

## Short Communication

# The Use of Comparative Duplex PCR in Monitoring of Patients with Non-Hodgkin's Lymphoma and Chronic Lymphocytic Leukaemia

( duplex PCR / quantitative molecular monitoring / clinical significance )

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**Abstract.** Various quantitative PCR approaches have been utilized during the last years to provide information about the treatment efficacy and the risk of recurrent disease in haematological malignancies. Apart from the frequently used real-time PCR, cost-saving modified standard PCR methods may be applied as well. This report evaluates the utility of the end-point comparative duplex PCR. We have used this method for monitoring of 35 patients with either NHL or CLL and observed a good correlation between quantitative molecular results and clinical outcome. There was also an agreement between comparative duplex PCR and real-time PCR in patients who were monitored by both methods. We therefore believe that use of this technique should be strongly considered instead of simple qualitative detection in monitoring of therapeutic outcome in NHL or CLL patients.

The polymerase chain reaction (PCR) has become an essential tool for molecular biologists in haematological oncology. Currently, attention is namely drawn to the exploitation of quantitative estimation. Quantitative data can be obtained quite comfortably by real-time PCR. This, however, is also the most expensive method. Thus, several cost-saving standard PCR methods have been established. We have described standard

comparative duplex PCR employing co-amplification of clone-specific markers and internal standards. We then evaluated relative quantitative changes of specific disease markers in consecutive DNA samples by gel densitometry. It is essential that duplex PCR is optimized so that amplicons of all samples are compared in sub-plateau phase of PCR. Using this approach, we have previously suggested co-amplification of the complementarity determining region (CDR3) of the immunoglobulin heavy chain gene (*IgH*) as the disease marker and a segment of the *Hras 1* gene containing codon 61 (ras) as the internal standard for monitoring B-lymphoproliferative disorders (Slavickova et al., 1999, 2000).

In the current study, we show other sets of disease-specific and normalizing markers, the agreement between results of comparative duplex and real-time PCR, and the applicability of the methodology in molecular monitoring of patients with non-Hodgkin lymphoma (NHL) and chronic lymphocytic leukaemia (CLL).

## Material and Methods

### Patient samples

Thirty-five patients were followed by quantitative PCR methodology. These patients were treated with either conventional therapy, rituximab (MabThera<sup>R</sup>, chimaeric anti-CD20 monoclonal antibody, F. Hoffmann-La Roche Ltd., Basel, Switzerland) in combination with fludarabine and cyclophosphamide, and several patients underwent high-dose therapy with autologous stem cell rescue. The group consisted of 23 chronic lymphocytic leukaemias, five mantle cell lymphomas, four follicular lymphomas and three diffuse large B-cell lymphomas. The diagnosis was established using clinical criteria, histology, histochemistry and immunophenotyping analysis according to the modified REAL classification. In all patients the clonal

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Abbreviations: CDR3 – the third complementarity determining region of the hypervariable domain of immunoglobulin heavy chain genes, CLL – chronic lymphocytic leukaemia, *IgH* – immunoglobulin heavy chain gene, mbr – the major breakpoint region of the *bcl-2* gene, mtc – major translocation cluster region of the *bcl-1* gene, NHL – non-Hodgkin lymphoma, PCR – polymerase chain reaction.

rearrangement of complementarity determining region 3 (CDR3) of the immunoglobulin heavy chain gene (*IgH*) was monitored. In addition, the interchromosomal translocation t(14;18-locus mbr) was used in three patients and the t(11;14) in two patients.

DNA was isolated from bone marrow aspirates and peripheral blood by the "salting out" procedure (Miller et al., 1988), dissolved in TE and stored at 4°C until use.

