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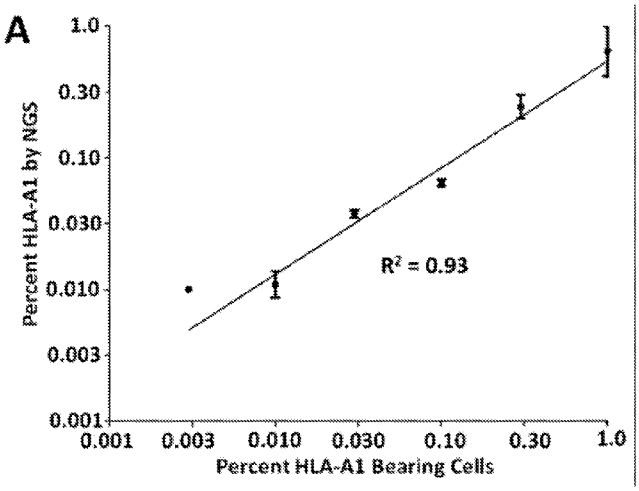
(54) Title: PLATFORM INDEPENDENT HAPLOTYPE IDENTIFICATION AND USE IN ULTRASENSITIVE DNA DETECTION

FIGURES 4A



(57) Abstract: The present invention provides methods for analyzing blocks of closely spaced SNPs, or haplotypes for use in identification of the origin of DNA in a sample. The methods comprise aligning common alleles of a gene of interest and identifying a region containing a plurality of SNPs which is flanked by non-polymorphic DNA which can be used for primer placement. Any sequencing method, including next generation sequencing methods can then be used to determine the haplotypes in the sample with a lower limit of detection of at least 0.01%. These inventive methods are useful, for example, for identification of hematopoietic stem cell transplantation patients destined to relapse, microchimerism associated with solid organ transplantation, detection of solid organ transplant rejection by detecting donor DNA in recipient plasma, forensic applications, and patient identification.

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PLATFORM INDEPENDENT HAPLOTYPE IDENTIFICATION AND USE IN ULTRASENSITIVE DNA DETECTION

REFERENCE TO RELATED APPLICATIONS

[0001] This application claims the benefit of U.S. Provisional Patent Application No. 62/033,254, filed on August 5, 2014, which is hereby incorporated by reference for all purposes as if fully set forth herein.

INCORPORATION-BY-REFERENCE OF MATERIAL SUBMITTED ELECTRONICALLY

[0002] The instant application contains a Sequence Listing which has been submitted in ASCII format via EFS-Web and is hereby incorporated by reference in its entirety. Said ASCII copy, created on August 5, 2014, is named P12978-01_ST25.txt and is 641 bytes in size.

BACKGROUND OF THE INVENTION

[0003] Myeloablative conditioning and allogeneic stem cell transplantation (alloSCT) has historically been limited to the treatment of lethal hematologic malignancies in children or young adults. More recently with the advent of highly immunosuppressive, non-myeloablative regimens, the clinical use of alloSCT has expanded to include older, less fit patients with hematologic malignancies as well as patients with non-malignant disorders such as sickle cell disease (SCD). Non-myeloablative conditioning regimens offer the additional safeguard of recovery of autologous hematopoiesis in the event of graft rejection and so may be a safer option in patients at risk for immune mediated rejection of the donor graft.

[0004] Chimerism testing at set intervals is an effective method for detecting graft rejection or recurrence of the original hematopoietic neoplasm after allogeneic HSCT (with either bone marrow or peripheral blood stem cells). Decades ago, bone marrow engraftment monitoring was performed using Southern blotting and minisatellite or Variable Number of Tandem Repeats (VNTR) loci. Today, short tandem repeat (STR), or microsatellite, loci are most commonly used for this purpose. STRs are composed of 10-60 tandemly repeated units, where each unit is 1-6 bases in length. They are widely distributed throughout the human genome, are highly variable between individuals, and therefore allow for excellent differentiation between individuals, including patient and donor, even if they are closely

related. Most laboratories use multiplex PCR based kits, originally developed for forensics analysis using Combined DNA Index System (CODIS) loci. STR analysis most commonly involves PCR amplification using fluorescently labeled primers, followed by amplicon separation by capillary electrophoresis.

