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Detection of Immunoglobulin *IGH* Gene Rearrangements on Formalin-Fixed, Paraffin Embedded Tissue in Lymphoid Malignancies

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Abstract

Human lymphomas are aggressive malignant diseases, which can be categorized based on their B and T cell lineage. B-cell lymphomas form around 90% of the total lymphoma cases, the remnants of malignancies arise from the T cell branch. Lymphomas are mostly characterized as clonal proliferations of specific tumor cells. The detection of malignant lymphomas are extensively investigated by their morphological features, immunohistochemistry and flowcytometric immunophenotyping, but in some of cases remained unknown. The BIOMED-2 protocols were used to determine the clonality of *IGH* gene rearrangements in patients with lymphoma. PCR amplification was performed on FFPE of 50 patients with B-cell lymphoma, which consisted of 11 cases with HLs, 25 cases of B-NHLs and 14 cases of B-LPD (lymphoproliferative disorders) that diagnosed as unclassifiable lymphoma. The rate of positive clonality was detected in 96% (24/25) of B-NHLs, whereas in 4% (1/25) of cases clonality was showed in a polyclonal pattern. In B-HLs, 82% (9/11) of cases showed clonality and 18% (2/11) of the cases showed polyclonality. The rate of positive clonality observed in 64.3% (9/14) of cases with B-LPD and 35.7% (5/14) of cases clonality was not detected in any of immunoglobulin gene family (FR1و FR2و FR3). In groups with DLBCL, clonality was detected in 95% (19/20) of the cases. In patients diagnosed with FL and MALTs 100% cases showed clonality for complete *IGH*. Our study revealed that EuroClonality BIOMED-2 protocols could be considered as a valuable and reliable method for clonality detection, especially in IGH analysis.

Key words: BIOMED-2, Clonality, DLBCL, HL, IGH, LPD, NHL.

Introduction

Human lymphomas are aggressive malignant diseases, which are categorized according to their B and T cell etiologies. Whilst the B cell subtype makes up around 90% of all lymphomas, the remnant malignancies originate from T cells (1). Based on the World Health Organization (WHO) lymphoproliferative disorders (LPD) classification, lymphomas are characterized into two groups; Hodgkin's lymphoma (HL) and non-Hodgkin's lymphoma (NHL) (2). In humans, B-cell differentiation is coordinated with the expression of particular antigen surface receptors called immunoglobulins (Ig). These molecules are multi-dimeric proteins which contain two indistinguishable heavy chains (H) and two identical light chain: kappa (κ) or lambda (λ) polypeptides, which are linked by disulfide bonds (3). Throughout the B-cell development process, all the genes which are responsible for encoding of the Ig and T-cell receptors (TCR), originate from the gremlin and are created by alternating segments of DNA (4). These gene segments consist of particular configuration of the V (variable), D (diversity), J (joining) and C (constant) regions, which when rearranged, sequentially mediate the V (D) J recombination procedure (5). During lymphoid differentiation, a vast assortment of antigen receptors is generated arbitrarily through the deletion or insertion of nucleotides at various binding sites. (6). As B-cell lymphomas are resulted from a particular neoplastic lymphocyte cell, the malignant cells of approximately all lymphoid tumors enclose one or multi clonal Ig gene rearrangements. Heterogeneity in Ig gene rearrangements refers to the existence of polyclonality, contradictory, the identical rearranged Ig genes; mirror monoclonal tumor cells (7). Lymphomas are mostly characterized as clonal proliferations of specific tumor cells. The detection of malignant lymphomas are largely evaluated by their morphological features, immunohistochemistry, and flow cytometric immunophenotyping (8). However, different circumstances such as samples with unclear characteristics, minimal residual diseases (MDR) and miscellaneous lesions render themselves indistinguishable with the aforementioned conventional analysis (9). In addition, identifying the special features of LPD remains a significant challenge. Routinely, morphological features along with immunopathological assessments are now the principle of diagnosis in neoplastic lymphoma, whereas, the availability of BIOMED-2 protocol further enhances our ability to diagnose and classify lymphoid malignancies. Furthermore, clonality assays of Ig gene rearrangement would be a valuable method for obtaining access to the accurate diagnosis of LPD (8). Currently, the BIOMED-2 multiplex PCR methods, based on clonality assays, are being widely

Table 1. BIOMED-2 multiplex primer mixes for *IGH* (frame work I, II, III) clonality assay.

