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Improved Clonality Detection in B-cell Lymphoma Using a Semi-nested Modification of the BIOMED-2 PCR Assay for *IGH* Rearrangement: A Paraffin-embedded Tissue Study

Short running title: Semi-nested modification to BIOMED-2 assay (43 characters)

Yuma Sakamoto,[1] Ayako Masaki,[1] Satsuki Aoyama,[1] Shusen Han,[1] Kosuke Saida,[1] Kana Fujii,[1] Hisashi Takino,[1] Takayuki Murase,[1] Shinsuke Iida,[2] and Hiroshi Inagaki[1]

[1]Department of Pathology and Molecular Diagnostics, Nagoya City University Graduate School of Medical Sciences, Nagoya, Japan.
[2]Department of Hematology and Oncology, Nagoya City University Graduate School of Medical Sciences, Nagoya, Japan.

Correspondence: Hiroshi Inagaki, MD, PhD, Department of Pathology and Molecular Diagnostics, Graduate School of Medical Sciences, Nagoya City University, 1 Kawasumi, Mizuho-cho, Mizuho-ku, Nagoya 467-8601, Japan.
TEL: +81-853-8161, FAX: +81-851-4166, E-mail: hinagaki@med.nagoya-cu.ac.jp

YS and AM equally contributed to this work.

2736 words, 21 References, 2 Tables, and 4 Figures

ABSTRACT (182 words)

The BIOMED-2 PCR protocol for targeting the *IGH* gene is widely employed for detecting clonality in B-cell malignancies. Unfortunately, the detection of clonality with this method is not very sensitive when paraffin sections are used as a DNA source. To increase the sensitivity, we devised a semi-nested modification of a *JH* consensus primer. The clonality detection rates of three assays were compared: the standard BIOMED-2, BIOMED-2 assay followed by BIOMED-2 re-amplification, and BIOMED-2 assay followed by semi-nested BIOMED-2. We tested more than 100 cases using paraffin-embedded tissues of various B-cell lymphomas, and found that the clonality detection rates with the above three assays were 63.9%, 79.6%, and 88.0%, respectively. While BIOMED-2 re-amplification was significantly more sensitive than the standard BIOMED-2, the semi-nested BIOMED-2 was significantly more sensitive than both the standard BIOMED-2 and BIOMED-2 re-amplification. An increase in sensitivity was observed in all lymphoma subtypes examined. In conclusion, tumor clonality may be detected in nearly 90% of B-cell lymphoma cases with semi-nested BIOMED-2. This ancillary assay may be useful when the standard BIOMED-2 fails to detect clonality in histopathologically suspected B-cell lymphomas.

Keywords: BIOMED-2 PCR assay, re-amplification, semi-nested modification, B-cell lymphomas, formalin-fixed paraffin-embedded tissues

INTRODUCTION

B-cell lymphomas account for more than 70% of non-Hodgkin's lymphomas and are composed of many tumor subtypes.[1] A significant subset of B-cell lymphomas (5-15%) show atypical morphological and immunophenotypic features, and differential diagnosis with respect to reactive lymphadenopathy is critically important. In addition, it is not uncommon for paraffin tumor sections to be the only materials available for pathological diagnosis. In these settings, the polymerase chain reaction (PCR)-based clonality assay targeting immunoglobulin genes has contributed much to an accurate diagnosis of B-cell malignancies.[2]

PCR-based clonality assays were first introduced in the early 1990s, and different PCR strategies using different primer sets have been developed for determining clonality in B-cell malignancies.[3,4] In 2003, a comprehensive work of the European BIOMED-2 collaborative study group led to new standardized PCR protocols for the clonality assay using multiple primer sets for immunoglobulin gene rearrangements.[2,5] Many studies have reported the successful application of BIOMED-2 clonality assays in a diagnostic setting,[6-8] and they are now employed worldwide as the standard PCR-based method for assessing clonality.[9] Unfortunately, when paraffin sections are used as a DNA source to detect *IGH* gene rearrangement with this method, clonality detection rates in B-cell malignancies are not very high (52.2%-87.5%, average 70.9%).[2,5,10-13] This is partly because the extracted DNA may be highly degraded[2,5] and/or only a small amount of DNA can be obtained from biopsied tumor samples, especially in cases of extranodal lymphoma.[2]

