Quantification of *bcl-2/JH* Fusion Sequences and a Control Gene by Multiplex Real-Time PCR Coupled with Automated Amplicon Sizing by Capillary Electrophoresis

Beatriz Sanchez-Vega, Francisco Vega,
L. Jeffrey Medeiros, Ming S. Lee, and
Rajyalakshmi Luthra

From the Division of Pathology and Laboratory Medicine, The
University of Texas M.D. Anderson Cancer Center,
Houston, Texas

Follicular lymphoma is characterized by the presence of the t(14;18)(q32;q21) chromosomal translocation which juxtaposes the *bcl-2* gene at 18q21 with the immunoglobulin heavy chain locus at 14q32. Quantification of t(14;18) carrying cells in FL patients can be achieved by real-time PCR, a highly sensitive technique for evaluating treatment efficacy and minimal residual disease. Despite the many advantages of real-time technology for this purpose, one disadvantage is that current real-time t(14;18) PCR assays amplify a control gene as a normalizer in a separate reaction. Since each PCR reaction has its own kinetics, separate PCR assays for target and control sequences can potentially result in inaccurate quantification of t(14;18)-positive cells. In addition, the real-time t(14;18) PCR assays do not determine the size of the amplified fusion sequence, which is helpful for excluding contamination and is commonly used to demonstrate clonal identity between pre- and post-treatment specimens from a patient. To address these limitations, we designed a multiplex real-time PCR protocol that allows amplification of control and target genes in the same reaction and precise size determination of *bcl-2/JH* fusion sequences by capillary electrophoresis. This multiplex PCR assay is equally sensitive to previous assays, allows more accurate quantification of *bcl-2/JH* fusion sequences, and is more convenient. (J Mol Diagn 2002, 4:223–229)

Follicular lymphoma (FL) accounts for approximately 25% of adult non-Hodgkin's lymphomas in western countries.¹ Follicular lymphoma is characterized by the t(14;18)(q32;q21), which juxtaposes the *bcl-2* gene at 18q21 with enhancers of the immunoglobulin heavy chain (*IgH*) locus at 14q32, resulting in overexpression of *Bcl-2*.^{2–4}

We have shown previously that quantification of t(14;18)-positive cells in FL patients can be achieved by real-time TaqMan PCR (Applied Biosystems, Foster City, CA).^{5,6} Since our original report,⁶ several investigators have designed real-time PCR assays to quantify the number of cells carrying the t(14;18), and these assays have proved to be equally sensitive and quantitatively more reliable than conventional single-round or nested PCR assays.^{7,8} Real-time TaqMan PCR assays are based on the 5'→3' exonuclease activity of *Taq* polymerase, and employ a non-extendable probe labeled with a fluorescent reporter dye at its 5' end and a quencher dye at its 3' end.^{9–13} The use of labeled probes complementary to target permits identification of specific PCR products. Thus, real-time PCR methods for detection of the t(14;18) are specific, highly sensitive, and reliable for evaluating treatment efficacy and following minimal residual disease in FL patients.

Real-time PCR assays need to be designed with a control reaction that allows direct comparison of DNA quantity in different samples. This control amplification allows normalization of quantitative results with respect to the total amount of amplifiable material in each sample. Currently, amplification of a housekeeping gene such as *β-actin* or glyceraldehyde phosphate dehydrogenase (*GAPDH*) in a second reaction is used to normalize real-time PCR results. However, co-amplification of target and control in the same reaction is considered more reliable because a second reaction has its own kinetics and set-up of second reaction increases the risk of pipetting error.^{11,14} This can be problematic when one uses TaqMan real-time PCR technology. Although this technology can be used to co-amplify two different sequences using specific probes labeled with different fluorescent reporter dyes, the sensitivity of detecting a few t(14;18)-positive cells in a background of numerous normal cells carrying the control gene is often compromised due to competition between abundant control gene and limited target.

We describe a multiplex real-time TaqMan PCR assay that addresses this limitation of assays described previ-

Accepted for publication May 9, 2002.

Address reprint requests to R. Luthra, Ph.D., University of Texas M.D. Anderson Cancer Center, Box 149, NA01.091, 8515 Fannin, Houston, TX 77030-4095. E-mail: rluthra@mdanderson.org.