IGH: FRs (VH-JH)		
	Forward *(5′ - 3′)* VH-FRs	**Reverse *(5′- 3′)* JH-FRs**
V_{H1}**-FRI**	GGCCTCAGTGAAGGTCTCCTGCAAG	
V_{H2}**-FRI**	GTCTGGTCCTACGCTGGTGAAACCC	
V_{H3}**-FRI**	CTGGGGGGTCCCTGAGACTCTCCTG	
V_{H4}**-FRI**	CTTCGGAGACCCTGTCCCTCACCTG	
V_{H5}**-FRI**	CGGGGAGTCTCTGAAGATCTCCTGT	
V_{H6}**-FRI**	TCGCAGACCCTCTCACTCACCTGTG	
V_{H1}**-FRII**	CTGGGTGCGACAGGCCCCTGGACAA	CTTACCTGAGGAGACGGTGACC
V_{H2}**-FRII**	TGGATCCGTCAGCCCCCAGGGAAGG	
V_{H3}**-FRII**	GGTCCGCCAGGCTCCAGGGAA	
V_{H4}**-FRII**	TGGATCCGCCAGCCCCCAGGGAAGG	
V_{H5}**-FRII**	GGGTGCGCCAGATGCCCGGGAAAGG	
V_{H6}**-FRII**	TTGGGTGCGACAGGCCCCTGGACAA	
V_{H7}**-FRII**	TGGATCAGGCAGTCCCCATCGAGAG	
V_{H1}**-FRIII**	TGGAGCTGAGCAGCCTGAGATCTGA	
V_{H2}**-FRIII**	CAATGACCAACATGGACCCTGTGGA	
V_{H3}**-FRIII**	TCTGCAAATGAACAGCCTGAGAGCC	
V_{H4}**-FRIII**	GAGCTCTGTGACCGCCGCGGACACG	
V_{H5}**-FRIII**	CAGCACCGCCTACCTGCAGTGGAGC	
V_{H6}**-FRIII**	GTTCTCCCTGCAGCTGAACTCTGTG	
V_{H7}**-FRIII**	CAGCACGGCATATCTGCAGATCAG	

applied in the diagnosis of suspected lymphoproliferative disorders (LPD) (8). This study was undertaken to employ clonality analysis of *IGH* gene rearrangements in the diagnosis of lymphoproliferative disorders.

Materials and methods

Samples collection

In this study, we enrolled a total number of 50 cases with malignant lymphoma which were referred to the department of pathology at Tabriz Imam Reza Hospital between 2010 and 2012. All the patients were evaluated by pathologists in terms of clinical parameters and conventional methods to verify existence of the malignant disorders. We performed clonality assays on formalin-fixed, paraffin embedded (FFPE) tissues in 11 cases (7 male and 4 female) diagnosed with HL, twenty cases (17 male and 3 female) with diffuse large B-cell lymphoma (DLBCL), three cases (2 male and1 female) with mucosa-associated lymphoid tissue (MALTs) and two cases (2 male) with follicular lymphoma (FL). In addition, we assessed fourteen cases (10 male and 4 female) with an unclassifiable lymphoma, which were categorized as Lymphoproliferative Disorders (LPD).

DNA extraction

Initially, all of the FFPE samples were deparaffinised with xylene, washed with two dilutions of ethanol and incubated overnight with proteinase K (20 mg/ml) for tissue digestion. We then extracted DNA according to the salting-out method (10). The extracted DNA from FFPE tissues was assessed using spectrophotometer (260/280 nm using the Nano-DropTM ND-1000, Nano-Drop Technology, Wilmington, DE, USA) to ensure the purity and concentration of the DNA. The average DNA samples had DNA concentration and OD260/280 ratios 250 ng/µl (ranging between 300-850 ng/µl) and 1.85. Samples with PCR products larger than 300 bp were applied as suitable and less than 300 bp were excluded for gene rearrangement clonality analysis. In addition, for the quality assessment of the DNA samples, we used control genes, which were proposed in the BIOMED-2 guideline (8). In order to validate monoclonality results and avoid pseudoclonality (non-reproducible clonal rearrangements), all samples were assessed two times. We used a gold standard multiplex PCR protocol (provided by European Biomedicine and Health (BIOMED-2) Concerted Action Project BMH4-CT98-3936) for improvement of diagnosis and analysis of clonality gene rearrangement in lymphoma malignancies.

