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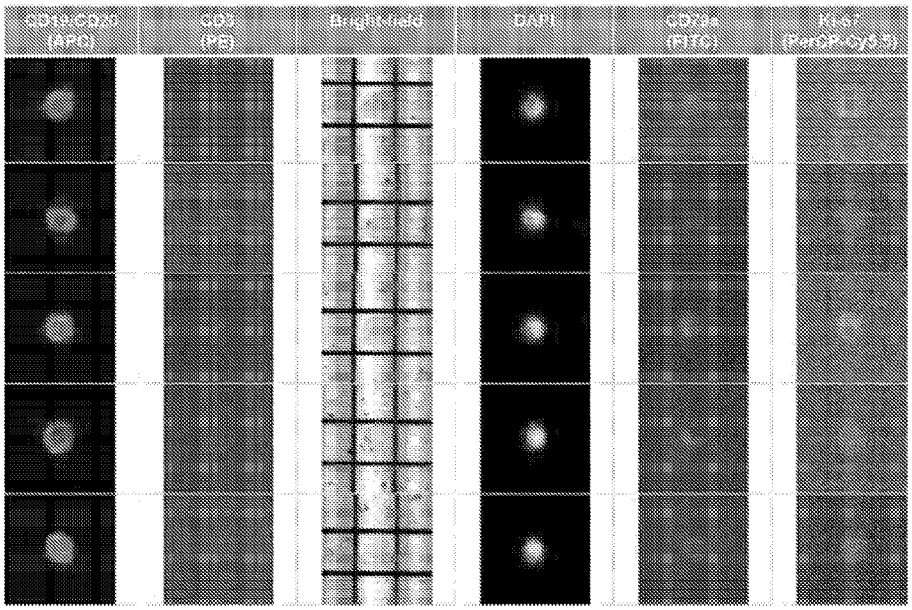

FIGURE 4

(57) Abstract: This invention relates to a methodology for single cell-based analysis of vitreous aspiration samples for diagnosis of vitreoretinal lymphoma (PVRL) such as primary vitreoretinal lymphoma (PVRL). Analysis of clonality, copy number aberration, BCL2/JH (t14;18) translocation and/or MYD88 mutations are performed on whole genomic amplified DNA derived from single B-cells to provide molecular-based diagnosis of VRL, which cannot be achieved by conventional cytology diagnosis approach.

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SINGLE CELLULAR DIAGNOSIS OF PRIMARY VITREORETINAL LYMPHOMA

FIELD OF THE INVENTION

This invention relates to a methodology for single cell-based analysis of liquid based (aqueous and vitreous) aspiration samples for diagnosis of lymphoproliferative disorder such as vitreoretinal lymphoma (VRL) and, in particular, primary vitreoretinal lymphoma (PVRL). Clonality analysis, copy number aberration, BCL2/JH (t14;18) translocation and/or MYD88 L265P mutation analyses are performed on genomic DNA derived from single B-cells to provide molecular-based diagnosis of lymphoproliferative disorder such as vitreoretinal lymphoma (VRL) and, in particular, primary vitreoretinal lymphoma (PVRL), which cannot be achieved by conventional cytological diagnosis.

BACKGROUND OF THE INVENTION

Vitreoretinal lymphoma (VRL) and, in particular, primary vitreoretinal lymphoma (PVRL) is a subset of central nervous system lymphoma (CNSL). The vast majority of VRL, and PVRL, are of B-cell origin, with more than 90% of the cases manifesting as diffuse large B-cell lymphoma (DLBCL) [Fend, F., *et al.*, *Br J Haematol.* 2016; 173(5): 680-92]. Due to the difficulty in diagnosis and thus under reporting of cases of VRL, there is currently a limited database or registry for such cases. In addition, there is no consensus protocol for the diagnosis, treatment, and monitoring of the disease [Fend, F., *et al.*, *Br J Haematol.* 2016; 173(5): 680-92; Touitou, V., et al., *Curr Opin Ophthalmol.* 2015; 26(6): 526-33]. However with increased awareness of this condition in ophthalmologists and oncologists, a rise in VRL, and PVRL, cases has been reported in both Europe and America in the past two decades [Sagoo, M.S., *et al.*, *Survey of Ophthalmology.* 2014; 59(5): 503-516; Schabet, M., *J Neurooncol.* 1999; 43(3): 199-201; Baehring, J.M., *et al.*, *Cancer.* 2005; 104(3): 591-7; Eby, N.L., *et al.*, *Cancer.* 1988; 62(11): 2461-5; Levy-Clarke, G.A., *et al.*, *Hematol Oncol Clin North Am.* 2005; 19(4): 739-49].

Various techniques such as cytology, immunohistochemistry, cytokine analysis, flow cytometry and molecular analysis are available for VRL, and PVRL, diagnosis [Fend, F., *et al.*, *Br J Haematol.* 2016; 173(5): 680-92; Reichstein, D., *Curr Opin Opthalmol.* 2016; 27(3): 177-84] but are limited by the expertise needed for interpretation (cytology, immunohistochemistry), or by limited number of cells (flow cytometry and cytology). Despite the fact that cytology is the current gold standard for VRL, and PVRL, diagnosis, the technique alone can only confirm 45 to 60% of the clinical cases [Davis, J.L., *et al.*, *Am J Ophthalmol.* 2005; 140(5): 822-829; Wittenberg, L.A., *et al.*, *Ophthalmology.* 2008; 115(11):

1944-50; Kimura, K., *et al.*, *Jpn J Ophthalmol.* 2012; 56(4): 383-9]. Furthermore, the yield of cells is typically low due to the fragility of the lymphoma cells and prognostication of the disease without additional samples is currently not possible. Precise diagnosis of VRL, and PVRL, still remains a challenge in the hands of non-specialty pathologists. Cellular morphology and immunocytochemistry are used to confirm the presence of atypical enlarged cells expressing pan B-cell markers such as CD20 and CD79a that have high proliferation Ki-67 index. In a paucicellular yield, these B cells may not be abundant and limit the threshold of diagnosis. Furthermore, in the absence of supporting mutational analysis, which may not be available due to low cell yield, a diagnosis of VRL, and PVRL, will be difficult if not almost impossible to make with certainty. In addition, chronic inflammatory vitritis from viral infections are also known to be able to induce restricted immune repertoire and inflammation induced atypia in B cells, making predominance of such B-cells difficult to differentiate from lymphomatous cells. Lymphomatous cells are from the same progenitors (or same clone) unlike polyclonal viral induced B cells [Sagoo, M.S., *et al.*, *Survey of Ophthalmology*. 2014; Coupland, S.E. and Damato B., *Clin Exp Opthalmol.* 2008; 36(6): 564-78; AlQahtani, A., *et al.*, *Ocul Immunol Inflamm*. 2014; 22(3): 189-96].

A low number of cells (<100 lymphoma cells per sample) also hinders the adoption of flow cytometry technology in VRL, and PVRL diagnosis, which in the presence of debris, necrotic cells, and reactive inflammatory immune cells can further confound the diagnosis [Coupland, S.E., *et al.*, *Graefes Arch Clin Exp Ophthalmol.* 2003; 241(10): 860-70]. Moreover, proper handling and fixation of the sample plays a critical role because lymphomatous cells are extremely fragile, and are only viable and remain intact for a short period [Chan, C.-C., *et al.*, *The Oncologist*. 2011; 16(11): 1589-1599; Gonzales, J.A. and C.C. Chan, *Int Ophthalmol.* 2007; 27(4): 241-50; Char DH., *et al.*, *Br J Ophthalmol.* 1988; 72(12): 905-11]. Therefore, if immediate assessment of the sample is not possible, they should be placed in the relevant fixative to preserve cytological details and DNA material to avoid a delay in confirmatory diagnosis and prevent a false negative diagnosis which can lead to high morbidity and mortality by delaying diagnosis [Whitcup, S.M., *et al.*, Opthalmology. 1993; 100(9): 1399-406; Coupland, S.E., *Dev Ophthalmol.* 2012; 49: 96-116; Davis, J.L., *Eye*. 2013; 27(2): 153-162].

To overcome some of these diagnostic challenges in differentiating VRL, and PVRL, from inflammatory disease, PCR-based molecular techniques focusing on B-cell clonality on bulk cells was developed. The clonality assay had reported sensitivity between 65% and 95%, and such approach had been successfully developed and harmonized by the Euroclonality (BIOMED-2) consortium [Baehring, J.M., *et al.*, *Cancer.* 2005; 104(3): 591-7; Kimura, K., *et*

al., *Jpn J Ophthalmol.* 2012; 56(4): 383-9; Coupland, S.E., *et al.*, *Graefes Arch Clin Exp Ophthalmol.* 2003; 241(10): 860-70; Coupland, S.E., *et al.*, *Invest Ophthalmol Vis Sci.* 2005; 46(10): 3507-14; Merle-Beral, H., *et al.*, *Br J Haematol.* 2004; 124(4): 469-73; Wang, Y., *et al.*, *Int J Mol Sci.* 2011; 12(9): 5684-97; van Dongen, J.J., *et al.*, *Leukemia.* 2003; 17(12): 2257-317]. However, limitations remain in this bulk-cell based clonality assay. Firstly, the frequency of clonal B-lymphoma cells must be above the detection threshold (>1% clonal cell in the bulk samples) as required by the clonality assay and false negative detection may occur if lymphoma cells are not pre-enriched to meet the requirement of detection threshold. More importantly, B-cell clonality as defined by the pairing of immunoglobulin heavy-chain (IgH, Chromosome 14) and light-chain (IgK, Chromosome 2) on each individual B-cell cannot be detected with these conventional clonality assays as they rely on a mixed DNA source from bulk cells and not individual cells. Thus, in order to truly determine the complete Ig-rearrangement (IgH + IgK; clonality), it is necessary to determine both heavy and light chain clonality of a single B-cell.

Myeloid differentiation primary response 88 (MYD88) is an adaptor protein involved in the Toll/interleukin-1 signaling for NF-$_{\kappa}$B (nuclear factor κ-light-chain-enhancer of activated B cells) expression [Deguine, J. and G.M. Barton, F1000Prime Rep, 2014. 6: p. 97]. Of the numerous reported MYD88 mutations, a variant (L265P) with non-synonymous point mutation and consequent amino acid substitution from leucine (C<u>T</u>G) to proline (C<u>C</u>G) at position 265 accounts for more than 60% of all mutants [Ngo, V.N., *et al.*, *Nature*, 2011. 470(7332): p. 115-9; Dubois, S., *et al.*, *Clin Cancer Res*, 2017. 23(9): p. 2232-2244]. In recent years, MYD88 L265P mutation is additionally reported to be present in several lymphomas including CNSL, such as PCNSL [Ngo, V.N., *et al.*, *Nature*, 2011. 470(7332): p. 115-9; Montesinos-Rongen, M., *et al.*, *Acta Neuropathol*, 2011. 122(6): p. 791-2] and VRL [Bonzheim, I., *et al.*, *Blood*, 2015. 126(1): p. 76-9]. Conventionally, the MYD88 L265P mutation was detected based on polymerase chain reaction (PCR), using specific primers coupled with Sanger sequencing of the purified amplicon. However, such an approach has a low detection sensitivity of 25% [Wang, C.Z., *et al.*, *Clin Biochem*, 2013. 46(4-5): p. 385-7]. To increase the sensitivity MYD88 L265P detection, newer approach such as allelic-specific PCR coupled with high resolution melt curve analysis was developed, but with a mere 5% improvement in sensitivity [Bonzheim, I., *et al.*, *Blood*, 2015. 126(1): p. 76-9]. With the modest improvement in sensitivity, the detection of MYD88 L265P mutation is still restricted to the ratio of wild type (WT) to mutant in bulk sample analysis.

There is a need for improved methods of detecting and/or diagnosing lymphoproliferative disorders such as VRL.

SUMMARY OF THE INVENTION

This invention provides a method of performing single cell-based genomic analysis of B-cell clonality by methods for single-B-cell isolation, determining copy number aberration, BCL2/JH (t14;18) translocation and/or presence of a MYD88 L265P mutation for the diagnosis and prognostication of a lymphoproliferative disorder such as vitreoretinal lymphoma (VRL) and, in particular, primary vitreoretinal lymphoma (PVRL). The method is capable of identifying monoclonality in a control Pfeiffer DLCBL cell line. The clinical utility is demonstrated by successful detection of clonal single B-cells in VRL, such as PVRL, positive cases. In addition to diagnosis, the detection of copy number aberration, BCL2/JH (t14;18) translocation and MYD88 L265P mutation also serve as prognostication markers.

According to one aspect, there is provided a method for the identification of clonal lymphoproliferative cells in a sample from a subject, the method comprising:

a) staining cells comprised in the sample of said subject with B-cell specific markers;

b) loading the cells from (a) onto a microfluidic cartridge that allows the separation of single cells into individual dielectrophoretic cages;

c) detecting individual positively-stained B cells and isolating each of said positively-stained B cells;

d) amplifying individually the whole genome of each of said isolated single cell;

e) performing a specific amplification on the product obtained from each single cell from (d) with primers directed to immunoglobulin heavy-chain (IgH) and/or immunoglobulin light-chain (IgK);

f) separating and isolating amplicons corresponding to IgH and/or IgK obtained for each single cell from (e) ;

g) sequencing said amplicons corresponding to IgH and/or IgK ,

and

h) determining whether there is a copy number aberration and/or a BCL2/JH t(14;18) translocation and/or a MYD88 L265P mutation in said isolated positively-stained B cell from c),

wherein the presence of IgH and/or IgK amplicons with the same sequence in said isolated positively-stained B cells and/or the presence of said copy number aberration and/or a BCL2/JH t(14;18) translocation indicates the presence of clonal lymphoproliferative cells and/or a MYD88 L265P mutation indicates the cells are lymphoproliferative.

In some embodiments, the cells in step a) are stained with DAPI and one or more B-cell markers selected from CD19, CD20, CD79a and Ki-67.

In some embodiments the cells in step a) are fixed prior to staining.

In some embodiments the cells in step b) and step c) are separated and isolated, respectively, using a DEPArray™ NxT system (Menarini Silicon Biosystems).

In some embodiments, in step c) B cells are detected based on their positivity for at least one of B-cell markers selected from CD19, CD20, CD79a and Ki-67.

In some embodiments, in step e) the specific amplification method can be selected from known methods such as polymerase chain reaction (PCR), loop mediated isothermal amplification (LAMP), nucleic acid sequence based amplification (NASBA), self-sustained sequence replication (3SR), rolling circle amplification (RCA) etc., most of which are isothermal nucleic acid amplification methods. In preferred embodiments the specific amplification method in step e) is PCR.

According to some embodiments, the primers used in step e) comprise at least one forward oligonucleotide primer and at least one reverse oligonucleotide primer selected from the group comprising:

IgK primers selected from:

Forward primers comprising the nucleotide sequence set forth in:

5'-TGTAAAACGACGGCCAGTTCAAGGTTCAGCGGCAGTGGATCTG-3' (SEQ ID NO: 1)

5'-TGTAAAACGACGGCCAGTGGCCTCCATCTCCTGCAGGTCTAGTC-3' (SEQ ID NO: 2)

5'-TGTAAAACGACGGCCAGTCCCAGGCTCCTCATCTATGATGCATCC-3' (SEQ ID NO: 3)

5'-TGTAAAACGACGGCCAGTCAACTGCAAGTCCAGCCAGAGTGTTTT-3' (SEQ ID NO: 4)

5'-TGTAAAACGACGGCCAGTCCTGCAAAGCCAGCCAAGACATTGAT-3' (SEQ ID NO: 5) and

5'-TGTAAAACGACGGCCAGTGACCGATTTCACCCTCACAATTAATCC-3' (SEQ ID NO: 6); and

Reverse primers comprising the nucleotide sequence set forth in:

5'- CAGGAAACAGCTATGACCCTTACGTTTGATCTCCACCTTGGTCCC-3' (SEQ ID NO: 7), and

5'- CAGGAAACAGCTATGACCCTTACGTTTAATCTCCAGTCGTGTCCC-3' (SEQ ID NO: 8)

and/or

IgK primers selected from

Forward primers comprising the nucleotide sequence set forth in:

5'-TGTAAAACGACGGCCAGTTCAAGGTTCAGCGGCAGTGGATCTG-3' (SEQ ID NO: 9)

5'-TGTAAAACGACGGCCAGTGGCCTCCATCTCCTGCAGGTCTAGTC-3' (SEQ ID NO: 10)

5'-TGTAAAACGACGGCCAGTCCCAGGCTCCTCATCTATGATGCATCC-3' (SEQ ID NO: 11)

5'-TGTAAAACGACGGCCAGTCAACTGCAAGTCCAGCCAGAGTGTTTT-3' (SEQ ID NO: 12)

5'-TGTAAAACGACGGCCAGTCCTGCAAAGCCAGCCAAGACATTGAT-3' (SEQ ID NO: 13)

5'-TGTAAAACGACGGCCAGTGACCGATTTCACCCTCACAATTAATCC-3' (SEQ ID NO: 14)

and

5'-TGTAAAACGACGGCCAGTCGTGGCACCGCGAGCTGTAGAC-3' (SEQ ID NO: 15)

Reverse primers comprising the nucleotide sequence set forth in:

