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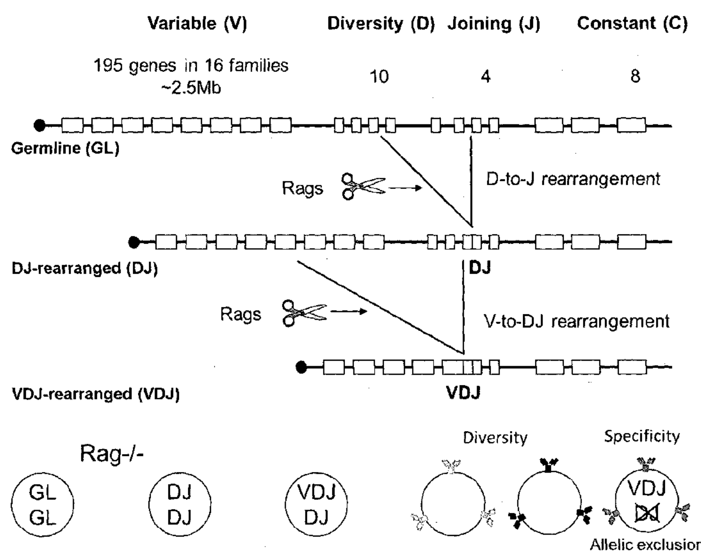


FIGURE 1

(57) **Abstract**: The invention relates to a method of identifying VDJ recombination products which comprises the use of sequence specific enrichment and specific restriction endonuclease enzymes or other DNA-shearing approaches to provide high resolution and high throughput interrogation of antigen receptor repertoires.

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# METHOD OF IDENTIFYING VDJ RECOMBINATION PRODUCTS

## FIELD OF THE INVENTION

The invention relates to a method of identifying VDJ recombination products which comprises the use of sequence specific enrichment and specific restriction endonuclease enzymes or other DNA-shearing approaches to provide high resolution and high throughput interrogation of antigen receptor repertoires.

## BACKGROUND OF THE INVENTION

VDJ recombination, also known as somatic recombination, is a mechanism of genetic recombination in the early stages of immunoglobulin (Ig) and T cell receptor (TCR) production of the immune system. VDJ recombination nearly-randomly combines Variable (V), Diverse (D) and Joining (J) gene segments of vertebrates, and because of its randomness in choosing different genes, is able to diversely encode proteins to match antigens from bacteria, viruses, parasites, dysfunctional cells such as tumor cells and pollens.

VDJ recombination of the mouse immunoglobulin heavy chain locus is pictorially shown in Figure 1. This is a large 3 Mb locus consisting of approximately 195 variable (V) genes, 10 diversity (D) genes and 4 joining (J) genes. These are the segments that participate in VDJ recombination. There are also 8 constant genes which, as their name suggests, do not undergo VDJ recombination. The first event in the VDJ recombination of this locus is that one of the D genes rearranges to one of the J genes. Following this, one of the V genes is appended to this DJ rearrangement to form the functional VDJ rearranged gene that then codes for the variable segment of the heavy chain protein. Both of these steps is catalysed by recombinase enzymes called Rags which delete out the intervening DNA. An analogous arrangement exists in the human genome, which instead comprises 95 variable (V) genes, 20 diversity (D) genes and 6 joining (J) genes.

This recombination process takes place in a stepwise fashion in progenitor B cells to produce the diversity required for the antibody repertoire but there is another requirement - that of specificity such that each B cell only produces one antibody. This specificity is fundamental for the function of the immune system

and is achieved by a process called allelic exclusion such that functional rearrangement of one allele signals via a currently unclear mechanism to prevent further recombination of the second allele.

5 The existing methodology uses PCR-based approaches to identify VDJ recombination products. This comprises pairs of primers, where one primer binds to one of the four J genes, common to all VDJ recombination products, or a sequence immediately downstream of a J gene, in combination with a primer or primers specific for the V gene component of the VDJ recombination product.

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There are a number of weaknesses with the existing methodology. For example, there are numerous V gene families (16 in the mouse Igh), and to ensure specificity of detection, different V gene primers must be designed for each family. This introduces a bias in quantitative comparative analysis, since 15 amplification of individual V gene families will depend on the relative efficiencies of the V gene primers designed for different V gene families, and differences in efficiency introduce inaccuracies in comparative analysis.

Even within V gene families, the V gene members have slightly different 20 sequences and thus, unless a primer can be designed that matches each V gene member sequence 100%, this will introduce bias in comparative amplification of V genes within a family. For larger V gene families, and thus the majority of V genes, it is virtually impossible to design a V gene primer that can detect all V family members. The only way to circumvent this is to design primers to 25 subsets of V genes within a family, but this introduces an additional bias again, due to different efficiency of amplification with different PCR primers. The combination of these two problems means that current methods cannot provide an unbiased and complete analysis of the VDJ recombination products in a sample.

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The current PCR-based methods also have a problem with scale. The usual step after PCR amplification is to clone and sequence the PCR products. As an example, there are almost 200 V genes in the mouse Igh. The most frequent aim is to determine how often these are used relative to each other in the

immunoglobulin repertoire. In order to detect each different V gene once, assuming they were recombined at equal efficiency and detected by PCR with equal efficiency (neither of which is the case), 200 clones would have to be sequenced. To actually determine relative usages of V genes in a population in 5 which they are used at frequencies that can differ by orders of magnitude, tens of thousands of clones would have to be generated and sequenced. This is currently prohibitive, both in terms of cost and labour.

Some attempts have been made to overcome the problem of scale by 10 incorporating next generation sequencing approaches into the methodology. Although several of these have been described recently, they all continue to use PCR primers for the V gene families as the starting point for detection of VDJ recombination products, and subsequently incorporate next generation sequencing as a method of 'cloning and sequencing' large numbers of PCR 15 products. Thus the inherent biases due to PCR primer efficiency remain.

There is therefore a great need to provide improved methods of identifying VDJ recombination products which overcome one or more of the aforementioned problems.

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**SUMMARY OF THE INVENTION**

According to a first aspect of the invention, there is provided a method of identifying a VDJ recombination product which comprises the following steps:

(a) obtaining a nucleic acid sample comprising a VDJ recombination 25 product;

(b) when said nucleic acid sample comprises DNA, fragmenting the VDJ recombination product either by sonication, shearing or performing a restriction endonuclease reaction at a first site downstream of each of the J genes or downstream of the constant region, and a second site within or immediately 30 upstream of the V gene to generate digested VDJ recombined fragments and unrecombined J fragments;

(c) when said nucleic acid sample comprises DNA, annealing oligonucleotides to the digested fragments at unique regions within or immediately downstream of each of the J genes, and when the nucleic acid

sample is cDNA or RNA, annealing oligonucleotides to the VDJ recombination product at a position specific to the constant region or J gene;

(d) when said nucleic acid sample comprises DNA, separating the digested VDJ recombined fragments from the unrecombined J fragments and the rest of the genome;

(e) sequencing the VDJ recombined fragments or products; and

(f) data processing of the sequencing data to identify each VDJ recombination product and quantify VDJ recombination frequencies.

According to a further aspect of the invention, there is provided a kit for identifying VDJ recombination products which comprises instructions to use said kit in accordance with the methods described herein.

According to a further aspect of the invention, there is provided a VDJ recombination product obtainable by the method as described herein.

According to a further aspect of the invention, there is provided a VDJ recombination product or a method of identifying a VDJ recombination product as described herein for use in monitoring an immunodeficiency disorder.

## BRIEF DESCRIPTION OF THE FIGURES

**Figures 1 and 2** provide an overview of the concept of VDJ recombination for the generation of antibody diversity.

**Figures 3-5** provide schematic overviews of alternative embodiments of the method of the invention.

**Figure 6** provides a schematic demonstrating the high resolution comparative frequency of recombination of V genes throughout the mouse Igh V region.

**Figure 7** provides a detailed picture of usage of each one of 195 individual V genes analysed by the method of the invention in accordance with the methodology described in Example 1.

**Figure 8** provides a detailed picture of the recombination frequency for Example 2 with sonication in Mouse proB cells and Igh; B1, B2, B3 are replicate samples; J4 indicates VDJ recombined sequences including J4.

## DETAILED DESCRIPTION OF THE INVENTION

According to a first aspect of the invention, there is provided a method of identifying a VDJ recombination product which comprises the following steps:

(a) obtaining a nucleic acid sample comprising a VDJ recombination product;

(b) when said nucleic acid sample comprises DNA, fragmenting the VDJ recombination product either by sonication, shearing or performing a restriction endonuclease reaction at a first site downstream of each of the J genes or downstream of the constant region, and a second site within or immediately upstream of the V gene to generate digested VDJ recombined fragments and unrecombined J fragments;

(c) when said nucleic acid sample comprises DNA, annealing oligonucleotides to the digested fragments at unique regions within or immediately downstream of each of the J genes, and when the nucleic acid sample is cDNA or RNA, annealing oligonucleotides to the VDJ recombination product at a position specific to the constant region or J gene;

(d) when said nucleic acid sample comprises DNA, separating the digested VDJ recombined fragments from the unrecombined J fragments and the rest of the genome;

(e) sequencing the VDJ recombined fragments or products; and

(f) data processing of the sequencing data to identify each VDJ recombination product and quantify VDJ recombination frequencies.

According to a second aspect of the invention, there is provided a method of identifying a VDJ recombination product which comprises the following steps:

(a) obtaining a nucleic acid sample comprising a VDJ recombination product;

(b) annealing a primer specific for a unique region within or immediately downstream of each of the J genes if the nucleic acid is a DNA

sample or a primer specific for the constant region if the nucleic acid is a cDNA sample;

(c) performing a primer extension reaction upon the primer annealed in step (b);

5 (d) fragmenting the DNA either by sonication or shearing or performing a restriction endonuclease reaction at a first site downstream of each of the J genes or downstream of the constant region primer and a second site within or immediately upstream of the V gene to generate recombined VDJ digested fragments and unrecombined J fragments;

10 (e) separating the recombined VDJ digested fragments from the unrecombined J fragments and the rest of the genome;

(f) sequencing the recombined VDJ digested fragments; and

(g) data processing of the sequencing data to identify each VDJ recombination product and quantify VDJ recombination frequencies.

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The identification method of the invention provides a number of significant advantages over conventional techniques employed to determine the nature of a repertoire. For example, the method of the invention offers a high resolution, high throughput interrogation of antigen receptor repertoires.

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It will be appreciated that the invention finds great utility in the interrogation of antigen receptor repertoires in mouse immunoglobulin heavy chain IgH and Igκ and human heavy chain IgH, as described in the experimental procedure and data presented herein. However, the method has applicability to other
25 repertoires and is also adaptable for other antigen receptor repertoires and other species, such as human, rabbit, rat and the like. The invention provides enrichment of VDJ and DJ recombined sequences along with the generation of large unbiased libraries of recombined sequences. The method of the invention allows the unambiguous identification of individual recombination events and
30 complete details of V, D, J gene usage, N nucleotide addition and the like. The invention also has surprisingly high resolution and in particular has been able to identify a previously unknown recombination mechanism and chromosomal translocations. More particularly, the method of the invention offers advantages with respect to scalability. The method may be performed in a high throughput

manner and as shown in the Examples described herein, approximately 100,000 unique VDJ recombinations were captured and identified which represents a 1000 fold increase over existing methods.

5 In one embodiment, the nucleic acid sample comprising a VDJ recombination product comprises a cell population. It will be appreciated that the cell population may be obtained from any species or organism containing transgenic antigen receptor loci. In one embodiment, the cell population is obtained from a mouse, such as a transgenic mouse. In an alternative embodiment, the cell 10 population is obtained from a human.

It will be appreciated that the cell population will comprise any suitable population of cells which would contain a VDJ recombination product. In one embodiment, the cell population comprises an immunoglobulin containing cell. In 15 a further embodiment, the immunoglobulin containing cell comprises a white blood cell. In a yet further embodiment, the white blood cell comprises a lymphocyte, such as a small lymphocyte. In a yet further embodiment, the small lymphocyte comprises a T cell or a B cell, such as a B cell.

20 In one embodiment, the VDJ recombination product is derived from a heavy chain immunoglobulin. In the developing B cell, the first recombination event to occur is between one D and one J gene segment of the heavy chain locus. Any DNA between these two genes is deleted. This D-J recombination is followed by the joining of one V gene, from a region upstream of the newly formed DJ 25 complex, forming a rearranged VDJ gene. All other genes between V and D segments of the new VDJ gene are now deleted from the cell's genome. Primary transcript (unspliced RNA) is generated containing the VDJ region of the heavy chain and both the constant *mu* and *delta* chains ($C_\mu$ and $C_\delta$). (i.e. the primary transcript contains the segments: V-D-J-$C_\mu$-$C_\delta$). The primary RNA is processed to 30 add a polyadenylated (poly-A) tail after the $C_\mu$ chain and to remove the sequence between the VDJ segment and this constant gene segment. Translation of this mRNA leads to the production of the Ig μ heavy chain protein.

In an alternative embodiment, the VDJ recombination product is derived from a light chain immunoglobulin. The kappa (κ) and lambda (λ) chains of the immunoglobulin light chain loci rearrange in a very similar manner to the heavy chain locus, except the light chains lack a D segment. For example, the first step of recombination for the light chains involves the joining of the V and J chains to give a VJ complex before the addition of the constant chain gene during primary transcription. Translation of the spliced mRNA for either the kappa or lambda chains results in formation of the Ig κ or Ig λ light chain protein. Assembly of the Ig μ heavy chain and one of the light chains results in the formation of membrane bound form of the immunoglobulin IgM that is expressed on the surface of the immature B cell.

In an alternative embodiment, the cell population comprises a T cell. In one embodiment, the VDJ recombination product is derived from a T cell receptor. During thymocyte development, the T cell receptor (TCR) chains undergo essentially the same sequence of ordered recombination events as that described for immunoglobulins. D-to-J recombination occurs first in the β chain of the TCR. This process can involve either the joining of the $D_{\beta}1$ gene segment to one of six $J_{\beta}1$ segments or the joining of the $D_{\beta}2$ gene segment to one of seven $J_{\beta}2$ segments. DJ recombination is followed (as described above) with $V_{\beta}$-to-$D_{\beta}J_{\beta}$ rearrangements. All genes between the $V_{\beta}$-$D_{\beta}$-$J_{\beta}$ genes in the newly formed complex are deleted and the primary transcript is synthesized that incorporates the constant domain gene ($V_{\beta}$-$D_{\beta}$-$J_{\beta}$-$C_{\beta}$). mRNA transcription splices out any intervening sequence and allows translation of the full length protein for the TCR $C_{\beta}$ chain.

The rearrangement of the alpha (α) chain of the TCR follows β chain rearrangement, and resembles V-to-J rearrangement described for Ig light chains (as described above). The assembly of the β- and α- chains results in formation of the αβ-TCR that is expressed on a majority of T cells.

T cell receptor (TCR) chains may also comprise gamma (γ) and delta (δ) chains which assemble to form γδ-TCR.

In an alternative embodiment, the nucleic acid sample comprising a VDJ recombination product comprises a library of VDJ recombined nucleic acids obtained from an *in vitro* antibody production system, such as ribosome display.

5 References herein to the term "identifying" refer to any step which enables one member of a repertoire of antigens to be differentially identified from that of another member of a repertoire of antigens.

References herein to the term "VDJ recombination product" refer to the product
10 of a recombination event between a variable (V), diversity (D) and joining (J) gene.

In one embodiment, the nucleic acid comprises DNA or RNA obtained from the sample. In a further embodiment, the nucleic acid comprises cDNA or mRNA. In
15 a yet further embodiment, the nucleic acid comprises cDNA. When the sample comprises a cell population, it will be appreciated that the process of obtaining nucleic acid from the cell population is readily apparent to the skilled person in accordance with standard molecular biology techniques.

20 In one embodiment, the nucleic acid sample is DNA. Thus, according to a further aspect of the invention, there is provided a method of identifying a VDJ recombination product which comprises the following steps:

(a) obtaining a DNA sample comprising a VDJ recombination product;

(b) fragmenting the VDJ recombination product either by sonication,
25 shearing or performing a restriction endonuclease reaction at a first site downstream of each of the J genes or downstream of the constant region, and a second site within or immediately upstream of the V gene to generate digested VDJ recombined fragments and unrecombined J fragments;

(c) annealing oligonucleotides to the digested fragments at unique
30 regions within or immediately downstream of each of the J genes;

(d) separating the digested VDJ recombined fragments from the unrecombined J fragments and the rest of the genome;

(e) sequencing the VDJ recombined fragments; and

(f) data processing of the sequencing data to identify each VDJ recombination product and quantify VDJ recombination frequencies.

It will be appreciated that when the nucleic acid sample comprises cDNA or RNA (i.e. mRNA), steps (b) and (d) of the first aspect of the invention may be omitted. Thus, according to a further aspect of the invention, there is provided a method of identifying a VDJ recombination product which comprises the following steps:

(a) obtaining a cDNA or RNA sample comprising a VDJ recombination product;

(c) annealing oligonucleotides to the VDJ recombination product at a position specific to the constant region or J gene;

(e) sequencing the VDJ recombined products; and

(f) data processing of the sequencing data to identify each VDJ recombination product and quantify VDJ recombination frequencies.