Re-amplification of the first-round PCR products and nested PCR strategies are techniques that are

frequently used to increase the sensitivity and specificity of PCR protocols.[14] To the best of our knowledge, applications of these techniques in BIOMED-2 clonality assays have not been described. In this study, we devised a semi-nested PCR protocol for a BIOMED-2 *IGH* rearrangement clonality assay (semi-nested BIOMED-2). Employing more than 100 cases of B-cell lymphoma, we compared clonality detection rates of the standard BIOMED-2 *IGH* clonality assay (BIOMED-2 assay), the BIOMED-2 *IGH* assay followed by re-amplification (BIOMED-2 re-amplification), and the BIOMED-2 *IGH* assay followed by semi-nested amplification (semi-nested BIOMED-2).

MATERIALS AND METHODS

Case selection and DNA extraction

B-cell lymphoma cases (n=126) were retrieved from the files of the Department of Pathology and Molecular Diagnostics, Nagoya City University Graduate School of Medical Sciences. Formalin-fixed, paraffin-embedded sections of these cases were subjected to DNA extraction. Non-neoplastic lymph nodes obtained from cancer patients during surgery (n=15) were used as polyclonal controls. To confirm DNA quality, a fragment of the *PLZF* gene (300 bp) in each case was amplified by PCR.[2] The quality of DNA was well preserved in all non-neoplastic lymph nodes but was not in 18 cases of the lymphoma, and the latter were excluded from the present study. In total, 108 cases of B-cell lymphoma (Table 1) and 15 cases of reactive lymphadenopathy were included. All cases were reviewed and after precise immunohistochemical evaluation, diagnoses were confirmed by hematopathologists (AM and HI) according to the WHO classification of malignant lymphomas.[15] Genomic DNA was extracted by incubating deparaffinized tissues overnight at 56 ºC in digestion buffer containing proteinase K. The samples were heated at 95 ºC for 10 minutes to inactivate proteinase K and were stored at −20 ºC until use. This study was approved by the Nagoya City University Institutional Review Board, and conducted in accordance with the Declaration of Helsinki.

BIOMED-2 assay for *IGH* rearrangement

The BIOMED-2 assay for *IGH* rearrangement was carried out according to a protocol as described previously,[2] with some modifications. Each PCR was carried out in a 25-μL volume. This procedure consists of tri-parallel multiplex PCRs carried out in three tubes. As shown in Figure 1, tube A contained six primers for the *FR1* region and a *JH* consensus primer. Similarly, tube B contained seven primers for the *FR2* region and a *JH* consensus primer, and tube C contained seven primers for the *FR3* region and a *JH* consensus primer. The *JH* consensus primer used for the *FR4* region was common to the above three PCRs.[2] The BIOMED-2 assay was carried out in triplicate and only cases showing consistently positive results were considered to be monoclonal.

In the preliminary experiment, we found that the BIOMED-2 assay when performed with more than 35 cycles of amplification (e.g., 40, 45, or 50 cycles) resulted in an increase in non-specific bands and primer dimers with no increase in the sensitivity of clonal detection.

Re-amplification using BIOMED-2 protocol

Lymphoma cases where clonality was not detected in the first-round BIOMED-2 assay were subjected to re-amplification. The first-round PCR products of those cases were re-amplified using the BIOMED-2 protocol (Figure 1), and PCR conditions used were the same as those of the original BIOMED-2 protocol.[2] The first-round PCR products were diluted with distilled water, and the amount corresponding to 0.02μL of the first-round PCR products was subjected to the PCR mixtures (25 μL) of the BIOMED-2 re-amplification.