[0005] Other polymorphic DNA that could be used to monitor bone marrow engraftment include single nucleotide polymorphisms (SNPs). SNPs are theoretically superior to STR based analyses because analysis of STR loci by capillary electrophoresis is relatively insensitive (limit of detection 1-5%) and microsatellite alleles of varying length amplify with different efficiencies, thus making them inherently biased. STR amplification can also be difficult in the setting of highly degraded DNA. However, SNPs are less attractive as targets due to their inherently lower informativity (e.g. only two possible bases for a bi-allelic SNP vs. 10 or more alleles for some microsatellites), requiring many more SNPs to be tested to identify those that distinguish donor from recipient. For example, we previously estimated that one would need to screen more than 20-30 individual SNPs to confidently identify one SNP where the donor is homozygous for one allele and an unrelated recipient is homozygous for the other. Fewer would need to be included if heterozygotes were included, but more would have to be analyzed for related individuals.

[0006] Recently emerging next generation sequencing (NGS) technologies along with their decreasing costs are now feasible for clinical testing. However, all NGS technologies currently have high error rates, in the range of 0.04% - 1% at each base, which precludes their use for ultrasensitive detection of a single SNP. One solution to this problem is sequencing blocks of closely spaced SNPs, i.e. haplotypes. Haplotypes are regions of the genome where polymorphic areas are sufficiently close that they are inherited together, including either genes (e.g. HLA-1 A, HLA-B, etc) within a locus, or multiple SNPs within a region of DNA.

SUMMARY OF THE INVENTION

[0007] In the present invention, the inventors first used the HLA-A locus as proof-of-principle to demonstrate a novel, inventive approach which permits high sensitivity, precision, and accuracy. These methods were then used to study bone marrow (BM) samples from a cohort of patients who engrafted after HSCT and tested as all donor by STRs, and found that low level patient DNA is commonly present. To identify additional loci that could be used for this purpose, the inventors used the inventive methods to comprehensively

analyze the human genome and identified other regions with highly informative haplotypes. These inventive methods can be used in many other situations where routine haplotyping of patient samples would improve patient safety.

[0008] In accordance with an embodiment, the present invention provides a method for identifying informative haplotypes useful for identity testing comprising: a) obtaining the DNA sequences of a plurality of individual genomes of a mammal; b) identifying within the genomes of a) haplotypes comprising both allelic DNA sequences of about 100 to 400 or more base pairs in length, having at least one or more polymorphic regions which are flanked at both the 5' and 3' ends with constant regions of at least about 20 base pairs in length; c) identifying within the haplotypes of b) those haplotypes which have at least about 2 or more single nucleotide polymorphism variants of the polymorphic regions; and d) identifying those haplotypes of c) as informative if at least 1 haplotype from a first individual genome has at least 2 or more single nucleotide polymorphism differences from both alleles of a second individual genome.

[0009] In accordance with another embodiment, the present invention provides a method for determining the DNA sequence of one or more informative haplotypes in a DNA sample of a mammal comprising: a) obtaining a sample containing a sufficient amount of DNA which comprises at least about 100 to about 100,000 genomes of the mammal; b) purifying the DNA from a); c) amplifying the DNA from b) using PCR and primers and probes specific for one or more informative haplotypes and for the sequencing method (including Sanger sequencing, pyrosequencing, etc.) being used to analyze the DNA sequences of the informative haplotypes; d) analyzing the plurality of DNA sequences of the amplified informative haplotypes for single nucleotide polymorphisms in c); e) comparing the DNA sequence single nucleotide polymorphisms found in d) to the DNA sequence single nucleotide polymorphisms for one or more reference informative haplotypes, wherein when a DNA sequence of the one or more informative haplotypes from d) does not contain all of the single nucleotide polymorphisms of the one or more reference informative haplotypes, the DNA sequence is discarded as erroneous; and f) establishing that when a DNA sequence of the one or more informative haplotypes from d) contains all of the single nucleotide polymorphisms of the one or more reference informative haplotypes, the DNA sequence is a match and the haplotype identity is confirmed.

