GENETIC TESTING AND MOLECULAR BIOMARKERS
Volume 00, Number 00, 2014

Pp. 1–4
DOI: 10.1089/gtmb.2014.0174

Invivoscribe BIOMED-2 Primer Mixes in B-Cell Immunoglobulin Gene Rearrangement Studies: Experience of a Molecular Diagnostics Laboratory in a Major Tertiary Care Center

Fatmeh Abbas,[1] Souha N. Yazbek,[2] Dina Shammaa,[1] Rouba Hoteit,[1] Puzant Fermanian,[1] and Rami Mahfouz[1]

Aims: To determine the frequency of positive reactions obtained using the Invivoscribe BIOMED-2 kit for B-cell gene rearrangement studies in leukemias and lymphomas. *Materials and Methods:* We reviewed the gel patterns for 192 samples tested, using the above-mentioned kit and matched the positive signal with the corresponding mix available in the assay kit. *Results:* 92.2% had immunoglobulin heavy-chain clonality, of which 74% were detected by the IgH VH − FR1 + JH primer set, 75.5% by IgH VH − FR2 + JH primer set, 65.1% by IgH VH − FR3 + JH primer set, 26% by IgH DH + JH primer set, and 2.1% by IgH DH7 + JH primer set. In addition, 55.7% had clonality in the kappa light chain, where 33.3% were positive by the IgK Vκ + Jκ primer set and 39.6% by IgK Vκ and INTR + Kde primer sets. Clonality in the lambda light chain of immunoglobulins was detected in 17.7% of specimens tested using the IgL Vλ + Jλ primer set. *Conclusion:* All primer mixes provided by the assay were positive. Thus, the Invivoscribe BIOMED-2 B-cell gene rearrangement kit is very reliable in adequately covering all targets represented by the master mixes. This assay is an integral part of the differential diagnosis of clonal populations of cells. Our report is the first in the literature that describes the full range of coverage of the BIOMED-2 primer mixes provided in this assay.

Introduction

GENE REARRANGEMENT STUDIES have proven to be useful as a diagnostic tool for leukemias and lymphomas and serve as a molecular marker for tumor cells derived from clonal B-lymphocyte expansion. Molecular clonality analysis is based on the fact that, in principle, all cells of a malignancy have a common clonal origin and show clonally (identically) rearranged immunoglobulin (Ig) genes (Fan and Robetorye, 2013). The diagnosis of malignant B-cell proliferations is therefore supported by the finding of Ig gene clonality, whereas reactive lymphoproliferations show polyclonally rearranged Ig genes (Cleary *et al.*, 1984; Sklar and Weiss, 1988; Cossman *et al.*, 1991; Langerak *et al.*, 2001; Sandberg *et al.*, 2005; González *et al.*, 2007; Noor *et al.*, 2013). The clonal IgH gene rearrangement was not demonstrated in any of the reactive lymphoid hyperplasia and nonlymphoid malignancies. Both heavy and light Ig chains are clonally rearranged in lymphomas (low, intermediate, and high grade) and leukemias (Cossman *et al.*, 1991; Aisenberg, 1993; Noor *et al.*, 2013). B lymphocytes generate diverse antibodies through the mechanism of immunoglobulin (Ig) gene rearrangement and somatic hypermutation. B lymphocytes generate diverse antibodies through the mechanism of immunoglobulin (Ig) gene rearrangement and somatic hypermutation. The immunoglobulin heavy-chain (IgH) gene consists of three highly variable complementarity determining regions (CDR) and three rather conserved framework regions (FR), interspersed among each other. The antigen-binding site, known as CDRIII, is the most variable region of the rearranged IgH gene. It is generated by recombining an assortment of 1 of the 200 to 300 variable (V) segments to 1 of the 20 to 30 diversity (D) segments and 1 of the 1 to 6 joining (J) segments. Further variability is introduced by random removal and addition of nucleotides at the junction between the segments. This rearrangement is specific to an individual B cell and its clonal progeny and, thus, may serve as a marker for clonally derived tumor cells. (Tai and Peh, 2003). Since the antigen receptor genes are polymorphic (consisting of a heterogeneous population of related DNA sequences), it is difficult to employ a single set of DNA primer sequences to target all of the conserved flanking

[1]Department of Pathology and Laboratory Medicine, American University of Beirut Medical Center, Beirut, Lebanon.
[2]Department of Health Sciences and Biochemistry, American University of Beirut, Beirut, Lebanon.

regions around the V-J rearrangement. N-region diversity and somatic mutation further scramble the DNA sequences in these regions. Therefore, multiplex master mixes, which target several FR, are required to identify the majority of clonal rearrangements. As indicated, clonal rearrangements are identified as prominent single sized. The Invivoscribe kit targeting IgH includes five master mixes. The IGH master mixes A, B, and C target the framework 1, 2, and 3 regions (respectively) within the variable region, and the joining region of the immunoglobulin heavy-chain locus. The IGH master mixes D and E target the diversity and joining regions.