Semi-nested modification to BIOMED-2 protocol

We carried out a semi-nested modification of the BIOMED-2 protocol. For this purpose, we designed the following three primers internal to the *JH* consensus primer: 5' - ACCTGAGGAGACGGTGACCAGG -3', 5' - ACCTGAGGAGACGGTGACCATT -3' and 5' - ACCTGAGGAGACGGTGACCGTG -3.' We designed these three nested primers 1) to be at least three bases internal, 2) not to have long runs of a single base in the 3' ends for avoiding mispriming, and 3) to minimize the number of primers used. In our modified assay (tri-parallel), we used these three multiple primers instead of the *JH* consensus primer, and the original *FR1/2/3* multiple primers were employed (Figure 1). In the preliminary experiment, a clonality assay employing primers described above detected clonal proliferation with a sensitivity similar to that for the original BIOMED-2 assay (data not shown). Lymphoma cases where clonality was not detected in the first-round BIOMED-2 were subjected to a second-round semi-nested BIOMED-2 PCR. The PCR conditions used for the semi-nested assay were the same as those used for the BIOMED-2 except for the primers employed. The first-round PCR products were diluted with distilled water, and the amount corresponding to 0.02μL of the first-round PCR products was subjected to the PCR mixtures (25 μL) of the semi-nested BIOMED-2.

Evaluation of clonality

In the preliminary experiment, two types of detection technique were compared; electrophoresis of the homoduplexes in a 6% polyacrylamide gel and DNA-chip-based electrophoresis (Agilent Bioanalyzer 2100, Agilent Technologies, Santa Clara, CA, USA). We found that the latter technique, which is rapid and simple, and can be performed at a low cost per sample, was more sensitive than the former.[16] The PCR products were subjected to the DNA-chip-based electrophoresis and the results were evaluated as follows: when one or two well-defined peaks were visualized as equivalent to the positive control, the case was judged as a monoclonal proliferation; when a smear or ladder pattern was observed, the case was considered as a polyclonal proliferation; and when no bands were observed, the case was judged to be non-amplifiable. When monoclonal bands were observed in at least one of the tri-parallel PCRs (tubes A-C), the case was judged to be a monoclonal proliferation.[2]

Statistical analysis

Differences between two groups were assessed by the sign test. A $P<0.05$ was considered as statistically significant.

RESULTS

The total of 108 B-cell lymphoma cases consisted of eight tumor subtypes: diffuse large B-cell lymphoma (DLBCL) (n=36), chronic lymphocytic leukemia/ small lymphocytic lymphoma (CLL/SLL) (n=11), nodal marginal zone lymphoma (MZL) (n=6), extranodal MZL of mucosa-associated lymphoid tissue (MALT) (n=15), follicular lymphoma (FL) (n=19), mantle cell lymphoma (MCL) (n=13), and Burkitt lymphoma (BL) (n=8). The PCR products were evaluated using DNA-chip-based electrophoresis (Figures 2,3) and the results are summarized in Table 1. Non-neoplastic lymph nodes (n=15) used as polyclonal controls showed a smear pattern in all three BIOMED-2-based assays.

Standard BIOMED-2 assay

The standard BIOMED-2 assay of the 108 cases of B-cell lymphoma detected clonal proliferation in 69 (63.9%) and failed to show clonality in 39 (36.1%): polyclonal proliferation was seen in 10 cases (9.3%) and non-amplification in the remaining 29 (26.9%) (Table 1 and Figures 2-4). Using tube A (*FR1*), tube B (*FR2*), and tube C (*FR3*), clonality was observed in 28 (25.9%), 39 (36.1%), and 46 (42.6%) cases, respectively (Table 2). High detection rates of clonality (>80%) were achieved in cases of CLL/SLL (9/11, 81.8%) and nodal MZL (5/6, 83.3%). The detection rates were lower in BL cases (3/8, 37.5%).

BIOMED-2 re-amplification

Thirty-nine cases judged as polyclonal or non-amplifiable in the first-round standard BIOMED-2 assay were subjected to BIOMED-2 re-amplification (Table 1 and Figures 2-4). Seventeen cases, all of which were non-amplifiable in the first-round BIOMED-2 assay, were judged to be monoclonal in the BIOMED-2 re-amplification. All 10 cases judged as polyclonal in the first-round BIOMED-2 assay remained polyclonal in the BIOMED-2 re-amplification. Consequently, clonality was detected in 86 cases (79.6%) of the B-cell lymphomas evaluated. Clonality was observed with tube A (*FR1*), tube B (*FR2*), and tube C (*FR3*) assays in 31 cases (28.7%), 48 (44.4%), and 59 (54.6%) cases, respectively (Table 2). High detection rates for monoclonality were achieved in CLL/SLL (10/11, 90.9%), nodal MZL (6/6, 100%), MCL (12/13, 92.3%), and BL (8/8, 100%). The detection rates were somewhat lower in DLBCL (26/36, 72.2%), MALT lymphoma (10/15, 66.7%) and FL cases (14/19, 73.3%).