5'- CAGGAAACAGCTATGACCCTTACGTTTGATCTCCACCTTGGTCCC-3' (SEQ ID NO: 16)

5'- CAGGAAACAGCTATGACCCTTACGTTTAATCTCCAGTCGTGTCCC-3' (SEQ ID NO: 17)

5'- CAGGAAACAGCTATGACCCCTCAGAGGTCAGAGCAGGTTGTCCTA-3' (SEQ ID NO: 18)

and/or

IgH primers selected from:

Forward primers comprising the nucleotide sequence set forth in:

5'-TGTAAAACGACGGCCAGTGGCCTCAGTGAAGGTCTCCTGCAAG-3' (SEQ ID NO: 19)

5'-TGTAAAACGACGGCCAGTGTCTGGTCCTACGCTGGTGAAACCC-3' (SEQ ID NO: 20)

5'-TGTAAAACGACGGCCAGTCTGGGGGGTCCCTGAGACTCTCCTG-3' (SEQ ID NO: 21)

5'-TGTAAAACGACGGCCAGTCTTCGGAGACCCTGTCCCTCACCTG-3' (SEQ ID NO: 22)

5'-TGTAAAACGACGGCCAGTCGGGGAGTCTCTGAAGATCTCCTGT-3' (SEQ ID NO: 23)

5'-TGTAAAACGACGGCCAGTTCGCAGACCCTCTCACTCACCTGTG-3' (SEQ ID NO: 24)

Reverse primer comprising the nucleotide sequence set forth in:

5'- CAGGAAACAGCTATGACCCTTACCTGAGGAGACGGTGACC-3' (SEQ ID NO: 25)

and/or

IgH primers selected from:

Forward primers comprising the nucleotide sequence set forth in:

5'-TGTAAAACGACGGCCAGTCTGGGTGCGACAGGCCCCTGGACAA-3' (SEQ ID NO: 26)

5'-TGTAAAACGACGGCCAGTTGGATCCGTCAGCCCCCAGGGAAGG-3' (SEQ ID NO: 27)
5'-TGTAAAACGACGGCCAGTGGTCCGCCAGGCTCCAGGGAA-3' (SEQ ID NO: 28)
5'-TGTAAAACGACGGCCAGTTGGATCCGCCAGCCCCCAGGGAAGG-3' (SEQ ID NO: 29)
5'-TGTAAAACGACGGCCAGTGGGTGCGCCAGATGCCCGGGAAAGG-3' (SEQ ID NO: 30)
5'-TGTAAAACGACGGCCAGTTGGATCAGGCAGTCCCCATCGAGAG-3' (SEQ ID NO: 31)
5'-TGTAAAACGACGGCCAGTTTGGGTGCGACAGGCCCCTGGACAA-3' (SEQ ID NO: 32)

Reverse primer comprising the nucleotide sequence set forth in:
5'- CAGGAAACAGCTATGACCCTTACCTGAGGAGACGGTGACC-3' (SEQ ID NO: 33)

and/or

IgH primers selected from:
Forward primers comprising the nucleotide sequence set forth in:
5'-TGTAAAACGACGGCCAGTTGGAGCTGAGCAGCCTGAGATCTGA-3' (SEQ ID NO: 34)
5'-TGTAAAACGACGGCCAGTCAATGACCAACATGGACCCTGTGGA-3' (SEQ ID NO: 35)
5'-TGTAAAACGACGGCCAGTTCTGCAAATGAACAGCCTGAGAGCC-3' (SEQ ID NO: 36)
5'-TGTAAAACGACGGCCAGTGAGCTCTGTGACCGCCGCGGACACG-3' (SEQ ID NO: 37)
5'-TGTAAAACGACGGCCAGTCAGCACCGCCTACCTGCAGTGGAGC-3' (SEQ ID NO: 38)
5'-TGTAAAACGACGGCCAGTGTTCTCCCTGCAGCTGAACTCTGTG-3' (SEQ ID NO: 39)
5'-TGTAAAACGACGGCCAGTCAGCACGGCATATCTGCAGATCAG-3' (SEQ ID NO: 40)

Reverse primer comprising the nucleotide sequence set forth in:
5'- CAGGAAACAGCTATGACCCTTACCTGAGGAGACGGTGACC-3' (SEQ ID NO: 41)

and/or

MYD88 forward primer comprising the nucleotide sequence set forth in:
5'- TGTAAAACGACGGCCAGTTGCAGGGGTTGGTGTAGT-3' (SEQ ID NO: 42) and

MYD88 reverse primer comprising the nucleotide sequence set forth in:
5'- CAGGAAACAGCTATGACCGTTGTTAACCCTGGGGTTG-3' (SEQ ID NO: 43);

wherein the specific IgK, IgH and MYD88 targeting sequences are not underlined.

According to some embodiments the PCR primers have a short oligonucleotide tail at the 5' end to facilitate sequencing of the PCR products.

According to some embodiments one of the PCR primers, such as the forward primer, has a short oligonucleotide tail at the 5' end comprising the nucleotide sequence 5'-TGTAAAACGACGGCCAGT-3' (SEQ ID NO: 44) and the other PCR primer, such as the reverse primer, has a short oligonucleotide tail at the 5' end comprising the nucleotide sequence 5'-CAGGAAACAGCTATGACC-3' (SEQ ID NO: 45) to facilitate sequencing of said amplicons. Such sequencing tails are shown underlined in SEQ ID Nos: 1-43. It would be understood that the sequence of the short oligonucleotide tails, used for sequencing according to the invention, could vary significantly from the nucleotide sequences set forth in SEQ ID Nos: 44 and 45 and still function for the purpose of sequencing the PCR amplicons.

According to some embodiments, in step g) the sequencing method is Sanger Sequencing or Next Generation Sequencing.

In some embodiments, the presence of IgH and/or IgK amplicons with the same sequence in said isolated positively-stained B cells and/or the presence of said MYD88 L265P mutation, copy number aberration or a BCL2/JH t(14;18) translocation in said isolated positively-stained B cells is diagnostic of a lymphoproliferative disorder in the subject.

In some embodiments, if there is determined to be at least 60%, preferably at least 70%, of single B-cells sharing the same IgH and IgK sequence; and/or at least 85%, preferably at least 90%, of single B-cells sharing the same IgH sequence in the absence of IgK sequence; and/or at least 5%, preferably at least 20%, of single B-cells sharing the same homozygous MYD88 L265P mutation; and/or at least 30%, preferably at least 50%, of single B-cells sharing the same BCL2/JH t(14;18) translocation or similar profiles of copy number aberration is diagnostic of a lymphoproliferative disorder in the subject.

In preferred embodiments the presence of at least 70% of single B-cells sharing the same IgH and IgK sequence; and/or at least 90% of single B-cells sharing the same IgH sequence in the absence of IgK sequence; and/or at least 20% of single B-cells sharing the same homozygous MYD88 L265P mutation; and/or at least 50% of single B-cells sharing the same BCL2/JH t(14;18) translocation or similar profiles of copy number aberration is diagnostic of a lymphoproliferative disorder in the subject.

In some embodiments, the lymphoproliferative disorder is selected from vitreoretinal lymphoma (VRL), central nervous system lymphoma (CNSL), VRL-specific minimal residue disease (MRD), or CNSL-specific MRD. In preferred embodiments, the lymphoproliferative

disease is VRL or VRL-specific MRD.

In some embodiments, the sample is from vitreous fluid, blood, bone marrow or cerebrospinal fluid.

According to a preferred embodiment, the sample is a vitreous fluid and the lymphoproliferative disease is VRL or VRL-specific MRD.

According to another preferred embodiment, the sample is a cerebrospinal fluid and the lymphoproliferative disease is CNSL or CNSL-specific MRD.

According to a preferred embodiment, the subject is a human.

According to a further aspect, the invention provides a method of monitoring a B-cell lymphoproliferative disease in a subject, comprising screening a sample from said subject using a method according to any aspect of the present invention.

According to a further aspect, the invention provides a kit for the identification of clonal lymphoproliferative cells or diagnosis of a lymphoproliferative disorder in a subject according to the method of the invention, said kit comprising:

- (i) at least one forward oligonucleotide primer and at least one reverse oligonucleotide primer selected from the groups of IgK primers and/or IgH primers and/or at least one MYD88 forward oligonucleotide primer and at least one MYD88 reverse oligonucleotide primer hereinbefore listed
- optionally, wherein at least one of the said primers is structurally and/or chemically modified from its corresponding natural nucleic acid.

It would be understood that oligonucleotides may be made more resistant to nuclease degradation during PCR and/or sequencing methods by modifying internucleoside linkages (e.g., methylphosphonates or phosphorothioates) or by incorporating modified nucleosides (e.g., 2'-0-methylribose or 1'-α-anomers). In some embodiments, the poly- or oligonucleotide primer or probe comprises a modified phosphate backbone synthesized from a nucleotide having, for example, one of the following structures: a phosphorothioate, a phosphoridothioate, a phosphoramidothioate, a phosphoramidate, a phosphordiimidate, a methylsphosphonate, an alkyl phosphotriester, 3'-aminopropyl and a formacetal or analog thereof. Various tags provide means for detection. In some embodiments, the structural and/or chemical modifications are selected from the group comprising the addition of tags,

such as fluorescent tags, radioactive tags, biotin, a 5' tail, the addition of methylphosphonate or phosphorothioate (PS) bonds, 2'-O-Methyl modifications and/or phosphoramidite C3 Spacers during synthesis.

In some embodiments, said kit further comprises at least one of the following reagents:

- (ii) at least one antibody targeting at least one B-cell marker selected from the group CD19, CD20, CD79a and Ki-67; and/or
- (iii) suspension buffer for single cell isolation using microfluidic cartridge that allows the separation of each single cells into individual dielectrophoretic cages; and/or
- (iv) reaction buffer, enzymes and reagents for whole genome amplification and -quality check; and/or
- (v) reaction buffer, enzymes and reagents for BCL2/JH t(14;18) translocation assay; and/or
- (vi) reaction buffer, enzymes and reagents to generate barcoded sequencing libraries suitable for genome-wide copy-number profiling.

In some embodiments, the kit further comprises at least one of the following reagents selected from the group comprising:

- (vii) methanol-based fixative buffer, which is preferably 35-55% methanol-based;
- (viii) 4',6-diamidino-2-phenylindole (DAPI);
- (ix) sequencing primers for DNA sequencing of IgH, IgK and MYD88 genes;
- (x) a positive control which comprises DNA from clonal B-cells (clonal DNA control); and
- (xi) a negative control which comprises DNA from at least two different B-cells (polyclonal DNA control).

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 shows that Shandon's fixative is not suitable for single cell-based clonality assay. Single or pooled B lymphoma cells were prefixed (red) or not (black) with Shandon's fixative. Single B cell was isolated by DEPArray™ NxT, and its genomic DNA was amplified before IgH PCR reaction. A single band near to 300 base pair ladder was observed in live single B cell (S1, S2, S3, S5) but not fixed single B cell (F1-F5). S1-S5: live single B-cell; P: pooled B-cells (n=1000); F1-F5: fixed single B-cell with Shandon's fixative; Pf: fixed of pooled B-cell (n=1000). +ve: clonal control; -ve: no template control.

Figures 2A-2B show selection of suitable fixative for the development of single B-cell based

clonality assay. B lymphoma cells were pre-fixed with PreservCyt (**Fig. 2A**) or Paxgene (**Fig. 2B**) fixative before immunophenotyping with flow cytometry. Cells were stained with antibodies (dashed line) against markers (CD3, CD8, CD19, CD20, CD79a, Ki-67, IL-10) or their respective isotype controls (solid line).

Figure 3 shows a experimental workflow of single-cell analysis using the DEPArray™ NxT system. Samples (either Pfeiffer cell line or vitreous fluid from the clinic) were first stained with antibodies as illustrated in table 1 prior to single-cell isolation on the DEPArray™ NxT system. Whole genome amplification and quality check were performed on isolated single cells to ensure that amplified genomic sequences were of satisfactory quality for downstream immunoglobulin heavy and light chain PCR, BCL2/JH t(14;18) translocation assay, MYD88 mutation and copy number aberration analysis. Sanger sequencing was employed to elucidate the clonality and MYD88 mutation of isolated single cells.

Figure 4 shows isolation of Pfeiffer single cell on the DEPArray™ NxT system. Immunophenotypic profiling of single Pfeiffer cells using DEPArray™ NxT system. Results show positive staining for diffuse-large B-cell lymphoma markers ($CD19^+CD20^+CD79a^+Ki\text{-}67^+$) and DAPI, but negative for T cell marker CD3.

Figure 5 shows IgH and IgK PCR products separated by gel electrophoresis. **(A)** IgH PCR. A single band corresponding to 300 base pairs was observed in all five single Pfeiffer cells isolated from the DEPArray™ NxT system. Amplicons were within the expected size range for IgH PCR products. **(B)** IgK PCR. A single band near the 200 base pairs ladder marker was observed in the same five cells, within the expected size range for IgK PCR products. PC (polyclonal control), CC (clonal control) and NTC (no template control) were used as controls.

Figures 6A-6B show a multiple sequence alignment and IgBlast of IgH sequencing data, respectively. **(Fig. 6A)** Multiple sequence alignment of five representative cells showed good alignment throughout the entire IgH read length of 300 base pairs. **(Fig. 6B)** Online database IgBlast showed 97.6% alignment to the IGHV3-7*01 allele, corresponding to framework region 1(FR1), complementarity-determining region (CDR1), FR2, CDR2, FR3 and CDR3 within the variable region of the immunoglobulin heavy chain.

Figures 7A-7B show a multiple sequence alignment and IgBlast of IgK sequencing data, respectively. **(Fig. 7A)** Multiple sequence alignment of five representative cells showed good

alignment throughout the entire IgK read length of 190 base pairs. **(Fig. 7B)** Online database IgBlast showed 90.8% alignment to the IGKV1-5*01 allele, corresponding FR3 and CDR3 within the variable region of the immunoglobulin light chain.

Figure 8 shows a comparison of dominant IgH allele frequencies in vitreoretinal lymphoma patient vs inflammatory uveitis. **(A)** Percentage of dominant IgH was plotted against number of single B cells isolated from vitreoretinal lymphoma (VRL) or inflammatory uveitis. Each dot represents individual patient. **(B)** Significant higher percentage of dominant IgH allele was detected in VRL lymphoma than inflammatory uveitis (*P<0.05, unpaired t-test.). Dotted line is the optimum cut-off value at 68.75% dominant IgH allele to achieve sensitivity and specificity of the test at 100% and 75% respectively.

Figures 9A-9B: Fig. 9A shows genome-wide analysis of copy number aberration (CNA) at single cell resolution. CNA was detected in single B cell from (A) VRL lymphoma but not from (B) inflammatory uveitis. Grey: gain of copy; Boxed: loss of copy. **Fig. 9B** shows copy number aberration (CNA) analysis of single B-cell isolated from PVRL patient. Global CNA analysis of 15 B-cells (S01-S15) from PVRL patient showing areas of chromosomal gains (grey) and loss (boxed). Each row represents individual single B-cell. Each column represents individual chromosome. Similar CNA profile is a good indication that isolated single cells are derived from the same origin.

Figure 10 shows an analysis of BCL2/IgH fusion gene of single B-cell. BCL2 translocation was detected in **(A)** PVRL patient but not in **(B)** inflammatory patient. BCL2/JH t(14;18) Translocation Assay was performed on 15 B-cells (1-15) from a PVRL patient. The existence of a BCL2/IgH translocation of the same size (~200bp) indicated cellular malignancy and potentially a common B-cell lymphoma origin. Positive control (+, arrow); negative control (-).

Figure 11 shows the detection limit of Sanger sequencing. **(A)** MYD88 PCR was conducted to amplify both WT and MYD88 L265P alleles. Sequencing results were verified to encode C<u>C</u>G and C<u>T</u>G corresponding to the mutant and WT MYD88 alleles. **(B)** PCR amplicons were mixed in titrating proportion to illustrate the limit of detection of Sanger sequencing. The detection limit of Sanger sequencing in detecting mutant and WT MYD88 alleles was around 30%.

Figure 12 shows testing of single cell-MYD88 PCR on clinical sample. **(A)** Single cell-MYD88 PCR was conducted a clinical sample (total of 15 cells). 14 out of 15 cells gave a PCR band detected around 200 bp. **(B)** Multiple sequence alignment of the excised and

sequenced PCR amplicons. T → C point mutation can be observed in the five representative cells. Pfeiffer cell line, a germinal center B-cell-like DLBCL exhibited WT MYD88 (C**T**G) allele. **(C)** Electropherogram showing the DNA trace of mutant MYD88 L265P and WT MYD88.

Figure 13 shows a comparison of homozygous $MYD88^{L265P}$ mutation frequencies in vitreoretinal lymphoma patient vs inflammatory uveitis**. (A)** Percentage of homozygous $MYD88^{L265P}$ mutation was plotted against number of single B cells isolated from vitreoretinal lymphoma (VRL) or inflammatory uveitis. Each dot represents individual patient. **(B)** Higher percentage of homozygous $MYD88^{L265P}$ mutation was detected in VRL lymphoma than inflammatory uveitis (p=0.07, one-tailed unpaired *t*-test.). Dotted line is the optimum cut-off value at 5.2% homozygous $MYD88^{L265P}$ mutation to achieve 100% sensitivity and specificity of the receiver-operating characteristic (ROC) test.