Table 1. Sequences of Primers and Probes Used in Real-Time PCR Assay

Primers	Sequences
<i>bcl-2 mbr</i> -F	5'-GCT TTA CGT GGC CTG TTT CA-3'
<i>bcl-2 mcr</i> -F	5'-CCT GGC TTC CTT CCC TCT GT-3'
JH-NED	5'- NED -ACC TGA GGA GAC GGT GAC C-3'
<i>cy</i> -F	5'-TGA GAC AGC AGA TAG AGC CAA GC-3'
<i>cy</i> -R	5'-TCC CTG CCA ATT TGA CAT CTT C-3'
<hr/>	
Probes	
<i>mbr</i> -P	5'- FAM -AGG GCT CTG GGT GGG TCT GTG TTG- TAMRA -3'
<i>mcr</i> -P	5'- FAM -TCT CTG IGG AGG AGT GGA AAG GAA GG- TAMRA -3'
<i>cy</i> -P	5'- VIC [®] -AGC ACC AAT ATT CAG TAC ACA GCT TAA AGC TAT AGG TAT- TAMRA -3'

Abbreviations: *mbr*, major breakpoint region; *mcr*, minor cluster region; JH, joining region of immunoglobulin heavy chain gene; F, forward; R, reverse; P, probe; *cy*, *cyclophilin*.

ously and allows co-amplification of the t(14;18) and a control gene, *cyclophilin*, in a single reaction. In this assay sensitivity is not compromised and the risk of pipetting error is reduced. In addition, the amplified *bcl-2/JH* fusion sequences are labeled with a fluorescent dye, NED (Applied Biosystems), in this real-time PCR assay as we described previously.¹⁵ The labeling of the amplification products allows rapid determination of the size of the *bcl-2/JH* fusion sequences following real-time PCR by capillary electrophoresis (CE). As amplicon size is helpful for excluding contamination and is also commonly used to demonstrate clonal identity between pre- and post-treatment specimens, multiplex real-time TaqMan PCR combined with CE provides a simple and rapid approach for monitoring minimal residual disease facilitating high-throughput analysis of multiple samples.

Materials and Methods

A total of 33 peripheral blood or bone marrow specimens from 22 patients with FL were analyzed. By conventional PCR methods, all patients had the t(14;18), 19 involving the major breakpoint cluster region (*mbr*) and three patients involving the minor breakpoint cluster region (*mcr*) of the *bcl-2* gene. All cases of FL had been diagnosed previously in tissue biopsy specimens according to criteria specified by the World Health Organization classification.¹⁶

DNA from t(14;18)-positive cell lines involving either the *mbr* and *mcr* of the *bcl-2* gene (gifts from Dr. Richard Ford, M. D. Anderson Cancer Center, Houston, TX) were used as positive controls and to generate standard curves. Five additional cell lines with known *bcl-2/JH* fusion sequences were also included in this study to assess the accuracy of size determination. DNA from the cell line HL60 was included as negative control. High-molecular weight DNA was isolated from cell lines, peripheral blood, and bone marrow specimens in accordance with standard proteinase K digestion and organic extraction procedures.

Preparation of Standards

DNA derived from cell lines harboring *bcl-2/JH* fusion sequences involving either *bcl-2 mbr* or *mcr* regions were

diluted to a concentration of 0.2 µg/µl in Tris 10 mmol/L, pH 8.0. This stock DNA was then serially diluted in Tris 10 mmol/L, pH 8.0, to achieve 10⁻¹- to 10⁻⁵-fold dilutions.

In a multiplex PCR, when t(14;18)-positive cell line DNA is serially diluted into t(14;18)-negative HL60 DNA, different dilutions will have a similar amount of *cyclophilin* and will not be useful to generate a standard curve for *cyclophilin*. Hence, we used DNA diluted into Tris buffer to create standard curves for both *bcl-2/JH* and *cyclophilin*. We have observed previously that standard curves generated for *bcl-2/JH* by diluting positive cell line DNA into negative HL60 DNA or Tris buffer result in similar threshold cycle (Ct) values (data not shown). The Ct value for *bcl-2/JH* fusion sequence and *cyclophilin* of each unknown sample was converted into a quantitative value using the respective standards included in each run. The amount of *bcl-2/JH* fusion sequences present in each sample was then normalized to the amount of *cyclophilin* and expressed as a ratio of *bcl-2/JH* fusion sequences to *cyclophilin*.