Semi-nested BIOMED-2

The 39 cases judged as polyclonal or non-amplifiable in the first-round BIOMED-2 assay were subjected to semi-nested BIOMED-2 (Table 1 and Figures 2-4). Twenty-six cases, all of which were from the non-amplifiable group of the first-round BIOMED-2, were judged to be monoclonal in the semi-nested

BIOMED-2. We repeated semi-nested BIOMED-2 assay in these 26 cases using newly extracted DNA, and obtained results identical to those of the initial semi-nested BIOMED-2 assay. All 10 cases judged as polyclonal in the first-round BIOMED-2 remained polyclonal in the semi-nested BIOMED-2. Consequently, 95 cases (88.0%) showed monoclonal proliferation. Monoclonality was observed in 33 (30.6%), 53 (49.1%), and 62 (57.4%) cases, using tube A (*FR1*), tube B (*FR2*), and tube C (*FR3*) assays, respectively (Table 2). The monoclonality detection rates exceeded 70% in all lymphoma subgroups examined. High detection rates were obtained in CLL/SLL (11/11, 100%), nodal MZL (6/6, 100%), MCL (13/13, 100%), BL (8/8, 100%) and MALT lymphoma (14/15, 93.3%), and the detection rates were somewhat lower in cases of DLBCL (28/36, 77.8%) and FL (15/19, 78.9%).

Comparison of clonality detection rates of the three assays

In the present series of B-cell lymphomas (n=108), BIOMED-2 re-amplification (86/108, P<0.0001) and semi-nested BIOMED-2 (95/108, P<0.0001) yielded higher clonality detection rates than the standard BIOMED-2 assay (69/108). In addition, a higher detection rate was obtained with the semi-nested BIOMED-2 than BIOMED-2 re-amplification (P=0.0039) (Figure 4). The clonality detection rates seen with BIOMED-2 re-amplification and semi-nested BIOMED-2 were higher than those with the standard BIOMED-2 in most of the lymphoma groups, although these differences were not statistically significant. Similarly, clonality detection with the semi-nested BIOMED-2 was higher than with BIOMED-2 re-amplification for most of the lymphoma groups, but the differences were also not statistically significant.

DISCUSSION

Since first described in 2003, BIOMED-2 clonality assays have been used worldwide, and now are considered as one of the standard means of PCR-based clonality testing.[2,9] Many studies on B-cell lymphoma employed BIOMED-2 clonality assay for *IGH* gene rearrangement using paraffin specimens.[2,5,10-13] Clonality detection in these studies has ranged from 52-88% (average 71%) and the detection rate obtained here (63.9%) was comparable to those of the previous reports. However, these detection rates appeared not to be sufficiently high, and certain modifications to the standard BIOMED-2 IGH clonality assay were thought necessary for increasing detection rates. For this purpose, we first increased the number of PCR cycles from the recommended 35 up to 50 in the preliminary analysis. Unfortunately, this approach failed to increase the detection sensitivity and only resulted in an amplification of primer dimers. Many factors have been presumed to contribute to this limited amplification, including inhibition of enzyme activity by an increasing pyrophosphate concentration and a reduction in the denaturation efficiency per cycle as well as exhaustion or inactivation of substrates (dNTPs or primers) and DNA polymerase.[14,17] In addition, the limited amplification may also reflect the multiplex approach of the BIOMED-2 assay[18] since the BIOMED-2 IGH clonality assay employs seven or eight primers, which may increase the possibility of primer complementarities at the 3' ends, resulting in the formation and amplification of primer dimers when performed under non-recommended PCR conditions. Twenty-nine

(26.9%) cases were judged as non-amplifiable in the first-round BIOMED-2 assay. Although DNA quality was confirmed in all lymphoma cases by amplifying a fragment of the *PLZF* gene (300 bp), non-amplifiable cases may be explained by difference in PCR efficiency, and the BIOMED-2 PCR was probably less efficient than that for *PLZF* gene.