Rearrangement of immunoglobulin genes is ordered during development in such a way that the immunoglobulin heavy-chain gene usually precedes rearrangement of the kappa light-chain gene, and kappa light-chain genes generally rearrange only if a prior successful rearrangement of one or the other allele for the immunoglobulin heavy-chain gene has taken place (Korsmeyer *et al.*, 1981; Van der Burg *et al.*, 2001, 2002). Lambda light-chain genes only rearrange when neither kappa allele has generated a viable kappa gene product (Hieter *et al.*, 1982; Van der Burg *et al.*, 2001, 2002). The Invivoscribe kit for IgK includes two master mixes. The IGK Master Mix A targets the variable (V) and joining (J) regions of the immunoglobulin kappa light-chain locus, and the IgK Master Mix B targets the variable, Jκ-Cκ intron, and Kde regions. The IGL Master Mix targets the variable and the joining regions of the immunoglobulin lambda light-chain locus. It is crucial to evaluate whether the multiple master mixes included in the Invivoscribe kit are useful in the detection of clonal rearrangements and to determine the detection rate of clonal rearrangement by each of the different mixes used.

Materials and Methods

Samples

This study was performed at the American University of Beirut Medical Center (AUBMC), which is a tertiary care center in Lebanon, accommodating patients from the different districts of the country and from different countries. It is a retrospective analysis of gel patterns from assays of referrals for immunoglobulin gene rearrangement studies in the Molecular Diagnostics Laboratory at AUB-MC.

We reviewed the gel pattern results of 192 samples to determine the detection rate of clonality by each master mix used.

Polymerase chain reaction and gel detection

The Invivoscribe kit was used; it employs multiple consensus DNA primers that target conserved genetic regions within the immunoglobulin heavy-chain gene. This assay is used to detect the vast majority of clonal B-cell malignancies from DNA.

The utilized primers cover for the following targets:

IgH: VH – FR1 + JH, VH – FR2 + JH, VH – FR3 + JH, DH + JH, DH7 + JH.

IgK: Vκ + Jκ, Vκ, and INTR + Kde

IgL: Vλ + Jλ

The protocol was followed as described by the manufacturer. The thermocycler program consists of an initial step of 95°C for 7 min, followed by 34 cycles of 95°C for 45 s, 60°C for 45 s, 72°C for 90 s, and a final extension step of 72°C for 10 min. Finally, a heteroduplex analysis is performed and products run on a polyacrylamide gel to differentiate clonal and nonclonal polymerase chain reaction (PCR) products. A heteroduplex analysis involves denaturing the PCR products at a high temperature, then quickly reannealing the DNA strands by suddenly reducing the temperature. This causes a large portion of DNA strands to incorrectly bind to other nonhomologous strands creating loops in the DNA. These loops cause a significant reduction in the ability of the DNA to migrate through a polyacrylamide gel. However, if the majority of the PCR products are clonal, when a heteroduplex analysis is performed, most of these PCR products will correctly reanneal with a homologous strand. These PCR products will run normally through the polyacrylamide gel. Therefore, in a clonal sample with a polyclonal background, a heteroduplex analysis will cause most of the polyclonal products to run much slower through the polyacrylamide gel, thereby increasing their separation and the ability to identify the clonal band(s).

Positive and negative (HPG) controls furnished with the kit are used each time the assay is performed to ensure proper performance of the assay. In addition, a no template control (e.g., water) is included to test for contamination of the master mix or cross contamination of PCRs due to improper sterile technique.

Interpretation of results

Bands detected by gel electrophoresis are compared to the positive controls for each mix. The size range for each of the master mixes has been determined by testing positive and negative control samples. For an accurate and meaningful interpretation, it is important to ignore peaks that occur outside of the valid size range for each of the master mixes.

Statistical analysis

Data were entered into the SPSS program where each master mix was considered as a nominal variable having a positive or negative result. Because B-cell immunoglobulins tend to rearrange differently, one sample may be positive for more than one specific primer mix, thus the corresponding mix was counted separately. Descriptive statistics were done for each of the master mixes used in testing for immunoglobulin gene rearrangements for both heavy and light chains, and frequency tables were obtained for each component.