Multiplex TaqMan PCR for t(14;18)(q32;q21) and Cyclophilin

PCR amplification of *bcl-2/JH* fusion sequences was carried out in duplicate using a JH consensus reverse primer (JH-R) and *bcl-2 mbr*-specific (*mbr*-F) or *mcr*-specific (*mcr*-F) forward primers (Table 1). A 93-bp segment of the *cyclophilin* gene was co-amplified using specific forward (Cy-F) and reverse (Cy-R) primers¹⁷ (Table 1). To allow analysis of *bcl-2/JH* fusion sequence size after PCR by CE, the JH-R primer was labeled with NED at its 5' end (JH-NED). The assay was performed using TaqMan technology in a PRISM 7700 Sequence Detector (Applied Biosystems). The 5' ends of the *bcl-2 mbr* and *mcr* probes were labeled with 6-carboxyfluorescein (6-FAM) and the 5' end of the *cyclophilin* probe was labeled with VIC. All probes were labeled with the quencher dye N,N,N',N'-tetramethyl-6-carboxyrhodamine (TAMRA) at their 3' ends.

PCR was performed in a final volume of 25 µl containing 1 µg of genomic DNA, 1X TaqMan buffer, 5 mmol/L MgCl₂, 0.2 µmol/L of *bcl-2 mbr* or *mcr* and JH primers, 0.1 µmol/L of *bcl-2 mbr*- or *mcr*-specific probe, 480 µmol/L dUTP, 240 µmol/L dATP, 240 µmol/L dCTP, 240 µmol/L

Figure 1. Standard curves showing the initial DNA quantity versus threshold cycle (Ct). The standard curves were generated by three different real-time PCR assay conditions using serial dilutions of DNA from t(14;18)-positive cell lines: **blue**, *mbr* or *mcr* alone; **red**, co-amplification of *mbr* or *mcr* with *cyclophilin*; **green**, co-amplification of *mbr* or *mcr* with *cyclophilin* and using a NED-labeled JH primer.

dGTP, 0.25 units of AmpErase uracil-N-glycosylase (UNG), and 0.625 units of AmpliTaq Gold polymerase. The *cyclophilin* gene was co-amplified using 40 nmol/L of the appropriate primers and 0.05 μ mol/L of fluorescently labeled *cyclophilin*-specific. The PCR thermal cycling conditions included a 2 minute 50°C UNG activation and a 10 minute 95°C TaqGold activation and template-denaturation, followed by 40 cycles: 95°C for 15 seconds, 57°C for 45 seconds, and 72°C for 45 seconds.

The fluorescence emission data for each sample was analyzed immediately after PCR using Sequence Detection Software (SDS version 1.7, Applied Biosystems). The Ct and fluorescence intensity (ΔRn) values of each sample and cell line were exported into Microsoft Excel software for further analysis.

GeneScan Analysis

Following real-time PCR, 0.25 μ l of amplification product was resolved by CE in an ABI-310 or ABI-3700 Genetic Analyzer (Applied Biosystems). GenoType-ROX 50 to 500 DNA ladder (Life Technologies, Gaithersburg, MD) was used as internal size standard. Local Southern option of GeneScan (GS) software (Applied Biosystems) was used to determine the size of the *bcl-2/JH* fusion sequences.

Results

In all 33 patient samples and in the five cell lines analyzed the real-time PCR t(14;18) results confirmed results obtained using standard gel-based PCR methods. There were no discrepancies in the results between real-time and gel-based PCR methods.

Sensitivity of the Multiplex PCR Assay for t(14;18) and Cyclophilin

We tested the effect of *cyclophilin* gene co-amplification on the sensitivity of the TaqMan real-time PCR assay for

t(14;18) by analyzing serially diluted DNA of t(14;18)-positive cell lines in the presence or absence of *cyclophilin* primers and probe. Standard curves generated from the amplification plots of these assays are shown in Figure 1. These curves show a linear correlation between the Ct and serial dilutions of t(14;18) *bcl-2 mbr*- or *mcr*-positive cell lines.

