The Korean Journal of Pathology 2011; 45: 582-588
http://dx.doi.org/10.4132/KoreanJPathol.2011.45.6.582

Detection Limit of Monoclonal B-Cells Using Multiplex PCR and Laser-Induced Fluorescence Capillary Electrophoresis

Sung Hak Lee · Yeonsook Moon[1] Byunghoo Song · Hyung Nam Lee Ahwon Lee · Eun Sun Jung Yeong-Jin Choi · Kyo Young Lee Chang Suk Kang · Gyeongsin Park

Department of Hospital Pathology, Seoul St. Mary's Hospital, The Catholic University of Korea College of Medicine, Seoul; [1]Department of Laboratory Medicine, Inha University School of Medicine, Incheon, Korea

Received: October 17, 2011
Accepted: October 25, 2011

Corresponding Author
Gyeongsin Park, M.D.
Department of Hospital Pathology, Seoul St. Mary's Hospital, The Catholic University of Korea College of Medicine, 505 Banpo-dong, Seocho-gu, Seoul 137-701, Korea
Tel: +82-2-2258-1620
Fax: +82-2-2258-1628
E-mail: gspark@catholic.ac.kr

*The authors wish to acknowledge the financial support of the Korean Society of Pathologists from Yang Moon Ho Research Foundation made in the program year of 2011.

Background: The identification of monoclonality has been widely used for making diagnoses of lymphoproliferative lesions. Awareness of the sensitivity and detection limit of the technique used would be important for the data to be convincing. **Methods:** We investigated the minimum requirement of cells and sensitivity of gel electrophoresis (GE) and laser-induced fluorescence capillary electrophoresis (LFCE) for identifying IgH gene rearrangement using BIOMED-2 protocols. DNA extracted from Raji cells were diluted serially with peripheral blood mononuclear cells (PBMNCs) DNA. DNA from mixtures of diffuse large B-cell lymphoma (DLBCL) and reactive lymph nodes were also serially diluted. **Results:** For Raji cells, the detection limit was 62 and 16 cell-equivalents for GE and LFCE, respectively. In the condition with PBMNCs mixture, 2.5% and 1.25% of clonal cells was the minimum requirement for GE and LFCE, respectively. In 23% of DLBCL cells in tissue section, the detection limit was 120 and 12 cell-equivalents for GE and LFCE, respectively. In 3.2% of DLBCL cells, that was 1,200 and 120 cell-equivalents for GE and LFCE, respectively. **Conclusions:** These results show that LFCE method is more sensitive than GE and the sensitivity of clonality detection can be influenced by the amount of admixed normal lymphoid cells.

Key Words: Electrophoresis, capillary; Monoclonality; Lymphoproliferative disorders; BIOMED-2

Malignant lymphomas are basically defined as clonal proliferations of certain lymphoid cells. The diagnosis of malignant lymphoma can be made largely by morphologic evaluation supplemented with immunohistochemistry or flow cytometric immunophenotyping.[1,2] However, in some conditions such as cases with ambiguous histology, limited tissue, minimal residual diseases or mixed lesion, it is not easy to make a definitive diagnosis based on conventional methods. The identification of clonal immunoglobulin (Ig) or T-cell receptor (TCR) gene rearrangement would be useful for making a decision in such cases.[3] To serve as a potent ancillary diagnostic tool, the methods for molecular clonality analysis have advanced.

A southern blot (SB) of the prototype method and the easier polymerase chain reaction (PCR)-based techniques have been used for gene rearrangement analysis.[4] SB analysis was used as the "gold standard" for clonality assessment because of its high reliability. However, this technique requires relatively large amounts of DNA isolated from fresh or frozen tissue in addition to radioactively labeled probes, and the process is complex. In contrast, PCR-based techniques are less labor intensive, and require significantly less high-molecular-weight DNA. Moreover, PCR can be performed on formalin-fixed paraffin-embedded (FFPE) tissue samples. Therefore, the SB method has been replaced by PCR-based techniques because of their efficiency and sensitivity.[2,5] Despite these advantages, PCR studies still harbor two main technical problems: false-negative results due to improper primer annealing and difficulties in discrimination between monoclonal and polyclonal Ig/TCR gene rearrangements.[2]