PCR-Amplification for IGH Gene Rearrangements

PCR amplifications were carried out in a final volume of 25µl reactions including150-200ng of genomic DNA, 2x master mix red with 0.2 Units/µl Ampliqon Taq DNA polymerase [Ampliqon A/S, Stenhuggervej 22, Denmark] and one picomole for each primer according to the BIOMED-2 protocol. The target reactions for *IGH* gene arrangement assays included three reactions targeting IGH_A (V_HF_{R1}-J_H), IGH_B (V_HF_{R2}-J_H) and IGH_C (V_HF_{R3}-J_H (Table 1).

Rearrangement assays of the target genes (three framework regions; FRI, FRII and FRIII) were amplified with the following PCR conditions: initial denaturation for 10 minutes at 95°C, followed by 35 cycles of 95°C for 30 seconds, 60 °C for 30 seconds and finally 72°C for 90 seconds. Before the end of the amplification cycles, a final extension step was performed at 72°C for 10 minutes. We used clonal control genes was used according to the proposed BIOMED-2 protocols in each run of the PCR amplification (InVivoScribe Technologies,SanDiego,CA, USA).

Heteroduplex analysis

For heteroduplex analysis, initially, the PCR products were denatured at 95ºC for 5minutes and subsequently

Table 2. Gene rearrangement analysis on patients with lymphoid malignancies to detect clonality and polyclonaliity.

DIAGNOSIS/IGH	FR1	FR2	FR3	FR1+FR2	FR2+FR3	FR1+FR3	FR1+FR2+FR3
DLBCL	4/20 (20%)	8/20 (40%)	7/20 (35%)	12/20 (60%)	15/20(75%)	11/20(55%)	19/20(95%)
FL	1/2 (50%)	0%	1/2 (50%)	1/2 (50%)	1/2(50%)	2/2(100%)	2/2(100%)
MALT	1/3 (33%)	0%	2/3 (66.6%)	1/3 (33%)	2/3(66.6%)	3/3(100%)	3/3(100%)
HL	0%	4/11 (36%)	5/11(45%)	4/11 (36%)	9/11(82%)	5/11(45%)	9/11(82%)
LPD	1/14 (7%)	5/14 (35.7%)	3/14(21%)	6/14(43%)	8/14(57%)	4/14(28.5%)	9/14(64.3%)
TOTAL n=50	7/50(14%)	17/50(34%)	18/50(36%)	24/50(48%)	35/50(70%)	25/50(50%)	42/50(84%)

DLBCL, diffuse large B-cell lymphoma; FL, follicular lymphoma; MALT, mucosa-associated lymphoid tissue lymphoma; HL, Hodgkin lymphoma; LPD, Lymphoproliferative disorders .

incubated at 4ºC to generate homoduplex. Next, the PCR products were immediately loaded on an 8% non-denaturing polyacrylamide gel, run at room temperature and visualized by silver nitrate staining. The observation of one or two bands within the expected size (between 310-360bp for FRI, between 250-295bp for FRII, and between 100–170 bp for FRIII) indicated clonality, while polyclonality patterns were revealed as rough smear in the gel (11).

Results

Gene rearrangement analysis revealed clonality in 96% (24/25) of B-NHLs, while a single case showed polyclonaliity (4%) (Table 2). In B-HLs, 9 out of 11 (82%) cases showed clonality for complete *IGH* whereas 2 out of 11 (18%) cases showed a polyclonal pattern. Positive clonality was observed in 9 out of 14 (64.3%) cases of B-LPD whereas in the rest of the 5 cases (35.7%), clonality was not detected in any of the immunoglobulin gene family (FR1 و FR2 و FR3) and showed a polyclonal pattern. In the DLBCL group, clonality was detected in 19 out of the 20 (95%) cases for complete *IGH*. Four (20%) cases showed clonality for FR1, eight (40%) cases for FR2 and seven (35%) cases for FR3). In patients diagnosed with FL and MALTs, 100% of cases showed clonality for complete *IGH*. Therefore, among 50 cases of B-cell lymphomas, 42 (84%) cases showed clonality for complete *IGH* (Figures 1-3).

