buffer, pH 7.5; concentrations were then checked again. DNA samples of the two homozygote patients were used to establish optimal PCR conditions and to test the linearity and the efficiency of each assay. The DNA extracted from fibroblasts was diluted in the heterozygous DNA obtained from the healthy patient according to the following v/v percentages: 2%, 5%, 10%, 18%, 25%, 35%, 55%, 75%, 85%, and 95%. The DNA solutions used were equimolar, thus v/v dilutions correspond to DNA concentrations. Dilutions have been used

as reference samples with nominal MRD values of 2%, 5%, 10%, 18%, 25%, 35%, 55%, 75%, 85%, and 95%. Solutions were mixed at 4°C for 30 minutes and subsequently subdivided in single-use aliquots stored at –20°C.

Quantification

The subpopulation of fibroblasts with the 7q deletion was quantified by RQ-PCR following two alternative strategies. In both strategies the comparative Ct method²⁰ has been applied, and DNA extracted from a healthy patient was used as blank. In the first strategy, here named Chr versus Chr strategy, for chromosome versus chromosome, a sequence around MID1064 on 7q35 was used to target the chromosome 7, and a second sequence on chromosome 14 was used as a reference. The name Chr versus Chr was used to emphasize that this strategy is based on the relative quantification of two markers mapped on two different chromosomes. The difference between the threshold cycles (ΔCt) of these two sequences is linked to the ratio between the amount of undeleted chromosome 7 ($aCh7$) and chromosome 14 ($aCh14$): $aCh7/aCh14 = 2^{-(\Delta Ct)}$. The amounts of the two chromosomes are related to the amount of altered cells (ρ) and to the total amount of cells in the sample (τ) by this relation: $aCh7 = \rho \cdot 1 + (\tau - \rho) \cdot 2$ and $aCh14 = \tau \cdot 2$. The percentage of cells with the 7q deletion over the total amount of cells in the sample ($\rho/\tau \cdot 100$) corresponds to the MRD. MRD has therefore been extrapolated as a ΔCt function:

$$MRD = 200 \cdot (1 - 2^{-(\Delta Ct)})$$

The second strategy, here named two alleles strategy, is based on the bi-allelic marker MID 1064. RQ-PCR-specific assays targeting the two alleles were developed using allele-specific forward primers. This strategy may be applied only in samples from patients heterozygous for the polymorphism so that one of the two alleles appears in the DNA extracted from both normal and abnormal cells. The other allele marks specifically only normal cells. The ratio between the amount of normal cells (aNC) and the total amount of cells under analysis (aTC) is given by the following relation:

$$aNC/aTC = 2^{-\Delta Ct}$$

where ΔCt is the difference between the threshold cycles of the two alleles. The percentage of cells with the 7q deletion (MRD) is given by the difference between the total amount of cells in the sample (100%) and the percentage of normal cells. MRD has been extrapolated as a ΔCt function: $MRD = 100 \cdot (1 - 2^{-(\Delta Ct)})$. To normalize the sample under analysis with the blank, the ΔCt variable of both functions has been replaced by the $\Delta \Delta Ct$. $\Delta \Delta Ct$ is the difference between the average ΔCt of the sample $\overline{\Delta Ct}_{sample}$ and the average ΔCt of the blank $\overline{\Delta Ct}_{blank}$: $\Delta \Delta Ct = \overline{\Delta Ct}_{sample} - \overline{\Delta Ct}_{blank}$. The functions in this paper used to calculate MRD are therefore: $MRD = 200 \cdot (1 - 2^{-(\Delta \Delta Ct)})$ for the Chr versus Chr strategy and $MRD = 100 \cdot (1 - 2^{-(\Delta \Delta Ct)})$ for the two alleles strategy. Each ΔCt

Figure 1. Illustration of the forward primers and the common reverse primer and probe. In **square brackets**, the MID1064 polymorphism on the partial sequence of 7q35.

value is the mean of nine ΔC_t measurements and $\sigma_{\Delta C_t}$ is the corresponding SD. The SD of $\Delta\Delta C_t$ ($\sigma_{\Delta\Delta C_t}$) is given by the following formula:

$$\sigma_{\Delta\Delta C_t} = \sqrt{\sigma_{\Delta C_t \text{ sample}}^2 + \sigma_{\Delta C_t \text{ blank}}^2}^{19}$$

