Journal of Molecular Diagnostics, Vol. 8, No. 1, February 2006

DOI: 10.2353/jmoldx.2006.040383

Consultations in Molecular Diagnostics

Limitations and Practical Procedure in *BclII-Ig Heavy Chain* Gene Rearrangement Real-Time Quantitative Polymerase Chain Reaction

Barbara Dessars,* Pierre Heimann,*
Stéphane Swillens,† and Hakim El Housni*

From the Department of Genetics, Erasme Hospital; and Institute of Interdisciplinary Research,† Free University of Brussels, Brussels, Belgium*

Follicular lymphoma is characterized by the t(14;18)(q32;q21) translocation, which juxtaposes *Ig heavy chain* gene (*IgH*) sequences with the *Bcl*II gene. Several publications have highlighted the importance of molecular follow-up in follicular lymphoma, demonstrating that the detection of cells bearing the *Bcl*II-*IgH* rearrangement by real-time quantitative polymerase chain reaction (RQ-PCR) can anticipate a clinical relapse. In this context, we developed a *Bcl*II-*IgH* RQ-PCR. We began with SYBR Green I detection technology but observed that this system does not allow an accurate measurement of the tumor load when working with genomic DNA. While we were designing the assay using Taqman technology, Moppett et al (Moppett J, van der Velden VHJ, Wijkhuijs AJM, Hancock J, van Dongen JJM, Goulden N: Inhibition affecting RQ-PCR-based assessment of minimal residual disease in acute lymphoblastic leukemia: reversal by addition of bovine serum albumin. Leukemia 2003, 17:268–270) reported PCR inhibition problems in around 15% of blood and bone marrow samples, affecting the DNA quantification and thus the assessment of minimal residual disease. They demonstrated that this PCR inhibition could be partially resolved by adding nonacetylated bovine serum albumin. In our studies, we observed the same phenomenon in a single follicular lymphoma case and extended our study to other available cases. As a result, we suggest a new RQ-PCR procedure that is based on Taqman probe technology and that takes into account the PCR inhibition problems, making this assay more reliable in a routine molecular laboratory. ***(J Mol Diagn 2006, 8:133–136; DOI: 10.2353/jmoldx.2006.040383)***

Follicular lymphoma (FL) is characterized by the juxtaposition of *Ig heavy chain* gene (*IgH*) sequences with *Bcl*II gene via the t(14;18)(q32;q21) translocation, which occurs in greater than 80% of cases.[1,2] The persistence of cells bearing the *Bcl*II-*IgH* rearrangement, which can be detected by real-time quantitative polymerase chain reaction (RQ-PCR), can anticipate a clinical relapse. As such, molecular follow-up is recommended in FL.[3] Therefore, we sought to develop a *Bcl*II-*IgH* RQ-PCR using SYBR Green I detection technology. However, we observed that this system does not accurately measure tumor load when working with genomic DNA. Moppett et al[4] have reported that about 15% of the blood and bone marrow samples examined by their lab exhibit PCR inhibition, which affects DNA quantification and assessment of minimal residual disease (MRD). They were partially able to resolve this PCR inhibition by addition of nonacetylated bovine serum albumin (BSA). We observed similar inhibition in a single FL case (outlined below) and extended our study to other available cases. As a result, we have developed a new procedure that improves the reliability of this assay in a routine molecular laboratory.

Case Reports

A 70-year-old male patient presented with an asymptomatic left inguinal mass of 10-cm length. Surgical examination disclosed several lymph nodes that were resected for pathological investigation. The histological findings were diagnostic of a diffuse large B-cell lymphoma arising from a follicular lymphoma. Bone marrow aspirate was normal on morphological grounds. Southern blot analysis was performed on fresh lymphomatous tissue, with the use of the S45/23 probe screening the *MBR* cluster (a generous gift from Dr. Bastard), demonstrating a *Bcl*II rearrangement. A *Bcl*II *MBR-IgH* PCR analysis was subsequently performed with specific *Bcl*II *MBR* and *IgH*

Accepted for publication August 16, 2005.