As shown in Figure 1, the Ct values for *bcl-2/JH* fusion sequences over a 5 log dynamic range of starting target copy number were similar whether *cyclophilin* primers and probe were present or absent in the same reaction. This indicates that *cyclophilin* co-amplification did not interfere with the real-time detection of *bcl-2/JH* fusion sequences, even at the low dilutions of *bcl-2/JH* fusion sequences. We were able to detect approximately one t(14;18)-positive cell in a background of 100,000 t(14;18)-negative cells.

The Ct values for *cyclophilin* remained similar in the presence of varying amounts of *bcl-2/JH* fusion sequences in the sample (data not shown) indicating that co-amplification of *bcl-2/JH* fusion sequences did not interfere with *cyclophilin* amplification.

Effect of *bcl-2/JH* Amplicon Size

We tested two t(14;18) *bcl-2/JH mbr*-positive cell lines with fusion sequences that differed in size by 125 bp to determine whether the efficiency of the multiplex PCR assay was influenced by amplicon size. As is shown in Figure 2, there was an almost perfect correlation ($r = 0.999$) between the Ct values using both cell lines at the same DNA concentrations indicating that the efficiency of the multiplex PCR was not altered by the amplicon size.

Effect of Fluorescent-Labeled Primer on the Sensitivity of Multiplex TaqMan t(14;18) PCR

We tested the effect of introducing a JH primer labeled with the fluorescent dye NED on the sensitivity of the multiplex TaqMan real-time PCR t(14;18) assay by per-

Figure 2. Correlation between the results of multiplex real-time PCR for two *bcl-2 mbr/JH*-positive cell lines with fusion sequences of 87 bp and 220 bp. There was an almost perfect correlation ($r = 0.999$) between the Ct values using both cell lines at the same concentrations indicating that the efficiency of the multiplex PCR was not altered by amplicon size. Each experiment was repeated three times and each sample was analyzed in duplicate.

forming PCR with the JH-NED primer and comparing the results with those obtained using an unlabeled JH primer. Ct values were identical (Figure 1), demonstrating that introduction of this fifth fluorescent dye to the real-time TaqMan PCR did not affect the efficiency or sensitivity of the t(14;18) assay.

Normalization with Cyclophilin Amplified in the Same or Separate Reactions

Table 2 shows the real-time PCR results from 33 patient samples analyzed for *bcl-2/JH* fusion sequences with the *cyclophilin* gene amplified in the same or separate reactions. The quantitative values obtained by both assays correlated well, with correlation coefficients of 0.981 and 0.973 for *bcl-2 mbr/JH* and *bcl-2 mcr/JH* fusion sequences, respectively (Figure 3).

We included two patients (patients 18 and 19) in this study with two and four sequential bone marrow samples, respectively, in which we could compare the clone in each specimen during the course of disease. In patient 18, the initial and subsequent bone marrow specimens, the latter obtained 56 months later showed an identical *bcl-2 mbr/JH* fusion sequence. In patient 19, the initial bone marrow specimen and subsequent samples obtained 6, 13, and 20 months later showed identically sized *bcl-2 mbr/JH* fusion sequences (Table 2 and Figure 4). At the time of diagnosis, the initial bone marrow aspirate specimen was extensively involved by tumor. This patient was treated with 6 cycles of fludarabine, mitoxantrone, dexamethasone, and rituximab and a partial clinical remission was achieved. Twenty months later, after completion of therapy, the last bone marrow aspirate specimen was minimally involved by tumor. The real-time assay performed at this time was in concordance with the bone marrow aspirate morphological findings and revealed a markedly decreased tumor burden (*bcl-2 mbr/JH* to *cyclophilin* ratio decreased by 98%).