Results

Using the Invivoscribe kit and out of 192 gels, 92.2% had immunoglobulin heavy-chain clonality, of which 74% were detected by the IgH VH – FR1 + JH primer set, 75.5% by IgH VH – FR2 + JH primer set, 65.1% by IgH VH-FR3 + JH primer set, 26% by IgH DH + JH primer set, and 2.1% by IgH DH7 + JH primer set (Table 1). In addition, 55.7% had clonality in the kappa light chain, where 33.3% were positive by the IgK Vκ + Jκ primer set and 39.6% by the IgK Vκ and INTR + Kde primer sets (Table 2). Clonality in the lambda light chain of immunoglobulins was detected in 17.7% of the specimens tested using the IgL Vλ + Jλ primer set (Table 3).

Discussion

Molecular diagnostics laboratories are adding tests that help clinicians and pathologists in identifying abnormal

Table 1. Frequency Distribution of All IgH Mixes Associated with the Invivoscribe BIOMED-2 Kit

Mix	*Positive*	*Negative*	*Frequency of positives (n = 192)*
IgH	177	15	92
IgH MIX A (VH − FR1 + JH)	142	50	74
IgH MIX B (VH − FR2 + JH)	145	47	76
IgH MIX C (VH − FR3 + JH)	125	67	65
IgH MIX D (DH + JH)	50	142	26
IgH MIX E (DH7 + JH)	4	188	2

populations of cells with a differential diagnosis ranging from atypical reactive infiltrate to malignancy (Hartmann *et al.*, 2013). In addition to morphological examination, immunohistochemistry, and flow cytometry, identifying clonal rearrangement is important in the diagnosis of malignant B-cell proliferation, since a subset of lymphomas lack detectable surface or cytoplasmic immunoglobulin; however, clonal immunoglobulin gene rearrangements were detectable. Studies indicated that detection of immunoglobulin gene rearrangements is a valuable method for diagnosis and classification of various lymphoproliferative disorders that are difficult to evaluate histologically or that lack distinctive antigenic markers. Moreover, clonal gene rearrangements distinguish the lymphomas from the reactive lymphoid hyperplasia and nonlymphoid malignancies, as reported by numerous studies in the international literature (Cleary *et al.*, 1984; Sklar and Weiss, 1988; Langerak *et al.*, 2001; González *et al.*, 2005, 2007; Sandberg *et al.*, 2005). The detection of a clonal B-cell population can facilitate diagnosis in patients, in whom clinical, histopathological, and immunophenotypical findings are consistent but not entirely typical of a malignant lymphoproliferation, thus the importance of this single molecular test in the workup of suspected lymphomas or leukemias (Sandberg *et al.*, 2005).

The BIOMED-2 B-cell Gene Rearrangement assay by Invivoscribe is an important test available in molecular laboratory services of different centers and institutions worldwide. The major attribute of this kit is its content of primer mixes, which have been consolidated and standardized according to the international consortium provided through BIOMED-2, reflecting basically all possible alternatives for the rearrangement patterns detected. In our experience with the Invivoscribe kit, we were able to detect immunoglobulin clonality by the entire provided multiple primer mixes that target different regions and rearrangements in the immunoglobulin heavy and light chains targeted for by this reliable assay. All provided primer mixes were positive; thus, an adequate coverage of all involved targets is secured using this BIOMED-2 assay. This kind of information and data are useful for the international users of this assay in terms of being reassured that any kind of described rearrangement pattern is covered by this BIOMED-2 protocol, which makes it a very important addition in the workup of a lymphoproliferative disorder.

Table 2. Frequency Distribution of All IgK Mixes Associated with the Invivoscribe BIOMED-2 Kit

Mix	*Positive*	*Negative*	*Frequency of positives (n = 192)*
Igk	107	85	56
Vκ + Jκ	63	129	33
IgK Vκ and INTR + Kde	77	115	40

Table 3. Frequency Distribution of All IgL Mixes Associated with the Invivoscribe BIOMED-2 Kit

Mix	*Positive*	*Negative*	*Frequency of positives (n = 192)*
IgL (Vλ + Jλ)	35	157	18

This technique provides a valuable method in identifying clonality and diagnosing malignancies, resulting in better patient care management. Also, this technique is employed not only as a clinical tool for diagnosing malignancies but also as a monitoring marker for minimal residual disease (MRD) and detection of early relapse. The trend for the use of this assay for MRD applications has been of great utility in terms of detecting the initial diagnostic molecular rearrangement upon follow-up for any subsequent specimen tested at a later clinical stage. For example, if the initial rearrangement pattern of the identified B-cell leukemia shows positivity for different mixes on the initial lymph node or bone marrow examined, then the same primers mixes can be used to detect any residual disease upon later clinical follow-up of the patient. Our practice also includes correlation with morphology on aspirates and biopsies, as well as other MRD testing panels, including flow cytometry or other molecular markers.