Address reprint requests to Barbara Dessars, Department of Genetics, Erasme Hospital, 808 Lennikstreet, 1070 Brussels, Belgium. E-mail: Bdessars@ulb.ac.be.

primers (see below) to obtain a sensitive molecular marker for quantitative follow-up. The patient was then treated with six 3-week cycles of Rituximab and CHOP (adriamycin, cyclophosphamide, vincristine and prednisone) regimen. The evolution was favorable, and at present, the patient is alive and well.

Molecular Analysis

Five hundred nanograms of genomic DNA (corresponding to 83,333 diploid genomes) was extracted by a standard phenol/chloroform method from a lymphomatous lymph node. This DNA was subjected to 40 cycles of PCR amplification using the ABI PRISM 7700 Sequence Detection System (Applied Biosystems, Foster City, CA). The PCR assay was performed using *MBR* and *IgH* primers and *MBR* probe according to Luthra et al[5] (*MBR*, 5′-ttagagagttgctttacgtggcc-3′; *IgH*, 5′-actcacctgaggagacggtgac-3′; *MBR* probe, 5′-tttcaacacagacccaccccagagcc-3′), at the final concentration of 300 and 200 nmol/L for primers and probe, respectively, and in a reaction mixture of 25 μl including the Taqman Universal PCR Master Mix (Applied Biosystems). The amplification protocol was as follows: initial denaturation of 10 minutes at 95°C followed by 40 cycles of 15-second denaturation at 95°C and 1-minute annealing/extension at 60°C.

The qualitative sensitivity we obtained in our follicular lymphoma cases was very similar to that described by Luthra et al,[5] 56% (18 cases of 32) versus 54% (13 cases of 24), respectively. A PCR assay was also designed targeting the *ABL* gene (see below), with the use of forward (5′-gctagtggtggttgcaggaga-3′) and reverse (5′-actcccttagaaacatgcagttgt-3′) primers and an *ABL* probe (5′-FAM-tggtaggtcaaaccgcaattcccagattt-TAMRA-3′). The PCR conditions as well as the amplification protocol were the same as for *Bcl*II *MBR-IgH* PCR (see above).

Theoretically, a difference of cycle threshold (Ct) values (ΔCt) of 3.3 should be observed between two 10-fold dilutions for a PCR showing 100% efficiency. In practice, a ΔCt ≥3 is acceptable.[6] In our PCR design, the equation curve obtained by diluting the *MBR-IgH* cell line, DoHH2,[7] (600 down to 0.06 ng diluted in normal DNA) in duplicates and in three independent assays was $Y = -3.4934 \times \text{Log}_{10}(X) + 39.057$ (where Y is the $Ct_{MBR\text{-}IgH}$ and X is the number of cells; $r^2 = 0.99$) (data not shown).

Regarding our patient, an amplification plot for *MBR-IgH* rearrangement was demonstrated with a Ct value of 26.13 (threshold, 0.1). To rule out a putative PCR inhibition phenomenon as described by Moppett et al,[4] a subsequent PCR reaction was performed using the same DNA quantity (500 ng), as well as 10- and 100-fold dilutions, with and without the addition of 0.04% nonacetylated BSA (Biolabs).

Results and Discussion

In our patient, we observed in two independent assays a strong *Bcl*II *MBR-IgH* PCR inhibition with a DNA quantity of 500 ng as demonstrated by a ΔCt between 50 and 500 ng (ΔCt_{50-500}) of 1.82 (data not shown). This inhibition was partially resolved with addition of 0.04% BSA ($\Delta Ct_{50BSA\text{-}500BSA} = 2.41$) and, furthermore, disappeared when the DNA quantity used was limited to 50 ng ($\Delta Ct_{5-50} = 3.1$).