Table 2. Summary of *bcl-2/JH* Real-Time PCR Quantitative Results from 33 Samples

Patient	Multiplex PCR	Separate PCR	Size bp
	<i>mbr/Cy</i>	<i>mbr/Cy</i>	
1	1.09910	0.97123	110
2	0.28478	0.25702	122
3	0.01415	0.02039	129
4	0.27353	0.35481	144
5	1.40069	0.99149	154
6a	0.00033	0.00013	161
6b	0.00132	0.00109	161
7	0.00049	0.00052	164
8	0.06534	0.06454	170
9	0.00111	0.00086	179
10	0.03861	0.04093	185
11	0.00421	0.00669	187
12	0.62250	0.70963	188
13a	0.09367	0.12161	204
13b	0.05287	0.06908	204
14a	0.84420	0.78098	215
14b	0.72251	0.61447	215
15	0.00005	0.00002	223
16	0.37027	0.29113	231
17	0.00020	0.00012	241
18a	0.00197	0.00193	131
18b ₁	0.00052	0.00035	131
18b ₂	0.03566	0.01311	131
19b ₁	0.93257	nd	108
19b ₂	0.31114	nd	108
19b ₃	0.12001	nd	108
19b ₄	0.00025	nd	108
<hr/>			
	<i>mcr/Cy</i>	<i>mcr/Cy</i>	pb
20a	0.00029	0.00025	144
20b	0.00012	0.00000	144
21a	0.16551	0.12498	132
21b	0.22363	0.22878	132
22a	0.08307	0.08059	136
22b	0.05413	0.06542	136

The amount of *bcl-2/JH* fusion sequences present in each sample normalized to *cyclophilin* and expressed as a *bcl-2/JH* to *cyclophilin* (MBR/Cy) ratio. The size of the amplicons is also shown.

Abbreviations: a, peripheral blood sample, b, bone marrow sample, subscripts indicate sequential samples, *mbr*, major breakpoint region; *mcr*, minor cluster region; *cy*, *cyclophilin*; Ct, threshold cycle; bp, base pairs; nd, not done.

Inter-Assay Variability

The performance of multiplex real-time PCR quantification was evaluated in 10 independent assays. In each

Figure 3. Correlation between multiplex and separate real-time PCR results for 33 *bcl-2 mbr/JH*-positive patient samples.

Figure 4. Patient 19 with four sequential bone marrow specimens. **A:** Amplification plots of real-time PCR assay for *bcl-2 mbr/JH* fusion sequences. **B:** Capillary electrophoresis electropherograms for each sample in **A**. **C:** Amplification plots of real-time PCR assay for cyclophilin. In **A**, note that a lower fluorescent signal was observed in the last bone marrow sample obtained (green) compared with the other specimens (yellow plot at time of diagnosis; red plot after 6 months, and blue plot after 13 months). As shown in **B**, the same size *bcl-2 mbr/JH* fusion sequence of 108 bp was obtained in all assays.

Figure 5. Capillary electrophoresis electropherograms showing the size of the *bcl-2/JH* fusion sequences obtained from one patient (patient 5) and a t(14;18)-positive cell line. *bcl-2/JH* fusion sequences of 154 bp and 87 bp were obtained, respectively.

Table 3. Inter-Assay Variability for 10 Independent Assays of Various DNA Dilutions.

Input DNA	Ct mean	SD
100 ng	23.02	0.58
10 ng	26.65	0.78
1 ng	30.28	0.62
0.1 ng	34.28	1.26
0.01 ng	36.99	1.29

Abbreviations: Ct, threshold cycle; SD, standard deviation.

assay, all dilutions of the standards were used to generate standard curves. Inter-assay coefficients of variation for all dilutions are shown in Table 3. Less than one Ct difference was observed with input DNA of 100 ng, 10 ng and 1 ng indicating the reproducibility of the assay. At dilutions of 0.1 ng and 0.01 ng of DNA representing roughly 10 copies or less, a Ct difference of 1.26 and 1.29 was observed, respectively.

Capillary Electrophoresis for t(14;18)

As we have described previously,¹⁵ after PCR we resolved the amplification products by CE and analyzed by GS. Peaks representing a unique amplicon size for each patient specimen (Table 2 and Figure 5) were identified corroborating that the fluorescent signal detected by real-time PCR was due to patient-specific clones. The HL60-negative control DNA showed no peak confirming the specificity of the assay. The size of the amplified *bcl-2/JH* fusion sequences ranged from 110 to 241 bp in different patient samples. In patients with multiple specimens (cases 6, 13, 14, 18, 19, 20, 21, and 22), identically sized *bcl-2/JH* fusion products were detected in each specimen of an individual patient. Moreover, *bcl-2/JH* fusion sequences were detected even in patient samples 7, 11, 15, and 20 in which low numbers of t(14;18)-positive cells were present.