BRIEF DESCRIPTION OF THE DRAWINGS

[0010] Figures 1A-1D depict an embodiment of the haplotype identification methods of the present invention. (1A) Shown is a theoretical locus containing 4 possible SNPs, where the donor (left) is homozygous adenine at each of the SNPs for both alleles and the patient (recipient, right) is homozygous cytosine for both alleles. (1B) In a post-transplant sample, one detects 10,000 reads that perfectly match the donor genotype, 10 reads with a cytosine (yellow) at the third SNP and 10 reads with a cytosine at the first SNP (yellow). The 20 reads with a single cytosine can be interpreted as PCR errors since they do not perfectly match either donor or patient alleles. Accordingly, these results would be interpreted as 100% donor. (1C) Another post-transplant sample contains 9,900 reads that perfectly match the donor haplotype, while 100 reads perfectly match the patient haplotype. Accordingly this sample is 1% patient. (1D) The distal region of HLA-A exon 3 (blue rectangle) and intron 3 (blue line) containing 18 potential SNPs (indicated with "X"s) and two HLA-A PCR primers (yellow rectangles) as shown. Note that the PCR primers are tailed with adapters (orange) for the DNA sequencing primer to bind (A-adapter) or to covalently anchor the amplicon onto the bead (P1-adapter). The total length of the HLA targeted region is 245 bp and the total length of the amplicon is 298 bp, including the adapters. Not shown is the 4-base library key located between the A-adaptor and the forward HLA-A primer.

[0011] Figure 2 depicts numbers of discriminating SNPs between common HLA-A alleles. Common European-origin HLA-A alleles were collected, aligned, and number of discriminating SNPs between them determined. The homozygous genotypes analyzed in Figure 3, A*01 and A*02, differ by 11 SNPs (double box). The genotypes from the dilution series in Figure 4 are A*01/A*02 into A*02/A*1 24, where the unique alleles vary by 7 SNPs (bold box). Other combinations vary by fewer SNPs, such as A*68 vs. A*02 (dashed box). Included are only alleles that occur at 1% or higher in the European origin population.

[0012] Figures 3A-3C show molecular specificity of the inventive methods. Samples homozygous for the A*01 allele (panel 3A) and the A*02 allele (panel 3B) were PCR amplified and NGS sequenced. Each was analyzed for the presence of the other allele and for hybrid molecules containing one or more SNPs of the other allele. (3C) "Waterfall" plot of the percent reads for each combination of the eleven SNP differences for the two samples. For example, the "10A1 + 1A2" bar reflects the mean number of erroneous reads (9/11=0.82), in the pure A*01 sample where any one of the 11 SNP positions matches the

A*02 allele (0.82/201,605, from panel 3A). Likewise the "9A1 +2A2" bar reflects the lack of any reads in the pure A*01 sample where 2 adjacent SNPs match the A*02 allele at any of the positions.

[0013] Figures 4A-4B show the linearity of using the inventive methods for NGS haplotyping and microsatellite analysis. (4A) Linearity of the SNP haplotyping approach. A*01/A*02 cells were serially diluted into A*02/A*24 cells, DNA isolated, and tested by HLA-A NGS. NGS HLA-A*01 counts expressed as a percentage, as a function of the percent HLA-A*01 bearing cells. Error bars: +/- 1 SEM. The error bar for 0.1% bearing cells is too small to be seen. The error bar for 0.003% is absent as only 1 out of 2 replicates amplified. Note that the NGS haplotyping data begins at 1% and is serially diluted down 1 from there. (4B) Post-transplant patient samples with various percentage of patient DNA were analyzed using the Profiler and Identifiler kits. Note the lack of the ability to detect DNA below the limit of detection of 3%.