Recently, the BIOMED-2 multiplex PCR methods based

clonal Ig and TCR gene rearrangement analysis was evaluated in a large series of B- or T-cell malignancies and was demonstrated to be a very reliable assay.[2] In addition, capillary electrophoresis (CE), an important technique in chemical cytometry, has been widely used in the analysis of single cells since the 1990s.[6] CE has also been used to assess the clonality in the lymphoid proliferation.[7] CE has better resolution efficiency and sensitivity and requires a smaller amount of sample than the conventional gel electrophoresis (GE) technique. Furthermore, it can be combined with sensitive detection techniques such as laser-induced fluorescence detection, electrochemical detection, and mass spectroscopic detection, which allow more precise determination of the product size.[8]

In most lymphomas, reactive lymphoid cells are intermixed to a variable extent, or even constitute dominant cellular components rather than neoplastic cells, and this will hinder the ability to make a correct diagnosis. Therefore, sensitive method for identifying clonal gene rearrangement would be helpful in the cases of lymphoma admixed with abundant reactive cells, or even in cases with limited tissue. For the data from the employed clonality tests to be convincing, especially with reactive results, minimal requirement of DNA or tumor cells should be elucidated both under the pure clonal condition such as cell lines and the condition of lymphoma mixed with reactive cells. To address this, in this study we evaluated the minimum cell requirement for clonality detection using gel electrophoresis and laser-induced fluorescence capillary electrophoresis (LFCE) under the conditions of various mixtures of lymphoma cells and normal reactive cells.

MATERIALS AND METHODS

Samples

The Raji cell line (Burkitt lymphoma) was used as a control of monoclonal B-lymphoma cells. The cells, purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA), were maintained under static conditions in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum at 37°C, 5% CO_2, and high humidity. Mononuclear cells were isolated from the peripheral blood of the healthy donor using Ficoll-Paque PREMIUM with a density of 1.077 g/mL (GE Healthcare Bio-Sciences Corporation, Piscataway, NJ, USA), and were used as polyclonal lymphoid cells. We calculated the number of cells with a cytometer. Raji cellular DNA was serially diluted either with, or without, polyclonal DNA from 1×10^4 of normal peripheral blood mononuclear cells (PBMNCs). Eighteen μL of a PCR mastermix of a BIOMED-2 multiplex PCR kit (InVivoScribe Technologies, San Diego, CA, USA) was added in each reaction to the manufacturer's recommendation (Table 1).[2]

Table 1. Minimum requirement of cell per PCR for clonality detection

	Raji cell line		DLBCL cells in FFPE tissue	
	Normal cells (-)	Normal cells (10,000)	23% Mixture	3.2% Mixture
GE	62	250	120	>1,200
LFCE	<16	125	12	120

PCR, polymerase chain reaction; DLBCL, diffuse large B-cell lymphoma; FFPE, formalin fixed paraffin embedded; GE, gel electrophoresis; LFCE, laser-induced fluorescence capillary electrophoresis.

Three FFPE lymph node tissue blocks, in which the tissue had been diagnosed as diffuse large B-cell lymphoma (DLBCL), and three FFPE lymph node tissue blocks, in which the tissue had been diagnosed as reactive hyperplasia, were checked previously for evaluating their DNA integrity and clonality while using the same BIOMED-2 multiplex PCR kit. Among these tissue blocks, one DLBCL case having shown the most clear monoclonal band on GE and monoclonal peak with minimal noise on LFCE, and one reactive lymph node (RLN) case having shown polyclonality without pseudoclonal band or peak were selected and studied as a monoclonal experimental sample and a polyclonal control, respectively. We counted the cell number at ten 1.0×10^4 μm^2 areas of each of the 4 μm-thick sections of the RLN and DLBCL tissue, which resulted in 156±10.8 cells (range, 137 to 175 cells) per 1.0×10^4 μm^2 and 102±10.6 cells (range, 85 to 129 cells) per 1.0×10^4 μm^2 in RLN and DLBCL, respectively (Fig. 1A). Based on these average cell numbers, the total cell numbers per 2 mm core of the 4 μm-thick sections of RLN and DLBCL were calculated at 4.9×10^4 and 3.2×10^4, respectively. We constructed tissue microarray blocks from the DLBCL tissue and from a RLN measuring 2 mm in diameter. After making the intended mixtures of DLBCL and RLN core sections according to the core numbers shown in Fig. 1C, DNA was harvested from each mixture. The study was performed after approval by the local Institutional Review Board (IRB).