To increase the sensitivity of PCR assays, re-amplification of first-round PCR products is frequently used.[14] Semi-nested PCR, which is a modification of the full-nested PCR strategy, is another technique that contributes to increasing not only the sensitivity but also the specificity of the PCR assay.[14] To the best of our knowledge, these techniques have not been applied to the BIOMED-2 assay for *IGH* rearrangement. With the re-amplification technique, the detection rate increased from 63.9% to 79.6% ($P<0.0001$). When we applied the semi-nested modification to the standard BIOMED-2 assay, the detection rate was further increased from 63.9% to 88.0% ($P<0.0001$). Notably, significantly higher rates were obtained with the semi-nested BIOMED-2, compared with BIOMED-2 re-amplification ($P=0.0039$), suggesting that the former technique may be more useful than the latter for detection of clonal proliferation in B-cell lymphomas. For our semi-nested modification of the standard BIOMED-2 assay, we searched a *JH* gene database and designed three different primers inner to the *JH* consensus primer of the BIOMED-2 protocol. These three *JH* primers cover all *JH* fragments of the human *IGH* gene. When amplifying immunoglobulin genes, it is necessary to take somatic hypermutation into account. Somatic hypermutation is a mechanism which improves the biding of immunoglobulins to their antigens, mainly as a result of single base substitutions of the immunoglobulin genes.[1] In some lymphoma cases, BIOMED-2 PCR amplification may be less efficient owing to imperfect annealing of the *JH* consensus primer caused by somatic hypermutation in *JH* regions. In such cases, it is intriguing to assume that the imperfect primer annealing may have been partly resolved by inner *JH* primers used in the semi-nested BIOMED-2 assay.

Most of the cases in which clonality was detected in the second-round assay were non-amplifiable and not polyclonal in the first-round BIOMED-2 assay. This finding suggests that amplification with the first-round PCR only was not sufficient for judging clonality. Indeed, clonality was detected in only 37.5% of BL cases in the first-round BIOMED-2. BL often presents as an extranodal high-grade lymphoma with fragile and necrotizing nuclear features,[15] and it is often difficult to obtain a sufficient amount of DNA for the clonality assay. Unfortunately, increasing the amount of template DNA may not always lead to an increase in the rate of clonality detection since PCR inhibitors are known to be present in DNA extracted from paraffin samples. Conversely, it has been reported that dilution of the DNA templates extracted from paraffin materials can result in successful PCR amplification.[2] This suggests that the second-round PCR strategy may be helpful in cases where the first-round BIOMED-2 results are negative.

The EuroClonality network has proposed an algorithm for target selection,[9] and in cases of suspected B-cell clonality, they have emphasized that the three different *IGH FR* targets should be chosen first, preferably with the *IGK* targets. *IGK* targets have been reported to be useful in germinal center (GC)/post-GC B-cell malignancies, especially follicular lymphomas, because of the high prevalence of somatic hypermutation in the rearranged immunoglobulin genes.[19-21] In this study, clonality detection rates were not

dramatically increased in follicular lymphomas with BIOMED-2 re-amplification or semi-nested BIOMED-2, and *IGK* targets may be useful when the BIOMED-2 *IGH* assay fails to show tumor clonality. However, it is usually difficult to know beforehand whether the suspected lymphoproliferative lesion to be tested is a GC/post-GC B-cell malignancy. In addition, as discussed above, *IGK* targeting may not always be successful when the false negative results in the BIOMED-2 *IGH* assay are associated with small amounts of template DNA obtained from the lymphoma tissues.

In conclusion, we showed that the clonality detection rates obtained with our semi-nested BIOMED-2 were significantly higher than those obtained with the standard BIOMED-2 and with BIOMED-2 re-amplification. The novel semi-nested modification of the standard BIOMED-2 *IGH* assay presented here may be a useful ancillary assay for detecting tumor clonality in B-cell lymphomas.

DISCLOSURE STATEMENT

None declared.