This report is the first in the literature that describes the completeness and appropriateness of the range of coverage of the BIOMED-2 primer mixes provided in this assay.

Author Disclosure Statement

No competing financial interests exist.

References

Aisenberg AC (1993) Utility of gene rearrangements in lymphoid malignancies. Annu Rev Med 44:75–84.

Cleary M, Chao J, Warnke R (1984) Immunoglobulin gene rearrangement as a diagnostic criterion of B-cell lymphoma. Proc Natl Acad Sci U S A 81:593–597.

Cossman J, Zehnbauer B, Garrett CT, *et al.* (1991) Gene rearrangements in the diagnosis of lymphoma/leukemia: guidelines for use based on a multiinstitutional study. Am J Clin Pathol 95:347–354.

Fan H, Robetorye RS (2013) Detection of clonal immunoglobulin heavy chain gene rearrangements by the polymerase chain reaction and capillary gel electrophoresis. Methods Mol Biol 999:151–167.

González D, González M, Balanzategui A, *et al.* (2005) Molecular characteristics and gene segment usage in IGH gene rearrangements in multiple myeloma. Haematologica 90:906–913.

González M, van Dongen J, San Miguel J, *et al.* (2007) Immunoglobulin gene rearrangements and the pathogenesis of multiple Myeloma. Blood 110:3112–3121.

Hartmann S, Helling A, Döring C, *et al.* (2013) Clonality testing of malignant lymphomas with the BIOMED-2 primers in a large cohort of 1969 primary and consultant biopsies. Pathol Res Pract 09:495–502.

Hieter PA, Korsmeyer SJ, Waldmann TA, Leder PA (1982) Human immunoglobulin kappa light-chain genes are deleted or rearranged in lambda-producing B cells. Nature 290:368–372.

Korsmeyer SJ, Hieter PA, Ravetch JV, *et al.* (1981) Developmental hierarchy of immunoglobulin gene rearrangements in human leukemic pro-B cells. Proc Natl Acad Sci U S A 78:7096—7100.

Langerak A, van Krieken J, Wolvers-Tettero I, *et al.* (2001) The role of molecular analysis of immunoglobulin and T cell receptor gene rearrangements in the diagnosis of lymphoproliferative disorders. J Clin Pathol 54:565–567.

Noor Haslina MN, Marini R, Rosnah B, *et al.* (2013) Immunoglobulin heavy chain gene rearrangement in non B-cell haematological malignancies. West Indian Med J 62:701–704.

Sandberg Y, van Gastel-Mol EJ, Verhaaf B, *et al.* (2005) BIOMED-2 multiplex immunoglobulin/T-cell receptor polymerase chain reaction protocols can reliably replace Southern Blot analysis in routine clonality diagnostics. J Mol Diagn 7:495–503.

Sklar J, Weiss LM (1988) Applications of antigen receptor gene rearrangements to the diagnosis and characterization of lymphoid neoplasms. Annu Rev Med 39:315–334.

Tai Y-C, Peh S-C (2003) Immunoglobulin gene rearrangement of lymphoid malignancies: application of the PCR technique to formalin-fixed, paraffin-embedded tissues. J Clin Exp Hematopathol 43:1–9.

Van der Burg M, Barendregt BH, Szczepañski T, *et al.* (2002) Immunoglobulin light chain gene rearrangements display hierarchy in absence of selection for functionality in precursor-B-ALL. Leukemia 16:1448–1453.

Van der Burg M, Tümkaya T, Boerma M, *et al.* (2001) Ordered recombination of immunoglobulin light chain genes occurs at the IGK locus, but seems less strict at the IGL locus. Blood 97:1001–1008.

Address correspondence to:
Rami Mahfouz, MD, MPH, IFCAP
Department of Pathology and Laboratory Medicine
American University of Beirut Medical Center
Cairo Street
Beirut 11-0236
Lebanon

E-mail: rm11@aub.edu.lb