In one embodiment of the first aspect of the invention, the oligonucleotide (*e.g.* a primer or *in vitro* transcribed RNA) used in step (c) is specific for a unique region within each of the J genes. In an alternative embodiment, the oligonucleotide used in step (c) is specific for a region immediately downstream of each of the J genes. It is beneficial for the oligonucleotide to be either within or very close to the J segment to prevent an excessive amount of bases being present when the final product is sequenced.

In one embodiment of the second aspect of the invention, the primer used in step (b) is specific for a unique region within each of the J genes. In an alternative embodiment, the primer used in step (b) is specific for a region immediately downstream of each of the J genes. It is beneficial for the primer to be either within or very close to the J segment to prevent an excessive amount of bases being present when the final product is sequenced.

It will be understood that references herein to the term "unique" refer to a sequence which is not present within the remainder of the genome. This

arrangement prevents other sequences being incorrectly identified by the method of the invention.

It will be appreciated that when the cell population comprises human cells, 6 different primers will be required for each of the 6 different human J genes. In the embodiment where the cell population comprises human cells, the primers are selected from any of the primers described in SEQ ID NOS: 51 to 56. In an alternative embodiment where the cell population comprises human cells, the 6 primers may be selected from other sequences within the six human J genes.

It will also be appreciated that when the cell population comprises mouse cells, 4 different primers will be required for each of the 4 different mouse J genes. In the embodiment where the cell population comprises mouse cells, the 4 primers may be selected from any of the primers described in SEQ ID NOS: 1 to 4. In an additional embodiment where the cell population comprises mouse cells, the 4 primers may be selected from any of the primers described in SEQ ID NOS: 47 to 50. In a further embodiment, where the cell population comprises mouse cells, the 4 primers may be selected from any of the primers described in SEQ ID NOS: 1 to 4. In an alternative embodiment where the cell population comprises mouse cells, the 4 primers may be selected from other sequences within the four mouse J genes.

In an alternative embodiment, when cDNA is used as a sample nucleic acid material, a single primer is used (*e.g.* in step (c) of the first aspect or in step (b) of the second aspect of the invention) which is specific for a region within an exon (*i.e.* any specific exon) of the constant region and thus all successfully VDJ recombined (and transcribed) products are captured regardless of J gene usage.

In a further alternative embodiment, when cDNA is used as a sample nucleic acid material, a single primer is used (e.g. in step (c) of the first aspect or in step (b) of the second aspect of the invention) which is specific for a region within the first exon of the constant region and thus all successfully VDJ recombined (and transcribed) products are captured regardless of J gene usage.

In one embodiment, step (c) of the first aspect of the invention comprises tagging the digested fragments with one member of a binding pair. In a particular embodiment when primers are used, the primer additionally comprises one member of a binding pair. This embodiment provides the advantage of allowing enrichment of the target J sequences over other sequences. Examples of suitable binding pairs include biotin and streptavidin or an antigen and an antibody. In one embodiment, the tag or primer additionally comprises biotin or streptavidin, such as biotin. It will be appreciated that enrichment of a biotinylated fragment will require a streptavidin containing moiety, such as a magnetic bead containing streptavidin, for example Dynabeads from the kilobaseBINDER kit (Invitrogen).

In one embodiment, step (c) of the first aspect of the invention additionally comprises the step of performing primer extension, oligonucleotide hybridization and/or reverse transcription.

It will be appreciated that the primer extension, oligonucleotide hybridization and reverse transcription techniques required in step (c) of the first aspect of the invention are readily apparent to the skilled person in accordance with standard molecular biology techniques.

In one embodiment, the nucleic acid sample is fragmented by sonication.

In an alternative embodiment, the nucleic acid sample is fragmented by performing a restriction endonuclease reaction.

In one embodiment, the restriction endonuclease enzyme is selected from *DpnII* and/or *NlaIII*.

In a particular embodiment, where the cell population comprises mouse cells and the IgH locus is being targeted, the restriction endonuclease enzyme used is selected from *DpnII* and/or *NlaIII*.

Restriction endonucleases are selected upon the following criteria: 4bp cutters are preferred to generate fragment sizes suitable for enrichment by magnetic beads. The restriction endonucleases must not cut anywhere within the J gene, or constant region, or 5' of the primers used in step (b) of the second aspect of the invention. Restriction endonucleases that generate overhangs are preferred because sticky-end ligation is more efficient than blunt-ended ligation. In human cells *DpnII* and/or *NlaIII* are also suitable for targeting the Igh locus. However there is a DpnII cut site within J2 leaving just 16bp in which to locate a primer.

In one embodiment, the method comprises the use of a third restriction endonuclease enzyme. When present, the third restriction endonuclease enzyme will be specific for a region upstream of the V gene.

It will be appreciated that the sequencing method described herein comprises an Illumina sequencing method, such as the Illumina sequencing by synthesis (SBS) technology which is available from Illumina. When the Illumina sequencing technology is used, the procedure will typically comprise addition of adapter molecules to each end of the VDJ recombination product, e.g. following restriction endonuclease treatment in step (d) of the second aspect of the invention. Thus, in one embodiment the method additionally comprises the addition of a first adapter molecule to one end of the VDJ recombination product. In a particular embodiment, the method additionally comprises the addition of a first adapter molecule to one end of the VDJ recombination product following restriction endonuclease treatment. In a further embodiment, the first adapter is added to the VDJ recombination product by ligation. It will be appreciated that the adapter molecule will be selected depending upon the restriction endonuclease used. For example, when the restriction endonuclease used is *NlaIII*, the adapter molecule will comprise a complementary paired end with *NlaIII*. In one embodiment, when the restriction endonuclease used is *NlaIII*, the adapter molecule may be selected from SEQ ID NO: 5. In an alternative embodiment, when the restriction endonuclease used is *DpnII*, the adapter molecule may be selected from SEQ ID NO: 6.

In one embodiment, when sonication is used to fragment the DNA, the T-overhang adapter is ligated to the sonicated DNA following end repair and A-tailing. In a further embodiment, when sonication is used to fragment the DNA, the adapter molecule may be selected from SEQ ID NOS: 22 and 23.

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In a further embodiment the method additionally comprises the addition of a second adapter molecule to a second end of the VDJ recombination product. In a further embodiment, the second adapter is incorporated into the VDJ recombination product by PCR. When the cell population comprises mouse cells,
10 the primers for the addition of the second adaptor may be selected from SEQ ID NOS: 7 to 11 or 57 to 65. In a further embodiment, when the cell population comprises mouse cells, the primers for the addition of the second adaptor may be selected from SEQ ID NOS: 7 to 11.

15 In an alternative embodiment, when the cell population comprises human cells and the primers for the addition of the second adaptor are selected from SEQ ID NOS: 66 to 72.

The advantage of incorporating the second adapter by PCR is that the resultant
20 amplified products will be free of the binding pair complexes if used for enrichment of the recombined VDJ products.

Step (d) of the first aspect of the invention (or step (e) of the second aspect of the invention) typically comprises the separation, or depletion, of the
25 recombined VDJ digested fragments from the unrecombined J fragments by any suitable molecular biology depletion means. For example, the methodology described herein relates to primer extension (performed in an analogous manner to that described for step (c) of the second aspect of the invention) of oligonucleotides designed to portions present on the unrecombined J fragment
30 but not on the recombined VDJ fragments. For example, in one embodiment step (d) of the first aspect of the invention (or step (e) of the second aspect of the invention) comprises the use of oligonucleotides specific to the upstream region of each of the 4 or 6 J genes on both strands. In a further embodiment, when the cell population comprises mouse cells, the oligonucleotides used in step (d)

of the first aspect of the invention (or step (e) of the second aspect of the invention) may be selected from SEQ ID NOS: 12 to 19 or 24 to 34. In a yet further embodiment, when the cell population comprises mouse cells, the oligonucleotides used in step (d) of the first aspect of the invention (or step (e)
5 of the second aspect of the invention) may be selected from SEQ ID NOS: 12 to 19.

In an alternative embodiment, when the cell population comprises human cells and the oligonucleotides used in step (d) of the first aspect of the invention (or
10 step (e) of the second aspect of the invention) are selected from SEQ ID NOS: 35 to 46.

As previously described herein, in one embodiment, the primer additionally comprises one member of a binding pair. This embodiment provides the
15 advantage of allowing depletion of the unrecombined J fragments over the recombined VDJ sequences. Examples of suitable binding pairs include biotin and streptavidin or an antigen and an antibody. In one embodiment, the primer additionally comprises biotin or streptavidin, such as biotin. It will be appreciated that depletion of a biotinylated fragment will require a streptavidin containing
20 moiety, such as a magnetic bead containing streptavidin, for example streptavidin coupled Dynabeads.

Once the depletion step is complete, the resultant VDJ recombination products may then be sequenced. It will be appreciated that any sequencing analysis
25 procedure may typically be used. In one embodiment, the sequencing step comprises standard Illumina 36bp paired end sequencing. In an alternative embodiment, the sequencing step comprises Illumina MiSeq 250bp single end sequencing.

30 When the Illumina sequencing technology is used, the procedure will typically require a sticky-end PCR reaction prior to sequence analysis in order to ensure sufficient binding to the flow cell. Such sticky-end PCR reactions may be conducted in accordance with the manufacturers protocol. When the method of

the invention comprises sticky-end PCR, the primers may be selected from SEQ ID NOS: 20 and 21.

Alternatively, sequencing may require the addition of paired end adapters, *e.g.* for paired-end sequencing. These adapters may be used to attach to an Illumina flow cell. When the method of the invention comprises the use of paired-end sequencing, the primers may be selected from SEQ ID NOS: 73 to 85 or 98 to 106.

Data may be processed in accordance with four main steps. Firstly the paired end reads are classified according to J segment identity and restriction endonuclease (if used for DNA fragmentation). Secondly the classified reads are filtered to identify unique recombination events and to exclude unrecombined reads and PCR duplicates. Thirdly the reads are mapped to either the genome or to a virtual array of known V, D and J segment sequences to identify the recombined V or D segments in each read. Fourthly the number of reads assigned to each V or D segment are quantified and displayed as a bar chart similar to Figure 7 or Figure 8.

According to a further aspect of the invention, there is provided a kit for identifying VDJ recombination products which comprises instructions to use said kit in accordance with the methods described herein.

In one embodiment, the kit additionally comprises nucleic acid extraction reagents configured to obtain the nucleic acid sample required for step (a) of the method of the invention.

In one embodiment, the kit additionally comprises oligonucleotides or primers specific for a unique region within or immediately downstream of each of the J genes or within the constant region as defined herein. In a further embodiment, the kit additionally comprises oligonucleotides or primers specific for a unique region within or immediately downstream of each of the J genes as defined herein.

In one embodiment, the kit additionally comprises primer extension reagents configured to perform the primer extension process of step (c) of the second aspect of the invention.

5 In one embodiment, the kit additionally comprises one or more restriction endonuclease enzymes as defined herein.

In one embodiment, the kit additionally comprises a computer readable storage medium configured to process the sequencing data obtained and generate a 10 visual representation of VDJ recombination frequencies in the sample.

Due to the unbiased nature of the assay any DNA sequence that has been recombined with a J segment can be identified. Several V genes have been found to be recombined by inversion of the intervening DNA sequence rather 15 than the typical deletion. VDJ recombination by inversion generates non-functional VD recombined products, however these Igh specific products have not been described in the literature before. Similarly the assay is also able to detect translocations, where aberrant recombination has taken place between a J gene and another region of the genome. Therefore the assay has the potential 20 to identify common Igh translocation partners. Thus, according to a further aspect of the invention, there is provided a VDJ recombination product obtainable by the method as described herein.

It will be appreciated that analysis of the VDJ repertoire of an individual as 25 described herein can be used to monitor immunodeficiency disorders. According to a further aspect of the invention, there is provided a VDJ recombination product or a method of identifying a VDJ recombination product as described herein for use in monitoring an immunodeficiency disorder. In one embodiment, the immunodeficiency disorder is selected from a lymphoma or leukemia. In a 30 further embodiment, the immunodeficiency disorder is selected from a clinically defined immunodeficiency disorder. In a yet further embodiment, the immunodeficiency disorder is selected from an ageing-related impairment of immune function.

By monitoring VDJ recombination products in accordance with methods of the invention, patient care can be tailored precisely to match the needs determined by the disorder and the pharmacogenomic profile of the patient.

5 The invention will now be described in more detail with reference to the following non-limiting Examples:

**Example 1: VDJ Recombination Product Identification Method in Mouse B Cells using restriction endonucleases**

10

***(A) Methods***

**(i) Primer extension using biotinylated J segment-specific oligonucleotides**

~ 7.5 µg of DNA was isolated from ~ 2 x $10^6$ FACS sorted fraction B/C B cells
15 and was divided into 8 aliquots to give ~ 1 µg per reaction. Primer extension reactions were assembled in 50 µl volumes using 2U of Vent (exo-) DNA polymerase (NEB) in the supplied 1x reaction buffer and 200 µm of dNTPs. A cocktail of 4 biotinylated oligonucleotides were used in the reaction and they were designed to anneal to each of the four J segments in the *Igh* locus:

20

*J segment biotinylated oligonucleotides*

J1 Rev Bio *-AGCCAGCTTACCTGAGGAGAC (SEQ ID NO: 1)

J2 Rev Bio *-GAGAGGTTGTAAGGACTCACCTG (SEQ ID NO: 2)

J3 Rev Bio *-AGTTAGGACTCACCTGCAGAGAC (SEQ ID NO: 3)

25 J4 Rev Bio *-AGGCCATTCTTACCTGAGGAG (SEQ ID NO: 4)

wherein * refers to the biotin moiety

The primer extension reaction was denatured at 94°C for 4 mins, annealed at 60 °C for 2 mins, and extended at 72 °C for 10 mins, then chilled on ice. The
30 primer extension reactions were combined and magnetically purified using 720 µl of Agencourt AMPure XP SPRI beads (Beckman Coulter) according to the manufacturers protocol, and eluted into 208 µl of water.

**(ii) Restriction endonuclease digestion**

The sample was mixed thoroughly and divided into 2 tubes, 1.2 µl of BSA, 12 µl of the appropriate NEB digestion buffer, and 20 units of either *DpnII* or *NlaIII* (NEB) restriction endonucleases were added to each tube. The samples were incubated for 3 hours at 37°C on a Thermomixer (Eppendorf) at 1200rpm.
5 Following digestion the reactions were purified using 216 µl of SPRI beads and eluted into 50 µl of water.

**(iii) Enrichment of VDJ recombined DNA**

The biotinylated VDJ recombined DNA fragments were enriched using
10 streptavidin coupled Dynabeads from the kilobaseBINDER kit (Invitrogen). 10 µl aliquots of Dynabeads were prepared according to the manufacturers protocol, to which the 50µl DNA samples were added and incubated overnight at 20 °C on a Thermomixer at 1200rpm.

15 The samples were placed on a magnet for 5 mins and the supernatant was removed and discarded. The pellet was washed twice in 100 µl of the kilobaseBINDER kit wash buffer, and washed once in 100 µl of 1x NEB digestion buffers appropriate to either *DpnII* or *NlaIII.* The pellets were resuspended in 1x NEB digestion buffer and 0.5 µl of either *DpnII* or *NlaIII* was added and
20 incubated at 37 °C for an hour to ensure complete digestion.

**(iv) Incorporation of a first Illumina adapter by ligation**

The digestion reactions were cleaned up by removal of the supernatant on a magnet, followed by 2 washes of 100 µl of kilobaseBINDER kit wash buffer, and
25 one wash in 100 µl of 10mM Tris-Cl, pH 8.5. The beads were resuspended in 50 µl of 1xNEB ligase buffer and incubated at 55 °C for 5 minutes, then rapidly chilled on ice.

In addition the *NlaIII*-Illumina adapter, and *DpnII*-Illumina adapter stocks were
30 also incubated at 55 °C for 5 minutes and rapidly chilled on ice. The beads were placed on a magnet and the supernatant was removed. To each tube 33 µl of water, 4µl of 10x NEB ligase buffer, and 1 µl (2000U) of T4 DNA ligase (NEB) was added along with 2 µl (200pmol) of either the *NlaIII*-, or *DpnII*-Illumina adapter appropriate to the sample:

*Paired-end adapter 1*

PE Adapter 1 – NlaIII

ACACTCTTTCCCTACACGACGCTCTTCCGATCTCATGTGTGAGAAAGGGATGTGCTGCG
5 AGAAGGCTAGAp (SEQ ID NO: 5)

PE Adapter 1 – DpnII

ACACTCTTTCCCTACACGACGCTCTTCCGATCTTGTGAGAAAGGGATGTGCTGCGAGAA
GGCTAGACTAGp (SEQ ID NO: 6)

10 The ligation reaction was incubated for 2 hours at room temperature on a rotating wheel. The ligation reaction was cleaned up by removal of the supernatant on a magnet followed by 2 washes of 100 µl of kilobaseBINDER kit wash buffer, and one wash in 100 µl of 10mM Tris-Cl, pH 8.5.