DETAILED DESCRIPTION OF THE INVENTION

As previously stated, this invention provides a method of performing single cell-based genomic analysis of B-cell clonality by methods for single-B-cell isolation, determining copy number aberration, BCL2/JH (t14;18) translocation and/or presence of a MYD88 L265P mutation for the diagnosis and prognostication of a lymphoproliferative disorder such as vitreoretinal lymphoma (VRL) and, in particular, primary vitreoretinal lymphoma (PVRL).

Definitions

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as is commonly understood by one of skill in the art to which the invention belongs. Certain terms employed in the specification, examples and appended claims are collected here for convenience.

The term "oligonucleotide," as used herein, refers to a nucleic acid sequence of at least about 6 nucleotides to 60 nucleotides, preferably about 15 to 30 nucleotides, and most preferably about 20 to 25 nucleotides, which can be used in PCR amplification or in a hybridization assay. As used herein, the term "oligonucleotide" is substantially equivalent to the terms "amplimers," "primers," "oligomers," and "probes," as these terms are commonly defined in the art.

The term "sample," as used herein, is used in its broadest sense. A biological sample suspected of containing lymphoproliferative cells may comprise a bodily fluid, an extract from a cell, chromosome, organelle, or membrane isolated from a cell, a cell, genomic DNA,

RNA, or cDNA (in solution or bound to a solid support), a tissue, a tissue print and the like.

The term "specific amplification", as used herein, refers to amplification of the number of copies of one or more specific (targeted) nucleic acids in a sample. For example, amplification of IgH and IgK nucleic acids with specific primers selected from SEQ ID Nos 1-41 as described herein for step e) of the method of the invention is a specific amplification, unlike the more general or non-specific amplification performed as whole genome amplification of step d) of the method of the invention.

The term "subject" is herein defined as vertebrate, particularly mammal, more particularly human. For purposes of research, the subject may particularly be at least one animal model, e.g., a mouse, rat and the like. In particular, for detecting and/or diagnosing lymphoproliferative disorders, preferably B cell lymphoproliferative disorders such as vitreoretinal lymphoma (VRL) and, in particular, primary vitreoretinal lymphoma (PVRL), the subject may be a human.

As used herein, the term 'comprising' does not preclude the presence of additional steps or substances in the methods and compositions, respectively, of the invention, and is understood to include within its scope the terms 'consisting of' and 'consisting essentially of' features defined in the claimed invention.

Having now generally described the invention, the same will be more readily understood through reference to the following examples which are provided by way of illustration, and are not intended to be limiting of the present invention.

EXAMPLES

Standard molecular biology techniques known in the art and not specifically described were generally followed as described in Sambrook and Russell, Molecular Cloning: A Laboratory Manual, Cold Springs Harbor Laboratory, New York (2001).

MATERIALS AND METHODS

1.1 Cell line

Pfeiffer cell line (ATCC® CRL2632™) was purchased from ATCC®. Cells were cultured in Roswell Park Memorial Institute (RPMI)-1640 medium supplemented with 10% (v/v) fetal bovine serum (FBS) and 1% (v/v) penicillin/streptomycin (Gibco™). All cells were cultured at 37°C in 5% CO_2 incubator. For cryopreservation, cells were resuspended in 1 ml of freeze medium (90% FBS and 10% dimethyl sulfoxide v/v), transferred into a cryogenic vial and

placed in a CoolCell® cell freezing container.

1.2 Clinical samples

All clinical samples were obtained with consent given for research according to the ethical approval from SingHealth Centralised Institutional Review Board (CIRB Ref: 2017/2494). Researchers involved in this study are blinded on the outcome of clinical diagnosis. All samples were anonymised and coded by a non-patient related identifier, in accordance with Singapore Personal Data Protection Act law.

1.3 DEPArray™ NxT sample preparation

Pfeiffer cell line and vitreous fluid were fixed with PreservCyt® for a minimum of 72 h, counted and washed twice with autoMACS® running buffer before staining with 4', 6-diamidino-2-phenylindole (DAPI) and a cocktail of different fluorophore-conjugated antibodies as illustrated in Table 1.

Table 1: List of conjugated antibodies

Target (clone)	Fluorophore	Company	Catalogue #
CD79a (HM47)	FITC	BioLegend	333512
CD3 (UCHT1)	PE	BioLegend	300408
CD19 (J3-119)	APC	Beckman Coulter	IM2470U
CD20 (B9E9)	APC	Beckman Coulter	A51076
Ki-67 (KI-67)	PerCP/Cy5.5	BioLegend	350520

Staining was incubated at 4°C for 1 h and washed twice with SB115 (Menarini Silicon Biosystems). A maximum of 3 X 10^5 cells (in 12 µl) and 2.5 ml of SB115 buffer were loaded into a DEPArray™ cartridge (Menarini Silicon Biosystems) for acquisition.

1.4 DEPArray™ NxT system operation

The DEPArray™ NxT system (Menarini Silicon Biosystems), is compatible with both live and fixed, single cell isolation [Boral, D., *et al.*, *Nature Communications*. 2017; 8(1): 196; Fontana, F., *et al.*, *Forensic Sci Int Genet*. 2017; 29: 225-241; Hansson, O. and Gill P., *Forensic Sci Int Genetics*. 2017: 30: 57-65; Mesquita, B., *et al.*, *Mol Oncol*. 2017; 11(12): 1687-1697; Palmirotta, R., *et al.*, *Cancer Genomics & Proteomics*. 2017; 14(3): 173-179]. The technology is based on an electrokinetic principle called diaelectrophoresis to immobilize cells at a specific location and has the capability to mobilize cells by alternating

the electric field of the electrodes within the cartridge's array. The cell selection technology is augmented with cell imaging capability for real-time visualization of cells in bright-field and fluorescent channels such as fluorescein isothiocyanate (FITC), Phycoerythrin (PE), allophycocyanin (APC) and peridinin chlorophyll-Cy5.5 (PerCP-Cy5.5).

A DEPArray™ cartridge with the loaded sample was inserted into the machine, and configuration to detect bright-field, FITC, PE, APC, PerCP-Cy5.5, and DAPI. Steps such as sample load, sample scan, and image analysis were automated processes on the DEPArray™ NxT system. Manual selection of $CD19^{+}CD20^{+}$ cells was performed before the cells were routing for eluting. Single cells were eluted in ~20 µl SB115 and collected in 0.2 ml PCR tubes. To remove the buffer, volume reduction was performed by adding 100 µl of 1 X PBS and centrifuged at 14,100 x g for 10 min using a 4 °C fixed rotor benchtop centrifuge. After centrifugation, the supernatant was carefully aspirated, leaving one to two microliter of the liquid containing the isolated cell. Isolated cells were stored at -20 °C prior to downstream molecular processing.

1.5 Whole genome amplification

Isolated single cells were thawed on ice prior to whole genome amplification (WGA) using Ampli1™ WGA kit (Menarini Silicon Biosystems). WGA was performed in accordance with manufacturer's protocol, involving steps such as cell lysis, deoxyribonucleic acid (DNA) digestion, adaptor ligation, and amplification to obtain 50 µl of amplified products for further analysis.

1.6 IgH and IgK PCR

IgH and IgK polymerase chain reaction (PCR) were performed using BIOMED-2 PCR protocol [van Dongen, J.J., *et al.*, *Leukemia*. 2003; 17(12): 2257-317], incorporated herein by reference. Briefly, for each of IgH and IgK, PCR of 10% of the WGA product was started with the preactivation step at 95 °C for 7 min. Thermal cycling for denaturation (95 °C for 45 s), annealing (60 °C for 45 s) and extension (72 °C for 90 s) was conducted for 50 cycles followed by a final extension at 72 °C for 10 min.

The primers used comprise at least one forward oligonucleotide primer and at least one reverse oligonucleotide primer. A unique short oligonucleotide tail (underlined sequence: 5'-TGTAAAACGACGGCCAGT-3' (SEQ ID NO: 44) or 5'-CAGGAAACAGCTATGACC-3' (SEQ ID NO: 45)) was added onto the 5' end of each forward and reverse primer, respectively, in order to perform Sanger sequencing after IgH IgK and MYD88 PCR reaction. Primers, with sequencing tail underlined, are selected from the group comprising:

IgK primers selected from:

Forward primers:

5'-TGTAAAACGACGGCCAGTTCAAGGTTCAGCGGCAGTGGATCTG-3' (SEQ ID NO: 1)

5'-TGTAAAACGACGGCCAGTGGCCTCCATCTCCTGCAGGTCTAGTC-3' (SEQ ID NO: 2)

5'-TGTAAAACGACGGCCAGTCCCAGGCTCCTCATCTATGATGCATCC-3' (SEQ ID NO: 3)

5'-TGTAAAACGACGGCCAGTCAACTGCAAGTCCAGCCAGAGTGTTTT-3' (SEQ ID NO: 4)

5'-TGTAAAACGACGGCCAGTCCTGCAAAGCCAGCCAAGACATTGAT-3' (SEQ ID NO: 5)

and

5'-TGTAAAACGACGGCCAGTGACCGATTTCACCCTCACAATTAATCC-3' (SEQ ID NO: 6);

and

Reverse primers:

5'-CAGGAAACAGCTATGACCCTTACGTTTGATCTCCACCTTGGTCCC-3' (SEQ ID NO: 7),

and

5'-CAGGAAACAGCTATGACCCTTACGTTTAATCTCCAGTCGTGTCCC-3' (SEQ ID NO: 8)

and/or

IgK primers selected from

Forward primers:

5'-TGTAAAACGACGGCCAGTTCAAGGTTCAGCGGCAGTGGATCTG-3' (SEQ ID NO: 9)

5'-TGTAAAACGACGGCCAGTGGCCTCCATCTCCTGCAGGTCTAGTC-3' (SEQ ID NO: 10)

5'-TGTAAAACGACGGCCAGTCCCAGGCTCCTCATCTATGATGCATCC-3' (SEQ ID NO: 11)

5'-TGTAAAACGACGGCCAGTCAACTGCAAGTCCAGCCAGAGTGTTTT-3' (SEQ ID NO: 12)

5'-TGTAAAACGACGGCCAGTCCTGCAAAGCCAGCCAAGACATTGAT-3' (SEQ ID NO: 13)

5'-TGTAAAACGACGGCCAGTGACCGATTTCACCCTCACAATTAATCC-3' (SEQ ID NO: 14)

and

5'-TGTAAAACGACGGCCAGTCGTGGCACCGCGAGCTGTAGAC-3' (SEQ ID NO: 15)

Reverse primers:

5'-CAGGAAACAGCTATGACCCTTACGTTTGATCTCCACCTTGGTCCC-3' (SEQ ID NO: 16)

5'-CAGGAAACAGCTATGACCCTTACGTTTAATCTCCAGTCGTGTCCC-3' (SEQ ID NO: 17)

5'-CAGGAAACAGCTATGACCCCTCAGAGGTCAGAGCAGGTTGTCCTA-3' (SEQ ID NO: 18)

and/or

IgH primers selected from:

Forward primers:

5'-TGTAAAACGACGGCCAGTGGCCTCAGTGAAGGTCTCCTGCAAG-3' (SEQ ID NO: 19)

5'-TGTAAAACGACGGCCAGTGTCTGGTCCTACGCTGGTGAAACCC-3' (SEQ ID NO: 20)

5'-TGTAAAACGACGGCCAGTCTGGGGGGTCCCTGAGACTCTCCTG-3' (SEQ ID NO: 21)

5'-TGTAAAACGACGGCCAGTCTTCGGAGACCCTGTCCCTCACCTG-3' (SEQ ID NO: 22)

5'-TGTAAAACGACGGCCAGTCGGGGAGTCTCTGAAGATCTCCTGT-3' (SEQ ID NO: 23)

5'-TGTAAAACGACGGCCAGTTCGCAGACCCTCTCACTCACCTGTG-3' (SEQ ID NO: 24)

Reverse primer:

5'-CAGGAAACAGCTATGACCCTTACCTGAGGAGACGGTGACC-3' (SEQ ID NO: 25)

and/or

IgH primers selected from:

Forward primers:

5'-TGTAAAACGACGGCCAGTCTGGGTGCGACAGGCCCCTGGACAA-3' (SEQ ID NO: 26)

5'-TGTAAAACGACGGCCAGTTGGATCCGTCAGCCCCCAGGGAAGG-3' (SEQ ID NO: 27)

5'-TGTAAAACGACGGCCAGTGGTCCGCCAGGCTCCAGGGAA-3' (SEQ ID NO: 28)

5'-TGTAAAACGACGGCCAGTTGGATCCGCCAGCCCCCAGGGAAGG-3' (SEQ ID NO: 29)

5'-TGTAAAACGACGGCCAGTGGGTGCGCCAGATGCCCGGGAAAGG-3' (SEQ ID NO: 30)

5'-TGTAAAACGACGGCCAGTTGGATCAGGCAGTCCCCATCGAGAG-3' (SEQ ID NO: 31)

5'-TGTAAAACGACGGCCAGTTTGGGTGCGACAGGCCCCTGGACAA-3' (SEQ ID NO: 32)

Reverse primer:

5'-CAGGAAACAGCTATGACCCTTACCTGAGGAGACGGTGACC-3' (SEQ ID NO: 33)

and/or

IgH primers selected from:

Forward primers:

5'-TGTAAAACGACGGCCAGTTGGAGCTGAGCAGCCTGAGATCTGA-3' (SEQ ID NO: 34)

5'-TGTAAAACGACGGCCAGTCAATGACCAACATGGACCCTGTGGA-3' (SEQ ID NO: 35)

5'-TGTAAAACGACGGCCAGTTCTGCAAATGAACAGCCTGAGAGCC-3' (SEQ ID NO: 36)

5'-TGTAAAACGACGGCCAGTGAGCTCTGTGACCGCCGCGGACACG-3' (SEQ ID NO: 37)

5'-TGTAAAACGACGGCCAGTCAGCACCGCCTACCTGCAGTGGAGC-3' (SEQ ID NO: 38)

5'-TGTAAAACGACGGCCAGTGTTCTCCCTGCAGCTGAACTCTGTG-3' (SEQ ID NO: 39)
5'-TGTAAAACGACGGCCAGTCAGCACGGCATATCTGCAGATCAG-3' (SEQ ID NO: 40)

Reverse primer:
5'-CAGGAAACAGCTATGACCCTTACCTGAGGAGACGGTGACC-3' (SEQ ID NO: 41)

1.6 MYD88 PCR

MYD88 PCR was performed using sense primer sequence (5'-TGTAAAACGACGGCCAGTTGCAGGGGTTGGTGTAGT-3'; SEQ ID NO: 42) and antisense primer sequence (5'-CAGGAAACAGCTATGACCGTTGTTAACCCTGGGGTTG-3'; SEQ ID NO: 43) (Integrated DNA Technologies). As per the amplification for IgH and IgK, the PCR primers comprised a unique short oligonucleotide sequence (underlined) at the 5' end of each primer for Sanger sequencing after MYD88 PCR amplification. PCR of 10% of the WGA product was performed in accordance with manufacturer's protocol (Menarini Silicon Biosystems). Briefly, PCR was started with the preactivation step at 95 °C for 10 min. Thermal cycling for denaturation (95 °C for 15 s), annealing (50 °C for 30 s) and extension (72 °C for 30 s) was conducted for 50 cycles followed by final extension at 72 °C for 10 min.

1.7 Gel extraction and Sanger sequencing

PCR amplicons were loaded onto a 2% Tris-acetate-ethylenediaminetetraacetic acid (TAE) gel and separated at constant voltage (100 V). Lanes with band corresponding to the expected size were excised and gel extracted with QIAquick® gel extraction kit (Qiagen). Samples were eluted with 20 µl of nuclease-free water (Sigma-Aldrich) before sending for Sanger sequencing (BioBasic) with either forward sequencing primer (5'-TGTAAAACGACGGCCAGT-3'; SEQ ID NO: 44) or reverse sequencing primer (5'-CAGGAAACAGCTATGACC-3'; SEQ ID NO: 45).

1.8 Sequencing data analysis

Quality of sequencing data was checked using FinchTV (Geospiza) and multiple sequence alignment was performed by CLC Sequence Viewer 7 software (Qiagen). Sequence database search was conducted using online resources blastn and/or IgBLAST.

1.9 Copy-number aberration analysis

Whole genome sequencing library for each single cell was prepared using Ampli1™ LowPass kit (Menarini Silicon Biosystems) according to manufacturer's protocol. Briefly, each sequencing library was tagged with a barcode which is unique to each single cell. Each barcoded sequencing library was clean up and quantified to generate an equimolar final

pooled library suitable for Ion Torrent or Illumina NGS sequencing.

1.10 BCL2/JH t(14;18) Translocation Assay

The presence of BCL2/IgH fusion gene was determined by BCL2/JH t(14;18) Translocation Assay (Invivoscribe Technologies), in accordance with manufacturer's protocol. Briefly, PCR master mixes targeting the joining region of the IgH and distinct region of the BCL2 gene was started with the preactivation step at 95 °C for 7 min. Thermal cycling for denaturation (94 °C for 30 s), annealing (55 °C for 30 s) and extension (72 °C for 60 s) was conducted for 35 cycles followed by a final extension at 72 °C for 10 min.