DNA Isolation

DNA extraction from FFPE tissues was performed using affinity purification of the DNA (QIAamp DNA mini kit, QIAGEN GmbH, Hilden, Germany) according to the manufactur-

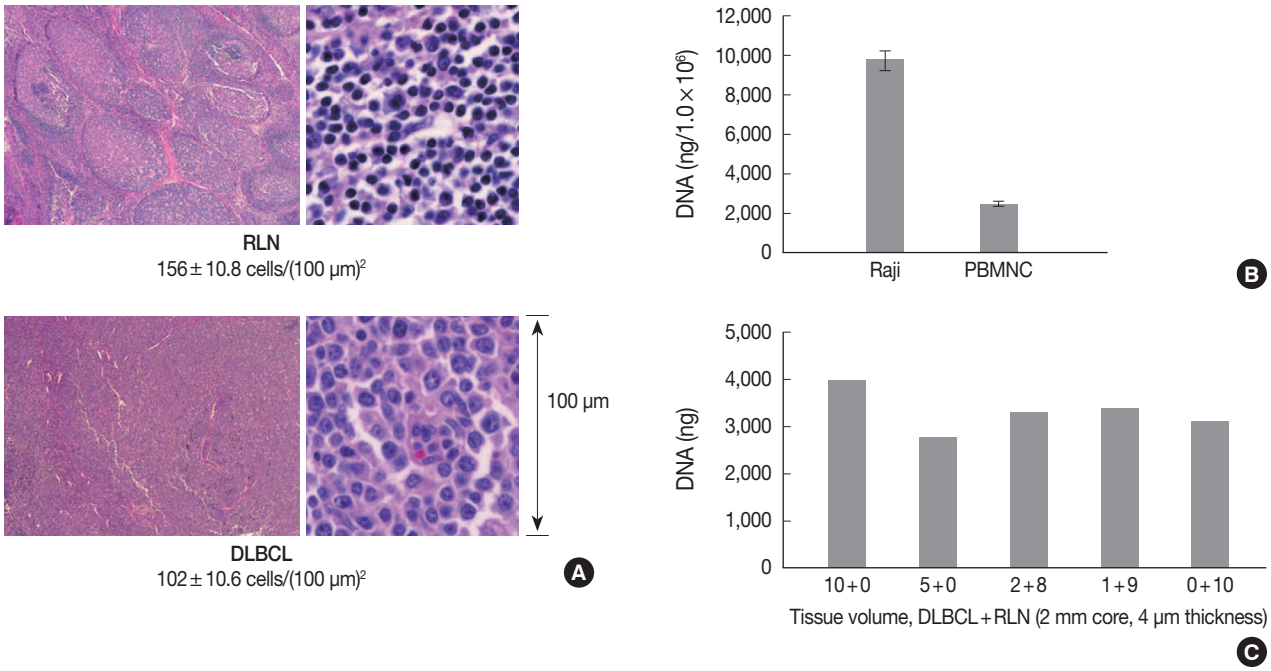

Fig. 1. (A) Estimated cell numbers in (100 μm)^2 of diffuse large B-cell lymphoma (DLBCL) tissue and reactive lymph node (RLN). There are 156.8 cells (range, 137 to 175) per 1.0×10^4 μm^2 in reactive lymph node samples; in contrast, there are 102.6 cells (range, 85 to 129) per 1.0×10^4 μm^2 in DLBCL samples. (B) The DNA extraction yields for Raji cells and peripheral blood mononuclear cells (PBMNC). The DNA yield of Raji cells is about 4 times higher than that of PBMNC (9,760±1,605 ng and 2,500±120 ng, respectively, per 10×10^6 cells, each) (n=3, each). (C) The DNA extraction yields for DLBCL and RLN tissue. The DNA yield of DLBCL tissue is higher than that of RLN (3,950 ng and 3,100 ng, respectively, per ten 4 μm thick-2 mm core sections, each). The DNA yields of mixed DLBCL-RLN tissue sections gradually declined along with the amount of RLN tissue.

er's protocol. DNA sample concentration and quality were assessed by spectrophotometry (260/280 nm using the NanoDrop™ ND-1000, NanoDrop Technology, Wilmington, DE, USA).

Multiplex PCR amplification and assessment of clonality

Detection of B-cell gene rearrangements was performed by amplification of IGH tube A (FR1-JH) and tube B (FR1-JH) using the multiplex PCR protocols developed by BIOMED-2 concerted action BMH4-CT98-3936.[2] Heteroduplex analysis by 3% NuSieve 3-1 Agarose Gel (Lonza Rockland Inc., Rockland, ME, USA) electrophoresis was used to identify the PCR products and was followed by ethidium bromide staining for detection of clonality. We accepted that monoclonality, as the distinguishable band, should be located at the same line as the positive control band. In addition, Multiplex