RQ-PCR Assays

Three real-time PCR assays were designed by Primer Express 2.0 (Applied Biosystems); details are shown in Figure 1. The first two assays (used in the two alleles strategy) use allele-specific forward primers to quantify the long or the short allele of the MID1064 polymorphism. The third assay (used in the Chr versus Chr strategy) targets chromosome 7 using a forward primer just outside the MID1064 polymorphic region. For each assay the same TaqMan Minor Groove Binder (MGB) probe and reverse primer have been used. The probe is 5'-labeled with 6-carboxyfluorescein (FAM) and purchased from Applied Biosystems. Amplifications were run in triplicate by ABI Prism 7000 SDS (Applied Biosystems). The reaction mixture contained 12.5 μ l of TaqMan Universal PCR master mix (Applied Biosystems), 900 nmol/L forward primer, 900 nmol/L reverse primer, 200 nmol/L probe, DNA (depending on the experiment), and nuclease-free water up to 25 μ l. PCR thermal profile was 2 minutes at 50°C followed by 10 minutes at 95°C and 40 amplification cycles (95°C for 15 seconds and 60°C for 60 seconds). The gene for the human ribonuclease P RNA component H1 (GenBank access number X16612) mapping to chromosome 14 was used as reference sequence in the Chr versus Chr strategy. This sequence was quantified by the TaqMan RNase P control reagents VIC (Applied Biosystems), following the same PCR conditions described for the other RQ-PCR assays. We choose this reference sequence because it is reported to be single copy²¹ and, thus, is directly linked to the amount of unaltered cells.

Linearity and Efficiency

The proportionality of the signal to the amount of DNA (linearity) was evaluated for each assay plotting the C_t values from series of samples (200, 20, 2, and 0.2 ng of homozygous DNA) versus the logarithm of the corresponding DNA amounts. Coefficients of determination (R^2) of the regression curves were calculated. PCR efficiencies were estimated by $E = 10^{(-1/b)} - 1$ ²² where b is the slope of the linear regression equation. R^2 and b

Figure 2. Plots of the MRD functions (**top**) and their derivatives (**bottom**). $\Delta\Delta C_t$ values range from 0 to 1 for the Chr versus Chr function and from 0 to $+\infty$ for the two alleles function.

parameters were estimated directly by the ABI Prism 7000 SDS analysis software (Applied Biosystems).

Accuracy and Precision

Limits (I_1, I_2) of the 95% confidence interval were calculated for the $\Delta\Delta C_t$ value of each reference sample (70 ng) by the following formula:

$$I_1, I_2 = \Delta\Delta C_t \pm t_{0.1} \cdot \sigma_{\Delta\Delta C_t} / \sqrt{N}$$

where N is the number of repetitions and $t_{0.05}$ the Student's t for the 5% probability at $N-1$ degrees of freedom. For each sample the $\Delta\Delta C_t$ value and its corresponding I_1, I_2 limits were introduced in the MRD function, obtaining the average calculated MRD value (μ_{best}) and its range of error at 95% probability (δ). The closeness of agreement between the nominal value of a reference sample (μ) and the corresponding μ_{best} (accuracy) has been assessed by their relative difference: relative difference = $100 \cdot (\mu_{\text{best}} - \mu) / \mu$. The degree of mutual agreement among MRD measurements of the same sample (precision) is inversely proportional to the corresponding δ . Furthermore μ_{best} versus μ plots were analyzed by regression analysis. Accuracy and precision of both strategies were estimated by using the same series of reference materials.

Results

Analysis of the Two Quantification Strategies

The MRD functions of the two strategies $\text{MRD} = 200 \cdot (1 - 2^{-(\Delta\Delta C_t)})$ for the Chr versus Chr strategy and $\text{MRD} = 100 \cdot (1 - 2^{-(\Delta\Delta C_t)})$ for the two alleles strategy) were plotted by Derive 6.0 (Texas Instruments Inc., Dallas, TX) and analyzed (Figure 2, top). Values of the inde-

Figure 3. Plots of Ct values of 100, 20, 2, and 0.2 ng of genomic DNA versus the logarithm of the corresponding DNA amount. For each plot the coefficient of determination (R^2), slope, and PCR efficiency are reported. Threshold was set at 0.1.

pendent variable (measured $\Delta\Delta Ct$ values) theoretically range from 0 to 1 for the Chr versus Chr strategy and from 0 to $+\infty$ for the two alleles strategy.

The limits (I_1, I_2) of the 95% confidence interval of probability of each $\Delta\Delta Ct$ value have been introduced in the MRD function to obtain the range of error of calculated MRD. Therefore, error is affected by the magnitude of the I_1 and I_2 limits and by the slope of the function. Theoretically, the Chr versus Chr strategy is less precise than the two alleles strategy because the slope of the first function is higher than the slope of the second, as shown by their derivatives (Figure 2, bottom). Furthermore, within each function, slopes decrease as $\Delta\Delta Ct$ values get higher; indeed measurements are expected to be more precise as the level of MRD increases.