Discussion

Molecular response of FL to therapy, as defined by conversion from PCR-positive to PCR-negative status inpatient samples, has been shown to be associated with improved failure-free survival in FL patients.¹⁸⁻²² Less well understood is the relationship between tumor burden and prognosis in patients with FL.²³ Accurate, reliable, and convenient methods to quantify t(14;18)-positive cells are necessary for this purpose.

To ensure that quantitative tumor burden values obtained by TaqMan assays are not affected by differences in the amount of DNA from sample to sample, or by the presence of PCR inhibitors in samples, control genes such as β -actin or GAPDH are amplified in parallel with t(14;18) real-time PCR reactions and values obtained for the reference gene are used to normalize patient samples. Ideally, it is preferable to perform both reactions in the same tube to avoid differences in the amount of DNA introduced by pipetting errors when performing two separate reactions. Nevertheless, reliable multiplex real-time

PCR assays to quantify tumor burden have been difficult to develop because of competition between the two targets, particularly with low levels of tumor, when the control target is much more abundant than tumor. To our knowledge, Kreuzer et al²⁴ is the only group who have designed a multiplex fluorescence real-time PCR assay that co-amplifies *bcr/abl* and β -actin transcripts in the same reaction, which they use to assess tumor burden in chronic myelogenous leukemia patients.

In our earlier studies, using limited primer concentrations we were able to co-amplify β -actin as an internal control to prove the presence of amplifiable DNA in a sample. However, we realized that β -actin was not a suitable normalizer for quantifying of t(14;18)-positive cells using our assay conditions, due to the inhibitory effect of β -actin on *bcl-2/JH* amplification, especially at low tumor burden. Thus, the goal of this study was to determine whether another gene, such as *cyclophilin*, would be more appropriate as a normalizer for this assay. The high overall concordance rate in this study between the multiplex and separate PCR assays to assess for *bcl-2/JH* fusion sequences indicates that co-amplification of *cyclophilin* does not diminish detection sensitivity for t(14;18)-positive cells in this assay.

Another disadvantage of the TaqMan PCR t(14;18) technique is that detection of PCR amplifiable *bcl-2/JH* fusion sequences by this method does not allow amplicon size determination. Thus, one cannot easily exclude contamination, particularly when different specimens are analyzed simultaneously. In addition, the size of PCR products, from either simultaneous or sequential specimens from a patient, is valuable presumptive evidence of clonal identity or disparity.²⁵ *bcl-2/JH* fusion sequence size generally varies from clone to clone, owing to variations in the exact location of the breakpoints and the number of N-region nucleotides.^{4,26,27} Although the most reliable method to compare two or more clones is to sequence the amplification products, this is an additional step that adds to turnaround time and workload. We have recently shown that PCR products labeled during real-time PCR by incorporation of a fluorescent dye-labeled primer can be resolved by CE, allowing precise size determination of each *bcl-2/JH* fusion sequence.¹⁵ This assay allowed us to recognize identically sized clones in sequential biopsy specimens from two patients. One patient (patient 19) had four sequential bone marrow samples in which we identified a clone of identical size, 108 bp, in each specimen during the course of the disease. Moreover, the real-time assay performed on the last specimen revealed a marked decrease in the quantity of the clone, in accordance with the decreased tumor burden in the bone marrow observed morphologically. Automated size determination of fluorescent-labeled amplification products using this technology allows the separation of fragments with a 1-bp size difference.²⁸ Presumably, the 108-bp amplicon is the same clone in each sample although confirmatory DNA sequencing was not performed.

In conclusion, we believe that this multiplex real-time PCR assay to detect the t(14;18) is another step toward our ultimate goal, that being routine quantification of t(14;

18)-positive cells in FL patients. The multiplex assay includes *cyclophilin* as an internal control in the same reaction tube. Co-amplification of *cyclophilin* using these assay conditions did not negatively impact assay sensitivity, even at low levels of t(14;18) target. This assay reduces the risk of pipetting errors and is convenient. Furthermore, labeling the JH primer at its 5' end with the fluorescent dye NED, which does not interfere with TaqMan reporter dyes or assay sensitivity, allows easy separation and size determination of amplified *bcl-2/JH* fusion sequences after real-time PCR by automated high throughput CE.