Discussion

Generally, lymphomas originate from single neoplastic lymphoid cells. As mentioned above, lymphoblastic cells contain identical Ig gene rearrangement patterns. Several routine diagnostic criteria for lymphoma detection exist in histopathology laboratories; however, these are not suitable for all cases with B-cell lymphoma. Use of the molecular genetics approach clonality testing in assumed B-cell malignancies, has been proposed to be a reliable method for atypical subjects. Euro Clonality BIOMED-2 Concerted Action developed a standard protocol for clonality detection (8, 12). In this study, we assessed the application value of BIOMED-2 clonality assays for the detection of clonality in B-cell malignan-

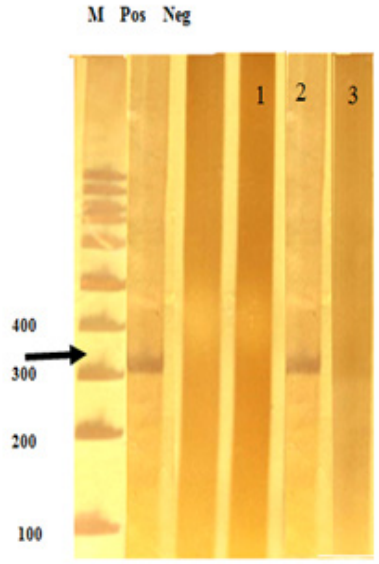

Figure 1. Gene rearrangements in FRI region. Lane 1; Negative: Lane (2, 3); Monoclonal band (320bp): N; Negative: Pos; Positive (IVS-0010), M; DNA marker 100bp.

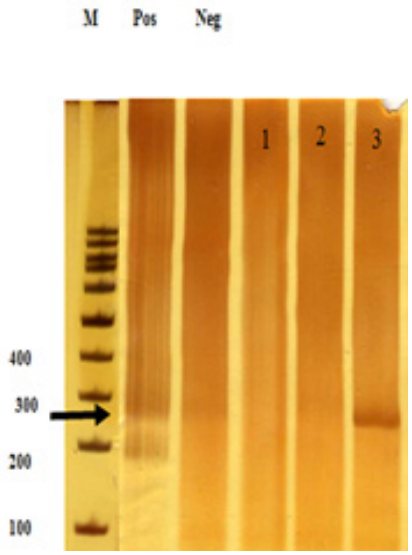

Figure 2. Gene rearrangements in FRII region. Lane (1, 2); Negative Lane 3; Monoclonal band (288bp): N; Negative: Pos; Positive (IVS-0010), M; DNA marker 100bp.

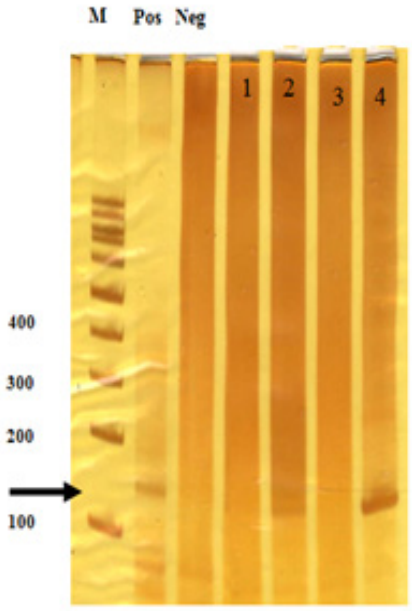

Figure 3. Gene rearrangements in FRIII region. Lane (1, 3); Negative: Lane (2, 4); Monoclonal band (155bp): N; Negative: Pos; Positive (IVS-0010), M; DNA marker 100bp.

cies including B-NHLs and HLs and compared the data between the two groups. The use of three complete *IGH* rearrangement assays detected total positive clonality in 42 of the 50 (84%) cases of B-cell malignancies. Our findings revealed positive clonality for FR1, FR2 and FR3 in 14% (7/50), 34% (17/50) and 36% (18/50of cases respectively. Of 20 cases diagnosed with DLBCL, 19 (95%) showed positive clonality for *IGH* which was higher than the previous studies which reported clonality in 67%-88% of cases (13-16). Further, positive clonality was detected in 20% (4/20) for FR1, which was similar to that reported by Catherwood *et al* (14), but significantly lower than that observed by others (12, 16). In addition, our results revealed a positive rate of 40% (8/20) in clonality detection for FR2 in DLBCL group, while other studies reported clonality ranging between 56%-61% (12, 14, 16). Some studies recorded a rate of positive clonality 50% for FR3 rearrangements (12, 16) although a significantly lower value (35%) was registered in the present study. The lower detection rates of clonality in FR1 and FR2 compared to FR3 could be due to the occurrence of SHM (somatic hypermutation) in the targeting site of the FR3 region (17) causing primer mismatching during Ig rearrangements assays (14).