15 **(v) Incorporation of the second Illumina adapter by PCR**

The samples were placed on a magnet and the previous Tris-Cl wash was removed.

The beads were washed once with 50 µl of 1x PCR mix (1x buffer and 200 µM of 20 each dNTP). Each sample was to be divided into 8 PCR reactions so sufficient PCR master mixes were prepared. Master mix 1 contained the following per reaction: 21µl of water, 1 µl of 10mM dNTP mix, 1.5 µl of 10 µM Illumina paired end adaptor 1 forward primer, and 1.5 µl of a 10 µM mix of 4 J segment specific reverse primers incorporating the Illumina paired end adapter 2 sequence at the 25 5′ end.

*PCR primers for incorporation of paired end adapter 2*

PE adaptor 1 forward

ACACTCTTTCCCTACACGACGCTCTTCCGATCT (SEQ ID NO: 7)

30 J1 PE PCR adapter 2 reverse

CTCGGCATTCCTGCTGAACCGCTCTTCCGATCTCCCAGACATCGAAGTACCAG (SEQ ID NO: 8)

J2 PE PCR adapter 2 reverse

CTCGGCATTCCTGCTGAACCGCTCTTCCGATCTTTGGCCCCAGTAGTCAAAG (SEQ ID NO: 9)

J3 PE PCR adapter 2 reverse

CTCGGCATTCCTGCTGAACCGCTCTTCCGATCTTGGCCCCAGTAAGCAAAC (SEQ ID
5 NO: 10)

J4 PE PCR adapter 2 reverse

CTCGGCATTCCTGCTGAACCGCTCTTCCGATCTCCCCAGTAGTCCATAGCATAG (SEQ ID NO: 11)

10 Master mix 2 contained the following per reaction: 19.25 µl of water, 5 µl of 10x Expand high fidelity buffer (Roche) and 0.75 µl of Expand high fidelity Taq (Roche, 2.6U).

The samples were placed on a magnet and the supernatant was removed. The
15 beads were resuspended in 200 µl of Master mix 1 and 25 µl was aliquoted into 8 PCR tubes. 25 µl of Master mix 2 was added to each tube and mixed. The PCR reaction was initially denatured at 94 °C for 2 minutes, but during the PCR cycling was denatured for 15 seconds. The primers were annealed at 60 °C for 30 seconds, and elongated for 1 minute at 72 °C. The PCR was cycled 15 times
20 followed by a final elongation step of 5 minutes at 72 °C. The 8 PCR reactions were pooled into a single Eppendorf and placed on the magnet. The 400 µl supernatant was removed and placed in a fresh tube and the beads were discarded. The pooled PCR reaction was cleaned up using 720 µl of SPRI beads according to the manufacturers protocol and eluted into 200 µl of water.

25

**(vi) Depletion of unrecombined J segments**

Each 200 µl sample was divided into eight 25 µl aliquots and placed into PCR tubes.

30 To each aliquot a primer extension reaction was assembled using 5 µl of the supplied NEB buffer, 1 µl of 10mM dNTP mix, 1µl (2U) of Vent (exo-) DNA polymerase (NEB), 17 µl of water and 1 µl of a 10 µM cocktail of 8 biotinylated oligos specific to the upstream regions of each of the 4 J segments on both strands:

*Depletion biotinylated oligonucleotides*

J1 dep bio F *-ATCTGAGTTTCTGAGGCTTG (SEQ ID NO: 12)

J1 dep bio R *-AAAACTCTCTCCACATCCTG (SEQ ID NO: 13)

5 J2 dep bio F *-CTAAAGGGGTCTATGATAGTGTG (SEQ ID NO: 14)

J2 dep bio R *-GTGTACAAAAACCCATCTACC (SEQ ID NO: 15)

J3 dep bio F *-CATTGTTGTCACAATGTGC (SEQ ID NO: 16)

J3 dep bio R *-TTAGACCCCTGACAATAAATG (SEQ ID NO: 17)

J4 dep bio F *-GTGGAACAATGACTTGAATG (SEQ ID NO: 18)

10 J4 dep bio R *-TGGGCAACTCAGACATTAT (SEQ ID NO: 19)

wherein * refers to the biotin moiety

The primer extension reaction was denatured at 94 °C for 4 mins, annealed at 55 °C for 2 mins, and extended at 72 °C for 10mins, then chilled on ice. The 8 15 primer extension reactions were pooled and magnetically purified using 720 µl of SPRI beads according to the manufacturers protocol, and eluted into 50 µl of water.

The biotinylated unrecombined DNA fragments were depleted using streptavidin 20 coupled Dynabeads from the kilobaseBINDER kit (Invitrogen). 10 µl aliquots of Dynabeads were prepared according to the manufacturers protocol, to which the 50µl DNA samples were added and incubated overnight at 20 °C on a Thermomixer at 1200rpm. The samples were placed on a magnet for 5 mins and the supernatant was removed and placed in a fresh tube, the beads containing 25 the unrecombined fragments were discarded. The depleted samples were magnetically purified using 108 µl of SPRI beads according to the manufacturers protocol, and eluted into 50 µl of water.

**(vii) Illumina size selection**

30 The *DpnII* and *NlaIII* samples were combined into a single tube, gel-loading buffer was added and the combined sample was run out on a 1.5% agarose TAE gel. The sample was sized selected by excision of a gel slice extending from 100 to 1000 bp.

The sample was isolated from the gel slice using the QIAGEN gel extraction kit according to the manufacturers protocol and was eluted in 50 µl of elution buffer.

5 **(viii) Sticky end PCR for Illumina flowcell binding**

Sufficient PCR Master mixes were prepared for 5 reactions. Master mix 1 contained the following per reaction: 11 µl of water, 1 µl of 10mM dNTP mix, 1.5 µl of 10 µM Illumina paired end PCR forward primer, and 1.5 µl of 10 µM Illumina paired end PCR reverse primer.

10

*Sticky-end PCR primers*

Illumina PE PCR forward

AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCT (SEQ ID NO: 20)

15 Illumina PE PCR reverse

CAAGCAGAAGACGGCATACGAGATCGGTCTCGGCATTCCTGCTGAACCGCTCTTCCGATCT (SEQ ID NO: 21)

Master mix 2 contained the following per reaction: 19.25 µl of water, 5 µl of 10x
20 Expand high fidelity buffer (Roche) and 0.75 µl of Expand high fidelity Taq (Roche, 2.6U).

75 µl of Master mix 1 was combined with the 50 µl sample, mixed and aliquoted into 5 PCR tubes. To each tube 25 µl of Master mix 2 was added and mixed. The
25 PCR reaction was initially denatured at 94 °C for 2 minutes, but during the PCR cycling was denatured for 15 seconds. The primers were annealed and elongated at 72 °C for 1 minute. The PCR was cycled 10 times followed by a final elongation step of 5 minutes at 72 °C. The PCR reactions were pooled and magnetically purified using 450 µl of SPRI beads according to the manufacturers
30 protocol, and eluted into 60 µl of 10mM Tris-Cl, pH 8.5.

**(ix) Illumina sequencing**

The sample was submitted for standard Illumina 36bp paired end sequencing.

***(B) Results***

The results of the analysis are shown in Figure 7 which depicts the recombination frequency data of V genes in the mouse Igh locus. All detectable V genes are shown in locus order on the x-axis, which has been split into two due to space constraints. The *DpnII* and *NlaIII* datasets have been normalised by dividing the total number of reads per dataset by the number of V segments detectable with that restriction enzyme giving number of reads per segment expected if the reads were evenly distributed. Finally, the number of reads observed for each V segment was divided by that expected number giving fold difference between number of reads observed compared to number of reads expected. A value of 1 corresponds to the number of reads expected if reads were evenly distributed.

**Example 2: VDJ Recombination Product Identification Method in Mouse B Cells using sonication**

***(A) Methods***

**(i) Sample material**

Bone marrow taken from 15 mice generally yields approximately 5 x $10^6$ fraction B/C VDJ recombined B cells following MACS depletion & FACS sorting. Approximately 16µg of DNA is typically isolated from this number of cells using the Qiagen DNeasy kit. For splenic B cells, deplete spleen of T cells, activated B cells and erythrocytes using CD43 biotin (1:1000) and Ter119 (1:400). One spleen typically gives 6-8 x $10^7$ cells at greater than 90% purity from 1-1.5 x $10^8$ starting cells. $10^7$ cells makes over 20µg DNA using the Qiagen DNeasy kit. Therefore, taking 10µg through is equivalent to 4-5 x $10^6$ cells. After eluting the DNA from the kit in buffer AE, precipitate with EtOH/NaOAc and spool out into 70% EtOH wash then into fresh tube. Resuspend immediately in the same volume of water – very little DNA is lost as a result of this (less than 5%). Nanodrop is used to determine DNA concentration and yield.

**(ii) Fragmentation & Repair**

DNA is fragmented using the Covaris E220 system using the manufacturer's protocol to generate 500bp peak fragments. The machine requires at least 1 hour of pre-cooling and degassing before use.

5 1. Resuspend up to 10μg of DNA in 130μl of water and transfer to Covaris crimp cap microtube (Cat No. 520052). Nanodrop should be relatively accurate for this DNA but you may want to quantify the DNA using picogreen assay.

2. Place tubes in Covaris E220 and run the following program for 500bp:

10

| | |
|---|---|
| Peak Incident Power (W) | 105 |
| Duty Factor | 5% |
| Cycles per Burst | 200 |
| Treatment time (s) | 80 |
| Temperature (°C) | 7 |
| Water Level | 6 |

15

3. Transfer sample from microtube to 1.5ml eppendorf. Run a small amount to check size although the migration kinetics of the DNA at this stage may be
20 different to the ladder due to ragged ends.

4. End repair DNA using the mix below:

130μl DNA
25 16μl 10x T4 DNA ligase buffer (use fresh aliquot)
4μl 10mM dNTP mix
5μl T4 DNA polymerase (NEB)
1μl Klenow (NEB)
5μl T4 PNK (NEB)
30 Total: 161μl volume

Incubate at 20°C for 30 minutes.

5. Purify reaction using Qiagen QIAquick columns using PCR purification protocol and elute in 50µl buffer EB.

**(iv) PE1 Adapter Ligation**

5 6. Create A-tails on the end of the repaired DNA using the mix below:

50µl of end repaired DNA
6µl 10x NEB buffer 2
1µl 10mM dATP
10 3µl Klenow (exo-) (NEB)
Total: 60µl volume

Incubate at 37°C for 30 minutes.

15 7. Purify reaction using Qiagen QIAquick columns using PCR purification protocol and elute in 30µl water twice.

8. Ligate Adapter oligo mix using the mix below:

20 30µl of A-tailed DNA
5µl 10x T4 DNA ligase buffer (NEB – use fresh aliquot)
4/8µl Adapter oligo mix (50pmol)
5µl T4 DNA ligase 2000U (NEB)
Total: 50µl volume

25

Incubate overnight at 16°C.

Adapter mix uses 2 oligos: “DpnII adapter F” & “Rev PE both adapter”, which anneal together to create an asymmetric PE1 adapter:

30

*PCR primers for Adapter oligo mix*

| Primer | Sequence | SEQ ID NO |
|---|---|---|
| DpnII adapter F | ACACTCTTTCCCTACACGACGCTCTTCCGATC*T | 22 |

| Rev PE both adapter | CGAGAAGGCTAG[Phos] | 23 |
|---|---|---|

wherein * refers to biotin moiety and [Phos] refers to a phosphorothioate moiety

Working stock of 100µM for each oligo are mixed together to give 50µM of adaptor. There is no need to heat and cool these, just mix equimolar amounts and use. 4µl of adaptor used in reaction = 200pmol which is approximately 6.67:1 ratio of adaptor ends (1x) to DNA ends (2x) (5µg of 500bp fragments = 15pmol x 2 = 30pmol). For 10µg DNA use 8µl adaptor.

9. Purify reaction using Qiagen QIAquick columns using PCR purification protocol, elute in 50µl water. When 1µl of this on a gel there was a shift of all fragments of approximately 40-50bp indicating highly efficient adapter ligation.

**(v) Depletion of unrecombined J segments**

10. Primer extension using biotinylated oligos annealing to upstream regions of each J segment. Split sample into separate primer extension reactions not exceeding 1µg DNA per reaction. For example, 8x reactions use less than 1µg per reaction accounting for loss of DNA through cleanup.

5µl 10x ThermoPol reaction buffer
1µl dNTPs (10mM each)
1µl Biotinylated primers (10mM mix)
1µl Vent exo- (NEB)
~25µl DNA (up to 2.5µg DNA per reaction)
~17µl water to 50µl
Total: 50µl volume

Thermal cycler conditions: 95°C for 4 minutes, 55°C for 5 minutes, 72°C for 15 minutes, 4°C pause.

A mixture of 8 biotinylated primers are used with Tms between 57.4°C to 59.4°C:

*Depletion biotinylated primers*

| Target | Primer | Sequence | SEQ ID NO |
|---|---|---|---|
| Mouse IgH | J1 dep F new | *-ACAGAGGCAGAACAGAGACT | 24 |
| | J1 dep R | *-AAAACTCTCTCCACATCCTG | 13 |
| | J2 dep F | *-CTAAAGGGGTCTATGATAGTGTG | 14 |
| | J2 dep R | *-GTGTACAAAAACCCATCTACC | 15 |
| | J3 dep F | *-CATTGTTGTCACAATGTGC | 16 |
| | J3 dep R | *-TTAGACCCCTGACAATAAATG | 17 |
| | J4 dep F new | *-AGAGGAAAAATCCACTATTGTG | 25 |
| | J4 dep R new | *-CCAGAGTCTGACTAGAATCACC | 26 |
| Mouse IgK | Jk1 F Dep | *-TACAGCCAGACAGTGGAGTAC | 27 |
| | Jk1 R Dep | *-CCTCACTGAAGAGGAACAGA | 28 |
| | Jk2 F Dep | *-TTGAGTGAAGGGACACCA | 29 |
| | Jk2 R Dep | *-CCCATACAAAAACTGAGCAT | 30 |
| | Jk4 F Dep | *-CTGAACTTAGCCTATCTAACTGG | 31 |
| | Jk4 R Dep | *-TTTACAAAAACCTGCCTGAG | 32 |
| | Jk5 F Dep | *-GCATGTCATAGTCCTCACTGT | 33 |
| | Jk5 R Dep | *-TCTCTACAAAAACCTGCCTG | 34 |
| Human IgH | Hu J1 F Dep | *-CAGGGCTGACTCACCGTG | 35 |
| | Hu J1 R Dep | *-CAGAAACCCACAGCCCG | 36 |
| | Hu J2 F Dep | *-GTGTTTTTGTATGGGAGAAGCAG | 37 |
| | Hu J2 R Dep | *-CACAGCCTCTGCCCTCCT | 38 |
| | Hu J3 F Dep | *-ACGGGCACAGGTTTGTGTC | 39 |
| | Hu J3 R Dep | *-GTCCCTGTTCCTGCCCAG | 40 |
| | Hu J4 F Dep | *-GTCGGAGAGTCAGGTTTTTGTG | 41 |
| | Hu J4 R Dep | *-AGTCACATTGTGGGAGGCC | 42 |
| | Hu J5 F Dep | *-GTCTGAGAGGGTCCCAGGG | 43 |
| | Hu J5 R Dep | *-GTGACAACAATGCCAGGACC | 44 |
| | Hu J6 F Dep | *-GGTGAGGATGGACATTCTGC | 45 |
| | Hu J6 R Dep | *-CAGCCACCCAGAGACCTTC | 46 |

wherein * refers to the biotin moiety

11. Pool multiple reactions and purify using Qiagen QIAquick columns using PCR purification protocol. Use pH indicator in buffer PB (*i.e.* PBI). Add 10µl 3M NaOAc and elute in 40µl water.

5 12. Prepare Dynabeads MyOne Streptavidin C1 beads (Invitrogen). Vortex Dynabead stock, transfer 20µl to eppendorf, add 1ml 1x B&W buffer(Binding & Washing), mix, place tube on magnet for 2 mins, discard supernatant. Resuspend beads in 20µl of 1x B&W buffer, place on magnet and discard supernatant. Repeat 20µl wash a total of 3 times. Resuspend beads in 40µl of 2x 10 B&W buffer.

*2x B&W (Binding & Washing) buffer*

| **Final concentration** | **Stock** | **Amount for 50ml** |
|---|---|---|
| 10mM Tris-HCl (pH 7.5) | 1M | 500µl |
| 1mM EDTA | 0.5M | 100µl |
| 2M NaCl | 5M | 20ml |
| 0.05% Tween-20 | 100% | 25µl |
| Water | - | 29.4ml |

15

20 13. Add 40µl of DNA sample from step 11 to beads, mix and incubate for 60 minutes, or overnight, on rotator at room temperature.

14. Place sample on magnet for 5 minutes, pipette supernatant into clean eppendorf. Wash beads by resuspending in 80µl EB buffer, place on magnet for 25 5 minutes and transfer supernatant to the same tube as before.

15. Purify reaction using Qiagen QIAquick columns using PCR purification protocol, elute in 50µl water.

30 **(vi) Enrichment of VDJ recombined fragments**

16. Primer extension using biotinylated oligos annealing within each J segment, approximately 8bp inset from the 5′ end. Split sample into separate primer extension reactions, not exceeding 1µg DNA per reaction.