[0014] Figure 5 depicts the identification of patient DNA in bone marrow samples. Unique patient DNA was detected in BM samples, which demonstrated all donor by conventional STR assays, after HSCT.

[0015] Figures 6A-6B show that HCT116 cells were serially diluted into DLD-1 cells and DNA co-isolated. HLA-A based haplotype counting was used to determine the true concentration (x-axis) and mutations detected in the samples using the AmpliSeq panel (Ion Torrent, Life Technologies, panel 6A) or ddPCR (Bio-Rad, panel 6B).

[0016] Figure 7 depicts donor molecules per million as a function of time after bone marrow infusion in a patient following limb transplantation.

[0017] Figure 8 is a graph showing Non-Relapse vs Relapse bone marrow transplant patients detected by haplotype counting. For relapse patients, the sample tested was immediately prior to that which tested positive by STR analysis. For non-relapse patients, the sample tested was followed by 291 days on average and remained fully engrafted.

[0018] Figure 9 shows alignments of novel *FARP1* alleles illustrate SNP positions (red) that either match or vary from the reference genome (*FARP1-1*). We have identified 5 new haplotypes (*FARP1-2* to *FARP1-6*) where bases that deviate from reference are highlighted in yellow.

[0019] Figure 10 depicts the haplotypes of *FARP1* locus compared. This figure demonstrates the number of SNPs that vary between any two *FARP1* haplotypes. For

example, if comparing 2 individuals, one homozygous for *FARP1-5* and another homozygous for *FARP1-3*, these will vary by 8 SNPs (highlighted in bold).

DETAILED DESCRIPTION OF THE INVENTION

[0020] A general schematic of an embodiment of the inventive methods for determining the DNA sequence of one or more informative haplotypes in a DNA sample of a mammal is demonstrated in Figure 1. Imagine a region of a gene that contains 4 SNPs, and two individuals: a donor who is homozygous for adenine at all 4 SNPs (designated homozygous haplotype A), and a patient (recipient) who is homozygous for cytosine at all 4 SNPs (homozygous haplotype C, Figure 1A). In a sample that is pure donor (top bar, 10,000 reads), due to the high error rates of NGS, mutations will be seen by NGS, some of which will coincidentally occur at SNP positions (Figure 1B, bottom 2 bars, 10 reads each). Since these involve only 1 of the 4 SNPs, these can be attributed as PCR errors and discarded since these molecules do not match either the donor or patient alleles. Note that if the strategy used only a single SNP, instead of a haplotype, one would be unable to distinguish between 10 true patient reads vs. 10 PCR error reads. In contrast, if 100 molecules are detected in the post-transplant sample with cytosine at all 4 SNPs, this would indicate the presence of true patient-specific DNA (Figure 1C).

[0021] By "nucleic acid" as used herein includes "polynucleotide," "oligonucleotide," and "nucleic acid molecule," and generally means a polymer of DNA or RNA, which can be single-stranded or double-stranded, synthesized or obtained (e.g., isolated and/or purified) from natural sources, which can contain natural, non-natural or altered nucleotides, and which can contain a natural, non-natural or altered internucleotide linkage, such as a phosphoroamidate linkage or a phosphorothioate linkage, instead of the phosphodiester found between the nucleotides of an unmodified oligonucleotide.

[0022] The nucleic acids used as primers in embodiments of the present invention can be constructed based on chemical synthesis and/or enzymatic ligation reactions using procedures known in the art. See, for example, Sambrook et al. (eds.), *Molecular Cloning, A Laboratory Manual*, 3rd Edition, Cold Spring Harbor Laboratory Press, New York (2001) and Ausubel et al., *Current Protocols in Molecular Biology*, Greene Publishing Associates and John Wiley & Sons, NY (1994). For example, a nucleic acid can be chemically synthesized using naturally occurring nucleotides or variously modified nucleotides designed to increase the biological