RESULTS

Identification of suitable fixative for the development of single B-cell based clonality assay

It is important to maximize the use of the limited vitreous biopsy material for different assays required for VRL diagnosis and in particular PVRL diagnosis. Vitreous biopsy specimens must be handled gently and quickly delivered for analysis as lymphoma cells undergo morphological degradation within an hour [Chan, C.-C., *et al.*, *The Oncologist*. 2011; 16(11): 1589-1599; Gonzales, J.A. and C.C. Chan, *Int Ophthalmol*. 2007; 27(4): 241-50; Char DH., *et al.*, *Br J Ophthalmol*. 1988; 72(12): 905-11]. In most cases immediate sample processing and diagnosis are challenging, as logistic times are needed to transport the sample for laboratory testing. Thus, vitreous samples are usually placed into fixative solution, such as Shandon™ Cytospin™ Collection Fluid (or Shandon's fixative), so as to preserve the integrity of cellular structure and morphology for cytological assay.

To investigate whether Shandon's fixative is able to preserve well the genomic DNA for downstream molecular assay, a clonality assay was performed on single B lymphoma cells isolated from DEPArray™ NxT. Results showed that Shandon's fixative did not affect the PCR reaction of bulk B-cell samples (n=1000, Pf, Fig. 1), but all single B-cells (5 out of 5) lost their PCR IgH amplicon after Shandon's fixation (F1-F5, Fig. 1). This result prompted a search for alternative preservatives suitable for the development of single-cell based VRL and, in particular, PVRL detection and/or diagnosis.

Next, flow cytometry was performed on a B lymphoma cell line pre-fixed in PreservCyt® or Paxgene®, which are two common fixatives used in the pathology laboratory. PreservCyt®-fixed B-cells showed positive staining of diffuse-large B-cell lymphoma markers ($CD19^+CD20^+CD79a^+Ki\text{-}67^+$), but negative staining of T cell markers CD3 and CD8 (Fig. 2A). In contrast, Paxgene®-fixed cells expressed low or no B-cell markers, and were stained with

an undesired high background of T cell markers CD3 (HIT3a and OKT3) (Fig 2B).

As good antibody staining is a pre-requisite step required by DEPArray to isolate single B-cells from heterogeneous vitreous samples, it is important to make sure that the fixative does not interfere with the antibody staining of B-cell markers. Because of this, PreservCyt® was chosen and used as the fixative in all samples for the subsequent experiments and molecular assays.

Isolation of Pfeiffer single cells using the DEPArray™ NxT system

Monoclonality is where cells derive from the same ancestral cell and is a clone of one another. It is a feature of cancer and is best exemplified by prolonged passaging of cell line [Tanooka, H., *Jpn J Cancer Res*. 1988; 79(6): 657-65; Nowell, P.C., *Science*. 1976; 194(4260): 23-8]. Pfeiffer cell line was selected to validate the feasibility of single cell IgH and IgK PCR (European BIOMED-2) as it is an established cell line; as well as being classified as DLBCL pathologically, a manifestation which is observed in 90% of VRL. To validate the feasibility of performing single cell PCR on Pfeiffer cells isolated from the DEPArray™ NxT system, the workflow as illustrated in Figure 3 was adopted. Briefly, Pfeiffer cells were harvested from culture, fixed with PreservCyt prior to antibody staining (Table 1) and loading onto the DEPArray™ cartridge. Using the DEPArray™ NxT system, single cells can be isolated for downstream applications such as WGA, IgH and IgK and MYD88 PCR.

As seen in Figure 4, the profile of five representative cells is illustrated. In view of the fact that Pfeiffer cell line is of B-cell origin, the expression of B-cell markers ($CD19^+CD20^+$) was observed in all five cells, but not CD3 - a marker specific for T cell. DAPI staining was also observed in all five cells owing to the fixation with PreservCyt that permitted nuclear staining with DAPI. Expression of CD79a and Ki-67 – markers for DLBCL, was also observed with varying expression in the isolated cells [Fend, F., et al., *Br J Haematol*. 2016; 173(5): 680-92].

Isolated cells were then subjected to Ampli1™ WGA according to manufacturer's protocol (data not shown) to increase the amount of genome DNA available for downstream IgH, IgK and MYD88 PCR.

Identical IgH and IgK sequences in multiple single cells indicates clonality

As shown in Figure 5A, a single band (around 300 base pairs ladder) was observed in all five lanes (labelled 1 to 5), corresponding to the five cells isolated in Figure 4. Observed PCR amplicons were within the expected size range (310 to 380 base pairs) as detailed in

the manufacturer's protocol. No band was observed in the lane containing no template control (NTC). To obtain paired IgH and IgK data, IgK PCR was performed on the same five cells. Unlike IgH PCR amplicons, the PCR product of IgK PCR was observed around the 200 base pair ladder, within the expected range of 190 to 210 base pairs.

To verify the clonality of Pfeiffer single cells isolated by the DEPArray™ NxT system, PCR amplicons shown in Figure 5A and 5B were excised and gel purified before Sanger sequencing. Sequencing results were then analyzed using CLC Sequence Viewer 7 software (Qiagen) and online database (IgBlast) for both IgH and IgK PCR amplicons. Based on the multiple sequence alignment of IgH in Figure 6A, 100% sequence alignment was observed in the five representative cells. When the consensus sequence was searched on the IgBlast database, the returned results showed 97.6% alignment to the allele IGHV3-7*01, with the detection of framework region 1 (FR1), complementarity-determining region (CDR1), FR2, CDR2, FR3 and CDR3 within the variable region of the immunoglobulin chain (Figure 6B).

Similarly, good IgK sequence alignment was also observed in the five representative cells (Figure 7A). In contrast to the greater read length (around 300 base pairs) as observed in IgH sequencing, a lower read length of around 190 base pairs was seen in IgK sequencing. Nonetheless, when the consensus was searched on the online database, 90.8% alignment to the IGKV1-5*01 allele of the light chain kappa was noted, with partial alignment to the FR3 and CDR3 (Figure 7B). Taken together, these data demonstrated that IgH and IgK sequencing using single cells can be achieved, and more importantly a proof of concept that the isolated Pfeiffer cells were clonal. As the ultimate test of the workflow, it is imperative to validate whether the clonality data obtained from vitreous fluid can corroborate with the diagnosis made by the pathologist.

To this end, the exact workflow which was conducted with a Pfeiffer cell line was applied onto clinical samples, with results summarized in Table 2. Results showed that 91% (10 out of 11) single B-cells isolated from a PVRL patient (VRL #1, Table 2) had identical dominant paired IgK and IgH alleles (IGHV5-51*01; IGKV1D-43*01). On the other hand, 3 to 6 B-cell clones with different paired IgK and IgH alleles (Percentage <60%) were found in vitreous sample from patients with chronic inflammation (Inflammation #1-3, Table 2). Bi-allelic of IgH and/or IgK alleles were also detected using our single B-cell clonality approach.

Higher frequency of dominant IgH allele was detected in vitreoretinal lymphoma (VRL)

The IgH allele frequencies from single B-cells isolated from another two VRL patients (VRL

#2, #3), were characterized. The dominant IgH alleles were IGHV3-7*01 and IGHV2-70, with frequencies of 100% (9 out of 9 cells) and 75% (12 out of 16 cells) respectively. Paired IgH:IgK clonality could not be established from these two VRL patients (VRL #2, #3) due to the unsuccessful detection and sequence of IgK allele (Table 2).

5

Table 2: IgH and IgK alleles of Pfeiffer cell line and clinical samples received from Singapore National Eye Center

Sample	**IgH allele**	**IgK allele**	**#Cells (%)**	**Frequency of BCL2/JH t(14;18)***
Pfeiffer cell line (DLBCL)	IGHV3-7*01	IGKV1-5*01	-	-
VRL #1	IGHV5-51*01 IGHV5-51*01	IGKV1D-43*01 IGKV1D-43*01 & IGKV3-11*01	10/11 (91%) 1/11 (9%)	15/15 (100%)
VRL #2	IGHV3-7*01	n.d.	9/9 (100% IgH)	0/13 (0%)
PVRL #3	IGHV2-70 IGHV2-5	n.d. n.d.	12/16 (75% IgH) 4/16 (25% IgH)	0/19 (0%)
Inflammation #1	IGHV3-7*01 IGHV1-46*03 &IGHV3-7*01 IGHV5-51*01	IGK1-5*01 IGKV3D-20*01 &IGKV1-5*01 IGKV3D-20*01 &IGKV1-5*01	4/7 (57%) 2/7 (29%) 1/7 (14%)	0/10 (0%)

Inflammation #2	IGHV3-7*01	IGKV1-5*01	2/7 (29%)	0/13 (0%)
	IGHV5-51*01	IGKV1-5*01	1/7 (14%)	
	IGHV3-7*01	IGKV1-39*01	1/7 (14%)	
	IGHV3-7*01	IGKV3D-20*01 &IGK1-5*01	2/7 (29%)	
	IGHV3-7*01	IGKV2D-26*01 &IGKV1-5*01	1/7 (14%)	
Inflammation #3	IGHV3-7*01	IGKV1-5*01	3/8 (37.5%)	0/13 (0%)
	IGHV3-7*01	IGKV1-5*03	1/8 (12.5%)	
	IGHV3-7*01	IGKV1-5*01 & IGKV3D-20*01	1/8 (12.5%)	
	IGHV1-46*03	IGKV1-5*01	1/8 (12.5%)	
	IGHV1-46*03	IGKV3D-20*01 &IGKV1-5*01	1/8 (12.5%)	
	IGHV3-7*01 & IGHV1-46*01	IGKV1-5*01	1/8 (12.5%)	

n.d. : not detected

*: number of B-cells with the same BCL2/JH t(14;18) translocation

Detection of a BCL2/JH t(14;18) translocation was found in all single B-cells from a PVRL patient (VRL #1, Table 2), but not in single B-cells from the patients with chronic inflammation (Inflammation #1-3, Table 2).

To examine the significance of the IgH alleles to discriminate VRL versus inflammatory uveitis patients, the frequencies of dominant IgH allele among all IgH alleles detected from individual cells were characterized and quantified. Results showed that VRL patients had significantly higher percentage of dominant IgH allele (75%-100%), as compared to inflammatory uveitis (57%-85%) (Figure 8A). Receiver-operating characteristic (ROC) analysis revealed that the optimum cut-off percentage of dominant IgH allele was at 68.75%, with sensitivity and specificity of the test at 100% and 75% respectively (Figure 8B).

Expression of MYD88 L265P indicates VRL

To illustrate the sensitivity of Sanger sequencing in detecting MYD88 L265P mutation in bulk samples, PCR was first conducted to isolate the both WT MYD88 and mutant (MYD88 L265P) alleles. As shown in Figure 11A (left), PCR amplicons corresponding to WT MYD88 and mutant were excised and verified by Sanger sequencing. As seen Figure 11A (right), black arrows were used to indicate the point mutation, with C**C**G (leucine) and C**T**G (proline) corresponding to mutant and WT alleles. The sequence identity of both alleles was checked against online database BLAST® Blastn software (NCBI, USA) and verified to be of human MYD88 origin (data not shown).

To test the limit of detection of Sanger sequencing, amplicons of both alleles were mixed in various proportion (amounting to a total of 100 ng) and sent for sequencing. In Figure 11B, a single red peak corresponding to thymidine of the WT allele was observed when the WT allele was spiked at 100% (100 ng equivalent). Similarly, a single blue peak corresponding to the mutant allele was observed when 100% of the L265P amplicon was added. When PCR amplicons of both alleles were mixed in equal proportion (50 ng WT: 50 ng L265P mutant), equal representation of both thymidine (WT) and cytosine (L265P mutant) was observed on the electropherogram. Based on the ability to detect both WT and mutant at their lowest proportion, it was observed that the detection limit/sensitivity of Sanger sequencing was approximately 30%.

Verification of single cell-MYD88 sequencing on a clinical sample.

With a surplus of WGA product left from the B-cell clonality, copy number aberration, and chromosomal translocation work, single-MYD88 PCR was attempted on a clinical sample with good DNA quality. As shown in Figure 12A, bands corresponding to 200 bp were

observed in 14 out of 15 single cells. Bands corresponding to 200 bp for WT MYD88 control were also observed, but not in no template control. To determine the sequence identity, bands were excised and sequenced. The sequence identity of all samples was checked against online BLAST® database Blastn and verified to be of human MYD88 origin (data not shown). Based on the multiple sequence alignment of MYD88 in Figure 12B, 100% sequence alignment was observed in the five representative cells. As indicated by the black arrows, a point mutation (C**C**G) representing MYD88 L265P was observed in all clinical samples but not WT control (C**T**G). Additionally, based on the electropherogram as seen in Figure 12C, good sequencing data with unambiguous DNA trace can be obtained.

Application of single cell-MYD88 PCR to clinical samples

With the success in single cell-MYD88 sequencing, all single cells isolated from 10 patients were screened for MYD88 L265P mutation. The number of single cells isolated and tested from each patient is the sum of the numbers shown in the respective wild type + homozygous + heterozygous columns. Cells with high background and ambiguous DNA trace were excluded from Table 3. Samples were grouped based on their clinical status and classified as WT MYD88, MYD88 L265P homozygous or MYD88 L265P heterozygous.

Table 3: MYD88-Sanger sequencing of single cell isolated from patients.

Patients	**Clinical status**	**$MYD88^{WT}$ Wild type**	**$MYD88^{L265P}$ T→C homozygous**	**$MYD88^{L265P}$ T→C heterozygous**
1	VRL (DLBCL)	0/14 (0%)	14/14 (100%)	0/14 (0%)
2	PVRL (DLBCL)	1/9 (11.1%)	4/9 (44.4%)	4/9 (44.4%)
3	PVRL (DLBCL)	5/10 (50%)	2/10 (20%)	3/10 (30%)
4	PVRL (DLBCL)	15/17 (88.2%)	1/17 (5.9%)	1/17 (5.9%)
5	PVRL (DLBCL)	6/11 (54.5%)	4/11 (36.4%)	1/11 (9.1%)
6	PVRL	15/22 (68.2%)	4/22 (18.2%)	3/22 (13.6%)

	(DLBCL)			
7	PVRL (DLBCL)	14/15 (93.3%)	1/15 (6.67%)	0/15 (0%)
8	Inflammation	5/5 (100%)	0/0 (0%)	0/0 (0%)
9	Inflammation	0/22 (0%)	1/22 (4.55%)	21/22 (95.5%)
10	Inflammation	0/13 (0%)	0/13 (0%)	13/13 (100%)

Higher frequency of homozygous MYD88^{L265P} mutation was detected in vitreoretinal lymphoma (VRL)

From the single-cell MYD88 sequencing approach, we found that patients with clinically confirmed VRL exhibited a mixed zygosity signature that contained homozygous MYD88^{L265P} mutations (patients 1-7, Table 3). Patients with clinically confirmed inflammatory uveitis exhibited zygosity signature predominantly consisted of both MYD88^{WT} and heterozygous MYD88^{L265P} mutation (patients 8-10, Table 3). To examine the significance of the homozygous MYD88^{L265P} mutations to discriminate VRL versus inflammatory uveitis patients, we characterized and quantified the frequencies of homozygous MYD88^{L265P} mutations derived from individual cells in each patient. Results showed that VRL patients had higher percentage of homozygous MYD88^{L265P} mutations (5.9%-100%), as compared to inflammatory uveitis (0%-4.5%) (Figure 13A). Receiver-operating characteristic (ROC) analysis revealed that the optimum cut-off percentage of homozygous MYD88^{L265P} mutations was at 5.2%, with sensitivity and specificity of the test at 100% and 100% respectively (Figure 13B). More clinical samples from both VRL and inflammation control groups were needed in order to improve the significance and power of the statistical test and ROC analysis.

SUMMARY

VRL, and in particular, PVRL is an ocular malignancy with more than 90% of the cases manifesting as DLBCL [Fend, F., *et al.*, *Br J Haematol.* 2016; 173(5): 680-92]. The diagnosis of VRL, such as PVRL, is challenging because of poor sample quality and uveitis-masquerading symptoms VRL, such as PVRL, displays. As such, prompt and accurate diagnostic approaches are required to prevent mistreatment, and to reduce morbidity and mortality associated with the disease. The advent of molecular clonality testing in VRL testing had generated much interest owing to its purported higher sensitivity than the standard cytological diagnosis [Baehring, J.M., *et al.*, *Cancer.* 2005; 104(3): 591-7; Kimura,

K., *et al.*, *Jpn J Ophthalmol.* 2012; 56(4): 383-9; Coupland, S.E., *et al.*, *Graefes Arch Clin Exp Ophthalmol.* 2003; 241(10): 860-70; Coupland, S.E., *et al.*, *Invest Ophthalmol Vis Sci.* 2005; 46(10): 3507-14; Merle-Beral, H., *et al.*, *Br J Haematol.* 2004; 124(4): 469-73; Wang, Y., *et al.*, *Int J Mol Sci.* 2011; 12(9): 5684-97; van Dongen, J.J., *et al.*, *Leukemia.* 2003; 17(12): 2257-317]. However, all of the molecular clonality testing methods to date were conducted on bulk sample (i.e. not sorted for single cell); and therefore risk the possibility of false positive diagnosis in the event that few cells present in the vitreous fluid have immunoglobulin amplicons which are of similar size, giving rise to a non-discernible 'single clonality' band.