PCR products of Ig targets were detected by GeneScan analysis software (GS) on a genetic analyzer ABI 3130 platform (Applied Biosystems, Foster City, CA, USA) with a laser-induced fluorescence detector. When the height of the peak is three times higher than the background, we considered the peak as monoclonality. Five μL and 1 μL from each PCR product were applied to LFCE, respectively.

RESULTS

Yield of DNA from lymphoid cells and tissue section

The DNA yield of Raji cells was about 4 times higher than that of PBMNCs (9,760±1,605 ng and 2,500±120 ng, respectively, per 10×10^6 cells, each) (n=3, each) (Fig. 1B). The estimated DNA per cell was 9.7 pg and 2.5 pg for Raji and PBMNC, respectively.

The DNA yield of DLBCL tissue was higher than that of RLN (3,950 ng and 3,100 ng, respectively, per ten 4 μm thick-2 mm core section). The estimated cellular DNA was 12.3 pg and 6.3 pg per DLBCL and reactive lymphoid cell, respectively. The DNA yields of mixed DLBCL-RLN tissue sections gradually declined along with the amount of RLN tissue (Fig. 1C).

IgH PCR and clonality assay in a lymphoma cell line

For the pure clonal cell line, the Raji cells, GE showed the detectable clonal band at 62 cell-equivalent (0.6 ng DNA) PCR, whereas LFCE showed a clonal peak at 16 cell-equivalent (0.15 ng DNA) PCR, which was the least cell number tested (Fig. 2A). Under the condition with 1.0×10^4 PBMNCs mixture, GE showed a clonal band at 250 cell-equivalent PCR (2.5% of clonal cells), whereas LFCE showed a clonal peak at 125 cell-equivalent PCR (1.25% of clonal cells). At cell levels lower than 125, the clonal peaks were masked by the polyclonal noise peak. The clonality detection level of LFCE was superior by one or two tiered-grades to that of conventional GE. However, the detection sensitivity decreased by admixed normal reactive cells for both methods (Table 1).