5μl 10x ThermoPol reaction buffer
1μl dNTPs (10mM each)
1μl Biotinylated primers (10mM mix)
1μl Vent exo- (NEB)
5 ~25μl DNA (up to 2.5μg DNA per reaction)
~17μl water to 50μl
Total: 50μl volume

Thermal cycler conditions: 95°C for 4 minutes, 59°C for 5 minutes, 72°C for 15
10 minutes, 4°C pause.

A mixture of biotinylated primers are used depending on the cell population. Tms are between 62.7°C to 63.7°C:

15 *Biotinylated primers for enrichment of VDJ recombined fragments*

| Target | Primer | Sequence | SEQ ID NO |
|---|---|---|---|
| Mouse IgH | J1 Rev Bio | *-AGCCAGCTTACCTGAGGAGAC | 1 |
| | J2 Rev Bio | *-GAGAGGTTGTAAGGACTCACCTG | 2 |
| | J3 Rev Bio | *-AGTTAGGACTCACCTGCAGAGAC | 3 |
| | J4 Rev Bio | *-AGGCCATTCTTACCTGAGGAG | 4 |
| Mouse IgK | Jk1 R Bio | *-GAAAGAGACTTTGGATTCTACTTACG | 47 |
| | Jk2 R Bio | *-GAACAAGAGTTGAGAAGACTACTTACG | 48 |
| | Jk3 R Bio | *-CACAAGTAAATGAGCAAAAGTCTACTT | 49 |
| | Jk4 R Bio | *-AAAGATGAGAAAAGTGTACTTACGTTTC | 50 |
| Human IgH | Hu J1 R Bio | *-CCAGACAGCAGACTCACCTG | 51 |
| | Hu J2 R Bio | *-TGCAGTGGGACTCACCTG | 52 |
| | Hu J3 R Bio | *-AGAAGGAAAGCCATCTTACCTG | 53 |
| | Hu J4 R Bio | *-CAGGAGAGAGGTTGTGAGGACT | 54 |
| | Hu J5 R Bio | *-AGGGGGTGGTGAGGACTC | 55 |

| | Hu J6 R Bio | *-CCATTCTTACCTGAGGAGACG | 56 |
|---|---|---|---|

wherein * refers to the biotin moiety.

17. Pool multiple reactions and purify using Qiagen QIAquick columns using PCR purification protocol. Use pH indicator in buffer PB (*i.e.* PBI) and elute in 40µl water.

18. Prepare Dynabeads MyOne Streptavidin C1 beads (Invitrogen) as before (see step 12).

19. Combine the DNA sample with the prepared Dynabeads, mix and incubate overnight on rotator at room temperature.

20. Place sample on magnet for 5 minutes, keep and clean up supernatant with Qiagen kit as depleted fraction. Wash beads twice in 100µl 1x B&W buffer, and wash once in 100µl EB (Qiagen). Resuspend beads in 46µl buffer EB.

**(vii) Incorporation of PE2 adapter by PCR**

21. PCR using Pwo master (Roche) with a mixture of four J reverse primers 10bp downstream of the unaltered recombination junction with PE2 sequence at the 5′ end and a single forward primer annealing to the PE1 sequence in the ligated adapter. Each sample is divided into four separate 25µl PCR reactions to increase library diversity. A negative, using water instead of DNA, is included to be used as a control for PCR contamination.

| **Mix** | **x5** |
|---|---|
| 12.5µl Pwo master | 62.5µl |
| 0.5µl F primer (10µM PE1 short) | 2.5µl |
| 0.5µl R primer mix (10µM J1-J4 PE2) | 2.5µl |
| 11.5µl DNA on beads (step 20) | 46µl |
| Total: 25µl volume | 125µl |

Place on the PTC100 using the following conditions:

94°C for 2 minutes, 15 cycles of: 94°C for 15 seconds, 61°C for 30 seconds, 72°C for 45 seconds, followed by 72°C for 5 minutes, and 4°C pause. The final number of PCR cycles required will need titrating to achieve optimal amplification, for example 10, 12 or 15 cycles. It is considered to be best to do 5 more cycles in the first round than the second (for example, 15x 1st round, 7x 2nd round). Ensure these primers are at least HPLC, ideally PAGE purified. Short PE1 primer Tm: 68.5°C; J primers Tm range: 68.7-69.8°C.

*Primer sequences for incorporation of PE adapters*

| **Target** | **Primer** | **Sequence** | **SEQ ID NO** |
|---|---|---|---|
| Mouse IgH | Short primer 1.0.1 | ACACTCTTTCCCTACACGACGCTCpT | 57 |
| | J1.10 PE2 PCR | GTGACTGGAGTTCAGACGTGTGCTCTTCCGA TCTCCCTGTGCCCCAGACATCGApA | 58 |
| | J2.10 PE2 PCR | GTGACTGGAGTTCAGACGTGTGCTCTTCCGA TCTAGTGGTGCCTTGGCCCCAGTApG | 59 |
| | J3.10 PE2 PCR | GTGACTGGAGTTCAGACGTGTGCTCTTCCGA TCTACCAGAGTCCCTTGGCCCCAGTApA | 60 |
| | J4.10 PE2 PCR | GTGACTGGAGTTCAGACGTGTGCTCTTCCGA TCTTGAGGTTCCTTGACCCCAGTAGTCCATpA | 61 |
| Mouse IgK | Jk1 PE2 PCR | GTGACTGGAGTTCAGACGTGTGCTCTTCCGA TCTTTTGATTTCCAGCTTGGTGCCTCpC | 62 |
| | Jk2 PE2 PCR | GTGACTGGAGTTCAGACGTGTGCTCTTCCGA TCTTTTATTTCCAGCTTGGTCCCCCpT | 63 |
| | Jk3 PE2 PCR | GTGACTGGAGTTCAGACGTGTGCTCTTCCGA TCTCGTTTTATTTCCAACTTTGTCCCCGpA | 64 |
| | Jk4 PE2 PCR | GTGACTGGAGTTCAGACGTGTGCTCTTCCGA TCTCAGCTCCAGCTTGGTCCCAGpC | 65 |
| Human IgH | Hu J1 PE2 PCR | GTGACTGGAGTTCAGACGTGTGCTCTTCCGA TCTGGTGCCCTGGCCCCAGTpG | 66 |
| | Hu J2 PE2 PCR | GTGACTGGAGTTCAGACGTGTGCTCTTCCGA TCTGGTGCCACGGCCCCAGAGpA | 67 |

| | | | |
|---|---|---|---|
| | Hu J3 PE2 PCR | GTGACTGGAGTTCAGACGTGTGCTCTTCCGA TCTACCATTGTCCCTTGGCCCCApG | 68 |
| | Hu J4 PE2 PCR | GTGACTGGAGTTCAGACGTGTGCTCTTCCGA TCTGACCAGGGTYCCYTGGCCCpC | 69 |
| | Hu J5 PE2 PCR | GTGACTGGAGTTCAGACGTGTGCTCTTCCGA TCTCAGGGTTCCYTGGCCCCAGpG | 70 |
| | Hu J6 PE2 PCR.1 | GTGACTGGAGTTCAGACGTGTGCTCTTCCGA TCTCCTTTGCCCCAGACGTCCATGTAGpT | 71 |
| | Hu J6 PE2 PCR.2 | GTGACTGGAGTTCAGACGTGTGCTCTTCCGA TCTTKSCCCCAGACGTCCATACCGpT | 72 |

wherein p refers to a phosphorothioate bond

22. Pool the 4 separate PCR reactions into a single 1.5ml tube. Place on magnet for 5 minutes pipette the supernatant containing the PE1-VDJ-PE2 products of interest to a new tube.

23. Wash beads once in 30µl buffer EB, and add supernatant to the previous supernatant. Keep the beads as PCR can be attempted again on these but first do 2x 1ml washes with water to remove any residual PCR products and resuspend in 46µl EB and keep frozen.

24. Purify and size select PCR reaction using 1x volume SPRI/Seramag beads (will be approximately 120µl). This removes primers/primer dimers and fragments less than 200bp. Binding capacity of SPRI beads is 3µg/µl and they are suspended in 20% PEG-8000, 2.5M NaCl (see, http://core-genomics.blogspot.co.uk/2012/04/how-do-spri-beads-work.html). Since the binding capacity is so great, it may be possible to dilute the beads down in this buffer to make them go further without affecting capture efficiency. Add beads, mix thoroughly by pipetting, incubate at room temp for 10 minutes or more, place on magnet for 5 minutes and discard supernatant. Wash twice with 500µl fresh 70% EtOH, keeping the sample on the magnet. Do not resuspend the beads. Remove final EtOH wash, air-dry bead pellet partially. Remove tube from magnet and resuspend beads thoroughly in 46µl buffer EB. Place tube back on

magnet for 2 minutes and transfer supernatant containing purified and size selected PCR to fresh tube.

**(viii) Incorporation of flowcell binding & barcoding sequences by PCR**

5 25. PCR using Pwo master (Roche) with universal Flowcell PE1 primer in combination with one of eight index primers + PE2 per sample. Each sample is divided into four separate 25µl PCR reactions to increase library diversity. Include negative control using 11.5µl water instead of DNA.

| 10 **Mix** | **x5** |
|---|---|
| 12.5µl Pwo master | 62.5µl |
| 0.5µl F primer (10µM Flowcell PE1) | 2.5µl |
| 0.5µl R primer (10µM Flowcell index PE2) | 2.5µl |
| 11.5µl DNA (1st round PCR) | 46µl |
| 15 Total: 25µl volume | 125µl |

Place on the PTC100 using the following conditions:
94°C for 2 minutes, 7 cycles of: 94°C for 15 seconds, 55°C for 30 seconds, 72°C for 45 seconds, followed by 72°C for 5 minutes, and 4°C pause. The final
20 number of PCR cycles will require titration to achieve optimal amplification but it may be good to keep the number of cycles in the second round PCR low, for example 5 or 7. Tms of regions annealing to target are 58.2°C for PE1, and 61.9°C for PE2.

25 Primers should be at least HPLC, preferably PAGE purified.

*Primers for incorporation of flowcell binding & barcoding sequences by PCR*

| Primer | Sequence | SEQ ID NO |
|---|---|---|
| Flowcell PE1 | AATGATACGGCGACCACCGAGATCTACACTCTTTCCC TACACGAC | 73 |
| Flowcell PE2 Index 1 | CAAGCAGAAGACGGCATACGAGATCGTGATGTGACT GGAGTTCAGACGTGT | 74 |
| Flowcell PE2 | CAAGCAGAAGACGGCATACGAGATACATCGGTGACT | 75 |

| | | |
|---|---|---|
| Index 2 | GGAGTTCAGACGTGT | |
| Flowcell PE2 Index 3 | CAAGCAGAAGACGGCATACGAGATGCCTAAGTGACT GGAGTTCAGACGTGT | 76 |
| Flowcell PE2 Index 4 | CAAGCAGAAGACGGCATACGAGATTGGTCAGTGACT GGAGTTCAGACGTGT | 77 |
| Flowcell PE2 Index 5 | CAAGCAGAAGACGGCATACGAGATCACTGTGTGACTG GAGTTCAGACGTGT | 78 |
| Flowcell PE2 Index 6 | CAAGCAGAAGACGGCATACGAGATATTGGCGTGACT GGAGTTCAGACGTGT | 79 |
| Flowcell PE2 Index 7 | CAAGCAGAAGACGGCATACGAGATGATCTGGTGACT GGAGTTCAGACGTGT | 80 |
| Flowcell PE2 Index 8 | CAAGCAGAAGACGGCATACGAGATTCAAGTGTGACTG GAGTTCAGACGTGT | 81 |
| Flowcell PE2 Index 9 | CAAGCAGAAGACGGCATACGAGATCTGATCGTGACTG GAGTTCAGACGTGT | 82 |
| Flowcell PE2 Index 10 | CAAGCAGAAGACGGCATACGAGATAAGCTAGTGACTG GAGTTCAGACGTGT | 83 |
| Flowcell PE2 Index 11 | CAAGCAGAAGACGGCATACGAGATGTAGCCGTGACT GGAGTTCAGACGTGT | 84 |
| Flowcell PE2 Index 12 | CAAGCAGAAGACGGCATACGAGATTACAAGGTGACTG GAGTTCAGACGTGT | 85 |

26. Purify and size select PCR reaction using 1x volume Ampure XP (SPRI) beads as in 24. Elute in 40µl EB. Ideally should be approximately 40-50ng of the final library.

5

27. Determine concentration by picogreen. Run 2µl out on a gel and perform QC PCRs checking for the presence of VDJ recombined products (*e.g.* VJ558.F to JR) and absence of non-*Igh* genes (*e.g.* B2M). Analyse enrichment of V-DJ and D-J products using J558FR3.F QPCR – J4.R QPCR assay and DSP/DFL.F - J4.R
10 QPCR in SYBR real-time, comparing library with sonicated input DNA (for example, using sonicated spleen DNA as a control). Analyse depletion of unrecombined J region and non-Igh sequences using SYBR real-time PCR. For

IgH J region use J3-J4 new int.F-mu0r2 = 141bp (IgH J) – this region will be lost if depletion has successfully occured. For non-Igh, B2M or actin can be used.

28. Bioanalyse results and perform QPCR in order to generate data.

5

***(B) Results***

The results of the analysis are shown in Figure 8 which depicts the recombination frequency data of V genes in the mouse Igh locus. All V genes are shown in locus order on the x-axis, which has been split into four due to space
10 constraints. Each V gene on the X axis is depicted by 3 bars representing the results from 3 biological replicates of mouse proB cells. The read count is normalized to the middle replicate to take into account that different sequencing libraries produce different numbers of sequences, such that the variation between the biological replicates reflects proportional differences in
15 recombination frequency, and not absolute read count. An improvement of this method compared to Example 1 is that all V genes are detectable, because sonication is used. In Example 1, a small fraction of genes are not detectable because the restriction enzyme does not cut within or near the V gene. In Figure 8, V genes in the locus that do not have reads are invariably V pseudogenes,
20 that have a defect that precludes VDJ recombination, and thus they do not contribute to the repertoire.

**Example 3: Oligonucleotide and RNA hybridization:**

Example 3 may be performed in an analogous manner to Examples 1 and 2
25 except for the following:

Instead of using primer extension to deplete unrecombined segments of DNA and enrich for V(D)J recombined regions an alternative would be to preferentially deplete unrecombined regions and enrich for V(D)J recombined DNA using
30 hybridisation, either with a set of strategically placed biotinylated oligonucleotides or biotinylated RNA, followed by separation using streptavidin magnetic beads.

**(i) Oligonucleotide hybridization**

In the biotinylated oligonucleotides methodology a series of oligonucleotides similar in position to those used for depletion and enrichment in Examples 1 and 2 are hybridised to denatured genomic DNA and used to pull-down first unrecombined then second recombined DNA regions using streptavidin magnetic beads. Library production following these steps would be the same as for the other Examples described herein. If this step is only used to deplete unrecombined regions and is followed by the standard primer extension to isolate V(D)J recombined regions it would only be necessary to target the sense strand using antisense oligonucleotides since only the sense strand is a template for the primer extension using antisense oligonucleotides. Similarly, if this method was used for enrichment with J-specific oligonucleotides following depletion using intergenic antisense oligonucleotides it would only be necessary to use J-specific antisense oligonucleotides to target the sense strand of the already depleted DNA and hence enrich for V(D)J recombined DNA.

**(ii) RNA hybridization**

In the biotinylated RNA methodology for depletion, regions just upstream of each J gene are amplified by PCR and cloned into a vector containing a T7 promoter sequence flanking the cloning site. These are then linearised at the opposite end of the insert to the T7 sequence and these are used as templates to produce biotinylated RNA using T7 polymerase. Large amounts of strand-specific biotinylated RNA can be made in this way as this enzyme is highly processive. This biotinylated RNA can then be used in a similar way to the oligonucleotides detailed above to hybridise to unrecombined regions in denatured genomic DNA and remove them using streptavidin magnetic beads. Depleting these regions would prevent them being targets for downstream primer extension reactions used to isolate V(D)J recombined DNA regions, as detailed in Examples 1 and 2. Again, if only used for the depletion step it would only be necessary to target the sense strand of the DNA using antisense RNA as the antisense strand of the DNA is not a template for the primer extension. This also means a larger region, and hence larger biotinylated RNA, can be used in the hybridisation step, which should aid hybridisation specificity and efficiency. RNA can easily be digested and removed from DNA following hybridisation.

For enrichment using the biotinylated RNA methodology, the J genes and regions just 3’ (100-200bp regions) could be cloned, linearised, used to produce biotinylated RNA and this then hybridised in a similar way to above but in this case to enrich for V(D)J recombined DNA. It would be best to do this after having already depleted for unrecombined regions. Again it would be best to use antisense RNA to target the sense strand in both depletion and enrichment steps as this would just leave unrecombined depleted, V(D)J enriched sense-strand DNA that would then be the perfect template for the standard PCR-based VDJ-seq library production as detailed in Examples 1 and 2, above.