IGH gene rearrangements analysis performed on FFPE samples in two patients with FL showed positive clonality in both, one for FR1 and the other for FR3. Amara *et al*. (13) and Liu *et al*. (16) had shown significantly lower 37% and 43% clonality for *IGH* respectively. Other investigators reported 76/7% and 96% positive clonality for *IGH* rearrangements (14, 18). A positive rate of detection of clonality for FR1 ranged from 30% to 73% and for FR3 from 13% to 54% (12, 14, 16, 18). In cases diagnosed with MALT, B-cell clonality by *IGH* multiplex PCR assays was detected in 84%, 86% and 100% respectively (12, 16, 19). Out of the three cases of MALT investigated in the present study two showed a clonal marker for FR3 and one for FR1. However, our analysis needs to be extended over a large number of patients.

Assessment of *IGH* gene rearrangement in 11 cases of HL revealed 82% (9/11) positive clonality in these cases. It is interesting that this rate of positive clonality was higher than previous studies, which reported a range between 21%-50% (15, 20, 21, 22). HL cases included mixed cellularity and nodular sclerosis sub-types and all samples consisted of high density of neoplastic CD30 positive cells. This could be the reason for a relatively higher percentage of clonality in our study. Among cases diagnosed with B-LPD (unclassifiable), the rate of positive clonality was observed in 7% for FR1, 35.5% for FR2, and 21% for FR3. However, with combination of three frame work regions in *IGH* assays, a total of 64.3% (9/14) clonality was detected. Liu *et al*. (16) achieved a 91% detection rate of Ig gene rearrangements when three reactions comprising of one targeting the *IGH* framework-2 region and two targeting the *IGK* locus were employed.

In conclusion, the combined use of three *IGHFR1, FR2 and FR3,* rearrangements to assay B-cell clonality, has provided a good diagnostic tool for the detection of LPD, especially in patients without a clear morphological status of lymphoma.

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References

1. Jaffe, E.S., Harris, N.L., Stein, H., Vardiman, J.W., Pathology & Genetics - Tumours of haematopoietic and lymphoid tissues. IARC. Lyon. 2001.

2. Swerdlow, S.H., WHO classification of tumours of haematopoietic and lymphoid tissues. IARC. Lyon. 2008.

3. Abbas, A.K., Lichtman, A.H., Pillai, S., Cellular and Molecular Immunology: with STUDENT CONSULT Online Access: Elsevier Health Sciences; 2011.[to check 2012? 8th ed.]

4. Gill, J.I., Gulley, M.L., Immunoglobulin and T-cell receptor gene rearrangement.*Hematology/oncology clinics of North America.* 1994, **8** (4): 751-770. doi:

5. Davis, M.M., Bjorkman, P.J., T-cell antigen receptor genes and T-cell recognition. *Nature.* 1988, **334** (6181): 395-402. doi: 10.1038/334395a0

6. Epstein, F.H., Küppers, R., Klein, U., Hansmann, M.L., Rajewsky, K., Cellular origin of human B-cell lymphomas. *New England Journal of Medicine.* 1999, **341** (20): 1520-1529. doi: 10. 1056/NEJM199911113412007

7. Langerak, A.W., Groenen, P.J.T.A., van Krieken, J.H.J.M., van Dongen, J.J.M., Immunoglobulin/T-cell receptor clonality diagnostics. Expert Opin. Med. Diagn. 2007, **1** (4): 451-461. doi: 10.1517/17530059.1.4.451

8. Van Dongen, J.J.M., Langerak, A.W., Brüggemann, M., Evans, P.A.S., Hummel, M., Lavender, F.L., Delabesse, E., Davi, F., Schuuring, E., Garcia-Sanz, R., *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** (12): 2257-2317. doi: 10.1038/sj.leu.2403202

9. Wolvers-Tettero, I.L., Analysis of immunoglobulin and T cell receptor genes. Part I: Basic and technical aspects. *Clinica Chimica Acta.* 1991, **198** (1): 1-92. doi: 10.1016/0009-8981(91)90246-9

10. Rivero, E.R., Neves, A.C., Silva-Valenzuela, M.G., Sousa, S.O., Nunes, F.D., Simple salting-out method for DNA extraction from formalin-fixed, paraffin-embedded tissues. Pathology-Research and Practice. 2006, **202** (7): 523-529. doi: 10.1016/j.prp.2006.02.007.