IgH PCR and clonality assay in FFPE tissue

Under the condition with 23% of DLBCL cells in tissue section, GE showed a detectable clonal band at 120 cell-equivalent PCR, whereas LFCE showed a clonal peak at 12 cell-equivalent PCR, which was the least cell number tested. Under the condition with 3.2% of DLBCL cells in tissue section, GE showed a detectable clonal band at 1,200 cell-equivalent PCR, whereas LFCE showed a clonal peak at 120 cell-equivalent PCR, which was the least cell number tested (Fig. 2B). The clonality detection level of LFCE was ten times better than that of conventional GE. Two thousand and four hundred DLBCL cells in 45,000 normal lymphoid cells, the LFCE peak was equivocal. However, a clear peak could be detected with 2,400 DLBCL cells in 5,000 normal lymphoid cells. Likewise, the detection

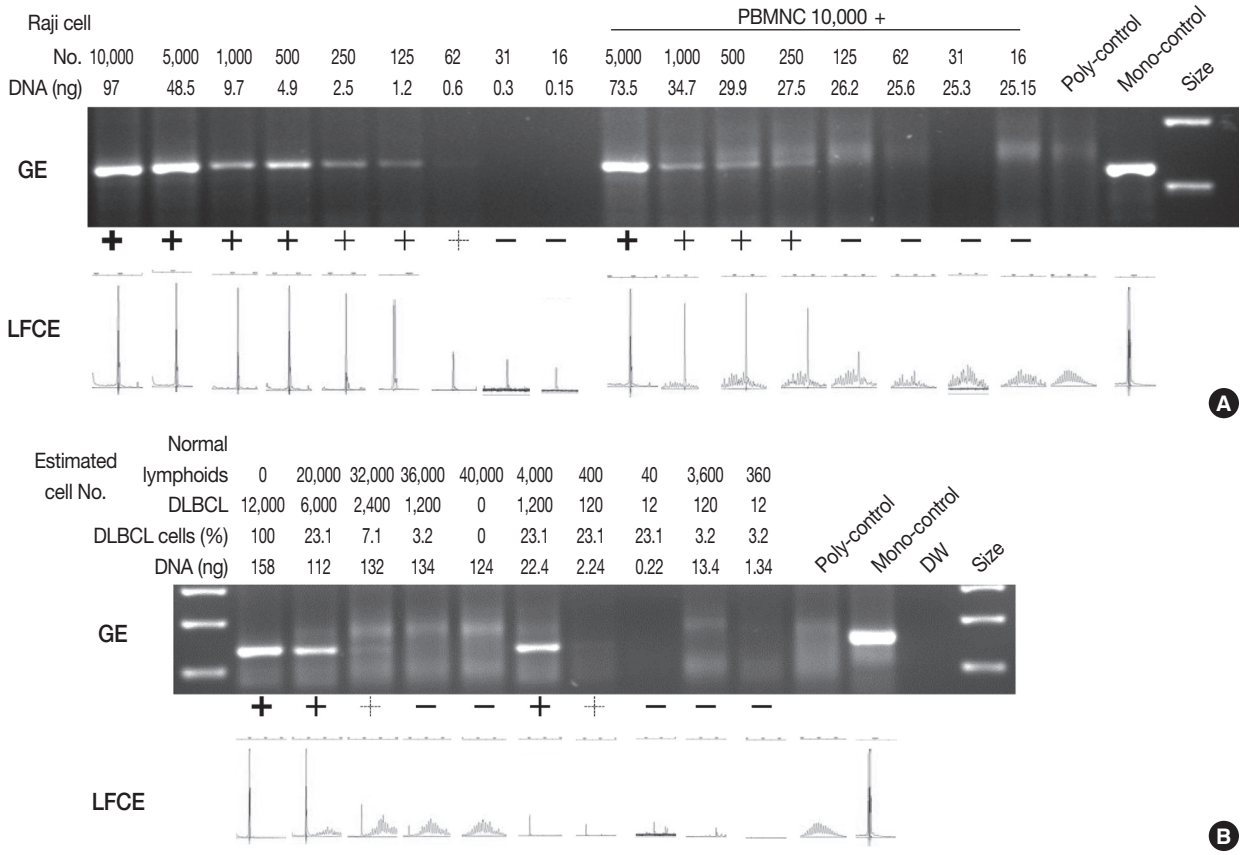

Fig. 2. (A) Clonality assay of lymphoma cells admixed with/without normal cells. For the pure clonal cell line, the Raji cells, gel electrophoresis (GE) shows the detectable clonal band at 62 cell-equivalent (0.6 ng DNA) polymerase chain reaction (PCR), whereas laser-induced fluorescence capillary electrophoresis (LFCE) shows clonal peak at 16 cell-equivalent (0.15 ng DNA) PCR, the least cell number tested. (B) Clonality assay of diffuse large B-cell lymphoma (DLBCL) tissue admixed with/without normal lymph node. Under the condition with 23% of DLBCL cells in tissue section, GE shows the detectable clonal band at 120 cell-equivalents PCR, whereas LFCE shows clonal peak at 12 cell-equivalents PCR, the least cell number tested. Under the condition with 3.2% of DLBCL cells in tissue section, GE shows the detectable clonal band at 120 cell-equivalents PCR, whereas LFCE shows clonal peak at 12 cell-equivalents PCR, the least cell number tested.
PBMNC, peripheral blood mononuclear cells; DW, distilled water; Size, size marker.