**Example 4: VDJ Recombination Product Identification Method using RNA**

***(A) Methods***

**(i) Sample material**

B cells are isolated from bone marrow or spleen from mice, or from human peripheral blood. RNA isolated by RNeasy or Trizol and DNase digested. Between 1-10μg RNA is used per library preparation.

**(ii) Reverse transcription & RNA degradation**

1. Set up RT reaction based upon Superscript III (Invitrogen) protocol in 0.5ml tube.

5μl RNA 1-10μg (up to 10μl)
1μl dNTPs (10mM each)
1μl primer mix* (2μM)
6μl water to 13μl
Total: 13μl volume

*Reverse Transcription primers*

| Primer | Sequence | SEQ ID NO |
|---|---|---|
| Human IgM RT | AAGGGTTGGGGCGGAT | 86 |
| Human IgG1-4+IgE RT | GGGAAGACSGATGGGC | 87 |

| Human IgA1&2 RT | CAGCGGGAAGACCTTGG | 88 |
|---|---|---|
| Human IgD RT | CCTGATATGATGGGGAACACA | 89 |

2. Incubate at 70°C for 5 minutes, then 55°C for 5 minutes.

3. Add the following mix, keeping the tube at 55°C:

5

4μl 5x FS buffer
1μl DTT
1μl RNasin
1μl SSIII
10 Total: 20μl volume

4. Mix, and incubate at 55°C for 60 minutes, then 70°C for 15 minutes.

5. Add 1μl RNase H or A/T mix.

15

6. Incubate at 37°C for 20 minutes.

7. Purify reaction using 1.0x SPRI beads and elute in 20μl water.

20 **(iii) Random octamer to add PE2 to 3' end cDNA**

8. Set up 20μl klenow exo-reaction in 0.5ml tube (without klenow to start with). Use half reaction as a negative control without the oligo, or set up 2x reaction both with oligo.

25 10μl cDNA
2μl 10x NEB buffer 2
1μl dNTPs (2mM each, therefore 1:5 dilution of usual concentration)
1μl random octamer+PE2 (100μM)
5μl water (to 19μl)

30

*Random octamer+PE2 oligo*

| Primer | Sequence | SEQ ID NO |
|---|---|---|
| PE2 temp switch block 8N | GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTN NNNNNNN[SpcC3] | 90 |

wherein [SpcC3] refers to a spacer with a 3′ modification that prevents elongation.

9. Heat to 65°C for 5 minutes (denature secondary structure) then 22°C for 5 minutes (anneal octamers).

10. Add 1µl Klenow exo- .

11. Incubate at 37°C for 30 minutes, then 95°C for 5 minutes to stop reaction and denature octamers from cDNA.

12. Transfer immediately to ice for 5 minutes.

13. Purify reaction using Qiagen QIAquick columns using PCR purification protocol or with 1.0x SPRI beads. Elute in 46µl EB.

**(iv) PCR to generate second strand & enrich VDJ sequences**

14. Perform PCR using Pwo master (Roche) with biotinylated constant region primers. Assemble reaction in PCR tubes. Each sample is divided into four separate 25µl PCR reactions to increase library diversity. Include negative to control for PCR contamination by using 11.5µl water instead of DNA.

| **Mix 1** | **x5** |
|---|---|
| 12.5µl Pwo master | 62.5µl |
| 0.5µl F primer (10µM Short PCR 2*) 4µl | 2.5µl |
| 0.5µl R primer mix (10µM C PE1 Bio*) | 2.5µl |
| 11.5µl DNA | 46µl |
| Total: 25µl volume | 125µl |

Place on the PTC100 using the following conditions:

94°C for 2 minutes, 12-15 cycles of: 94°C for 15 seconds, 58°C for 30 seconds, 72°C for 45 seconds, followed by 72°C for 5 minutes, and 4°C pause. The final number of PCR cycles required may need titrating to achieve optimal amplification.

5

Primer sequences only include constant region primers of interest in the primer mix. These contain 8 random nucleotides to increase complexity at the start of the run. Short PE2 primer Tm: 61.87°C; Constant-J primers Tm range: 61 - 63°C.

10

*Primer sequences to generate second strand & enrich VDJ sequences*

| **Primer** | **Sequence** | **SEQ ID NO** |
|---|---|---|
| Short PCR primer 2.0 | GTGACTGGAGTTCAGACGTGT | 91 |
| Constant region + PE1 IgM | ACACTCTTTCCCTACACGACGCTCTTCCGATCTNNNNNNNNCGGATGCACTCCCTGA | 92 |
| Constant region + PE1 IgG1&2 | ACACTCTTTCCCTACACGACGCTCTTCCGATCTNNNNNNNNCCTTGGTGGAGGCTGA | 93 |
| Constant region + PE1 IgD | ACACTCTTTCCCTACACGACGCTCTTCCGATCTCCTTGGTGGGTGCTGA | 94 |
| Constant region + PE1 IgA1&2 | ACACTCTTTCCCTACACGACGCTCTTCCGATCTNNNNNNNNGGTCGGGGATGCTGA | 95 |
| Constant region + PE1 IgE | ACACTCTTTCCCTACACGACGCTCTTCCGATCTGCTCTGTGTGGAGGCTGA | 96 |
| Constant region + PE1 IgG3&4 | ACACTCTTTCCCTACACGACGCTCTTCCGATCTCCTTGGTGGAAGCCTGA | 97 |

15. Pool the four separate PCR reactions into a single 1.5ml tube. Purify using 0.8x SPRI beads to size select and remove primers/primer dimers. Elute in 46µl

15 water.

16. Prepare Dynabeads MyOne Streptavidin C1 beads (Invitrogen).

Vortex Dynabead stock, transfer 20µl to eppendorf, add 1ml 1x B&W +Tween buffer*, mix, place tube on magnet for 2 mins, discard supernatant. Resuspend beads in 20µl of 1xB&W+Tween buffer, place on magnet and discard supernatant. Repeat 20µl wash again. Resuspend beads in 20µl of 1x B&W (no Tween) buffer, place on magnet and discard supernatant. Resuspend beads in 40µl of 2x B&W (no Tween) buffer.

*2x B&W (Binding & Washing) buffer (with and without Tween-20)

| **Final concentration** | **Stock** | **Amount for 50ml** |
|---|---|---|
| 10mM Tris-HCl (pH 7.5) | 1M | 500µl |
| 1mM EDTA | 0.5M | 100µl |
| 2M NaCl | 5M | 20ml |
| 0.05% Tween-20100% | | 25µl (Only include in Tween containing buffer) |
| Water | - | 29.4ml |

17. Add 40µl of DNA sample (step 15) to beads, mix and incubate for 60 mins, or overnight, on rotator at room temperature.

18. Place sample on magnet for 5 mins, discard supernatant. Wash beads twice in 100µl 1x B&W (No Tween) buffer, and wash once in 100µl EB (Qiagen). Resuspend beads in 50µl buffer EB.

19. PCR using Expand Taq (Roche) with Short PCR 1 to Short PCR 2 primers. Assemble reaction in 200µl tubes. Each sample is divided into 4 separate 50µl PCR reactions to increase library diversity.

| **Mix 1** | **x4** | **Mix 2** | **x4** |
|---|---|---|---|
| 1µl dNTPs (10mM each) | 4µl | 5µl 10x PCR buffer | 20µl |
| 1µl F primer (10µM Short PCR 1*) | 4µl | 0.75µl Expand Taq | 3µl |
| 1µl R primer (10µM Short PCR 2*) | 4µl | 19.25µl water | 77µl |
| 12.5µl DNA | 50µl | | |
| 9.5µl water | 38µl | 25µl volume | 100µl |

| | |
|---|---|
| 25µl volume | 100µl |

Aliquot 25µl of Mix 1 into 4 separate PCR tubes on ice, add 25µl Mix 2, mix, and quickly place on the Thermal cycler using the following conditions:

94°C 2mins, 5 cycles of: 94°C 15 secs, 55°C 30 secs, 72°C 45 secs, followed by 72°C 5mins, and 4°C pause.

* Primer sequences, Tms 61.8°C and 58.2°C respectively:

| | |
|---|---|
| Short PCR primer 2.0 | GTGACTGGAGTTCAGACGTGT (SEQ ID NO: 91) |
| Short PCR primer 1.0 | ACACTCTTTCCCTACACGAC (SEQ ID NO: 98) |

20. When PCR finished, combine 4 reactions into a single 1.5ml tube. Place sample on magnet for 5 mins, pipette supernatant into clean eppendorf. Wash beads by resuspending in 30µl EB buffer, place on magnet for 5 mins and transfer supernatant to the same tube as before. The tube containing the beads can be discarded (or resupended in 50µl EB and kept).

21. Purify reaction using Qiagen QIAquick columns using PCR purification protocol, elute in 40µl buffer EB.

**(v) Incorporation of flowcell binding & barcoding sequences by PCR**

22. PCR using Pwo master (Roche) with universal Flowcell PE1 primer in combination with one of twelve index primers + PE2 per sample. Each sample is divided into four separate 25µl PCR reactions to increase library diversity. Include negative control with 11.5µl water instead of DNA.

| **Mix** | **x5** |
|---|---|
| 12.5µl Pwo master | 62.5µl |
| 0.5µl F primer (10µM Flowcell PE1) | 2.5µl |
| 0.5µl R primer (10µM Flowcell index PE2) | 2.5µl |
| 11.5µl DNA (1st round PCR) | 46µl |
| Total: 25µl volume | 125µl |

Place on the PTC100 using the following conditions:
94°C for 2 minutes, 7-9 cycles of: 94°C for 15 seconds, 55°C for 30 seconds, 72°C for 45 seconds, followed by 72°C for 5 minutes, and 4°C pause. The final number of PCR cycles will require titration to achieve optimal amplification. Tms
5 of regions annealing to target are 58.2°C for PE1, and 61.9°C for PE2.

*Flowcell Primer sequences*

| Primer | Sequence | SEQ ID NO |
|---|---|---|
| Flowcell PE1 | AATGATACGGCGACCACCGAGATCTACACTCTTTCCC TACACGAC | 73 |
| Flowcell PE2 Index 1 | CAAGCAGAAGACGGCATACGAGATCGTGATGTGACT GGAGTTCAGACGTGT | 74 |
| Flowcell PE2 Index 2 | CAAGCAGAAGACGGCATACGAGATACATCGGTGACT GGAGTTCAGACGTGT | 75 |
| Flowcell PE2 Index 3 | CAAGCAGAAGACGGCATACGAGATGCCTAAGTGACT GGAGTTCAGACGTGT | 76 |
| Flowcell PE2 Index 4 | CAAGCAGAAGACGGCATACGAGATTGGTCAGTGACT GGAGTTCAGACGTGT | 77 |
| Flowcell PE2 Index 5 | CAAGCAGAAGACGGCATACGAGATCACTGTGTGACTG GAGTTCAGACGTGT | 78 |
| Flowcell PE2 Index 6 | CAAGCAGAAGACGGCATACGAGATATTGGCGTGACT GGAGTTCAGACGTGT | 79 |
| Flowcell PE2 Index 7 | CAAGCAGAAGACGGCATACGAGATGATCTGGTGACT GGAGTTCAGACGTGT | 80 |
| Flowcell PE2 Index 8 | CAAGCAGAAGACGGCATACGAGATTCAAGTGTGACTG GAGTTCAGACGTGT | 81 |

See DNA protocol from Examples 1 and 2 for further index sequences.

10 23. Purify using 0.8x SPRI beads to size select and remove primers/primer dimers.

24. Determine concentration by picogreen or bioanalyser, perform QPCR checking for the presence of VDJ recombined products (*e.g.* V4-1 to JR) and
15 absence of house-keeping genes (*e.g.* B2M).

## CLAIMS

1. A method of identifying a VDJ recombination product which comprises the following steps:

5 (a) obtaining a nucleic acid sample comprising a VDJ recombination product;

(b) when said nucleic acid sample comprises DNA, fragmenting the VDJ recombination product either by sonication, shearing or performing a restriction endonuclease reaction at a first site downstream of each of the J genes or 10 downstream of the constant region, and a second site within or immediately upstream of the V gene to generate digested VDJ recombined fragments and unrecombined J fragments;

(c) when said nucleic acid sample comprises DNA, annealing oligonucleotides to the digested fragments at unique regions within or 15 immediately downstream of each of the J genes, and when the nucleic acid sample is cDNA or RNA, annealing oligonucleotides to the VDJ recombination product at a position specific to the constant region or J gene;

(d) when said nucleic acid sample comprises DNA, separating the digested VDJ recombined fragments from the unrecombined J fragments and the 20 rest of the genome;

(e) sequencing the VDJ recombined fragments or products; and

(f) data processing of the sequencing data to identify each VDJ recombination product and quantify VDJ recombination frequencies.

25 2. The method as defined in claim 1, wherein the nucleic acid sample comprising a VDJ recombination product comprises a cell population.

3. The method as defined in claim 2, wherein the cell population is obtained from a human or a mouse, such as a transgenic mouse.

30

4. The method as defined in claim 2 or 3, wherein the cell population comprises an immunoglobulin containing cell, such as a white blood cell, in particular a small lymphocyte such as a T cell or a B cell.

5. The method as defined in claim 1, wherein the nucleic acid sample comprising a VDJ recombination product comprises a library of VDJ recombined nucleic acids obtained from an *in vitro* antibody production system, such as ribosome display.

6. The method as defined in any one of claims 1 to 5, wherein the VDJ recombination product is derived from a heavy chain immunoglobulin.

7. The method as defined in any one of claims 1 to 5, wherein the VDJ recombination product is derived from a light chain immunoglobulin.

8. The method as defined in any one of claims 1 to 5, wherein the VDJ recombination product is derived from a T cell receptor.

9. The method as defined in any one of claims 1 to 8, wherein the nucleic acid comprises DNA or RNA obtained from the sample, such as cDNA or mRNA, in particular cDNA.

10. The method as defined in any one of claims 2 to 9, wherein the cell population comprises mouse cells and the primers are selected from any of the primers described in SEQ ID NOS: 1 to 4 or 47 to 50.

11. The method as defined in any one of claims 2 to 9, wherein the cell population comprises human cells and the primers are selected from any of the primers described in SEQ ID NOS: 51 to 56.

12. The method as defined in any one of claims 1 to 11, wherein the nucleic acid sample is fragmented by sonication.

13. The method as defined in any one of claims 1 to 11, wherein the nucleic acid sample is fragmented by performing a restriction endonuclease reaction.

14. The method as defined in claim 13, wherein the restriction endonuclease enzyme used in step (b) is selected from *DpnII* and/or *NlaIII*.

15. The method as defined in claim 13 or claim 14, wherein step (b) comprises the use of a third restriction endonuclease enzyme, such as an enzyme which is specific for a region upstream of the V gene.

5

16. The method as defined in any one of claims 1 to 15, wherein step (c) additionally comprises the step of performing primer extension, oligonucleotide hybridization and/or reverse transcription.

10 17. The method as defined in any one of claims 1 to 16, wherein step (c) comprises tagging the digested fragments with one member of a binding pair, such as biotin and streptavidin or an antigen and an antibody.

18. The method as defined in any one of claims 1 to 16, which additionally
15 comprises the addition of a first adapter molecule to one end of the VDJ recombination product following step (c).

19. The method as defined in claim 18, wherein the restriction endonuclease used in step (b) is *NlaIII* and the adapter molecule is selected from SEQ ID NO:
20 5.

20. The method as defined in claim 18, wherein the restriction endonuclease used in step (b) is *DpnII* and the adapter molecule is selected from SEQ ID NO: 6.

25

21. The method as defined in any one of claims 18 to 20, which additionally comprises the addition of a second adapter molecule to a second end of the VDJ recombination product.

30 22. The method as defined in claim 21, wherein the cell population comprises mouse cells and the primers for the addition of the second adaptor are selected from SEQ ID NOS: 7 to 11 or 57 to 65.

23. The method as defined in claim 21, wherein the cell population comprises human cells and the primers for the addition of the second adaptor are selected from SEQ ID NOS: 66 to 72.

5 24. The method as defined in any one of claims 1 to 23, wherein step (d) comprises the use of oligonucleotides specific to the upstream region of each of the J genes on both strands.

25. The method as defined in claim 24, wherein the cell population comprises
10 mouse cells and the oligonucleotides used in step (d) are selected from SEQ ID NOS: 12 to 19 or 24 to 34.

26. The method as defined in claim 24, wherein the cell population comprises human cells and the oligonucleotides used in step (d) are selected from SEQ ID
15 NOS: 39 to 46.

27. A kit for identifying VDJ recombination products which comprises instructions to use said kit in accordance with the methods as defined in any one of claims 1 to 26.

20

28. The kit as defined in claim 27, which additionally comprises nucleic acid extraction reagents configured to obtain the nucleic acid sample required for step (a) of the method defined in claim 1.

25 29. The kit as defined in claim 27 or claim 28, which additionally comprises oligonucleotides or primers specific for a unique region within or immediately downstream of each of the J genes, or within the constant region.

30. The kit as defined in any one of claims 27 to 29, which additionally
30 comprises one or more restriction endonuclease enzymes.