These results prompted us to verify the possible presence of inhibitors in DNA extracted from 21 snap-frozen benign lymphoid hyperplasia cases. The *ABL* gene was used as an internal reference to assess the amount of amplifiable target DNA, hence the number of detectable cells in a given sample. A large spectrum of Ct values for the *ABL* gene was obtained when identical amounts (500 ng based on absorbance at 260 nm measurement) of target DNA extracted from our 21 samples were used. The 99% confidence interval of the Ct_{ABL} was 19.7 to 24.5 (data not shown). Because an identical amount of DNA was loaded for each sample, this broad range of Ct

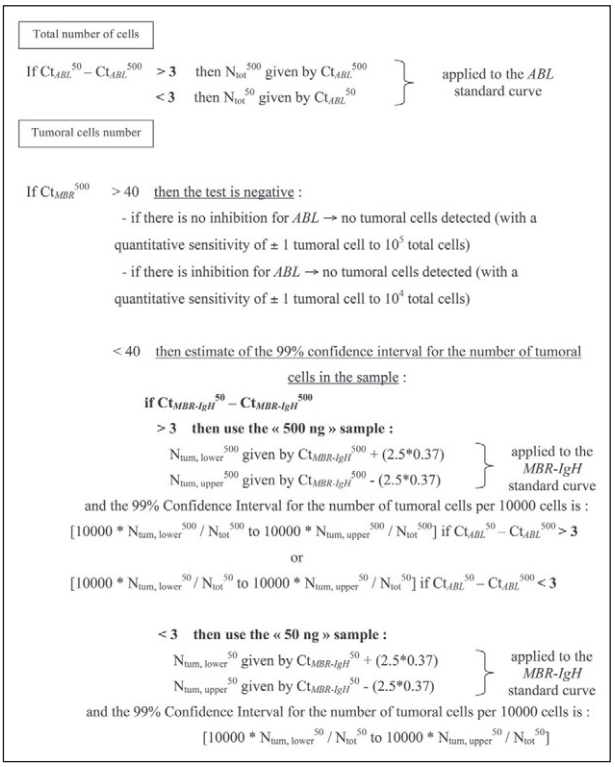

Figure 1. Algorithm for determining DNA quantification and assessment of MRD. This algorithm aims to produce reliable quantitative results of the MRD by considering parameters such as PCR inhibition. For each sample, four PCR reactions were performed, leading to *ABL* and *MBR-IgH* quantifications with either 500 or 50 ng of loaded genomic DNA. Thus, from these PCR reactions, four Ct values are estimated: Ct_{ABL}^{500}, Ct_{ABL}^{50}, $Ct_{MBR\text{-}IgH}^{500}$, and $Ct_{MBR\text{-}IgH}^{50}$. The number of total detectable cells in a given sample (N_{tot}^{500} and N_{tot}^{50}) is obtained by applying the corresponding Ct_{ABL}^{500} and Ct_{ABL}^{50} values to the *ABL* standard curve. If the difference ($Ct_{ABL}^{50} - Ct_{ABL}^{500}$) is less than 3, then only the 50-ng DNA sample will be used to avoid the detrimental effects of PCR inhibition. If $Ct_{MBR\text{-}IgH}^{500}$ and $Ct_{MBR\text{-}IgH}^{50}$ are >40, then the test is negative, according to the sensitivity of the assay to putative inhibition in the PCR reactions (quantitative sensitivity of ±1 tumor cell to 10^5 if inhibition is present or 10^4 if inhibition is absent). For a given DNA content, if $Ct_{MBR\text{-}IgH}$ <40, the range of tumor cells is extrapolated from the 99% confidence interval of the $Ct_{MBR\text{-}IgH}$ obtained experimentally: the $Ct_{MBR\text{-}IgH}$ ± (2.5 × 0.37) values, where 0.37 is the average SD observed in our case, are applied to the *MBR-IgH* standard curve by using the equation $N_{tum} = 10^{(39.057\ -\ Ct)/3.4934}$. Thus, $N_{tum,\,lower}^{500\text{ or }50}$ and $N_{tum,\,upper}^{500\text{ or }50}$ correspond to the lower and upper limit values of tumor cell numbers for either 500 or 50 ng of loaded genomic DNA. The final result is then expressed as a range of tumor cell numbers per 10,000 cells.