To address such unmet clinical need, the present invention combines the use of the DEPArray™ NxT system with molecular clonality testing steps to increase the sensitivity of VRL, such as PVRL, diagnosis. Here, a Pfeiffer DLBCL cell line was used to simulate PVRL and a WGA step was incorporated into the method so as to increase the availability of genome materials for downstream IgH, IgK and MYD88 PCR. Using the method of the invention the successful sequencing of IgH, IgK and MYD88 alleles of single cells isolated from the DEPArray™ NxT system was achieved. Moreover, it was further demonstrated that isolated Pfeiffer single cells were clones of each other based on the same IgH and IgK sequences upon multiple sequence alignment. With the establishment of the method, vitreous fluids were analysed with detection of clonal cells in clinical samples. While the detection of monoclonal B cells can serve as a distinguishing feature of VRL, such as PVRL; it is equally important to obtain non-cancerous cases (e.g. uveitis) as control, and to show that polyclonal B cells are detected (Table 2).

The invention presented herein demonstrates that IgH, IgK and MYD88 sequencing is possible with single cells and that molecular clonality and/or the detection of MYD88 L265P mutation can serve as a viable consideration for VRL, such as PVRL, diagnosis. The employment of WGA is a critical step which greatly increases the starting material. In fact, IgH and IgK PCR only utilized 20% of the WGA products, leaving 40 µl of amplified DNA available for other molecular exploration such as copy number aberration analysis (Figure 9), BCL2 translocation assay (Figure 10) and MYD88 L265P mutation assay (Figures 11-12). The study of copy number aberration enables the detection of chromosomal number abnormality, and at the same time permits investigation of the relationship between cells based on their chromosomal number profile. Data obtained from such study can further strengthen immunoglobulin-based molecular clonality. The BCL2 translocation assay can indicate poor prognosis in DLBCL, appending additional information that can be obtained from molecular analysis [Kawamoto, K., *et al.*, *Cancer Science.* 2016; 107(6): 853-861;

Zhang, H.W., *et al.*, *Chin J Cancer Res.* 2011; 23(2): 160-164]. Moreover, it can be determined within a single isolated B cell whether the cell is normal for MYD88, homozygous for MYD88 L265P mutation or heterozygous for MYD88 L265P mutation, a stand-alone or complementary indicator of VRL.

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The invention claimed is:

1. A method for the identification of clonal lymphoproliferative cells in a sample from a subject, the method comprising:

a) staining cells comprised in the sample of said subject with B-cell specific markers;

b) loading the cells from (a) onto a microfluidic cartridge that allows the separation of single cells into individual dielectrophoretic cages;

c) detecting individual positively-stained B cells and isolating each of said positively-stained B cells;

d) amplifying individually the whole genome of each of said isolated single cell;

e) performing a specific amplification on the product obtained from each single cell from (d) with primers directed to immunoglobulin heavy-chain (IgH) and/or immunoglobulin light-chain (IgK);

f) separating and isolating amplicons corresponding to IgH and/or IgK obtained for each single cell from (e) ;

g) sequencing said amplicons corresponding to IgH and/or IgK ,

and

h) determining whether there is a copy number aberration and/or a BCL2/JH t(14;18) translocation and/or a MYD88 L265P mutation in said isolated positively-stained B cell from c)

wherein the presence of IgH and/or IgK amplicons with the same sequence in said isolated positively-stained B cells and/or the presence of said copy number aberration and/or a BCL2/JH t(14;18) translocation indicates the presence of clonal lymphoproliferative cells and/or a MYD88 L265P mutation indicates the cells are lymphoproliferative.

2. The method of claim 1, wherein the cells in step a) are stained with DAPI and one or more B-cell markers selected from CD19, CD20, CD79a and Ki-67.

3. The method of claim 1 or 2, wherein the cells in step a) are fixed prior to staining.

4. The method of any one of the preceding claims, wherein in step c) B cells are detected based on their positivity for at least one B-cell marker selected from the group comprising CD19, CD20, CD79a and Ki-67.

5. The method of any one of the preceding claims, wherein in step e) the specific

amplification method can be selected from known methods such as polymerase chain reaction (PCR), loop mediated isothermal amplification (LAMP), nucleic acid sequence based amplification (NASBA), self-sustained sequence replication (3SR), rolling circle amplification (RCA).

6. The method of any one of the preceding claims, wherein in step e) the specific amplification method is polymerase chain reaction (PCR).

7. The method of any one of the preceding claims, wherein the primers used in step e) comprise at least one forward oligonucleotide primer and at least one reverse oligonucleotide primer selected from the groups comprising:

IgK primers selected from:
Forward primers comprising the nucleotide sequence set forth in SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5 and SEQ ID NO: 6, and
Reverse primers comprising the nucleotide sequence set forth in SEQ ID NO: 7 and SEQ ID NO: 8; and/or

IgK primers selected from:
Forward primers comprising the nucleotide sequence set forth in SEQ ID NO: 9, SEQ ID NO: 10, SEQ ID NO: 11, SEQ ID NO: 12, SEQ ID NO: 13, SEQ ID NO: 14 and SEQ ID NO: 15, and
Reverse primers comprising the nucleotide sequence set forth in SEQ ID NO: 16, SEQ ID NO: 17 and SEQ ID NO: 18; and/or

IgH primers selected from:
Forward primers comprising the nucleotide sequence set forth in SEQ ID NO: 19, SEQ ID NO: 20, SEQ ID NO: 21, SEQ ID NO: 22, SEQ ID NO: 23 and SEQ ID NO: 24, and
Reverse primer comprising the nucleotide sequence set forth in SEQ ID NO: 25; and/or

IgH primers selected from:
Forward primers comprising the nucleotide sequence set forth in SEQ ID NO: 26, SEQ ID NO: 27, SEQ ID NO: 28, SEQ ID NO: 29, SEQ ID NO: 30, SEQ ID NO: 31 and SEQ ID NO: 32, and
Reverse primer comprising the nucleotide sequence set forth in SEQ ID NO: 33; and/or

IgH primers selected from:

Forward primers comprising the nucleotide sequence set forth in SEQ ID NO: 34, SEQ ID NO: 35, SEQ ID NO: 36, SEQ ID NO: 37, SEQ ID NO: 38, SEQ ID NO: 39 and SEQ ID NO: 40, and

Reverse primer comprising the nucleotide sequence set forth in SEQ ID NO: 41; and/or

MYD88 forward primer comprising the nucleotide sequence set forth in SEQ ID NO: 42, and MYD88 reverse primer comprising the nucleotide sequence set forth in SEQ ID NO: 43.

8. The method of claim 7, wherein the forward primer has a short oligonucleotide tail at the 5' end comprising the sequence set forth in SEQ ID NO: 44 and the reverse PCR primer has a short oligonucleotide tail at the 5' end comprising the sequence set forth in SEQ ID NO: 45 to facilitate sequencing of said amplicons.

9. The method of any one of the preceding claims, wherein in step g) the sequencing is Sanger Sequencing or Next Generation Sequencing.

10. The method of any one of the preceding claims, wherein the presence of IgH and/or IgK amplicons with the same sequence in said isolated positively-stained B cells and/or the presence of said copy number aberration or a BCL2/JH t(14;18) translocation in said isolated positively-stained B cells and/or the presence of a MYD88 L265P mutation is diagnostic of a lymphoproliferative disorder in the subject.

11. The method of claim 10, wherein the presence of at least 60% of single B-cells sharing the same IgH and IgK sequence; and/or at least 85% of single B-cells sharing the same IgH sequence in the absence of IgK sequence; and/or at least 5% of single B-cells sharing the same homozygous MYD88 L265P mutation; and/or at least 30% of single B-cells sharing the same BCL2/JH t(14;18) translocation or similar profiles of copy number aberration is diagnostic of a lymphoproliferative disorder in the subject.

12. The method of any one of the preceding claims wherein the lymphoproliferative disorder is selected from vitreoretinal lymphoma (VRL), central nervous system lymphoma (CNSL), VRL-specific minimal residue disease (MRD), or CNSL-specific MRD.

13. The method of any one of the preceding claims, wherein the sample is from: vitreous fluid, blood, bone marrow or cerebrospinal fluid.

14. The method of any one of the preceding claims, wherein the sample is a vitreous fluid

and the lymphoproliferative disease is VRL or VRL-specific MRD.

15. The method of any one of claims 1 to 13, wherein the sample is a cerebrospinal fluid and the lymphoproliferative disease is CNSL or CNSL-specific MRD.

16. The method of any one of the preceding claims, wherein the subject is a human.

17. A method of monitoring a B cell lymphoproliferative disease in a subject, comprising screening a sample from said subject using a method according to any one of claims 1 to 16.

18. A kit for the identification of clonal lymphoproliferative cells or diagnosis of a lymphoproliferative disorder in a subject according to the method of claim 10 or 11, said kit comprising:

- (i) at least one forward oligonucleotide primer and at least one reverse oligonucleotide primer selected from the groups of IgK primers and/or IgH primers and/or at least one MYD88 forward oligonucleotide primer and at least one MYD88 reverse oligonucleotide primer listed in claim 7.

19. The kit of claim 18, said kit further comprising at least one of the following reagents:

- (ii) at least one antibody targeting at least one B-cell marker selected from the group CD19, CD20, CD79a and Ki-67; and/or
- (iii) suspension buffer for single cell isolation using microfluidic cartridge that allows the separation of each single cells into individual dielectrophoretic cages; and/or
- (iv) reaction buffer, enzymes and reagents for whole genome amplification and -quality check; and/or
- (v) reaction buffer, enzymes and reagents for BCL2/JH t(14;18) translocation assay; and/or
- (vi) reaction buffer, enzymes and reagents to generate barcoded sequencing libraries suitable for genome-wide copy-number profiling.

20. The kit according to claim 18 or 19, further comprising at least one of the following reagents selected from the group comprising:

- (vii) methanol-based fixative buffer, which is preferably 35-55% methanol-based;
- (viii) 4',6-diamidino-2-phenylindole (DAPI);

- (ix) sequencing primers for DNA sequencing of IgH and IgK genes;
- (x) a positive control which comprises DNA from clonal B-cells (clonal DNA control); and
-(xi) a negative control which comprises DNA from at least two different B-cells (polyclonal DNA control).

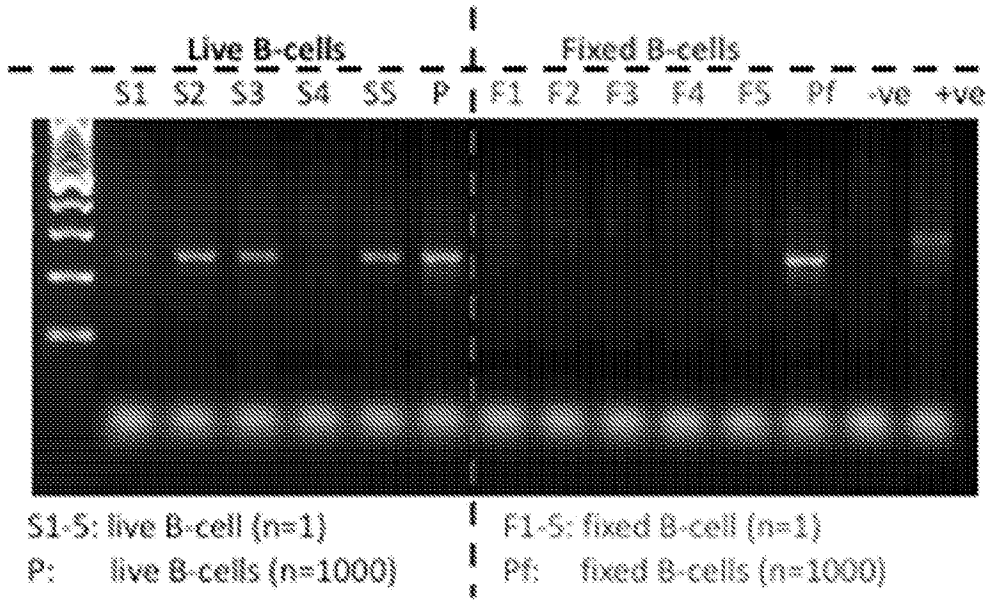
Live B-cells
Fixed B-cells
S1 S2 S3 S4 S5 P F1 F2 F3 F4 F5 Pf -ve +ve
S1-5: live B-cell (n=1)
P: live B-cells (n=1000)
F1-5: fixed B-cell (n=1)
Pf: fixed B-cells (n=1000)

FIGURE 1

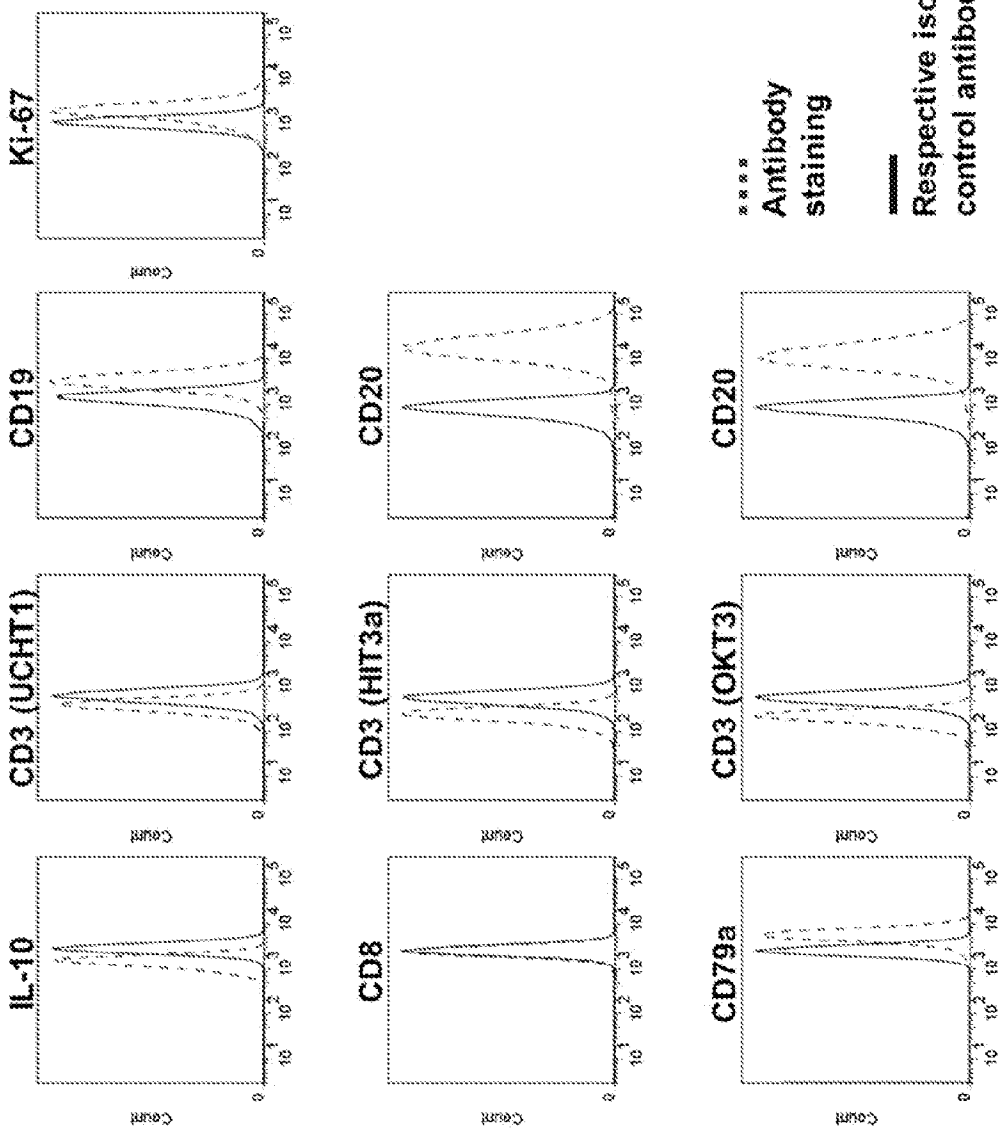
IL-10
CD3 (UCHT1)
CD19
Ki-67
CD8
CD3 (HIT3a)
CD20
CD79a
CD3 (OKT3)
CD20
Count
Antibody staining
Respective isotype control antibody