31. The kit as defined in any one of claims 27 to 30, which additionally comprises a computer readable storage medium configured to process the

sequencing data obtained in step (e) and generate a visual representation of VDJ recombination frequencies in the sample.

32. A VDJ recombination product obtainable by the method as defined in any 5 one of claims 1 to 26.

33. The method as defined in any one of claims 1 to 26 or the VDJ recombination product as defined in claim 32, for use in monitoring an immunodeficiency disorder.

10

34. The method or VDJ recombination product as defined in claim 33, wherein the immunodeficiency disorder is selected from a lymphoma or leukemia.

AMENDED CLAIMS
received by the International Bureau on 05 August 2013 (05.08.2013)

**CLAIMS**

1. A method of identifying a VDJ recombination product which comprises the following steps:

(a) obtaining a DNA sample comprising a VDJ recombination product;

(b) fragmenting the VDJ recombination product either by sonication, shearing or performing a restriction endonuclease reaction at a first site downstream of each of the J genes or downstream of the constant region, and a second site within or immediately upstream of the V gene to generate digested VDJ recombined fragments and unrecombined J fragments;

(c) annealing oligonucleotides to the digested fragments at unique regions within or immediately downstream of each of the J genes;

(d) separating the digested VDJ recombined fragments from the unrecombined J fragments and the rest of the genome;

(e) sequencing the VDJ recombined fragments or products; and

(f) data processing of the sequencing data to identify each VDJ recombination product and quantify VDJ recombination frequencies.

2. The method as defined in claim 1, wherein the DNA sample comprising a VDJ recombination product comprises a cell population.

3. The method as defined in claim 2, wherein the cell population is obtained from a human or a mouse, such as a transgenic mouse.

4. The method as defined in claim 2 or 3, wherein the cell population comprises an immunoglobulin containing cell, such as a white blood cell, in particular a small lymphocyte such as a T cell or a B cell.

5. The method as defined in claim 1, wherein the DNA sample comprising a VDJ recombination product comprises a library of VDJ recombined DNA samples obtained from an *in vitro* antibody production system, such as ribosome display.

6. The method as defined in any one of claims 1 to 5, wherein the VDJ recombination product is derived from a heavy chain immunoglobulin.

7. The method as defined in any one of claims 1 to 5, wherein the VDJ recombination product is derived from a light chain immunoglobulin.

8. The method as defined in any one of claims 1 to 5, wherein the VDJ recombination product is derived from a T cell receptor.

9. The method as defined in any one of claims 2 to 8, wherein the cell population comprises mouse cells and the primers are selected from any of the primers described in SEQ ID NOS: 1 to 4 or 47 to 50.

10. The method as defined in any one of claims 2 to 8, wherein the cell population comprises human cells and the primers are selected from any of the primers described in SEQ ID NOS: 51 to 56.

11. The method as defined in any one of claims 1 to 10, wherein the DNA sample is fragmented by sonication.

12. The method as defined in any one of claims 1 to 10, wherein the DNA sample is fragmented by performing a restriction endonuclease reaction.

13. The method as defined in claim 12, wherein the restriction endonuclease enzyme used in step (b) is selected from *DpnII* and/or *NlaIII*.

14. The method as defined in claim 12 or claim 13, wherein step (b) comprises the use of a third restriction endonuclease enzyme, such as an enzyme which is specific for a region upstream of the V gene.

15. The method as defined in any one of claims 1 to 14, wherein step (c) additionally comprises the step of performing primer extension, oligonucleotide hybridization and/or reverse transcription.

16. The method as defined in any one of claims 1 to 15, wherein step (c) comprises tagging the digested fragments with one member of a binding pair, such as biotin and streptavidin or an antigen and an antibody.

17. The method as defined in any one of claims 1 to 15, which additionally comprises the addition of a first adapter molecule to one end of the VDJ recombination product following step (c).

18. The method as defined in claim 17, wherein the restriction endonuclease used in step (b) is *NlaIII* and the adapter molecule is selected from SEQ ID NO: 5.

19. The method as defined in claim 17, wherein the restriction endonuclease used in step (b) is *DpnII* and the adapter molecule is selected from SEQ ID NO: 6.

20. The method as defined in any one of claims 17 to 19, which additionally comprises the addition of a second adapter molecule to a second end of the VDJ recombination product.

21. The method as defined in claim 20, wherein the cell population comprises mouse cells and the primers for the addition of the second adaptor are selected from SEQ ID NOS: 7 to 11 or 57 to 65.

22. The method as defined in claim 20, wherein the cell population comprises human cells and the primers for the addition of the second adaptor are selected from SEQ ID NOS: 66 to 72.

23. The method as defined in any one of claims 1 to 22, wherein step (d) comprises the use of oligonucleotides specific to the upstream region of each of the J genes on both strands.

24. The method as defined in claim 23, wherein the cell population comprises mouse cells and the oligonucleotides used in step (d) are selected from SEQ ID NOS: 12 to 19 or 24 to 34.

25. The method as defined in claim 23, wherein the cell population comprises human cells and the oligonucleotides used in step (d) are selected from SEQ ID NOS: 39 to 46.

26. A kit for identifying VDJ recombination products which comprises instructions to use said kit in accordance with the methods as defined in any one of claims 1 to 25.

27. The kit as defined in claim 26, which additionally comprises extraction reagents configured to obtain the DNA sample required for step (a) of the method defined in claim 1.

28. The kit as defined in claim 26 or claim 27, which additionally comprises oligonucleotides or primers specific for a unique region within or immediately downstream of each of the J genes, or within the constant region.

29. The kit as defined in any one of claims 26 to 28, which additionally comprises one or more restriction endonuclease enzymes.

30. The kit as defined in any one of claims 26 to 29, which additionally comprises a computer readable storage medium configured to process the sequencing data obtained in step (e) and generate a visual representation of VDJ recombination frequencies in the sample.

31. A VDJ recombination product obtainable by the method as defined in any one of claims 1 to 25.

32. The method as defined in any one of claims 1 to 25 or the VDJ recombination product as defined in claim 31, for use in monitoring an immunodeficiency disorder.

33. The method or VDJ recombination product as defined in claim 32, wherein the immunodeficiency disorder is selected from a lymphoma or leukemia.