11. Langerak, A.W., Groenen, P.J.T.A., Brüggemann, M., Beldjord, K., Bellan, C., Bonello, L., Boone, E., Carter, G.I., Catherwood, M., Davi, F., *et al*.: EuroClonality/BIOMED-2 guidelines for interpretation and reporting of Ig/TCR clonality testing in suspected lymphoproliferations. *Leukemia.* 2012, **26** (10): 2159-2171. doi: 10.1038/leu.2012.246

12. Evans, P., Pott, C., Groenen, P., Salles, G., Davi, F., Berger, F., Garcia, J., Van Krieken, J., Pals, S., Kluin, P., 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** (2): 207-214. doi: 10.1038/sj.leu.2404479

13. Amara, K., Trimeche, M., Ziadi, S., Sriha, B., Mokni, M., Korbi, S., PCR-based clonality analysis of B-cell lymphomas in paraffin-embedded tissues: Diagnostic value of immunoglobulin κ and λ light chain gene rearrangement investigation. *Pathology-Research and Practice.* 2006, **202** (6):425-431. doi: 10.1016/j.prp.2005.09.005

14. Catherwood, M.A., Gonzalez, D., Patton, C., Dobbin, E., Venkatraman, L., Alexander, H.D., Improved clonality assessment in germinal centre/post-germinal centre non-Hodgkin's lymphomas with high rates of somatic hypermutation. *Journal of clinical pathology.* 2007, **60** (5): 524-528. doi: 10.1136/jcp.2006.038984

15. McClure, R., Kaur, P., Pagel, E., Ouillette, P., Holtegaard, C., Treptow, C., Kurtin, P., Validation of immunoglobulin gene rearrangement detection by PCR using commercially available BIOMED-2 primers. *Leukemia.* 2006, **20** (1): 176-179. doi: 10.1038/sj.leu.2404049

16. Liu, H., Bench, A.J., Bacon, C.M., Payne, K., Huang, Y., Scott, M.A., Erber, W.N., Grant, J.W., Du, M.Q., A practical strategy for the routine use of BIOMED-2 PCR assays for detection of B-and Ttcell clonality in diagnostic haematopathology. *British journal of haematology.* 2007, **138** (1): 31-43. doi: 10.1111/j.1365-2141.2007.06618.x

17. Jolly, C.J., Wagner, S.D., Rada, C., Klix, N., Milstein, C., Neuberger, M.S., The targeting of somatic hypermutation. *Seminars in immunology: 1996*: Elsevier; 1996: 159-168. doi: 10.1006/smim.1996.0020

18. Berget, E., Helgeland, L., Molven, A., Vintermyr, O.K., Detection of clonality in follicular lymphoma using formalin-fixed, paraffin-embedded tissue samples and BIOMED-2 immunoglobulin primers. *Journal of clinical pathology.* 2011, **64** (1): 37-41.doi: 10.1136/jcp.2010.081109

19. Kim, Y., Choi, Y.D., Choi, C., Nam, J.H., Diagnostic Utility of a Clonality Test for Lymphoproliferative Diseases in Koreans Using the BIOMED-2 PCR Assay. *Korean journal of pathology.* 2013, **47** (5): 458-465. doi: 10.4132/KoreanJPathol.2013.47.5.458

20. Burack, W.R., Laughlin, T.S., Friedberg, J.W., Spence, J.M., Rothberg, P.G., PCR assays detect B-lymphocyte clonality in formalin-fixed, paraffin-embedded specimens of classical hodgkin lymphoma without microdissection. *American journal of clinical pathology.* 2010, **134** (1): 104-111. doi: 10.1309/AJCPK6SBE0XODHB

21. Hebeda, K., Van Altena, M., Rombout, P., Van Krieken, J., Groenen, P., PCR clonality detection in Hodgkin lymphoma. *Journal of hematopathology.* 2009, **2** (1): 34-41. doi: 10.1007/s12308-009-0024-1

22. Chute, D.J., Cousar, J.B., Mahadevan, M.S., Siegrist, K.A., Silverman, L.M., Stoler, M.H., Detection of immunoglobulin heavy chain gene rearrangements in classic Hodgkin lymphoma using commercially available BIOMED-2 primers. *Diagnostic Molecular Pathology.* 2008, **17** (2): 65-72. doi: 10.1097/PDM.0b013e318150d69