FIGURE 2A

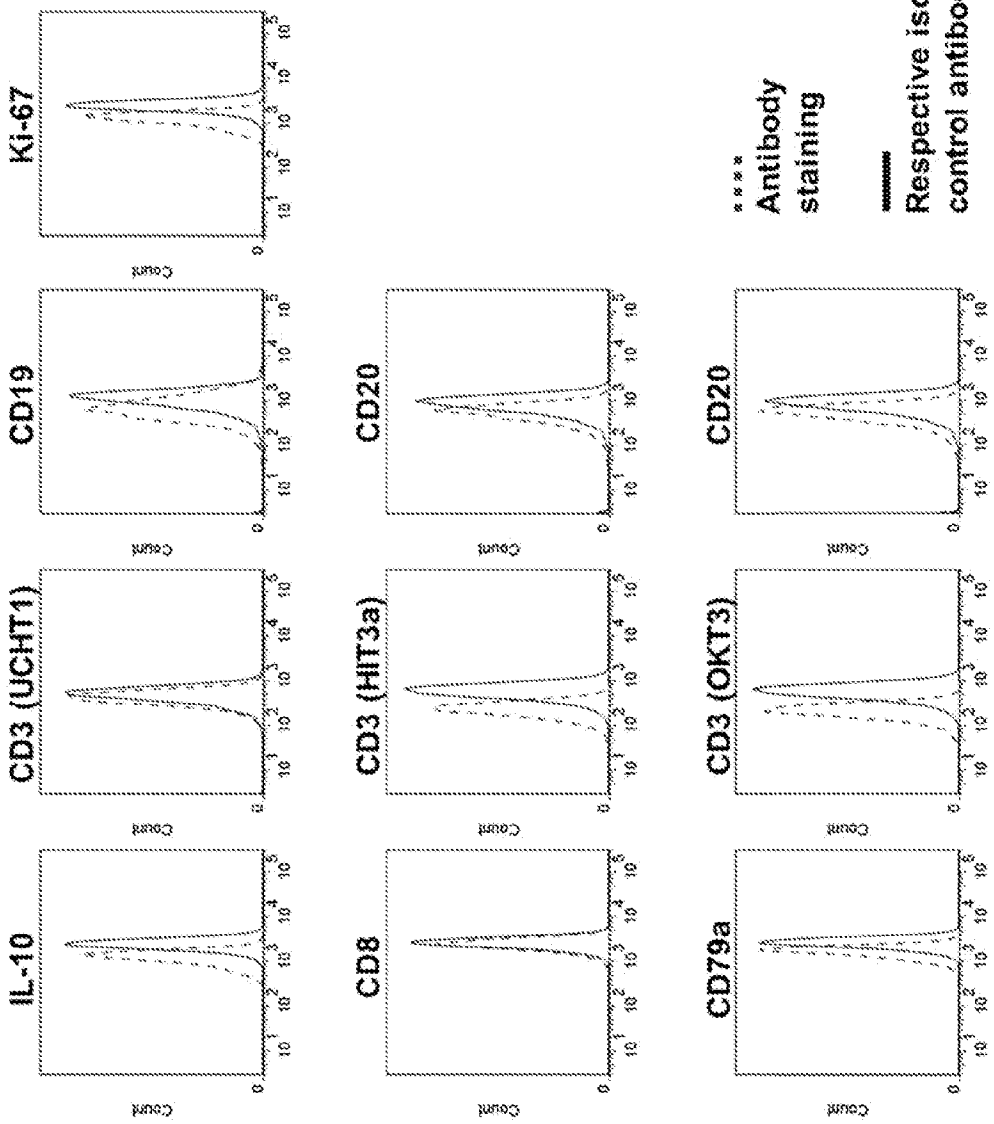
IL-10
CD3 (UCHT1)
CD19
Ki-67
CD8
CD3 (HIT3a)
CD20
CD79a
CD3 (OKT3)
CD20
Count
Antibody staining
Respective isotype control antibody

FIGURE 2B

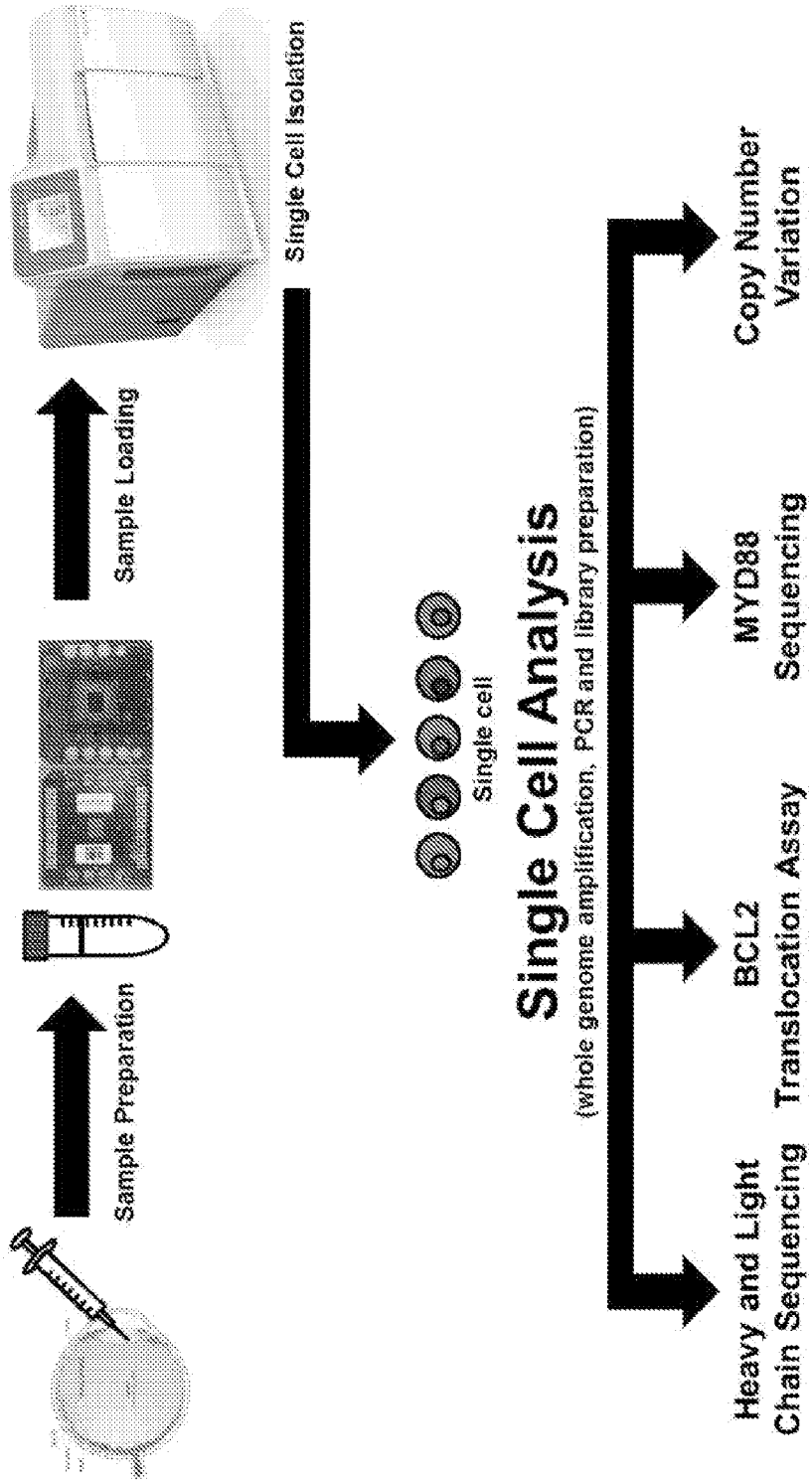
Sample Preparation
Sample Loading
Single Cell Isolation
Single cell
Single Cell Analysis
(whole genome amplification, PCR and library preparation)
Heavy and Light Chain Sequencing
BCL2 Translocation Assay
MYD88 Sequencing
Copy Number Variation

FIGURE 3

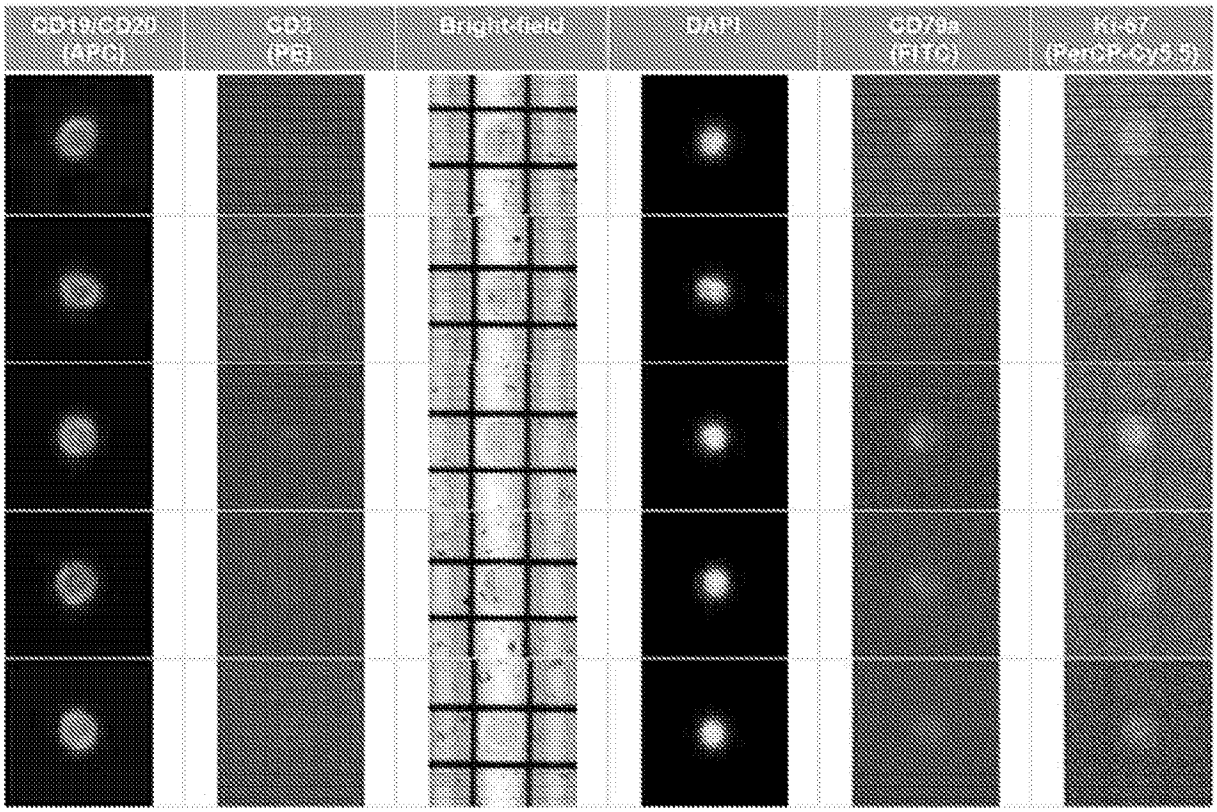
CD19/CD20
(APC)
CD3
(PE)
Bright-field
DAPI
CD79a
(FITC)
Ki-67
(PerCP-Cy5.5)

FIGURE 4

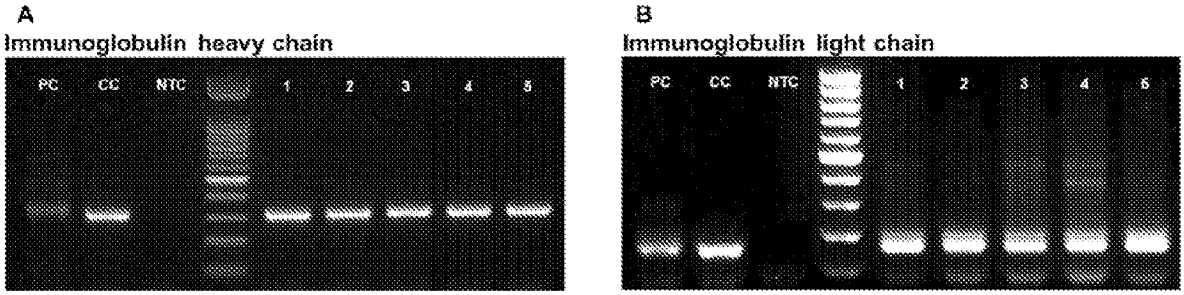
A
Immunoglobulin heavy chain
PC
CC
NTC
1
2
3
4
5
B
Immunoglobulin light chain
PC
CC
NTC
1
2
3
4
5

FIGURE 5

Cell #1 TGTATTGACT TCCTATAGGG AGGTCCCTGA GACTCTCCTG TGCAGCCTCT GGATTCACCT TTAGTAGGTA TTGGATGAGC TGGGTCCGCC 99 SEQ ID NO: 46
Cell #2 TGTATTGACT TCCTATAGGG AGGTCCCTGA GACTCTCCTG TGCAGCCTCT GGATTCACCT TTAGTAGGTA TTGGATGAGC TGGGTCCGCC 99 SEQ ID NO: 47
Cell #3 TGTATTGACT -CCTATAGGG AGGTCCCTGA GACTCTCCTG TGCAGCCTCT GGATTCACCT TTAGTAGGTA TTGGATGAGC TGGGTCCGCC 88 SEQ ID NO: 48
Cell #4 TGTATTGACT TCCTATAGGG AGGTCCCTGA GACTCTCCTG TGCAGCCTCT GGATTCACCT TTAGTAGGTA TTGGATGAGC TGGGTCCGCC 99 SEQ ID NO: 49
Cell #5 ---------- -CCTATAGGG AGGTCCCTGA GACTCTCCTG TGCAGCCTCT GGATTCACCT TTAGTAGGTA TTGGATGAGC TGGGTCCGCC 79 SEQ ID NO: 50
Conservation
Cell #1 AGGCTCCAGG GAAGGGGCTG GAGTGGTGGG CCAACATAAA GCAAGATGGA AGTGAGAAAT ACTATGTGGA CTCTGTGAAG GGCCGATTCA 198 SEQ ID NO: 46
Cell #2 AGGCTCCAGG GAAGGGCCTG GAGTGGTGGG CCAACATAAA GCAAGATGGA AGTGAGAAAT ACTATGTGGA CTCTGTGAAG GGCCGATTCA 198 SEQ ID NO: 47
Cell #3 AGGCTCCAGG GAAGGGGCTG GAGTGGTGGG CCAACATAAA GCAAGATGGA AGTGAGAAAT ACTATGTGGA CTCTGTGAAG GGCCGATTCA 178 SEQ ID NO: 48
Cell #4 AGGCTCCAGG GAAGGGGCTG GAGTGGTGGG CCAACATAAA GCAAGATGGA AGTGAGAAAT ACTATGTGGA CTCTGTGAAG GGCCGATTCA 198 SEQ ID NO: 49
Cell #5 AGGCTCCAGG GAAGGGGCTG GAGTGGGTGG CCAACATAAA GCAAGATGGA AGTGAGAAAT ACTATGTGGA CTCTGTGAAG GGCCGATTCA 169 SEQ ID NO: 50
Conservation
Cell #1 CCATCTCCAG AGACAACGCC AAGAACTCAT TGTATTTGCA AATGAACAGC CTGAGAGCCG AGGACACGGC TGTGTATTAT TGTGCGAGAC 278 SEQ ID NO: 46
Cell #2 CCATCTCCAG AGACAACGCC AAGAACTCAT TGTATTTGCA AATGAACAGC CTGAGAGCCG AGGACACGGC TGTGTATTAT TGTGGGAGAC 278 SEQ ID NO: 47
Cell #3 CCATCTCCAG AGACAACGCC AAGAACTCAT TGTATTTGCA AATGAACAGC CTGAGAGCCG AGGACACGGC TGTGTATTAT TGTGGGAGAC 258 SEQ ID NO: 48
Cell #4 CCATCTCCAG AGACAACGCC AAGAACTCAT TGTATTTGCA AATGAACAGC CTGAGAGCCG AGGACACGGC TGTGTATTAT TGTGGGAGAC 278 SEQ ID NO: 49
Cell #5 CCATCTCCAG AGACAACGCC AAGAACTCAT TGTATTTGCA AATGAACAGC CTGAGAGCCG AGGACACGGC TGTGTATTAT TGTGGGAGAC 259 SEQ ID NO: 50
Conservation
Cell #1 GATACAACGG TATGGACGTC TGGGGCCAAG GGACCACGGT CACCGCCTCC -TCAGGTAA 328 SEQ ID NO: 46
Cell #2 GATACAACGG TATGGACGTC TGGGGCCAAG GGACCACGGT CACCGCCTCC -TCAGGT-- 326 SEQ ID NO: 47
Cell #3 GATACAACGG TATGGACGTC TGGGGCCAAG GGACCACGGT CACCGCCTCC -TCAGGTA- 326 SEQ ID NO: 48
Cell #4 GATACAACGG TATGGACGTC TGGGGCCAAG GGACCACGGT CACCGCCTCC -TCAGGT-- 326 SEQ ID NO: 49
Cell #5 GATACAACGG TATGGACGTC TGGGGCCAAG GGACCACGGT CACCGCCTCC ATCAGGTAA 318 SEQ ID NO: 50
Conservation

FIGURE 6A

FR1-IMGT
CDR1-IMGT
FR2-IMGT
CDR2-IMGT
FR3-IMGT
CDR3-IMGT
Query_1
IGHV2-70*01
V 97.6% (246/252)
SEQ ID NO: 52
SEQ ID NO: 51
SEQ ID NO: 53