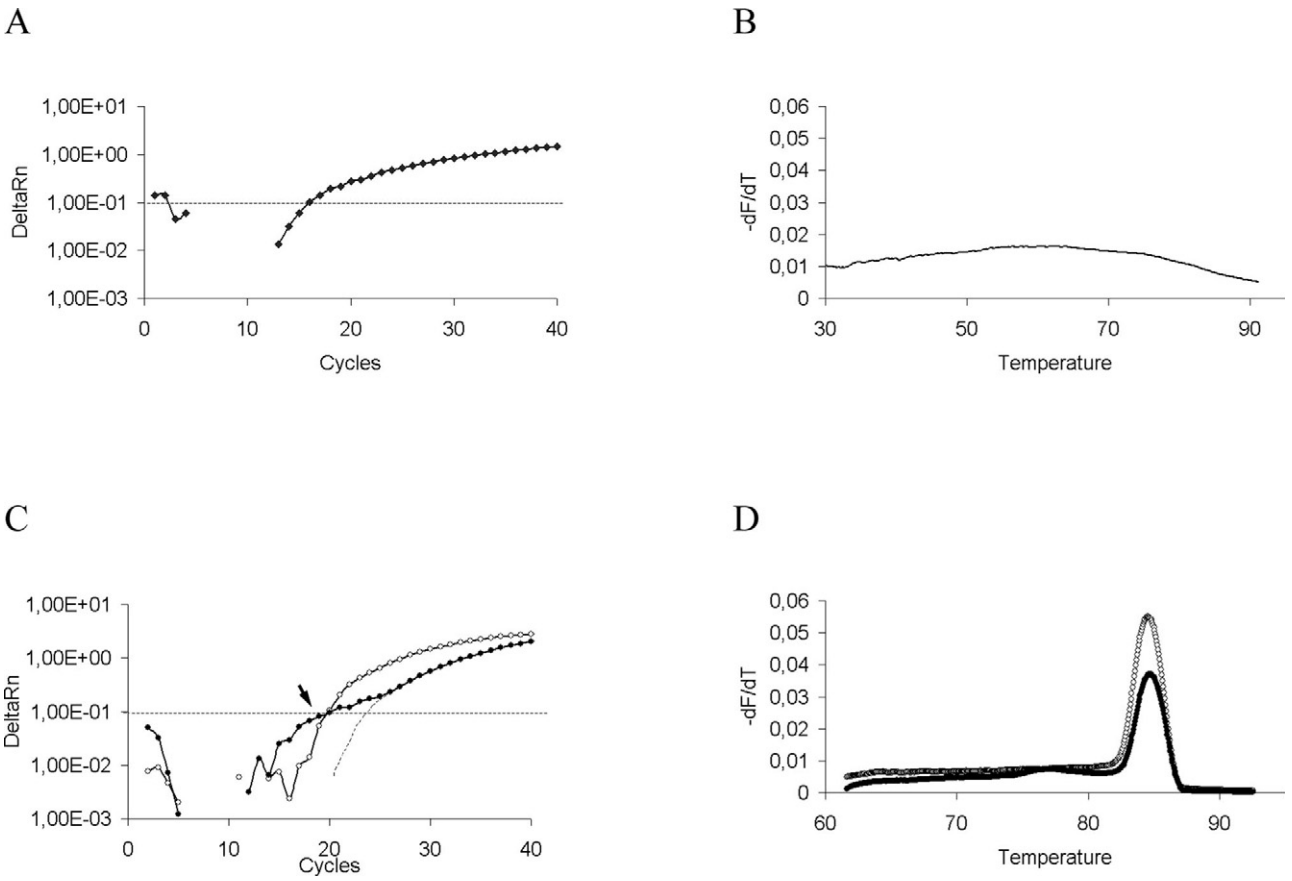

Figure 2. RQ-PCR for *MBR-IgH* rearrangement using SYBR Green I detection. **A:** Amplification curve observed with RQ-PCR using SYBR Green I detection when the PCR mix buffer is added to 500 ng of genomic DNA, with or without primers. **B:** Corresponding melting curve showing a flattened profile (see text). **C:** Amplification curves obtained from pure (○) and 10^{-2} diluted (●) DoHH2 cell line DNA in normal DNA. Each sample was tested in duplicate. The **horizontal dotted line** represents the threshold. Although the amplification plot obtained with the pure cell line shows a classical profile, the curve generated with the 10^{-2} diluted cell line displays a "bumpy profile," most likely due to genomic DNA autoamplification (see **arrow**). In the absence of this artifact, the ΔCt between two subsequent 10-fold dilutions should be around 3.3 (see the **dotted line**, which is extrapolated from the amplification plot generated by the 10^{-2} diluted cell line). As observed in this figure, the Ct value will therefore be altered by this artifact, leading to an incorrect estimate of the tumor cell number. **D:** Corresponding melting curves. As observed, a specific melting curve is always obtained for a 10^{-2} dilution (as well as for a 10^{-3} dilution; data not shown).

values should be due to PCR inhibitors. In keeping with this hypothesis, the inclusion of nonacetylated BSA (Biolabs) in the reaction mixture greatly reduced the 99% confidence interval of the Ct_{ABL} (20.8 to 22.8, data not shown). However, as shown in our reported case, adding nonacetylated BSA is not always sufficient to prevent completely the inhibition.

A second source of potential error in quantification results from the fact that PCR efficacy is known to depend on the size and/or the sequence of the template DNA. In molecular oncology laboratories, RQ-PCR is usually mostly used to quantify fusion transcripts displaying the same sequence and size in all patients. In *Bcl*II-*IgH* rearrangements, a Ct value could vary from sample to sample despite an identical starting DNA amount because both the size and sequence