sensitivity decreased by admixed normal reactive cells for both methods (Table 1).

Distinct clonal peak according to the size of PCR product

We investigated a distinct clonal peak pattern according to the size of DLBCL tissue, Raji cells and mixture of both (Fig. 3). DLBCL and Raji samples, each containing a reactive component of RLN and PBMNC, showed their specific clonal peaks at 305 base pair (bp) and 343 bp, respectively. After mixing both samples and performing PCR, the mixed PCR product showed two distinct clonal peaks corresponding to their original peaks.

DISCUSSION

The usefulness of clonality assessment for lymphoma diagnosis has been emphasized and the risks of false positive or false negative have also been discussed.[2,9,10] Awareness of the clonality detection limit according to the employed methods is important for the result data to be convincing, particularly for a negative result. In terms of the amount of template DNA for each PCR with 20 μL reaction volume, the minimum requirement of cells was 62 and 16 cells for the conventional GE and LFCE, respectively. The amounts of template DNA were 0.6 ng and 0.15 ng, respectively. Concerning the detection limit, Elenitoba-Johnson *et al.*[11] suggested that at least 10 to 20 B-cell genomic equivalents should be applied for the IgH PCR assay. The cell number was calculated from the serially diluted amount of template DNA, based on the estimate of 6 pg of DNA per diploid cell.[11] Theoretically, the calculated genomic DNA is about 3.3 ng per cell (3×10^9 bp$\times$660 MW/bp/6×10^{23}). The total cellular DNA was estimated as 6-13 pg per cell.[12,13] In our data, the amount of cellular DNA was varied, from 2.5 pg per resting PBMNC to 12.3 pg per DLBCL cell, which suggests that the size and status of cell should be considered for calculating cell number based on DNA amount.