stability of the molecules or to increase the physical stability of the duplex formed upon hybridization (e.g., phosphorothioate derivatives and acridine substituted nucleotides). Examples of modified nucleotides that can be used to generate the nucleic acids include, but are not limited to, 5-fluorouracil, 5-bromouracil, 5-chlorouracil, 5-iodouracil, hypoxanthine, xanthine, 4-acetylcytosine, 5-(carboxyhydroxymethyl) uracil, 5-carboxymethylaminomethyl-2-thiouridine, 5-carboxymethylaminomethyluracil, dihydrouracil, beta-D-galactosylqueosine, inosine, N^6-isopentenyladenine, 1-methylguanine, 1-methylinosine, 2,2-dimethylguanine, 2-methyladenine, 2-methylguanine, 3-methylcytosine, 5-methylcytosine, N^6-substituted adenine, 7-methylguanine, 5-methylaminomethyluracil, 5-methoxyaminomethyl-2-thiouracil, beta-D-mannosylqueosine, 5'-methoxycarboxymethyluracil, 5-methoxyuracil, 2-methylthio-N^6-isopentenyladenine, uracil-5-oxyacetic acid (v), wybutoxosine, pseudouracil, queosine, 2-thiocytosine, 5-methyl-2-thiouracil, 2-thiouracil, 4-thiouracil, 5-methyluracil, uracil-5-oxyacetic acid methylester, 3-(3-amino-3-N-2-carboxypropyl) uracil, and 2,6-diaminopurine. Alternatively, one or more of the nucleic acids of the invention can be purchased from companies, such as Macromolecular Resources (Fort Collins, CO) and Synthegen (Houston, TX).

[0023] The nucleotide sequences used herein are those which hybridize under stringent conditions preferably hybridizes under high stringency conditions. By "high stringency conditions" is meant that the nucleotide sequence specifically hybridizes to a target sequence (the nucleotide sequence of any of the nucleic acids described herein) in an amount that is detectably stronger than non-specific hybridization. High stringency conditions include conditions which would distinguish a polynucleotide with an exact complementary sequence, or one containing only a few scattered mismatches from a random sequence that happened to have a few small regions (e.g., 3-10 bases) that matched the nucleotide sequence. Such small regions of complementarity are more easily melted than a full-length complement of 14-17 or more bases, and high stringency hybridization makes them easily distinguishable. Relatively high stringency conditions would include, for example, low salt and/or high temperature conditions, such as provided by about 0.02-0.1 M NaCl or the equivalent, at temperatures of about 50-70 °C.

[0024] "Probe" as used herein may mean an oligonucleotide capable of binding to a target nucleic acid of complementary sequence through one or more types of chemical bonds, usually through complementary base pairing, usually through hydrogen bond formation. Probes may bind target sequences lacking complete complementarity with the probe

sequence depending upon the stringency of the hybridization conditions. There may be any number of base pair mismatches which will interfere with hybridization between the target sequence and the single stranded nucleic acids described herein. However, if the number of mutations is so great that no hybridization can occur under even the least stringent of hybridization conditions, the sequence is not a complementary target sequence. A probe may be single stranded or partially single and partially double stranded. The strandedness of the probe is dictated by the structure, composition, and properties of the target sequence. Probes may be directly labeled or indirectly labeled such as with biotin to which a streptavidin complex may later bind.

[0025] In accordance with one or more embodiments of the present invention, it will be understood that the term "biological sample" or "biological fluid" includes, but is not limited to, any quantity of a substance from a living or formerly living patient or mammal. Such substances include, but are not limited to, blood, serum, plasma, urine, cells, organs, tissues, bone, bone marrow, lymph, lymph nodes, synovial tissue, chondrocytes, synovial macrophages, endothelial cells, and skin. In a preferred embodiment the biological sample is a breast tissue sample, and more preferably, a breast tumor tissue sample. In other embodiments, the samples can be derived from potential donor organs for use in tissue typing for transplantation, for example, kidney, heart and other organs can be sampled and tested using the inventive methods.