FIGURE 6B

Cell #1 AATTGTAAAA CGACGGCCAG TTCAAGGTTC AGCGGCAGTG GATCTGGGAC AGAGATCAGT CTCACCATCT GCAGCCTGTA GCCTGGTGAT 90 SEQ ID NO: 54
Cell #2 AATTGTAAAA CGACGGCCAG TTCAAGGTTC AGCGGCAGTG GATCTGGGAC AGAGATCAGT CTCACCATCT GCAGCCTGTA GCCTGGTGAT 90 SEQ ID NO: 55
Cell #3 --TTGTAAAA CGACGGCCAG TTCAAGGTTC AGCGGCAGTG GATCTGGGAC AGAGATCAGT CTCACCATCT GCAGCCTGTA GCCTGGTGAT 88 SEQ ID NO: 56
Cell #4 AATTGTAAAA CGACGGCCAG TTCAAGGTTC AGCGGCAGTG GATCTGGGAC AGAGATCAGT CTCACCATCT GCAGCCTGTA GCCTGGTGAT 90 SEQ ID NO: 57
Cell #5 --TTGTAAAA CGACGGCCAG TTCAAGGTTC AGCGGCAGTG GATCTGGGAC AGAGATCAGT CTCACCATCT GCAGCCTGTA GCCTGGTGAT 88 SEQ ID NO: 58
Conservation
Cell #1 TTTGCAACTT CTTACTGCCA CCATTATAAG ACTCACAGTT TTGGCCAGGG GACCAAGGTG GAGATCAAAC GTAAGGGTCA TAGCTGTTTC 180 SEQ ID NO: 54
Cell #2 TTTGCAACTT CTTACTGCCA CCAGTATAAG ACTCACAGTT TTGGCCAGGG GACCAAGGTG GAGATCAAAC GTAAGGGTCA TAGCTGTTTC 180 SEQ ID NO: 55
Cell #3 TTTGCAACTT CTTACTGCCA CCAGTATAAG ACTCACAGTT TTGGCCAGGG GACCAAGGTG GAGATCAAAC GTAAGGGTCA TAGCTGTTTC 178 SEQ ID NO: 56
Cell #4 TTTGCAACTT CTTACTGCCA CCAGTATAAG ACTCACAGTT TTGGCCAGGG GACCAAGGTG GAGATCAAAC GTAAGGGTCA TAGCTGTTTC 180 SEQ ID NO: 57
Cell #5 TT-GCAACTT CTTACTGCCA CCAGTATAAG ACTCACAGTT TTGGCCAGGG GACCAAGGTG GAGATCAAAC GTAAGGGTCA TAGCTGTTTC 177 SEQ ID NO: 58
Conservation
Cell #1 CTGA------ 194 SEQ ID NO: 54
Cell #2 CTGATGCTGA 190 SEQ ID NO: 55
Cell #3 CTGATCCT-- 186 SEQ ID NO: 56
Cell #4 CTGATC-TGA 189 SEQ ID NO: 57
Cell #5 CTGA-CCTGA 186 SEQ ID NO: 58
Conservation

FIGURE 7A

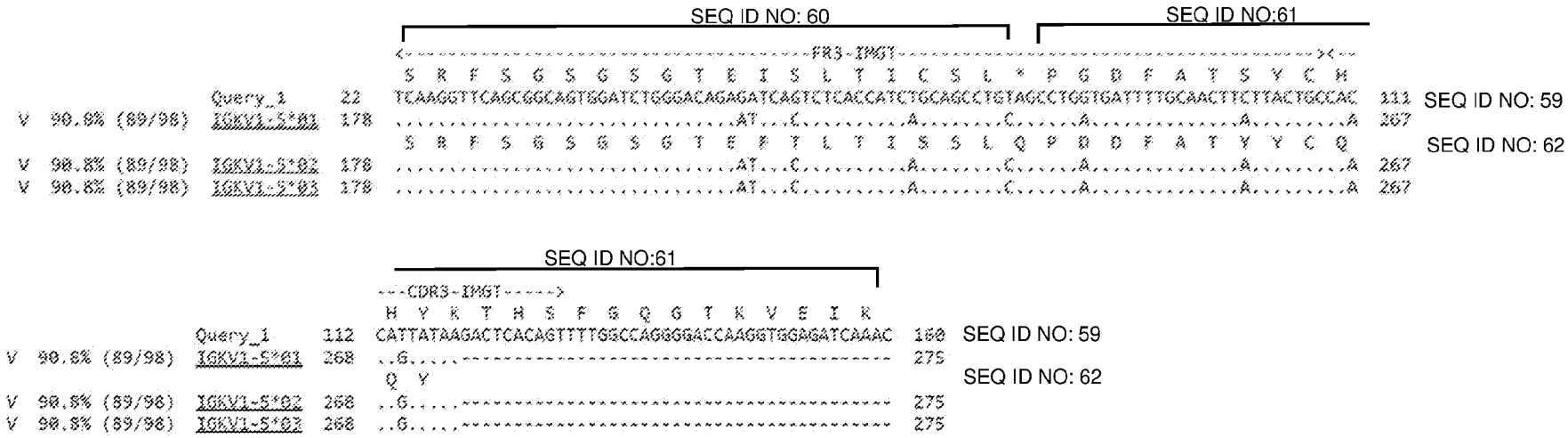
SEQ ID NO: 60
SEQ ID NO:61
<----------FR3-IMGT----------><--
S R F S G S G S G T E I S L T I C S L * P G D F A T S Y C H
Query_1 22 TCAAGGTTCAGCGGCAGTGGATCTGGGACAGAGATCAGTCTCACCATCTGCAGCCTGTAGCCTGGTGATTTTGCAACTTCTTACTGCCAC 111
V 90.8% (89/98) IGKV1-5*01 178 .....AT...C.....A.....C.....A.....A.....A 267
S R F S G S G S G T E F T L T I S S L Q P D D F A T Y Y C Q
V 90.8% (89/98) IGKV1-5*02 178 .....AT...C.....A.....C.....A.....A.....A 267
V 90.8% (89/98) IGKV1-5*03 178 .....AT...C.....A.....C.....A.....A.....A 267
SEQ ID NO:61
---CDR3-IMGT----->
H Y K T H S F G Q G T K V E I K
Query_1 112 CATTATAAGACTCACAGTTTTGGCCAGGGGACCAAGGTGGAGATCAAAC 160
V 90.8% (89/98) IGKV1-5*01 268 ..G.....~~~~~~ 275
Q Y
V 90.8% (89/98) IGKV1-5*02 268 ..G.....~~~~~~ 275
V 90.8% (89/98) IGKV1-5*03 268 ..G.....~~~~~~ 275
SEQ ID NO: 59
SEQ ID NO: 62
SEQ ID NO: 59
SEQ ID NO: 62

FIGURE 7B

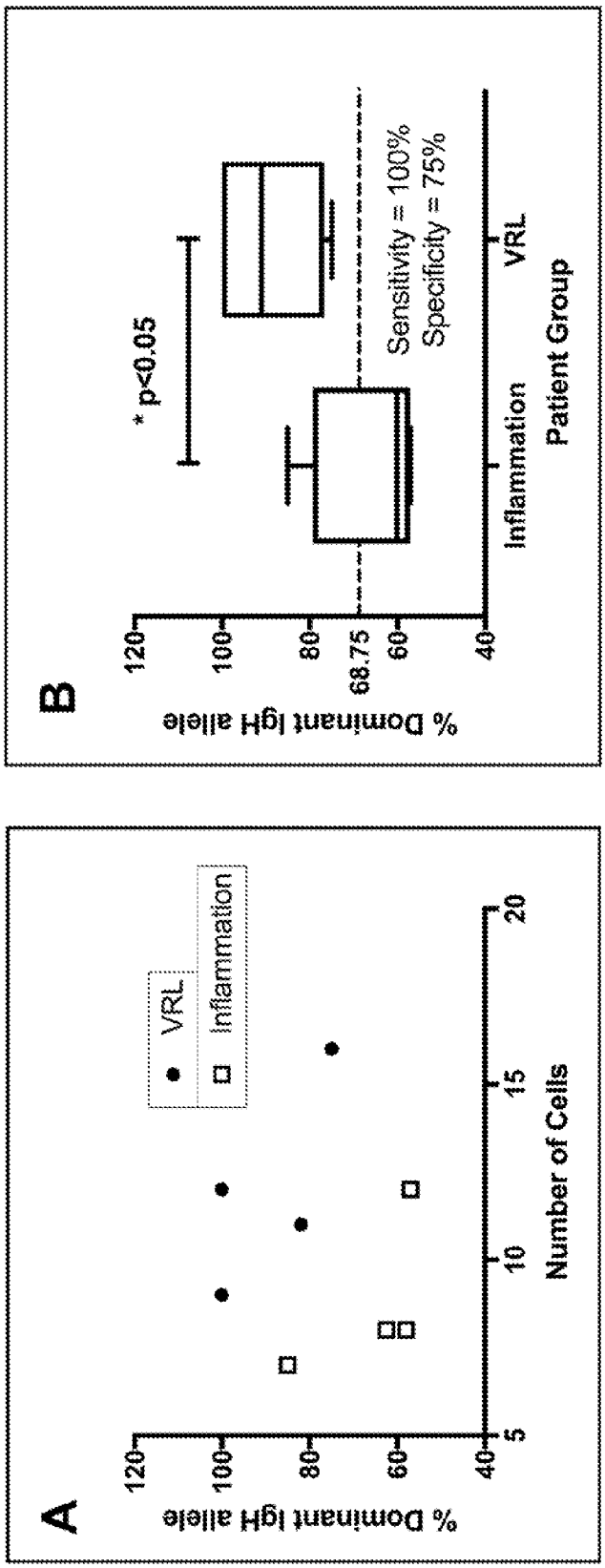
A
% Dominant IgH allele
120
100
80
60
40
5
10
15
20
Number of Cells
VRL
Inflammation
B
% Dominant IgH allele
120
100
80
68.75
60
40
* p<0.05
Sensitivity = 100%
Specificity = 75%
Inflammation
VRL
Patient Group

FIGURE 8

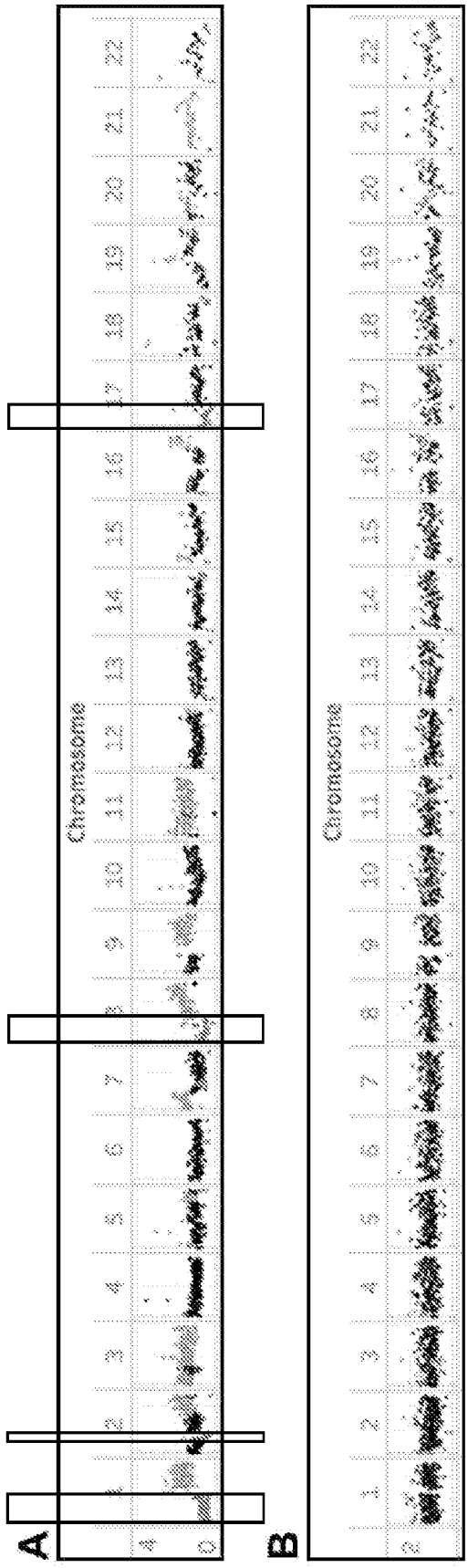

FIGURE 9A

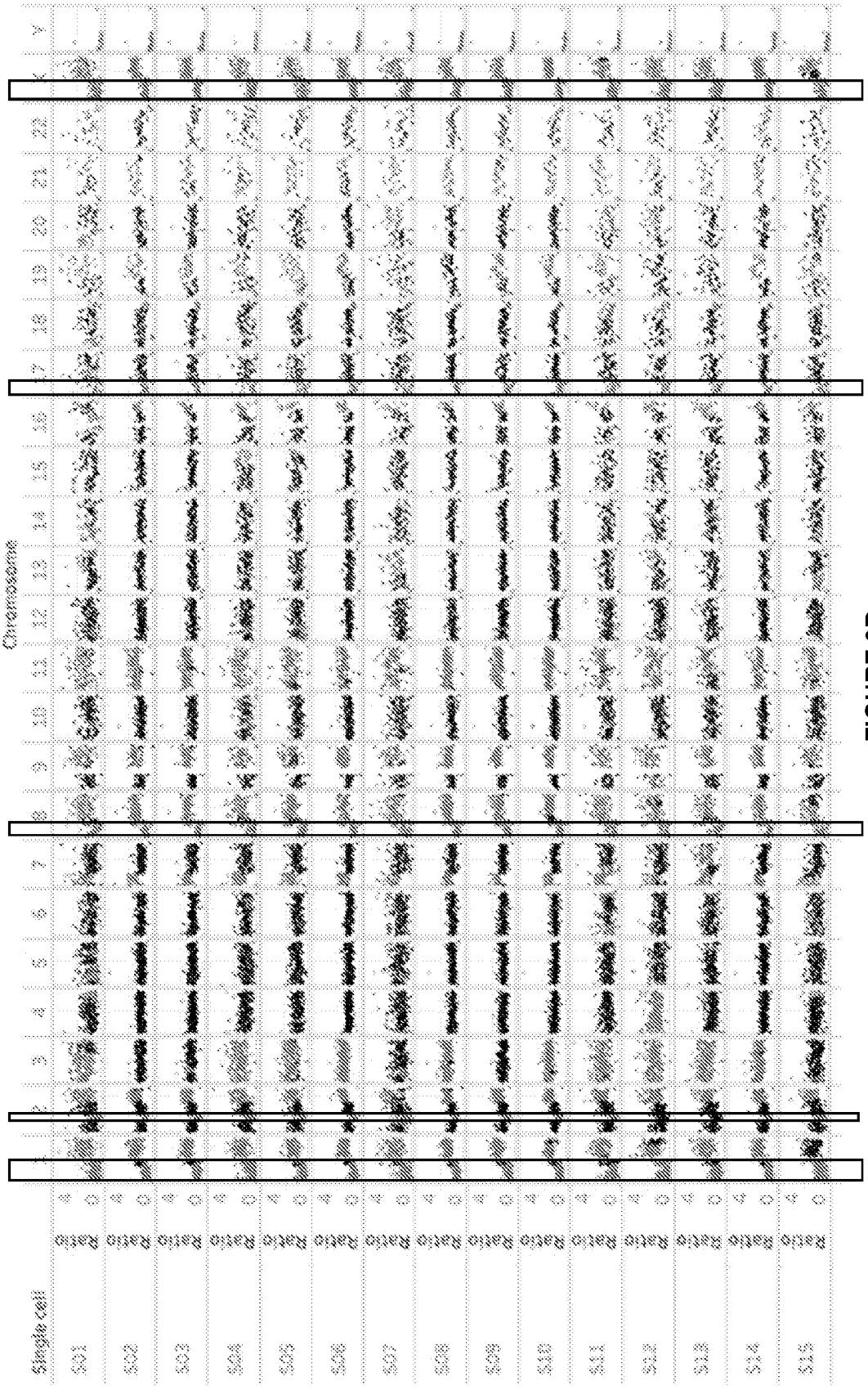
Chromosome
Single cell
S1 Ratio
S2 Ratio
S3 Ratio
S4 Ratio
S5 Ratio
S6 Ratio
S7 Ratio
S8 Ratio
S9 Ratio
S10 Ratio
S11 Ratio
S12 Ratio
S13 Ratio
S14 Ratio
S15 Ratio