of the *Bcl*II-*IgH* rearrangement may vary among different patients. For this reason, we believe it would be better to report the results as a range of tumor cells relative to the whole detectable cell population present in the PCR reaction instead of an inaccurate single value. A set of three dilution curves derived from the DoHH2 cell line was then tested in duplicate (from 600 down to 0.06 ng, diluted in normal DNA), to define a standard curve as well as an average SD of the $Ct_{MBR\text{-}IgH}$ values (data not shown). The number of lymphoma cells was then estimated for each sample, based on the 99% confidence interval calculated from the experimental $Ct_{MBR\text{-}IgH}$ value ± (2.5 × the average SD) (legend of Figure 1).

Regarding the putative PCR inhibition, the *ABL* gene was used to normalize the lymphoma cell number to the amount of amplifiable cells in the sample. Two different DNA samples were then used to establish a set of five dilution curves, again ranging from 600 down to 0.06 ng of DNA (corresponding to 10^5 down to 10 normal cells). An ABL mean standard curve was generated from the different plots derived from the five dilution curves (data not shown). On the basis of the standard curve equation $Y = -3.6114 \times \mathrm{Log}_{10}(X) + 38.632$ (where Y is the Ct_{ABL} and X is the number of cells), the amount of cells with amplifiable target DNA can then be determined from the Ct_{ABL} obtained experimentally, as $X = 10^{(38.632 - Ct_{ABL})/3.6114}$ cells.

This strategy allowed us to establish and propose an algorithm (described in Figure 1) that can be routinely applied to give reliable quantitative assessments of the MRD. To disclose potential inhibition phenomena, two

DNA quantities per sample were used: 50 and 500 ng. Both DNA amounts were chosen to ensure sufficient sensitivity for the MRD detection as well as to control the problems of inhibition: 500 ng is the minimum required to obtain a sensitivity of one tumor cell on 10^5 total cells (loading 500 ng corresponds to 83,333 diploid genomes), whereas a 10-fold dilution should significantly reduce the concentration of potential inhibitors.