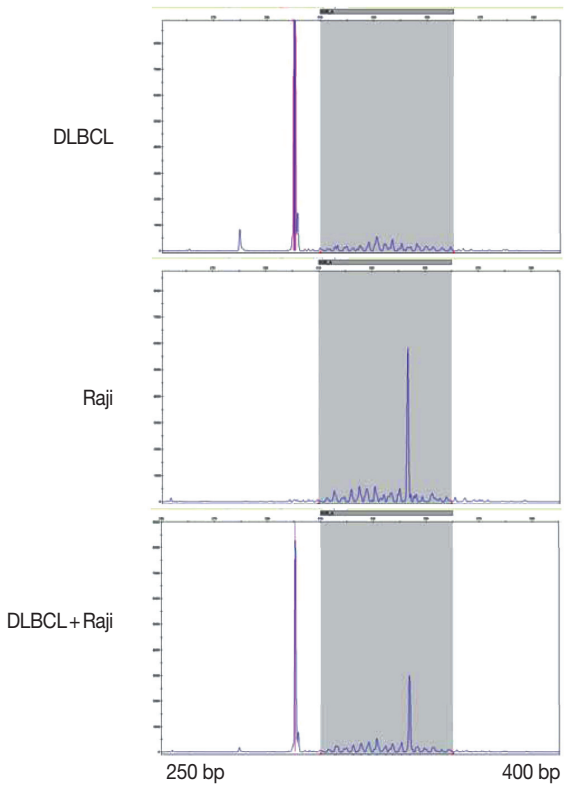

Fig. 3. Distinct clonal peak pattern according to the size of diffuse large B-cell lymphoma (DLBCL) tissue, Raji cells and two mixtures. DLBCL and Raji samples contain normal lymphoid component of lymph node tissue and peripheral blood mononuclear cells, respectively and showed their specific clonal peaks as 305 base pair (bp) and 343 bp. After mixing both samples and polymerase chain reaction (PCR) purification, the mixed PCR product shows two distinct clonal peaks corresponding with their original peaks.

The detection of monoclonality in lymphoproliferative disease has become an important ancillary technique. Especially, molecular methods need to be employed for the diagnosis in cases of: 1) inconclusive morphology and immunophenotyping; 2) immune-alteration associated lymphoproliferative lesions including post-transplant patients; 3) evaluation of the clonal relationship between two simultaneous lymphoid malignancies or distinction of disease relapse and de novo malignancy; 4) further classification of a lymphoid malignancy via Ig/TCR gene rearrangement patterns or particular chromosome aberrations; 5) staging of lymphomas; and 6) minimal residual lesion for follow-up.[2] Therefore, a more sensitive technique should be more helpful. The BIOMED-2 multiplex PCR technique is a rapid and reliable procedure that is significantly more sensitive than SB analysis in the evaluation of clonality in suspect lymphoproliferative lesions.[1] Of the methods for clonality detection, two techniques are widely used: heteroduplex analysis and GS. Heteroduplex analysis uses double-stranded PCR products and takes advantage of the length and composition of the junctional regions, whereas GS single-stranded PCR products are separated in a high resolution gel or polymer according to their length only.[2] GS analysis has been known to be a rapid, relatively simple and sensitive method, but requires expensive equipment.[4]

Most of the previous studies of BIOMED-2 multiplex PCR combined with LFCE have suggested the advantages of LFCE

over other clonality detection methods. However, so far, minimal study has been carried out on the minimum requirement of clonal cells at the various mixture levels. Further studies are needed for validation of the detection limit of IgH PCR assay.

Meanwhile, although PCR-based methods have the advantages of rapidity and the requirement of minimal sample material, the potential false-negative rate is a considerable weakness. A significant proportion of B-cell lymphomas do not demonstrate clonal IgH amplification due to the suboptimal annealing of primers and/or the presence of somatic hypermutation arising in stimulated germinal centre B-cells.[14] Such mutations reduce the binding efficiency of IgH rearrangement primers. Therefore, the detection of clonality by IgH assays in post-germinal center B-cell malignancies, such as follicular lymphomas and some cases of DLBCLs, is more affected than in other lymphoma types.[15] According to Berget *et al*.,[15] Igκ gene rearrangements have been previously studied as additional markers for the detection of B-cell clonality. Hence, by combining the Igκ and IgH PCR methods, the detection rate for clonality in B-cell lymphomas increased from 81 to 90%, with this effect being most evident among follicular lymphomas and DLBCLs.[14,16]

Our results show that the sensitivity of clonality assay for IgH rearrangement could be influenced by the status of admixed normal lymphoid cells, and the LFCE method was more sensitive than the GE methods. Hence, the LFCE method would be useful not only for identifying monoclonality, but also for follow-up of malignant lymphoma. In the cases of recurrence or secondary lymphoid malignancy, these may be easily detected in the form of another separated peak.

In conclusion, the molecular analysis of B-cell clonality based on the detection of IgH rearrangement by the BIOMED-2 based GS technology is a valuable tool for the diagnosis and monitoring of DLBCL.