References

- Armitage JO, Weisenburger DD: New approach to classifying non-Hodgkin's lymphomas: clinical features of the major histologic subtypes. Non-Hodgkin's Lymphoma Classification Project. *J Clin Oncol* 1998, 16:2780-2795
- Bakhshi A, Jensen JP, Goldman P, Wright JJ, McBride OW, Epstein AL, Korsmeyer SJ: Cloning the chromosomal breakpoint of t(14;18) human lymphomas: clustering around JH on chromosome 14 and near a transcriptional unit on 18. *Cell* 1985, 41:899-906
- Yunis JJ, Oken MM, Kaplan ME, Ensrud KM, Howe RR, Theologides A: Distinctive chromosomal abnormalities in histologic subtypes of non-Hodgkin's lymphoma. *N Engl J Med* 1982, 307:1231-1236
- Cleary ML, Smith SD, Sklar J: Cloning and structural analysis of cDNAs for bcl-2 and a hybrid bcl-2/immunoglobulin transcript resulting from the t(14;18) translocation. *Cell* 1986, 47:19-28
- Estalilla OC, Medeiros LJ, Manning Jr JT, Luthra R: 5'→3' exonuclease-based real-time PCR assays for detecting the t(14;18)(q32;q21): a survey of 162 malignant lymphomas and reactive specimens. *Mod Pathol* 2000, 13:661-666
- 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
- Dolken L, Schuler F, Dolken G: Quantitative detection of t(14;18)-positive cells by real-time quantitative PCR using fluorogenic probes. *Biotechniques* 1998, 25:1058-1064
- Johnson PW, Swinbank K, MacLennan S, Colomer D, Debuire B, Diss T, Gabert J, Gupta RK, Haynes A, Kneba M, Lee MS, Macintyre E, Mensink E, Moos M, Morgan GJ, Neri A, Johnson A, Reato G, Salles G, van't Veer MB, Zehnder JL, Zucca E, Selby PJ, Cotter FE: Variability of polymerase chain reaction detection of the bcl-2-IgH translocation in an international multi-centre study. *Ann Oncol* 1999, 10:1349-1354
- Holland PM, Abramson RD, Watson R, Gelfand DH: Detection of specific polymerase chain reaction product by utilizing the 5'→3' exonuclease activity of *Thermus aquaticus* DNA polymerase. *Proc Natl Acad Sci USA* 1991, 88:7276-7280
- Higuchi R, Fockler C, Dollinger G, Watson R: Kinetic PCR analysis: real-time monitoring of DNA amplification reactions. *Biotechnology (NY)* 1993, 11:1026-1030
- Higuchi R, Dollinger G, Walsh PS, Griffith R: Simultaneous amplification and detection of specific DNA sequences. *Biotechnology (NY)* 1992, 10:413-417
- Heid CA, Stevens J, Livak KJ, Williams PM: Real-time quantitative PCR. *Genome Res* 1996, 6:986-994
- Gibson UE, Heid CA, Williams PM: A novel method for real-time quantitative RT-PCR. *Genome Res* 1996, 6:995-1001
- Meijerink J, Mandigers C, van De Locht L, Tonnissen E, Goodsaid F, Raemaekers J: A novel method to compensate for different amplification efficiencies between patient DNA samples in quantitative real-time PCR. *J Mol Diagn* 2001, 3:55-61
- Sanchez-Vega B, Vega F, Hai S, Medeiros LJ, Luthra R: Real-time t(14;18)(q32;q21) PCR assay combined with high resolution capillary electrophoresis: a novel and rapid approach that allows accurate quantification and size determination of bcl-2/JH fusion sequences. *Mod Pathol* 2002, 15:448-453
- Nathwani BM, Piris MA, Harris NL, Berger F, Weisenburger D, Muller-Hermelink HK, Isaacson PG, Swerdlow SH: Follicular lymphoma. World Health Organization Classification of Tumours. Pathology and Genetics of Tumors of Hematopoietic and Lymphoid Tissues. Edited by Jaffe ES, Harris HL, Stein H, Vardiman JW. Lyon, France, IARC Press, 2001, pp 162-167