Optimization of the Real-Time PCR Assays

PCR conditions of both the allele-specific assays used in the two alleles strategy and the assays used in the Chr versus Chr strategy have been optimized. Increasing concentrations of forward and reverse primers were tested independently up to 1200 nmol/L. For each assay, steeper amplifications were achieved using both primers at a final concentration of 900 nmol/L (data not shown). The specificity was confirmed by agarose gel electrophoresis and direct sequencing (data not shown). DNA samples extracted from the two MID1064 homozygotes were amplified by both allele-specific assays used in the two alleles strategy. Each sample provided detectable signals only when amplified by its own corresponding assay; nonspecific signals were still under the detection

threshold (threshold was set at 0.1) after 40 amplification cycles.

Linearity and Efficiency

To evaluate the linearity and the efficiency of each assay, samples containing 200, 20, 2, and 0.2 ng of genomic DNA were amplified (for the two allele-specific assays, DNA homozygote for the corresponding allele was used). Average Ct values were plotted versus the logarithm of the corresponding DNA amount. As shown in Figure 3 each assay is linear over three orders of magnitude, ranging from 200 to 0.2 ng of DNA, with efficiencies close to the average value of 95.8%.

Precision and Accuracy

The MRD value of each reference sample (70 ng) was determined from the measured value of $\Delta\Delta Ct$ following both the Chr versus Chr strategy and the two alleles strategy. For each calculated value the corresponding range of error at the 95% confidence interval was obtained from the SD of the measured $\Delta\Delta Ct$ ($\sigma_{\Delta\Delta Ct}$). The accuracy and the precision of quantifications were assessed by the relative difference between calculated and nominal values of MRD and by the corresponding ranges of error, respectively. Results are summarized in Figure 4A. Figure 4, B and C, show the plots and the regression analysis of the calculated versus nominal values of MRD for the two strategies. Performances were considered acceptable in the range from 18 to 95% for the Chr versus

Nominal MRD	"Chr vs. Chr strategy"				"Two alleles strategy"			
	Calculated MRD	Relative difference	$\sigma_{\Delta\Delta Ct}$	Range of Error	Calculated MRD	Relative difference	$\sigma_{\Delta\Delta Ct}$	Range of Error
2%	10.2%	410%	0.083	1.6-18.4 %	2.9%	45%	0.148	0-10.2 %
5%	10.4%	108%	0.102	0-20.4 %	4.5%	-10%	0.085	0.1-8.8 %
10%	18.8%	88%	0.143	4.5-32 %	7%	-29%	0.109	1.6-12.3 %
18%	15.3%	-15%	0.173	0.7-28.7 %	18.1%	0.6%	0.115	12.9-22.9 %
25%	29.3%	17.2%	0.108	19.2-38.9 %	23%	-7.6%	0.076	19.9-26.1 %
35%	35.2%	0.6%	0.083	27.7-42.3 %	28.8%	-16.6%	0.147	23.5-34.5 %
55%	56.8%	3.3%	0.098	49.1-64.1 %	54.4%	-1.5%	0.133	50.9-57.3 %
75%	69.7%	-7.1%	0.106	62.1-76.8 %	73%	-2.7%	0.112	71.4-74.6 %
A 85%	79.9%	-6%	0.096	73.7-85.9 %	84.4%	-1.1%	0.141	82.9-85.2 %
95%	94.9%	-0.1%	0.101	89.1-100 %	93.8%	-0.1%	0.107	94.6-95.2 %

Figure 4. Calculated MRD values. **A:** The relative difference between nominal and calculated MRD values, SD of measured $\Delta\Delta Ct$ ($\sigma_{\Delta\Delta Ct}$), and range of error at the 95% confidence interval are reported for each reference sample. Threshold was set at 0.1. Plots of calculated versus nominal values of MRD are reported in **B** and **C** for the Chr versus Chr strategy and the two alleles strategy, respectively. Stippled lines enclose the range of error. Slope and coefficient of determination (R^2) of the plots are reported.

Chr strategy and from 5 to 95% for the two alleles strategy. As expected the second strategy is more precise. Furthermore, quantifications of MRD are more precise as the value for the MRD gets higher.