#### *Principle of comparative duplex PCR, evaluation of results*

Serial dilutions of consecutive DNA samples were submitted to the same duplex PCR. The primer sets 5' AGA CGT GCC TGT TGG ACA TC 3' with 5' CGC ATG TAC TGG TCC CGC AT 3' or 5' CAA CTT CAT CCA CGT TCA CC 3' with 5' GAA GAG CCA AGG ACA GGT AC 3' were used for *ras* and  $\beta$ -globin, respectively, as normalizing markers in co-amplification with the primers for disease-specific markers. The Jh consensus primer 5' ACC TGA GGA GAC GGT GAC CAG GGT 3' and 5' TAT GGT GGT TTG ACC TTT AG 3' were used for detection of the t(14;18-mbr) translocation. The Jh consensus primer and 5'ACA CGG CC/TG/C TGT ATT ACT GT 3' were used for the amplification of clonal CDR3. For detection of the t(11-mtc; 14) translocation the Jh consensus was then used with either 5' ATA TTC GGT TAG ACT GTG ATT AGC 3' or 5'CTA CTG AAG GAC TTG TGG GTT GCT 3'. All primers were products of Genosys Biotechnologies, Inc. (The Woodlands, TX). Amplification products were analysed on 10% polyacrylamide gels stained with ethidium bromide (Fig. 1). The amount of PCR products was determined by gel densitometry using gel documentation system (Ultra-Lum Inc., Claremont, CA) and gel analysis software (Media Cybernetics, Silver Spring, MD), as described earlier (Slavickova et al., 2000). The values obtained from sub-plateau phase of both amplifications were analysed further, that is, the amount of the specific product was related to the respective amount of the normalizer and the differences of specific markers in two or more successive samples (found either directly or by extrapolation at identical amounts of normalizing products) were used in the assessment of molecular regression (decrease of the specific marker in the later of two consecutive DNA samples) and molecular progression or relapse (increase of the specific marker). The molecular results obtained from stored DNA samples were then correlated with the corresponding clinical observations retrospectively.

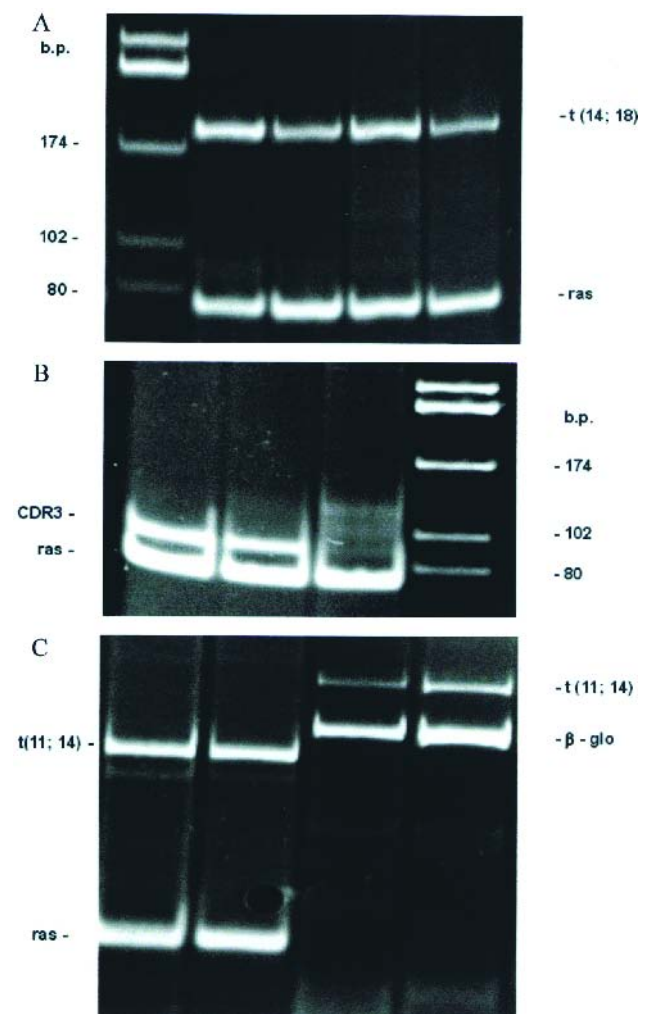
#### *Real-time PCR*

The real-time PCR quantification was performed using the LightCycler and the t(14;18-mbr) Quantification Kit of Roche Molecular Biochemicals exactly as recommended by the manufacturer.