- Medhurst AD, Harrison DC, Read SJ, Campbell CA, Robbins MJ, Pangalos MN: The use of TaqMan RT-PCR assays for semi-quantitative analysis of gene expression in CNS tissues and disease models. *J Neurosci Methods* 2000, 98:9-20
- Gribben JG, Neuberger D, Freedman AS, Gimmi CD, Pesek KW, Barber M, Saporito L, Woo SD, Coral F, Spector N: Detection by polymerase chain reaction of residual cells with the bcl-2 translocation is associated with increased risk of relapse after autologous bone marrow transplantation for B-cell lymphoma. *Blood* 1993, 81:3449-3457
- Lazzarino M, Arcaini L, Bernasconi P, Alessandrino EP, Gargantini L, Cairoli R, Orlandi E, Astori C, Brusamolino E, Pagnucco G, Colombo AA, Calatroni S, Iacona I, Regazzi MB, Morra E: A sequence of immuno-chemotherapy with rituximab, mobilization of in vivo purged stem cells, high-dose chemotherapy, and auto-transplant is an effective and non-toxic treatment for advanced follicular and mantle cell lymphoma. *Br J Haematol* 2002, 116:229-235
- Rambaldi A, Lazzari M, Manzoni C, Carlotti E, Arcaini L, Baccarani M, Barbui T, Bernasconi C, Dastoli G, Fuga G, Gamba E, Gargantini L, Gattei V, Lauria F, Lazzarino M, Mandelli F, Morra E, Pulsoni A, Ribersani M, Rossi-Ferrini PL, Rupolo M, Tura S, Zagonel V, Zaja F, Zinzani P, Reato G, Foa R: Monitoring of minimal residual disease after CHOP and rituximab in previously untreated patients with follicular lymphoma. *Blood* 2002, 99:856-862
- Colombat P, Salles G, Brousse N, Eftekhari P, Soubeyran P, Delwail V, Deconinck E, Haioun C, Foussard C, Sebban C, Stamatoullas A, Milpied N, Boue F, Taillan B, Lederlin P, Najman A, Thieblemont C, Montestruc F, Mathieu-Boue A, Benzohra A, Solal-Celigny P: Rituximab (anti-CD20 monoclonal antibody) as single first-line therapy for patients with follicular lymphoma with a low tumor burden: clinical and molecular evaluation. *Blood* 2001, 97:101-106
- 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
- Ladetto M, Sametti S, Donovan JW, Ferrero D, Astolfi M, Mitterer M, Ricca I, Drandi D, Corradini P, Coser P, Pileri A, Gribben JG, Tarella C: A validated real-time quantitative PCR approach shows a correlation between tumor burden and successful ex vivo purging in follicular lymphoma patients. *Exp Hematol* 2001, 29:183-193
- Kreuzer KA, Bohn A, Lupberger J, Solassol J, le Coutre P, Schmidt CA: Simultaneous absolute quantification of target and control templates by real-time fluorescence reverse transcription-PCR using 4-(4'-dimethylaminophenylazo)benzoic acid as a dark quencher dye. *Clin Chem* 2001, 47:486-490
- Gribben JG, Freedman A, Woo SD, Blake K, Shu RS, Freeman G, Longtine JA, Pinkus GS, Nadler LM: All advanced stage non-Hodgkin's lymphomas with a polymerase chain reaction amplifiable breakpoint of bcl-2 have residual cells containing the bcl-2 rearrangement at evaluation and after treatment. *Blood* 1991, 78:3275-3280
- Cotter F, Price C, Zucca E, Young BD: Direct sequence analysis of the 14q+ and 18q- chromosome junctions in follicular lymphoma. *Blood* 1990, 76:131-135
- Bakhshi A, Wright JJ, Graninger W, Seto M, Owens J, Cossman J, Jensen JP, Goldman P, Korsmeyer SJ: Mechanism of the t(14;18) chromosomal translocation: structural analysis of both derivative 14 and 18 reciprocal partners. *Proc Natl Acad Sci USA* 1987, 84:2396-2400
- Mayrand PE, Corcoran KP, Ziegler JS, Robertson JM, Hoff LB, Kronick MN: The use of fluorescence detection and internal lane standards to size PCR products automatically. *Appl Theor Electrophor* 1992, 3:1-11