Discussion

Cytogenetic and molecular prognostic tools are critical for the therapeutic decisions in the treatment of hematological malignancies. RQ-PCR is a time-saving, cost-effective, and accurate technique, but it has been applied only to target fusion products. In the present study we aimed to apply the RQ-PCR to those cases characterized by unbalanced chromosome changes, such as monosomies and deletions. We considered two different strategies: the Chr versus Chr and the two alleles strategies. For both approaches PCR protocols were established and optimized. Suitable MRD functions were derived by adapting the comparative Ct method described by Livak and Schmitteng.²⁰ To ensure that these functions could be properly applied, the efficiency and linearity of each PCR assay were tested. As shown in Figure 3, the efficiencies were approximately equal (close to the average value of 95.8%), and each assay was linear at least more than a 1000-fold range between 200 ng and 0.2 ng of DNA. Taking advantage of the comparative Ct method,

the assays we developed do not require standard curves, resulting in additional time and cost benefits. A series of samples with nominal MRD values ranging from 2 to 95% have been prepared and used as reference samples to test the accuracy and the precision of both strategies, thus also avoiding the variability because of the use of different standard series. It should be noted that the aim of this study was to explore the applicability of the RQ-PCR to those cases of unbalanced changes that usually cannot be investigated by RQ-PCR. Thus, problems such as different DNA extraction protocols, different specimen sources, and inhibition were not considered. The MRD value of each reference sample was calculated (Figure 4) and plotted against the corresponding nominal value. The regression curve of the Chr versus Chr strategy had a slope of 0.8915 and a coefficient of determination (R^2) of 0.986. Slope and R^2 values of the two alleles strategy were 1.0004 and 0.997, respectively. The precision of each calculated MRD value was assessed by the corresponding range of error at the 95% confidence interval (Figure 4).

A standard procedure to assess the accuracy and precision limits of a real-time PCR assay is not feasible because performance requirements must be adapted, case-by-case, to the goal for which the assay has been planned. The Community Reference Laboratory for GM

Food and Feed of the European Union proposes that the accuracy of a real-time PCR assay should be $\pm 25\%$ of the accepted reference value. In this study, the range of MRD concentrations that the two strategies could be applied within (dynamic range) was characterized by allowing for the above-mentioned guidelines and the specific clinical and biological implications of the data reported in Figure 4. The dynamic range of the Chr versus Chr strategy has been restricted to the interval of concentrations from 18 to 95% because the accuracy and precision of the reference samples at the 2%, 5%, and 10% of MRD were not considered satisfactory. The 2% reference sample was quantified through the two alleles strategy with an acceptable level of accuracy, but its range of error (from 0 to 10.2%) demonstrated that the method could not significantly discriminate between a sample that contains as few as 2% monosomic cells and a blank sample (containing no monosomic cells). One-way analysis of variance (data not shown) demonstrated significant differences between the ΔCt values of the blank sample and the reference samples containing 5% or more monosomic cells. For these reasons the dynamic range of the two alleles strategy was established from 5 to 95%.

The range of error of each calculated MRD value is affected by three variables: the slope of the function used to calculate the MRD, the SD of the measured $\Delta\Delta Ct$ ($\sigma_{\Delta\Delta Ct}$), and the number of measurements. The last two variables do not explain the different levels of precision reported in Figure 4; in fact each sample was measured nine times and we recorded similar values of $\sigma_{\Delta\Delta Ct}$, regardless of the sample analyzed or the strategy applied. The different values of the slope of the function used deeply influenced the level of precision. As expected from the study of the two MRD functions (Figure 2), the Chr versus Chr strategy is less precise than the two alleles strategy. Furthermore, both approaches are more precise as the value of the MRD gets higher because the slope of the functions decreases.

In conclusion, we show clearly that our method is relevant for clinical purposes and in particular that our two alleles strategy should be the strategy of choice. In this study we used the MID1064 polymorphism as a model, but a list of polymorphisms showing the same level of heterozygosity have been identified throughout the human chromosomes (a list is reported within the Marshfield Centre for Medical Genetics database of short insertion/deletion (available at <http://research.marshfieldclinic.org/genetics/indels/default.asp>), allowing the use of the two alleles strategy for any chromosome unbalanced anomaly. Furthermore, RQ-PCR results are obtained faster and more easily than conventional techniques, allowing frequent measurements and data handling by statistical tools. Butturini and colleagues²³ demonstrated that the major obstacles to assay accuracy are an inadequate sampling and, only marginally, the sensitivity of the assay used. The use of our PCR method enables the analysis of a larger number of cells than can be assessed with cytogenetics. In fact, the number of mitoses that can be surveyed by conventional chromosome analysis is routinely in the range of 10s to 100s, and

up to ~1000 nuclei can be cored by FISH. The analysis of a sample of 70 ng of DNA by RQ-PCR (as we did in our study) refers to ~9500 cells (according to the human genome size reported by the Technical University of Denmark, <http://www.cbs.dtu.dk/databases/DOGS/>).

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