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FIGURE LEGENDS

Figure 1

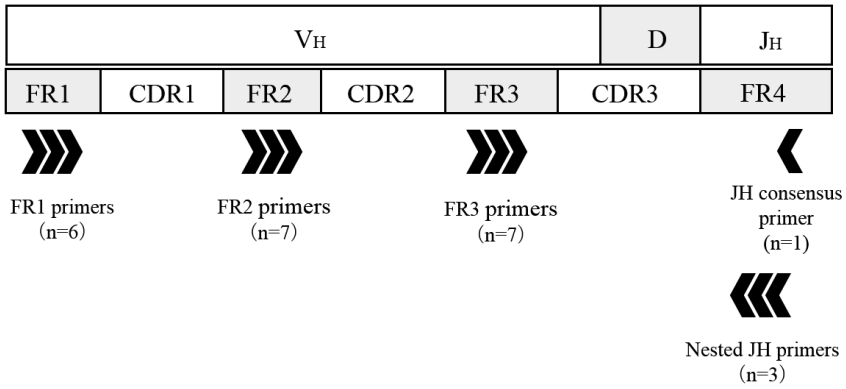

Figure 1. BIOMED-2 *IGH* gene clonality assay and its nested modification. The original BIOMED-2 assay was performed in a tri-parallel manner using the *JH* consensus primer and either *FR1* (tube A), *FR2* (tube B), or *FR3* (tube C) primers. In the nested modification, a nested *JH* primer set (three primers) was used instead of a *JH* consensus primer.

Figure 2

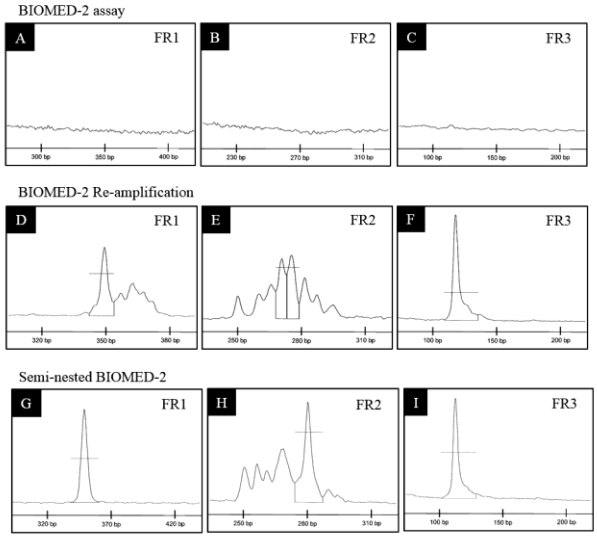

Figure 2. Detection of monoclonal *IGH* rearrangement using DNA-chip-based electrophoresis (case 1). With the original BIOMED-2 assay, no PCR product was obtained by assays targeting either *FR1* (A), *FR2* (B), or *FR3* (C). In BIOMED-2 re-amplification, monoclonal peaks were detected in assays targeting *FR1* (D) and *FR3* (F), but not in that targeting *FR2* (E). In semi-nested BIOMED-2, monoclonal peaks were obtained in assays targeting *FR1* (G), *FR2* (H), and *FR3* (I).

Figure 3

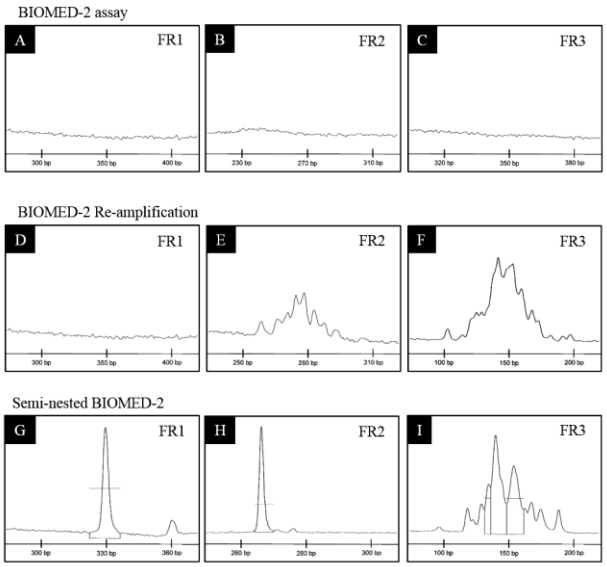

Figure 3. Detection of monoclonal *IGH* rearrangement using DNA-chip-based electrophoresis (case 2). In the original BIOMED-2 assay, no PCR product was obtained by assays targeting either *FR1* (A), *FR2* (B), or *FR3* (C) primers. In BIOMED-2 re-amplification, no PCR product was obtained in the assay targeting *FR1* (D) and polyclonal patterns were found in those targeting *FR2* (E) and *FR3* (F). In semi-nested BIOMED-2, monoclonal peaks were detected in assays targeting *FR1* (G) and *FR2* (H), but not that targeting *FR3* (I).