A parallel study was also performed using the cheaper SYBR Green I detection technology. However, we observed an amazing atypical amplification curve generated with 500 ng of genomic DNA from healthy patients even in the absence of primers (Figure 2A). The corresponding melting curve showed a flattened profile (Figure 2B), probably due to accumulation of PCR products of different sizes and sequences, generated by hybridization between repetitive sequences scattered throughout the genome or between small degraded DNA fragments (explaining why no primers were needed to generate such an amplification profile). This spurious amplification curve persisted when decreasing the amount of target DNA down to 100 ng (data not shown). We did not test less than 100 ng (corresponding to 16,666 diploid genomes) because this DNA amount is the minimum requirement for achieving a quantitative sensitivity of at least one tumor cell among 10^3 total cells. This feature was not observed with the pure DoHH2 cell line but was detected with low dilution (one tumor cell among 10^2 total cells) of this cell line DNA in normal DNA (Figure 2C). This artifact did not hinder the detection of one tumor cell among a total of 1000 cells because the melting curve obtained from this dilution could be superimposed on the one observed with the diagnostic sample (Figure 2D). However, the estimated Ct value did not fit the starting amount of tumor cells present in a tube (Figure 2C), rendering this approach unusable.

In conclusion, the SYBR Green I detection method should be avoided for quantification of genomic DNA when working with samples containing little target sequences. This limitation can be circumvented by using the Taqman probe technology. However, even if the real-time quantitative PCR offers clear advantages in the molecular follow-up of patients, it should be remembered that PCR inhibition can give rise to inaccurate quantitative results. For this reason, we have proposed an algorithm aimed at producing reliable quantitative assessment of the MRD with respect to putative PCR inhibition problems.

References

1. Godon A, Moreau A, Talmant P, Baranger-Papot L, Geneviève F, Milpied N, Zandecki M, Avet-Loiseau H: Is t(14;18)(q32;q21) a constant finding in follicular lymphoma? An interphase FISH study on 63 patients. Leukemia 2003, 17:255–259
2. Cleary ML, Galili N, Sklar J: Detection of a second t(14;18) breakpoint cluster region in human follicular lymphomas. J Exp Med 1986, 164:315–320
3. Lopez-Guillermo A, Cabanillas F, McLaughlin P, Smith T, Hagemeister F, Rodriguez MA, Romaguera JE, Younes A, Sarris AH, Preti HA, Pugh W, Lee MS: The clinical significance of molecular response in indolent follicular lymphomas. Blood 1998, 91:2955–2960
4. Moppett J, van der Velden VHJ, Wijkhuijs AJM, Hancock J, van Dongen JJM, Goulden N: Inhibition affecting RQ-PCR-based assessment of minimal residual disease in acute lymphoblastic leukemia: reversal by addition of bovine serum albumin. Leukemia 2003, 17:268–270
5. Luthra R, McBride JA, Cabanillas F, Sarris A: Novel 5′ exonuclease-based real-time PCR assay for the detection of t(14;18)(q32;q21) in patients with follicular lymphoma. Am J Pathol 1998, 153:63–68
6. van der Velden VHJ, Hochhaus A, Cazzaniga G, Szczepanski T, Gabert J, van Dongen JJM: Detection of minimal residual disease in hematologic malignancies by real-time quantitative PCR: principles, approaches, and laboratory aspects. Leukemia 2003, 17:1013–1034
7. Kluin-Nelemans HC, Limpens J, Meerabux J, Beverstock GC, Jansen JH, de Jong D, Kluin PM: A new non-Hodgkin's B-cell line (DoHH2) with a chromosomal translocation t(14;18)(q32;q21). Leukemia 1991, 5:221–224