[0026] In accordance with an embodiment, the present invention provides a method for identifying informative haplotypes useful for identity testing comprising: a) obtaining the DNA sequences of a plurality of individual genomes of a mammal; b) identifying within the genomes of a) haplotypes comprising both allelic DNA sequences of about 100 to 400 or more base pairs in length, having at least one or more polymorphic regions which are flanked at both the 5' and 3' ends with constant regions of at least about 20 base pairs in length; c) identifying within the haplotypes of b) those haplotypes which have at least about 2 or more single nucleotide polymorphism variants of the polymorphic regions; and d) identifying those haplotypes of c) as informative if at least 1 haplotype from a first individual genome has at least 2 or more single nucleotide polymorphism differences from both alleles of a second individual genome.

[0027] As used herein, the term "single nucleotide polymorphism or SNP" means a DNA sequence variation occurring commonly within a population (e.g. 1%) in which a single nucleotide — A, T, C or G — in the genome (or other shared sequence) differs between

members of a biological species or paired chromosomes. For example, two sequenced DNA fragments from different individuals, AAGCCTA to AAGCTTA, may contain a difference in a single nucleotide. In this case this would be defined as two alleles. The vast majority of common SNPs have only two alleles. The genomic distribution of SNPs is not homogenous; SNPs occur in non-coding regions more frequently than in coding regions or, in general, where natural selection is acting and fixating the allele of the SNP that constitutes the most favorable genetic adaptation. Other factors, like genetic recombination and mutation rate, can also determine SNP density. In accordance with the present invention, the inventive methods are able to detect two or more SNPs between haplotypes of a specific gene locus of interest.

[0028] As used herein, the term "haplotype" means an allelic DNA sequence of interest having about 100 to 400 or more base pairs in length, and having at least one or more polymorphic regions which are flanked at both the 5' and 3' ends with constant regions of at least about 20 base pairs in length; and wherein the haplotypes have at least about 1 or 2 or more single nucleotide polymorphism variants of the polymorphic regions.

[0029] The term "constant region" means the portion of the haplotype sequence where there are no polymorphisms or SNPs found.

[0030] As used herein, the term "next generation sequencing, or NGS" refers to a number of different modern sequencing technologies including Illumina (Solexa, MiSeq, HiSeq and NextSeq) sequencing, Roche 454 sequencing, Ion torrent: Proton / PGM sequencing, and ABI/Life Technologies SOLiD sequencing, Oxford Nanopore sequencing, Pacific Biosciences'RS II (Menlo Park, CA) which are known in the art.

[0031] As used herein, it will be understood that any high-throughput technique for sequencing nucleic acids can be used in the methods of the present invention. DNA sequencing techniques include classic dideoxy sequencing reactions (Sanger method) using labeled terminators or primers and gel separation in slab or capillary, sequencing by synthesis using reversibly terminated labeled nucleotides, pyrosequencing, 454 sequencing, allele specific hybridization to a library of labeled oligonucleotide probes, sequencing by synthesis using allele specific hybridization to a library of labeled clones that is followed by ligation, real time monitoring of the incorporation of labeled nucleotides during a polymerization step, polony sequencing, and SOLiD sequencing. Sequencing of the separated molecules has more recently been demonstrated by sequential or single extension reactions using polymerases or ligases as well as by single or sequential differential hybridizations with libraries of probes.

These reactions have been performed on many clonal sequences in parallel including demonstrations in current commercial applications of over 100 million sequences in parallel. These sequencing approaches can thus be used to study the identified haplotypes of the present invention in any gene locus of interest.

[0032] In an embodiment of the present invention, high-throughput methods of sequencing are employed that comprise a step of spatially isolating individual molecules on a solid surface where they are sequenced in parallel. Such solid surfaces may include nonporous surfaces (such as in Solexa sequencing, e.g. Bentley et al, Nature, 456: 53-59 (2008) or Complete Genomics sequencing, e.g. Drmanac et al, Science, 327: 78-81 (2010)), arrays of wells, which may include bead- or particle-bound templates (such as with 454, e.g. Margulies et al, Nature, 437: 376-380 (2005) or Ion Torrent sequencing, U.S. patent publication 2010/0137143 or 2010/0304982), micromachined membranes (such as with SMRT sequencing, e.g. Eid et al, Science, 323: 133-138 (2009)), or bead arrays (as with SOLiD sequencing or polony sequencing, e.g. Kim et al, Science, 316: 1481-1414 (2007)).