Figure 4

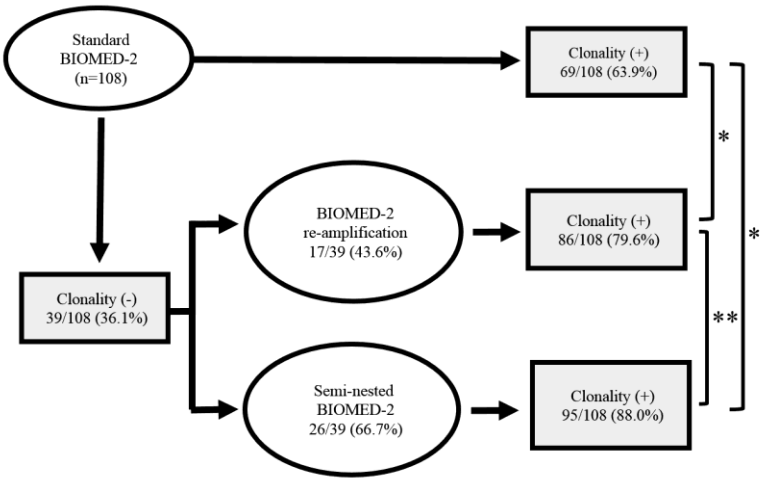

Figure 4. Summary of the present study. *, P<0.0001 and **, P=0.0039

Table 1. Clonality detection rates in B-cell lymphomas by the standard BIOMED-2, BIOMED-2 re-amplification, and semi-nested BIOMED-2

Lymphoma type	Standard BIOMED-2	BIOMED-2 re-amplification	Semi-nested BIOMED-2
DLBCL (n=36)	22/36 (61.1%)	26/36 (72.2%)	28/36 (77.8%)
CLL/SLL (n=11)	9/11 (81.8%)	10/11 (90.9)	11/11 (100.0%)
Nodal MZL (n=6)	5/6 (83.3%)	6/6 (100.0%)	6/6 (100.0%)
MALT (n=15)	9/15 (60.0%)	10/15 (66.7%)	14/15 (93.3%)
FL (n=19)	14/19 (73.7%)	14/19 (73.7%)	15/19 (78.9%)
MCL (n=13)	7/13 (53.8%)	12/13 (92.3%)	13/13 (100.0%)
BL (n=8)	3/8 (37.5%)	8/8 (100.0%)	8/8 (100.0%)
Total (n=108)	69/108 (63.9%)	86/108[a] (79.6%)	95/108[b,c] (88.0%)

DLBCL, diffuse large B-cell lymphoma; CLL, chronic lymphoid leukemia; SLL, small lymphocytic lymphoma; MZL, marginal zone lymphoma; MALT, mucosa-associated lymphoid tissue lymphoma; FL, follicular lymphoma; MCL, mantle cell lymphoma; and BL, Burkitt lymphoma. a, significantly higher than standard BIOMED-2 ($P<0.0001$); b, significantly higher than standard BIOMED-2 ($P<0.0001$); and c, significantly higher than BIOMED-2 re-amplification ($P=0.0039$).

Table 2. Clonality detection rates in B-cell lymphomas for VH primer sets

PCR	VH primer sets				
	FR1 (tube A)	FR2 (tube B)	FR3 (tube C)	Total	
BIOMED-2	28/108 (25.9%)	39/108 (36.1%)	46/108 (42.6%)	69/108 (63.9%)	
BIOMED-2 re-amplification*	3/39 (7.7%)	9/39 (23.1%)	13/39 (33.3%)	17/39 (43.6%)	**
Semi-nested BIOMED-2*	5/39 (12.8%)	14/39 (35.9%)	16/39 (41.0%)	26/39 (66.7%)	

*, 39 cases that were negative in the first-round BIOMED-2 were subjected to these second-round BIOMED-2 assays, **, P=0.0039