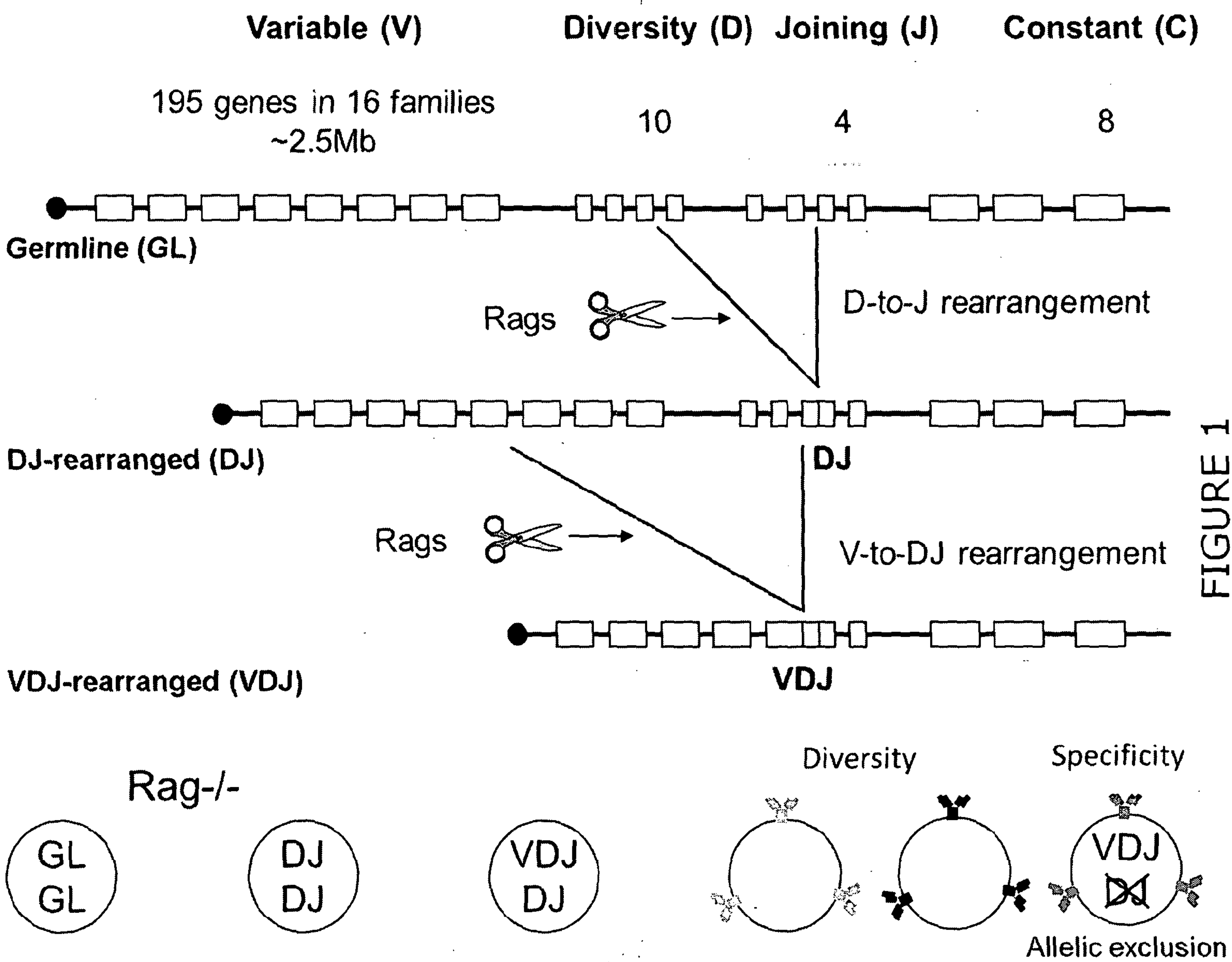


FIGURE 1

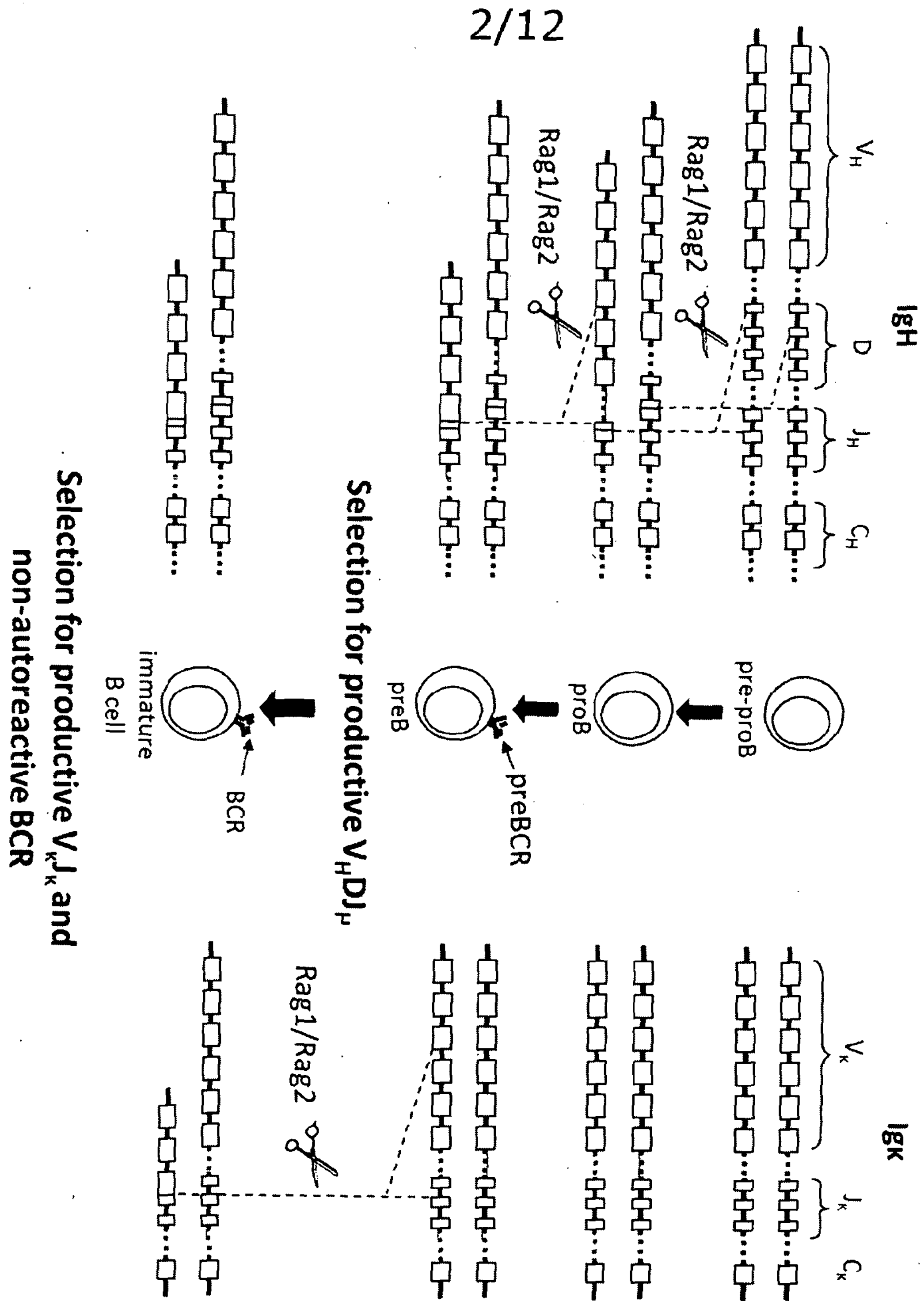


FIGURE 2

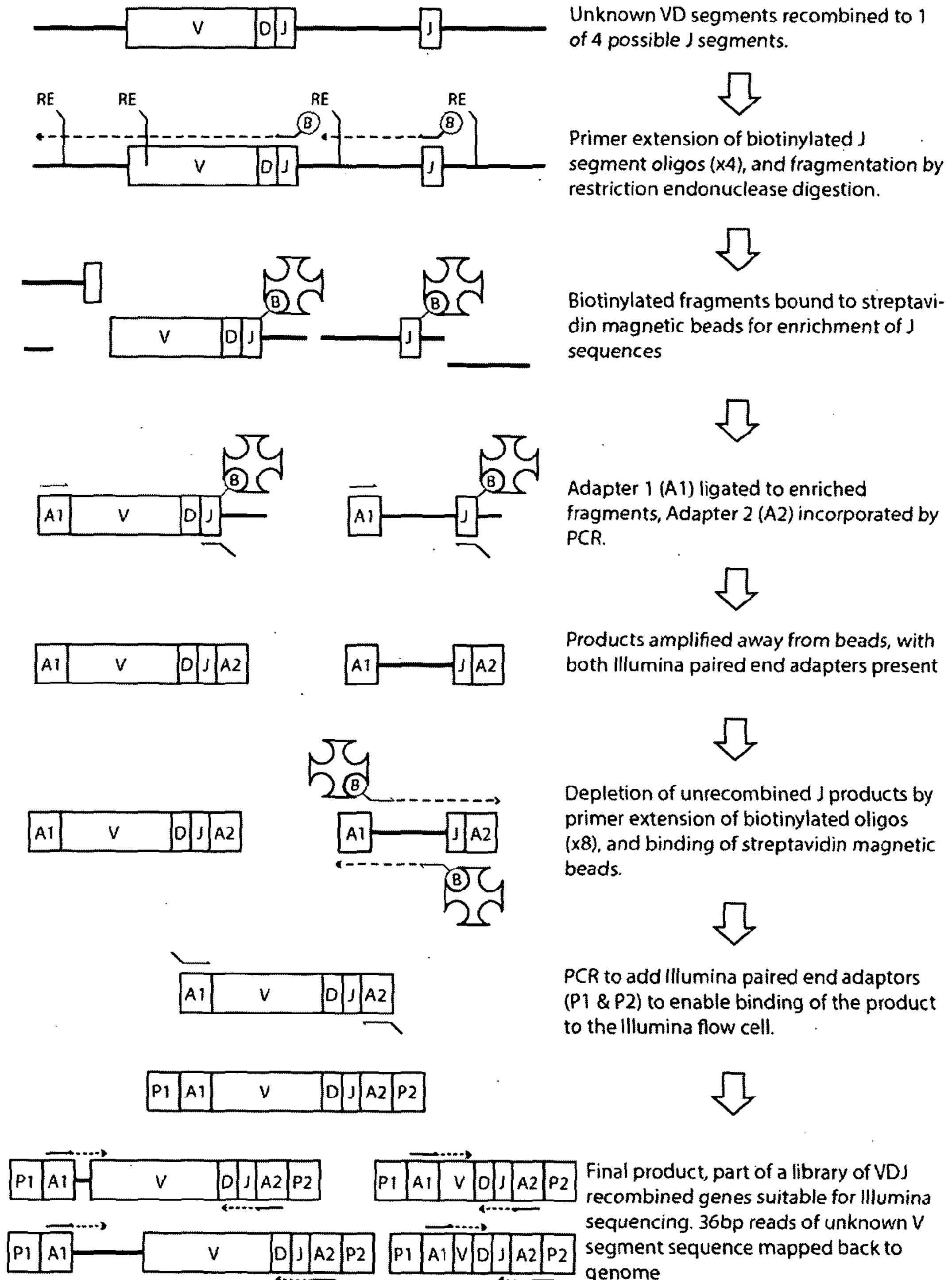


FIGURE 3

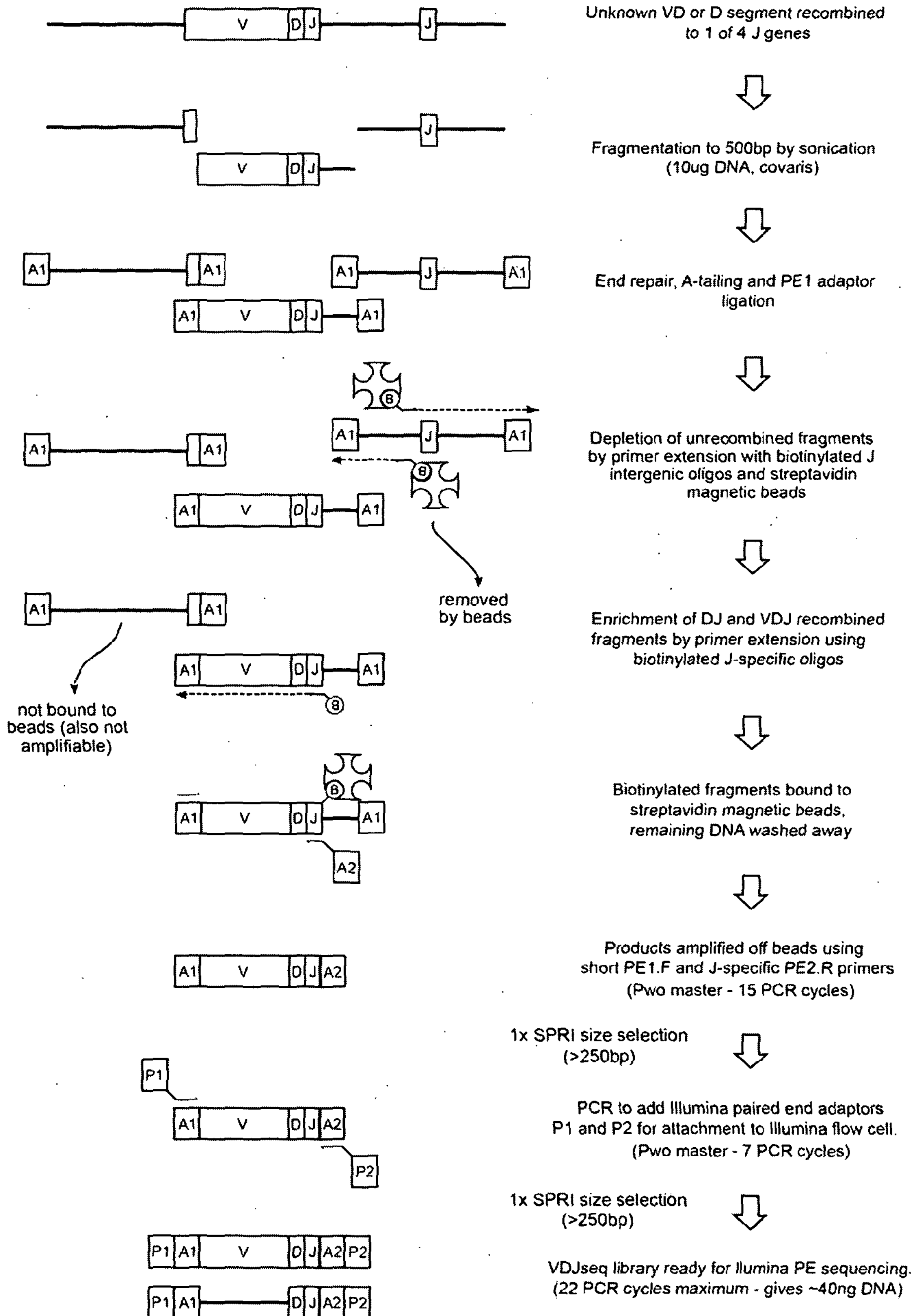


FIGURE 4

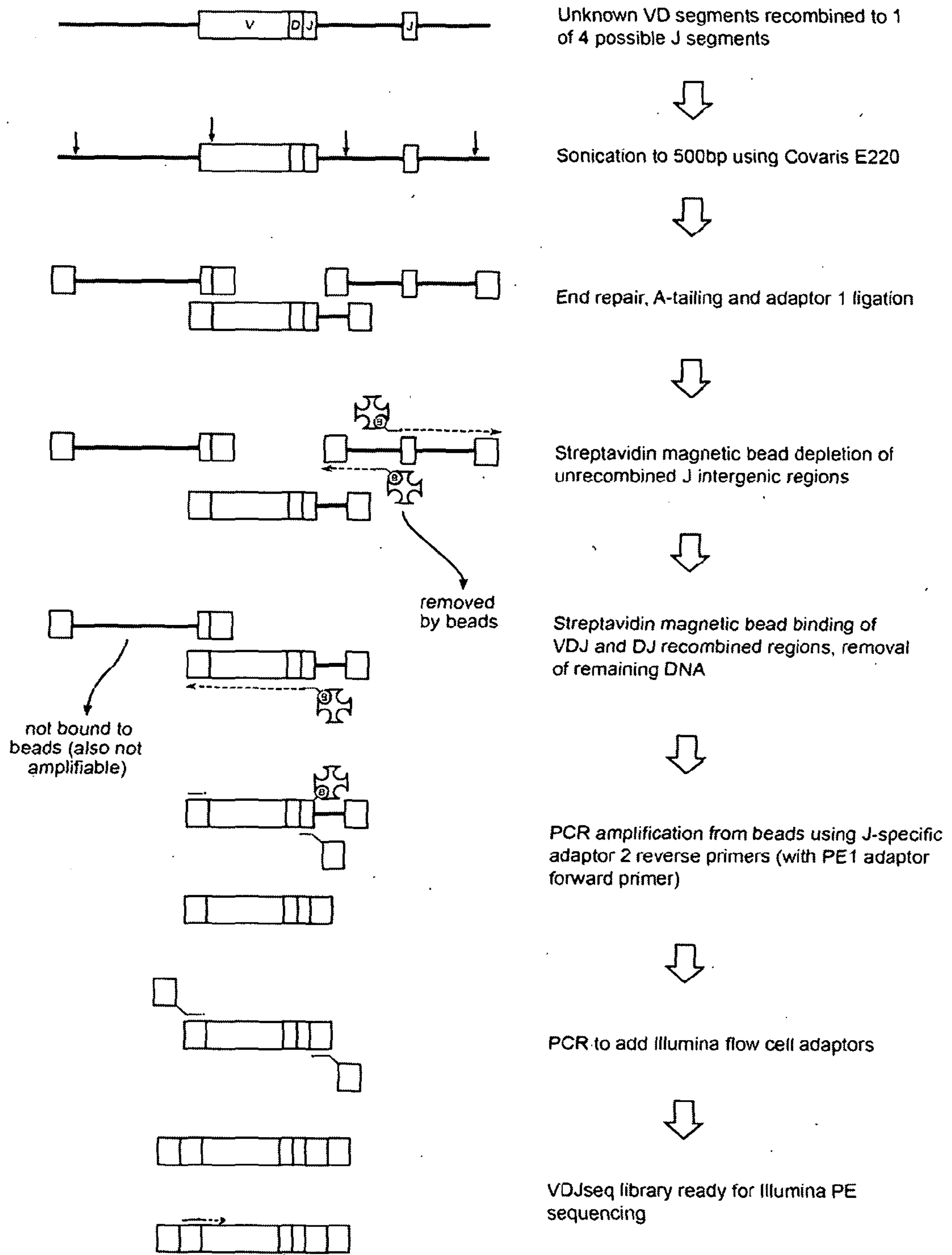


FIGURE 5

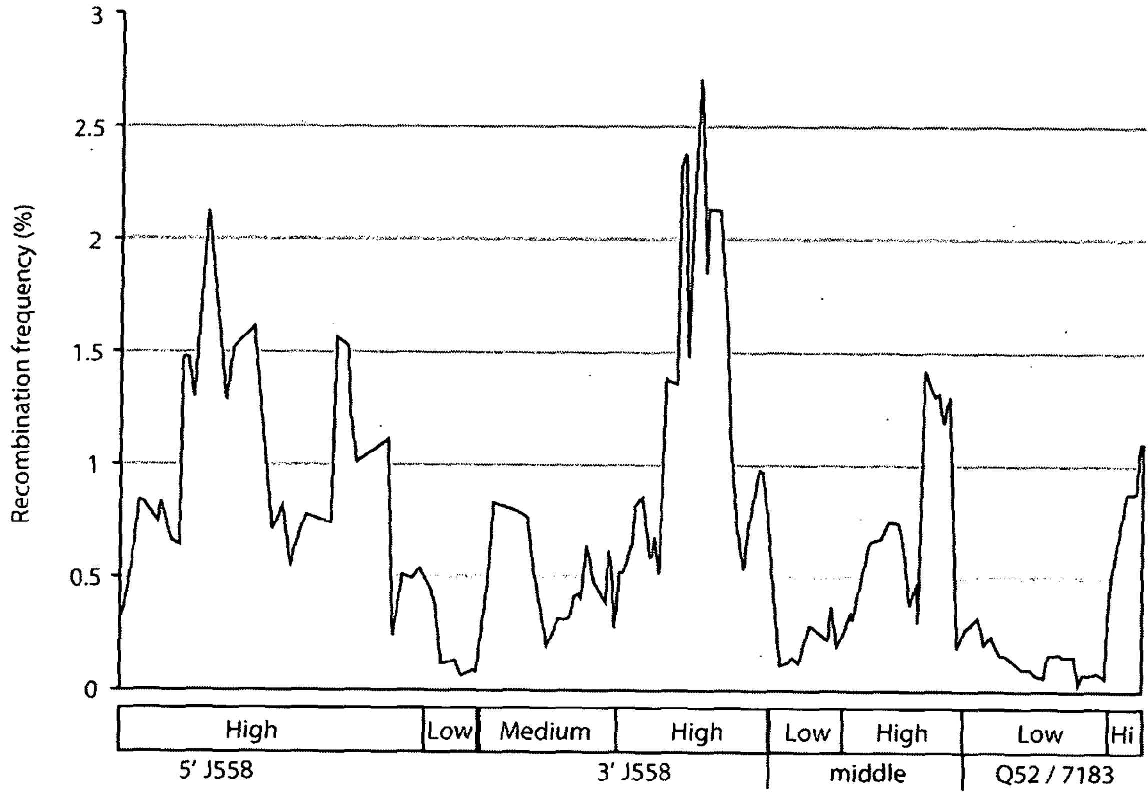


FIGURE 6

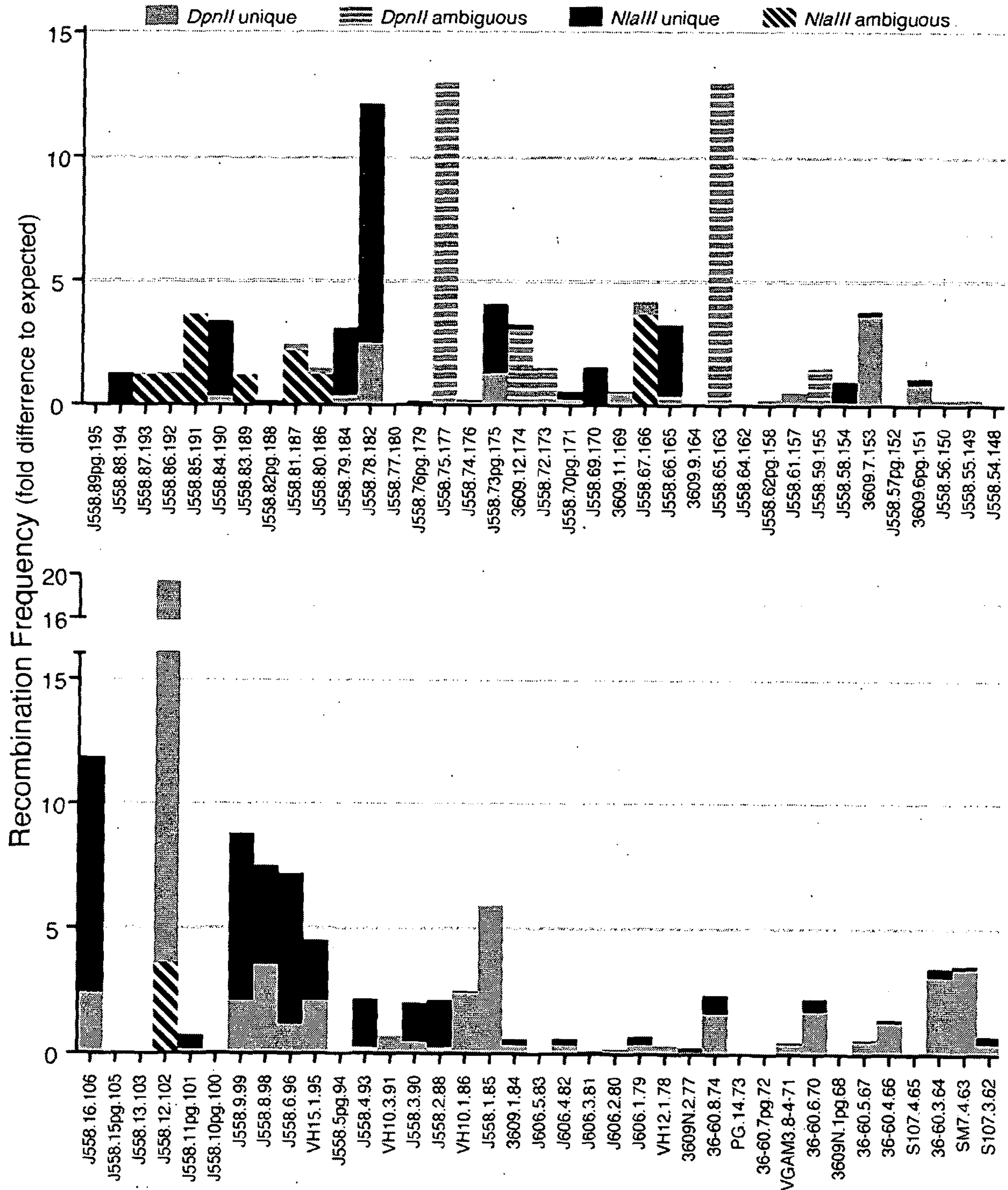


FIGURE 7

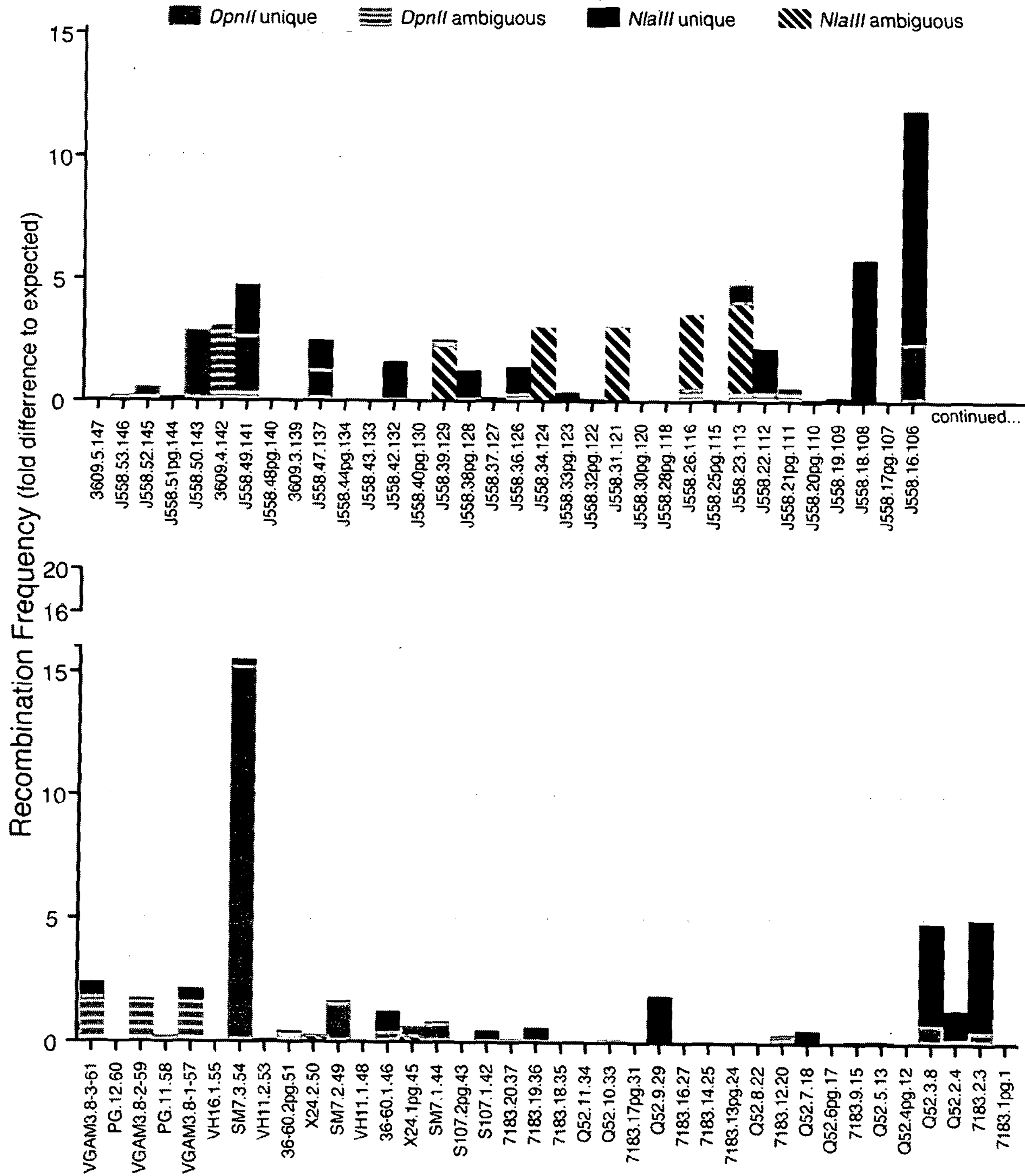


FIGURE 7 (ctd)