## Results and Discussion

### *Correlation of clinical outcome and quantitative molecular monitoring*

We have observed a good correlation between molecular follow-up and clinical outcome in a total of hundred intervals (ranging from 1 to 18 months) when a molecular regression/progression (quantitative decrease/increase of the clonospecific marker) was in accordance with the clinical state (regression/ progression). In a group of 17 patients with clinically stable disease, but increasing markers as detected by our quantitative molecular method, ten patients developed disease progression and the remaining seven relapse. In seven of 33 cases (21%) of clinical complete remissions the molecular residual disease was still detected. Besides, in available 14 cases of paired samples of bone marrow and peripheral blood no significant difference



*Fig. 1.* 10% PAGE of duplex PCR. The products of duplex PCR for *ras* as a normalizing marker and the t(14;18-mbr) translocation as the disease-specific marker (A); the products of duplex PCR for *ras* and the clonal CDR3 (B); the products of duplex PCR for *ras* and t(11-mtc;14) translocation (C left) and  $\beta$ -globin and the t(11-mtc;14) translocation (C right).

was observed, indicating that the molecular evaluation of peripheral blood may also be informative, where it is applicable.

We have also shown other suitable combinations of disease-specific and internal standards (Fig. 1). Furthermore, the agreement between comparative duplex and real-time PCR was demonstrated (Fig. 2). Interestingly, while a good correlation of molecular results in general (increase or decrease) was observed, the methods display different sensitivities as shown in Fig. 2. That is, the lower sensitivity of IgH/ras PCR was sufficient for describing kinetics of the disease as well as the t(14;18)-real time or duplex PCR for mbr/ras. Similar results were already observed in monitoring NHL patients after stem cell transplantation (Mitterbauer et al., 2001).

Summarized, it seems that the comparative duplex PCR may be a method of choice for monitoring NHL and CLL patients as it may provide prognostic informa-

tion. The predictive value of this method depends on the frequency of sample taking as well as on the assay sensitivity. The sensitivity should be checked in any single assay (Dölken, 2001). Despite the increasing pool of data on molecular monitoring and its prognostic role in lymphoproliferative diseases the issue of quantitative molecular monitoring has not been settled in a large clinical study and neither guidelines nor standards were established. Thus it is suggested that PCR amplification techniques and their correlation with clinical outcome need further observation and should remain investigational for the present (Gribben, 2002). This is in contrast with chronic myelogenous leukaemia, where it was already suggested that quantitative molecular monitoring of leukaemic cells might be helpful in individualizing therapeutic strategies (Lion, 1994).

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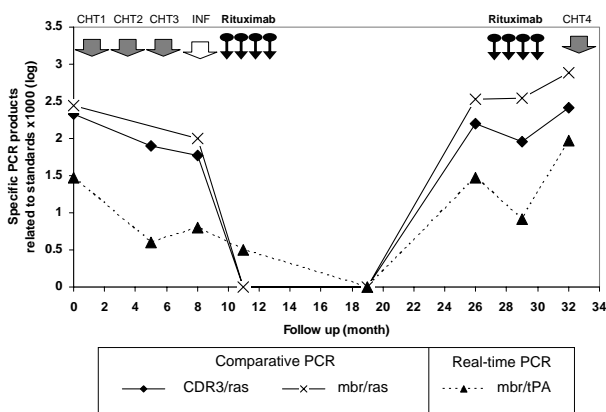


Fig. 2. Monitoring of treatment impact by comparative duplex and real-time PCR. The patient with follicular lymphoma treated with chemotherapy (CHT), interferon  $\alpha$  (INF) and rituximab achieved PCR negativity at clinical complete remission. The negativity, however, was disproved by hybridization with a probing sequence from the mbr region labelled with digoxigenin. The disease progressed after several months, further therapeutic effort failed and the molecular quantification was in accordance with this.