FIGURE 9B

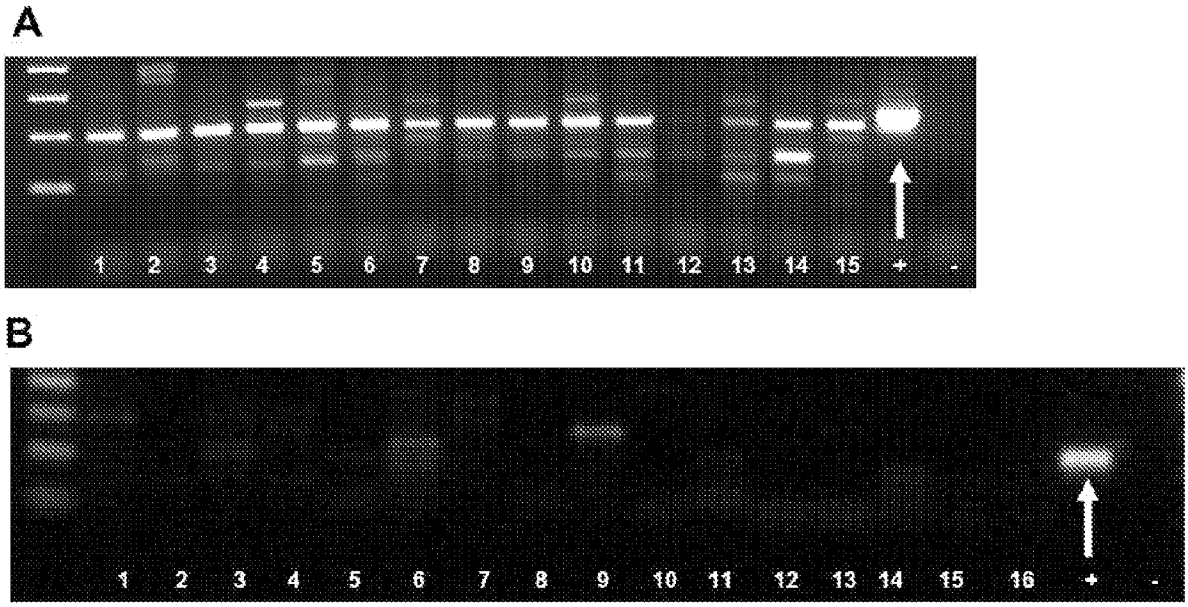

FIGURE 10

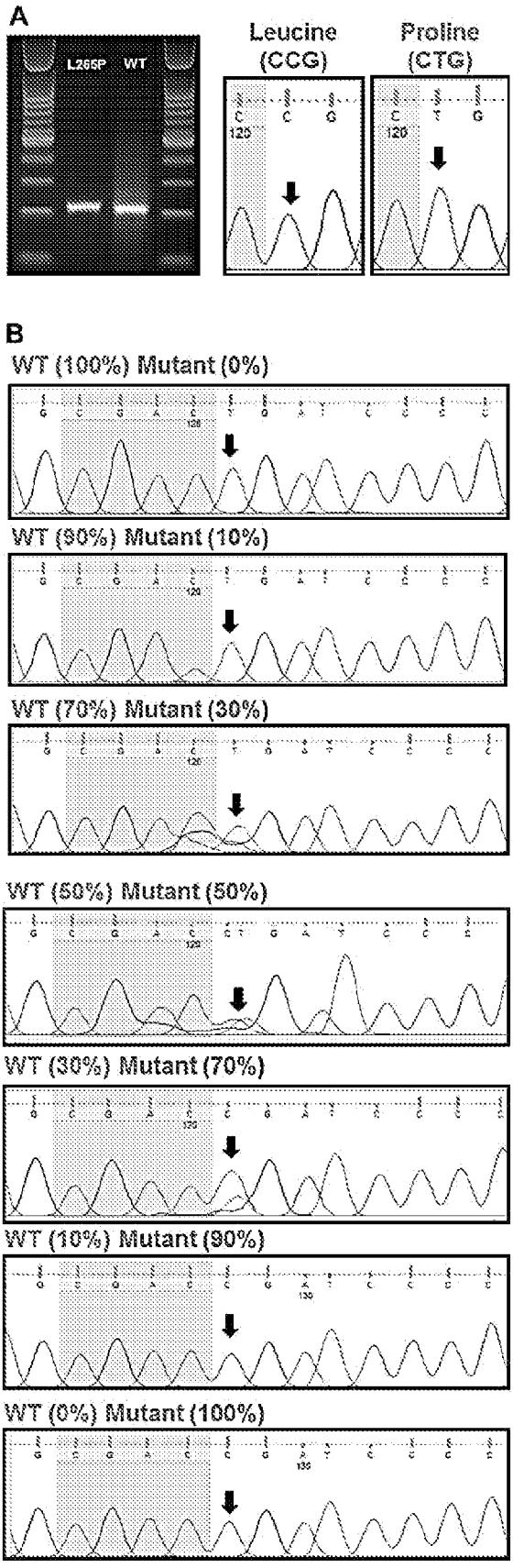
A
L265P
WT
Leucine
(CCG)
Proline
(CTG)
B
WT (100%) Mutant (0%)
WT (90%) Mutant (10%)
WT (70%) Mutant (30%)
WT (50%) Mutant (50%)
WT (30%) Mutant (70%)
WT (10%) Mutant (90%)
WT (0%) Mutant (100%)

FIGURE 11

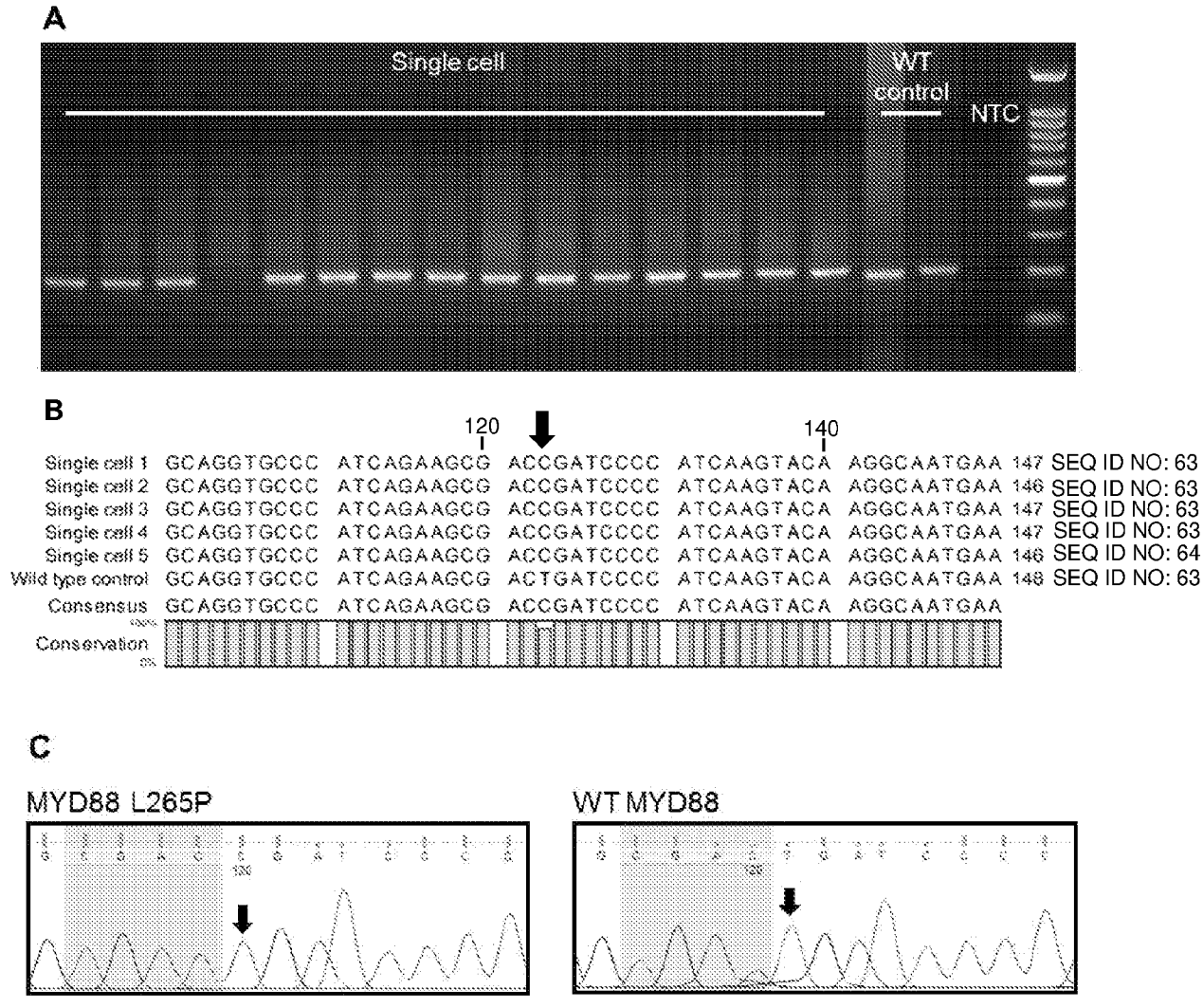
A
Single cell
WT control
NTC
B
120
140
Single cell 1 GCAGGTGCCC ATCAGAAGCG ACCGATCCCC ATCAAGTACA AGGCAATGAA 147 SEQ ID NO: 63
Single cell 2 GCAGGTGCCC ATCAGAAGCG ACCGATCCCC ATCAAGTACA AGGCAATGAA 146 SEQ ID NO: 63
Single cell 3 GCAGGTGCCC ATCAGAAGCG ACCGATCCCC ATCAAGTACA AGGCAATGAA 147 SEQ ID NO: 63
Single cell 4 GCAGGTGCCC ATCAGAAGCG ACCGATCCCC ATCAAGTACA AGGCAATGAA 147 SEQ ID NO: 63
Single cell 5 GCAGGTGCCC ATCAGAAGCG ACCGATCCCC ATCAAGTACA AGGCAATGAA 146 SEQ ID NO: 64
Wild type control GCAGGTGCCC ATCAGAAGCG ACTGATCCCC ATCAAGTACA AGGCAATGAA 148 SEQ ID NO: 63
Consensus GCAGGTGCCC ATCAGAAGCG ACCGATCCCC ATCAAGTACA AGGCAATGAA
Conservation
C
MYD88 L265P
WT MYD88

FIGURE 12

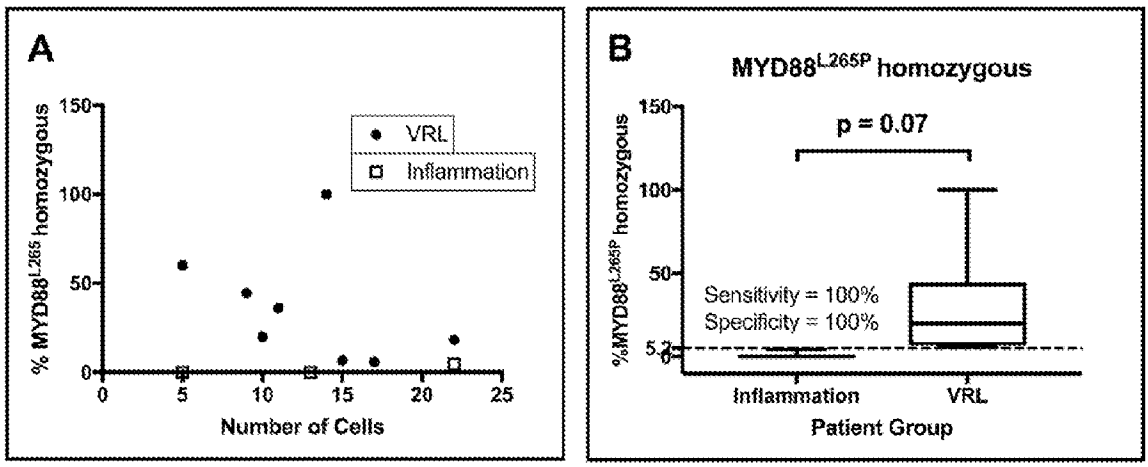
A
% MYD88L265 homozygous
150
100
50
0
0
5
10
15
20
25
Number of Cells
VRL
Inflammation
B
MYD88L265P homozygous
p = 0.07
%MYD88L265P homozygous
150
100
50
5.2
0
Sensitivity = 100%
Specificity = 100%
Inflammation
VRL
Patient Group

FIGURE 13

INTERNATIONAL SEARCH REPORT

International application No.
PCT/SG2019/050410

A. CLASSIFICATION OF SUBJECT MATTER

G01N 27/26 (2006.01) C12Q 1/686 (2018.01) G01N 33/48 (2006.01)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Databases: PATENW (All English Language databases), CAplus, BIOSIS, EMBASE, MEDLINE, Google, Google Scholar, Google Patents, Patentscope.

Keywords: Lymphoproliferative, lymphoma, leukaemia, leukemia, myeloma, vitreoretinal, B cell, dielectrophoresis, DEP, DEPArray, IgH, IgK, Clonality, clonal, copy number, single cell, MYD88, BCL2/JH, whole genome amplification, WGA, and similar keywords.

Applicant/Inventor Search: Menarini, Singapore Health Services, Wei Jian Tan, Meng Wang, Paola Ricciardi Castagnoli, Anita Sook Yee Chan, Tong Seng Lim; were searched in Google, Google Scholar, Google Patents and Patentscope

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
	Documents are listed in the continuation of Box C	

[X] Further documents are listed in the continuation of Box C [X] See patent family annex

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"D" document cited by the applicant in the international application

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
18 November 2019	18 November 2019
Name and mailing address of the ISA/AU	**Authorised officer**
AUSTRALIAN PATENT OFFICE PO BOX 200, WODEN ACT 2606, AUSTRALIA Email address: pct@ipaustralia.gov.au	Dr Brad Hoad AUSTRALIAN PATENT OFFICE (ISO 9001 Quality Certified Service) Telephone No. +61262832586

INTERNATIONAL SEARCH REPORT		International application No.
C (Continuation).	DOCUMENTS CONSIDERED TO BE RELEVANT	PCT/SG2019/050410

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Paoletti, C, et al.,'Comprehensive mutation and copy number profiling in archived circulating breast cancer tumor cells documents heterogeneous resistance mechanisms', 2018, Cancer research, Vol. 78, pages 1110-1122. Title; Methods, page 113, Single Cell Purification and Isolation, and CTC Genomic Profiling and Data Analysis.	1-20
Y	Peeters, D, et al., 'Semiautomated isolation and molecular characterisation of single or highly purified tumour cells from CellSearch enriched blood samples using dielectrophoretic cell sorting', 2013, British journal of cancer, Vol. 108, pages 1358-1367. Abstract; Materials and Methods; Figure 2.	1-20
Y	Carter, L, et al., 'Molecular analysis of circulating tumor cells identifies distinct copy-number profiles in patients with chemosensitive and chemorefractory small-cell lung cancer', 2017, Nature medicine, Vol 23, pages 1-6. Abstract; Page 1, last paragraph to page 2, 4th paragraph.	1-20
Y	Burbat, L, et al., 'Purification of Hodgkin and Reed-Sternberg cells from FFPE tissue sections using the DEPArray', Virchows Archiv, 2017, Vol. 471, Suppl 1, OFP-14-011, Conference Abstract. Abstract	1-20
Y	Avet-Loiseau, H, et al., 'Prognostic significance of copy-number alterations in multiple myeloma', Journal of Clinical Oncology, 2009, Vol. 27, pages 4585-4590. Figure 1; Tables 2 and 4.	1-20
Y	Delfau-Larue, M, et al., 'Total metabolic tumor volume, circulating tumor cells, cell-free DNA: distinct prognostic value in follicular lymphoma', 2018, Blood advances, Vol. 2, pages 807-816. Methods, Quantification of BCL2/JH translocated alleles in peripheral blood; Figure 4).	1-20
Y	Mahfouz, R, et al., 'Molecular frequency of BCL2/JH t (14; 18) using PCR among Lebanese patients with follicular lymphoma: another piece of the geographical map revealed', 2007, Molecular biology reports, Vol. 34, pages 271-274. Table 1.	1-20
Y	WO 2013/006443 A2 (DANA-FARBER CANCER INSTITUTE, INC.) 10 January 2013 Claims 1, 5	1-20
Y	Fernández-Rodríguez, C, et al., 'MYD88 (L265P) mutation is an independent prognostic factor for outcome in patients with diffuse large B-cell lymphoma', 2014, Leukemia, Vol. 28, pages 2104-2106. Whole document, particularly Figure 1.	1-20
Y	WO 2017/004599 A1 (NEOGENOMICS LABORATORIES, INC.) 05 January 2017 Abstract; Claims 1, 9-10; SEQ ID NOs: 1-2.	1-20
P,X	Raspadori, A, et al., 'A High-Throughput Workflow for the Detection, Isolation and Genomic Analysis of Single Circulating Multiple Myeloma Cells', 2018, Blood, The Journal of the American Society of Hematology, Vol. 132, Abstract only. Entire document	1-20
P,X	Tan, W, et al., 'Single-cell MYD88 sequencing of isolated B cells from vitreous biopsies aids vitreoretinal lymphoma diagnosis', 2019, Blood, The Journal of the American Society of Hematology, Vol. 134, pages 709-712. Entire document	1-20

INTERNATIONAL SEARCH REPORT C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		International application No. **PCT/SG2019/050410**
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.

INTERNATIONAL SEARCH REPORT Information on patent family members	International application No. **PCT/SG2019/050410**

This Annex lists known patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document/s Cited in Search Report		Patent Family Member/s	
Publication Number	**Publication Date**	**Publication Number**	**Publication Date**
WO 2013/006443 A2	10 January 2013	WO 2013006443 A2	10 Jan 2013
		CA 2840687 A1	10 Jan 2013
		EP 2726634 A2	07 May 2014
		EP 2726634 B1	22 Feb 2017
		US 2014249142 A1	04 Sep 2014
		US 10465247 B2	05 Nov 2019
WO 2017/004599 A1	05 January 2017	WO 2017004599 A1	05 Jan 2017
		US 2017002427 A1	05 Jan 2017
		US 10227657 B2	12 Mar 2019

End of Annex

Due to data integration issues this family listing may not include 10 digit Australian applications filed since May 2001.