REFERENCES

1. Sandberg Y, van Gastel-Mol EJ, Verhaaf B, Lam KH, van Dongen JJ, Langerak AW. BIOMED-2 multiplex immunoglobulin/T-cell receptor polymerase chain reaction protocols can reliably replace Southern blot analysis in routine clonality diagnostics. J Mol Diagn 2005; 7: 495-503.
2. van Dongen JJ, Langerak AW, Brüggemann M, *et al*. Design and standardization of PCR primers and protocols for detection of clonal immunoglobulin and T-cell receptor gene recombinations in suspect lymphoproliferations: report of the BIOMED-2 Concerted Action BMH4-CT98-3936. Leukemia 2003; 17: 2257-317.
3. van Dongen JJ, Wolvers-Tettero IL. Analysis of immunoglobulin and T cell receptor genes. Part I: Basic and technical aspects. Clin Chim Acta 1991; 198: 1-91.
4. Derksen PW, Langerak AW, Kerkhof E, *et al*. Comparison of different polymerase chain reaction-based approaches for clonality assessment of immunoglobulin heavy-chain gene rearrangements in B-cell neoplasia. Mod Pathol 1999; 12: 794-805.
5. Christensen M, Funder AD, Bendix K, Soerensen FB. Comparative investigations of T cell receptor gamma gene rearrangements in frozen and formalin-fixed paraffin wax-embedded tissues by capillary electrophoresis. J Clin Pathol 2006; 59: 645-54.
6. Deng B, Wang Z, Song J, Xiao Y, Chen D, Huang J. Analysis of doxorubicin uptake in single human leukemia K562 cells using capillary electrophoresis coupled with laser-induced fluorescence detection. Anal Bioanal Chem 2011; 401: 2143-52.
7. Luo V, Lessin SR, Wilson RB, *et al*. Detection of clonal T-cell receptor gamma gene rearrangements using fluorescent-based PCR and automated high-resolution capillary electrophoresis. Mol Diagn 2001; 6: 169-79.
8. Jeon S, Lee MJ, Park J, Kang SH. Fast molecular diagnostics of canine T-cell lymphoma by PCR and capillary gel electrophoresis with laser-induced fluorescence detector. J Chromatogr B Analyt Technol Biomed Life Sci 2007; 854: 268-72.
9. Park JY, Lee MH, Kwak EK, Kim DJ, Park TI, Bae HI. Molecular diagnosis of ctaneous T cell lymphoproliferative diseases. Korean J Pathol 2000; 34: 941-9.
10. Cho EY, Ko YH, Kim DS, Han JJ, Ree HJ. Diagnostic utility of polymerase chain reaction-based clonality analysis for immunoglobulin heavy chain gene and T-cell receptor gamma chain gene rearrangement in lymphoid neoplasms. Korean J Pathol 2001; 35: 461-9.
11. Elenitoba-Johnson KS, Bohling SD, Mitchell RS, Brown MS, Robetorye RS. PCR analysis of the immunoglobulin heavy chain gene in polyclonal processes can yield pseudoclonal bands as an artifact of low B cell number. J Mol Diagn 2000; 2: 92-6.
12. Isola J, DeVries S, Chu L, Ghazvini S, Waldman F. Analysis of changes in DNA sequence copy number by comparative genomic hybridization in archival paraffin-embedded tumor samples. Am J Pathol 1994; 145: 1301-8.
13. Jeffreys AJ, Wilson V, Neumann R, Keyte J. Amplification of human minisatellites by the polymerase chain reaction: towards DNA fingerprinting of single cells. Nucleic Acids Res 1988; 16: 10953-71.
14. Evans PA, Pott Ch, Groenen PJ, *et al*. Significantly improved PCR-based clonality testing in B-cell malignancies by use of multiple immunoglobulin gene targets: report of the BIOMED-2 Concerted Action BHM4-CT98-3936. Leukemia 2007; 21: 207-14.

15. Berget E, Helgeland L, Molven A, Vintermyr OK. Detection of clonality in follicular lymphoma using formalin-fixed, paraffin-embedded tissue samples and BIOMED-2 immunoglobulin primers. J Clin Pathol 2011; 64: 37-41.

16. Pai RK, Chakerian AE, Binder JM, Amin M, Viswanatha DS. B-cell clonality determination using an immunoglobulin kappa light chain polymerase chain reaction method. J Mol Diagn 2005; 7: 300-7.