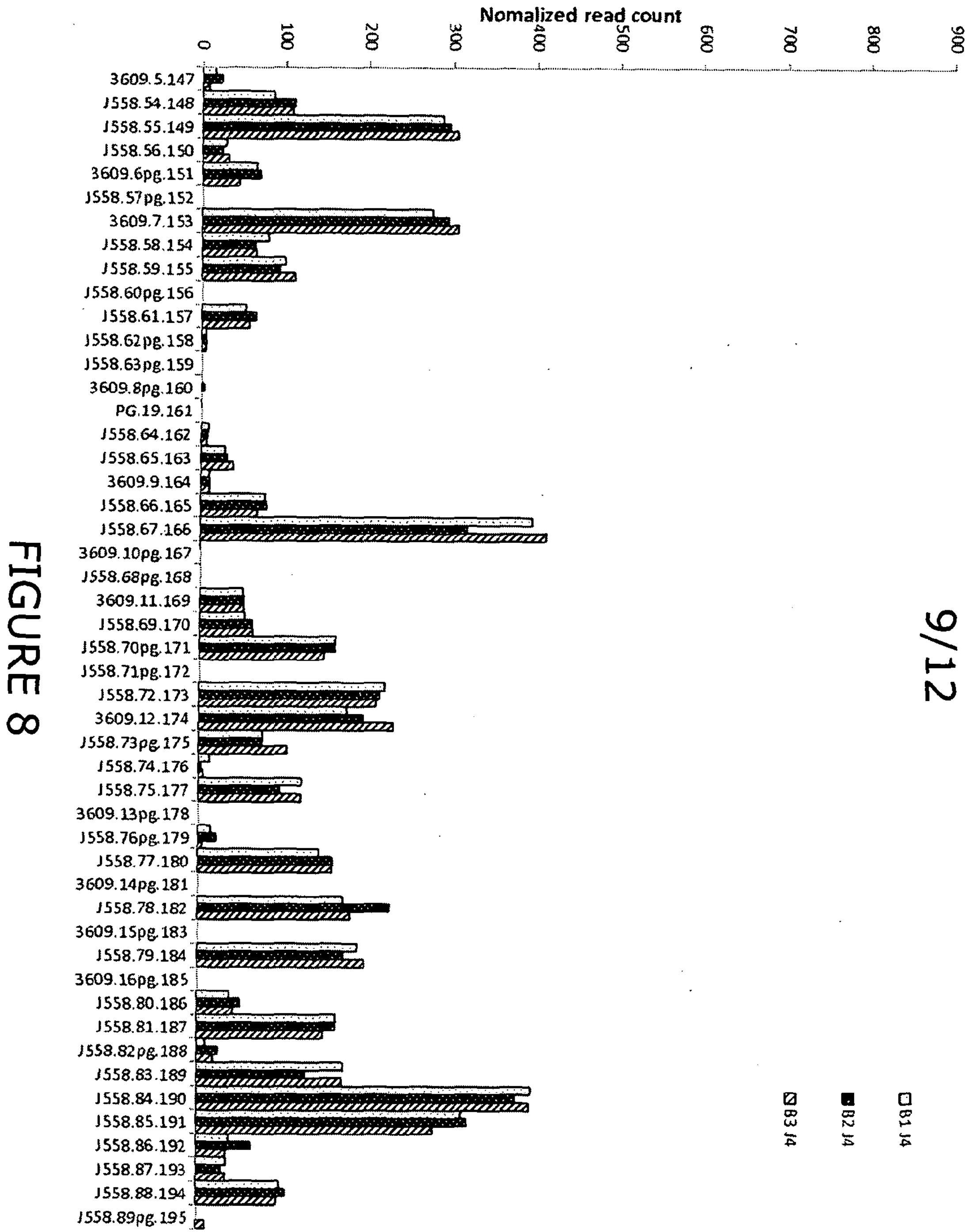


FIGURE 8

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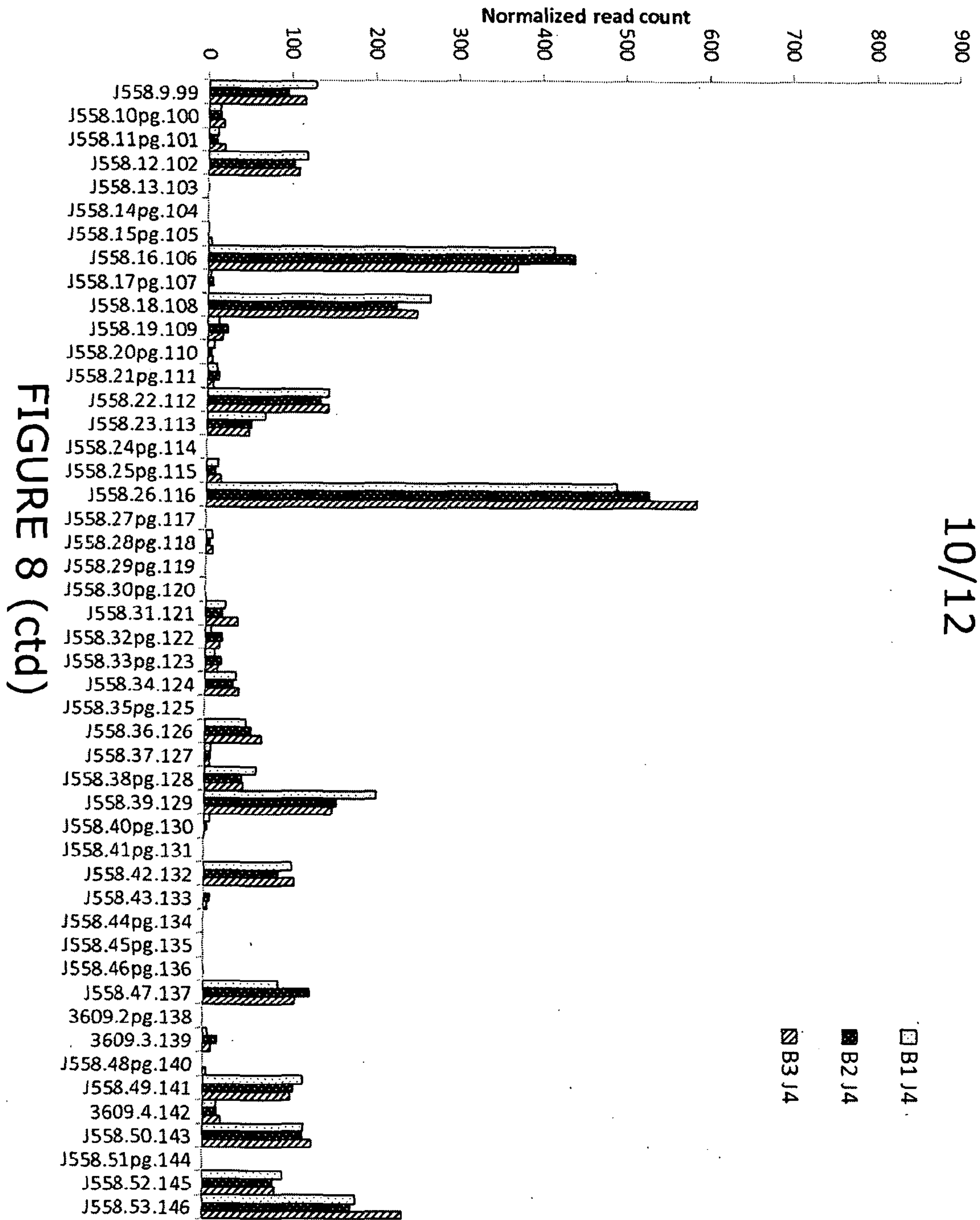


FIGURE 8 (ctd)

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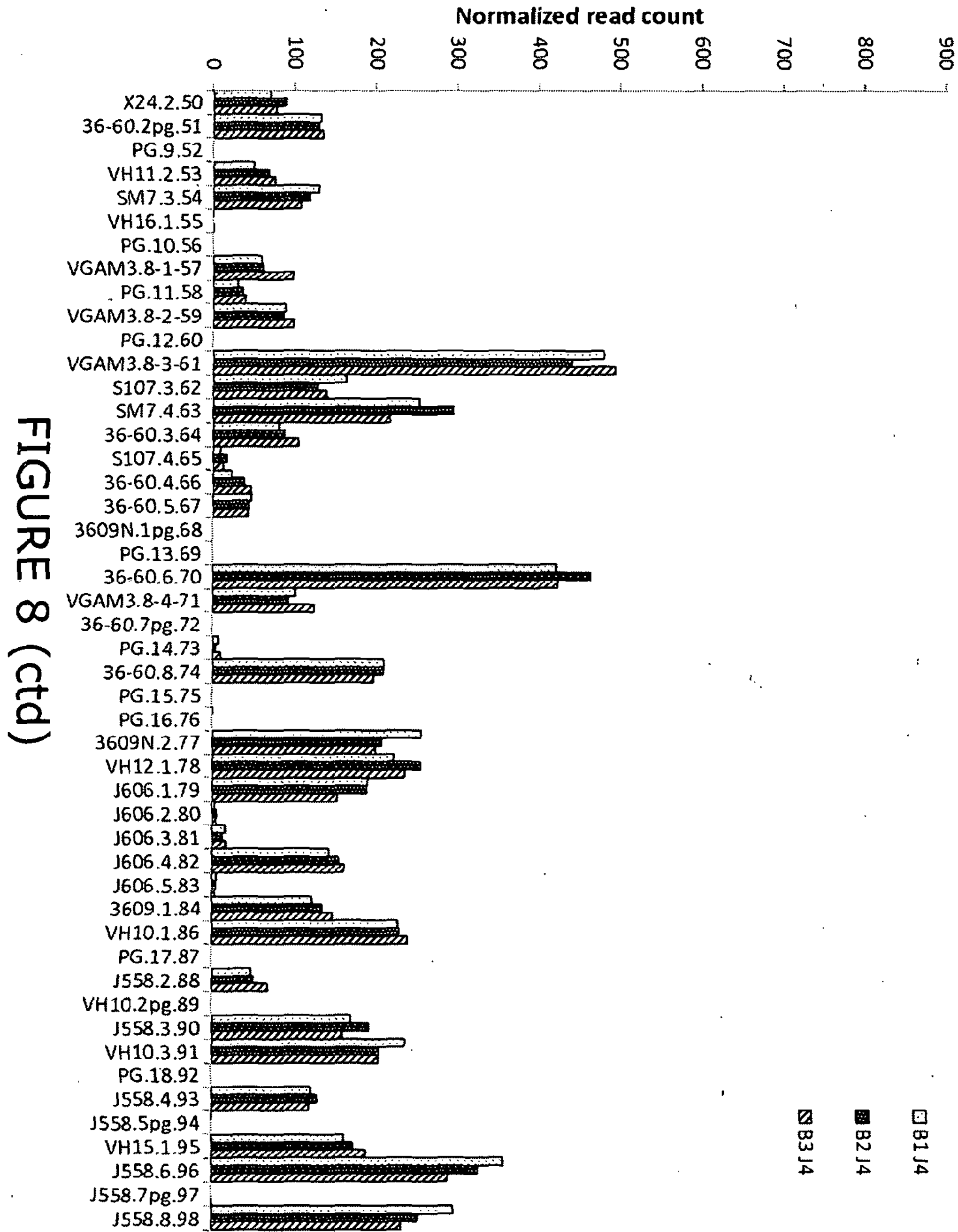


FIGURE 8 (ctd)

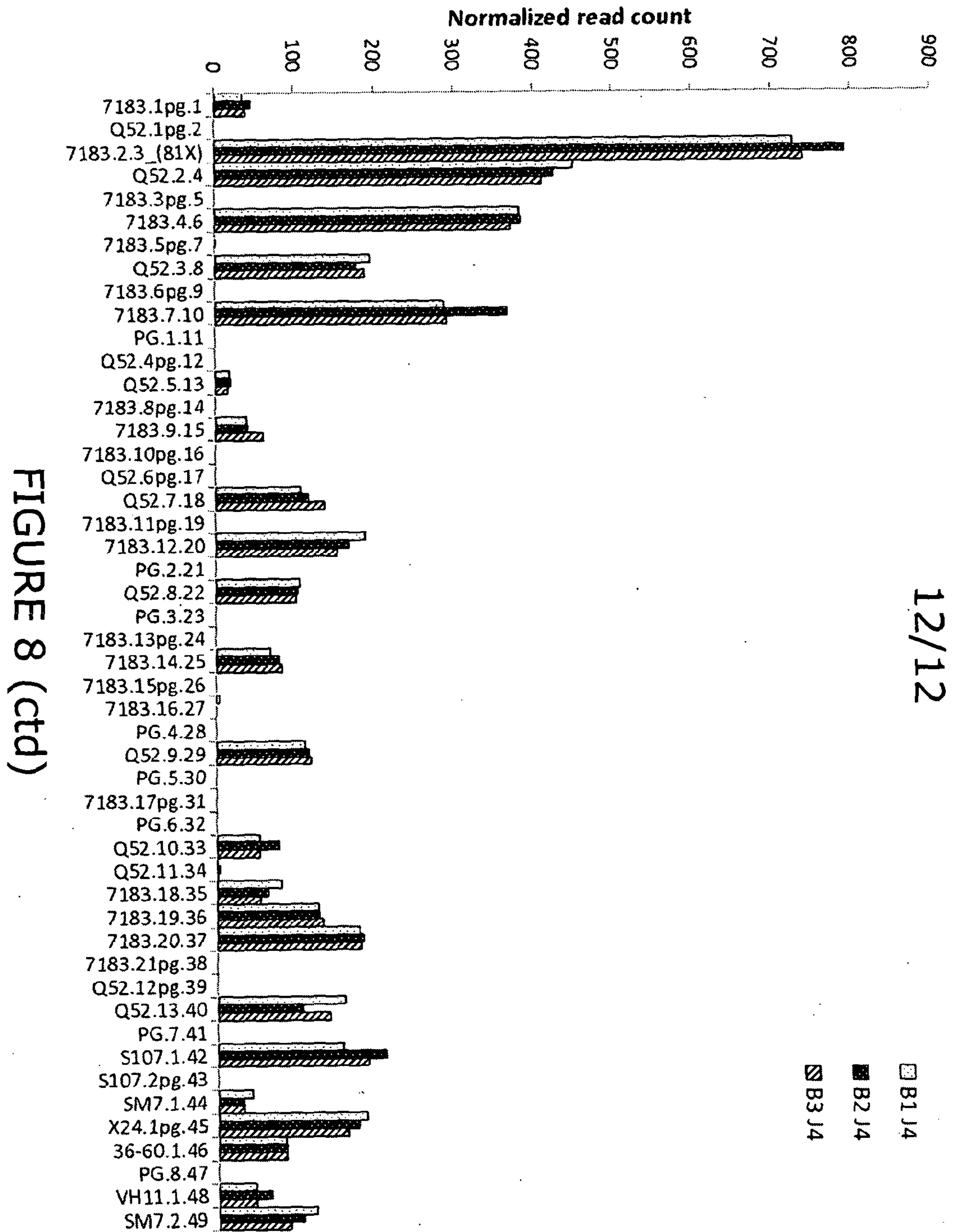


FIGURE 8 (ctd)

# INTERNATIONAL SEARCH REPORT

International application No
PCT/GB2013/050516

**A. CLASSIFICATION OF SUBJECT MATTER**
INV. C12Q1/68 C07K16/00
ADD.

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)
C12Q C07K

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)

EPO-Internal, BIOSIS, WPI Data

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[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

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"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 |
|---|---|
| 30 May 2013 | 05/06/2013 |

| Name and mailing address of the ISA/ | Authorized officer |
|---|---|
| European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016 | Bruma, Anja |

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Information on patent family members

International application No
PCT/GB2013/050516

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