[0033] In another embodiment, such methods comprise amplifying the isolated molecules either before or after they are spatially isolated on a solid surface. Prior amplification may comprise emulsion-based amplification, such as emulsion PCR, or rolling circle amplification. These methods can also include Solexa-based sequencing where individual template molecules are spatially isolated on a solid surface, after which they are amplified in parallel by bridge PCR to form separate clonal populations, or clusters, and then sequenced, as described in Bentley et al (cited above) and in manufacturer's instructions (e.g. TruSeq™ Sample Preparation Kit and Data Sheet, Illumina, Inc., San Diego, Calif., 2010); and further in the following references: U.S. Pat. Nos. 6,090,592; 6,300,070; 7,115,400; and EP0972081B1; which are incorporated by reference.

[0034] In an embodiment, individual molecules can be disposed and amplified on a solid surface form clusters in a density of at least 10^5 clusters per cm^2; or in a density of at least 5×10^5 per cm^2; or in a density of at least 10^6 clusters per cm^2. In another embodiment, sequencing chemistries are employed having relatively high error rates. In such embodiments, the average quality scores, produced by such chemistries are monotonically declining functions of sequence read lengths. In some embodiments, the decline corresponds to 0.5 percent of sequence reads have at least one error in positions 1-75; 1 percent of sequence reads have at least one error in positions 76-100; and 2 percent of sequence reads

have at least one error in positions 101-125. The overall error rates vary and can be as low as 0.1 % at every base position to every other base

[0035] In one or more embodiments, the methods of the present invention are able to detect mixtures of human DNA down to a LD of 0.01% (1 in 10,000) using haplotype counting by NGS, 100-fold more sensitive than current STR based methods. False positives from NGS are avoided by using haplotypes because if they vary from one another by enough SNPs, they should demonstrate no crosstalk, even with a mutation frequency of 0.1% to 1% per base. Although HLA-A was used as an illustrative proof-of-concept in the present invention, other haplotype loci from the 1000 Genomes database were identified using the inventive methods that can also be used for this purpose.

[0036] In accordance with some embodiments, selection of suitable loci may be influenced by the patient's ethnic background, number of discriminating SNPs and ease of primer placement. The set of haplotypes used for this purpose ideally would be suitable for transplant analysis of all patient ethnicities. Patient DNA is consistently detected bone marrow samples that test all donor by the conventional STR assay.

[0037] Detection of minimal residual disease (MRD) in cases such as acute promyelocytic leukemia (APL) and subsequent early intervention has been associated with significantly higher survival.

[0038] In accordance with an embodiment, the haplotype counting based assay of the present invention can be valuable to detect relapse in HSCT patients earlier than the existing microsatellite based assays. One of the other non-chromosome 6 (containing human HLA) loci (Table 1) could be better for this purpose, as one mechanism that leukemic cells can escape donor anti-leukemic T-cells is through the loss of the mismatched HLA allele, estimated to occur in 29.4 to 66.7% of such patients. Another limitation of the HLA-A haplotype approach is that some transplant donors are HLA-identical and loci other than HLA would be required to monitor such patients.

[0039] Table 1: Alternative loci for use with haplotype identification

Chr*	Gene†	Start‡	Stop	bp	#SNPs	#Haps	CEU	JPT	YRI	Mean
1	----	238853189	238853428	240	9	7	0.63	0.46	0.63	0.57
1	----	238416454	238416673	220	9	11	0.39	0.46	0.43	0.43
2	----	106142506	106142791	286	10	8	0.63	0.61	0.66	0.63
2	*VIT*	36927840	36928071	232	9	6	0.58	0.59	0.61	0.59
3	*ST6GAL1*	186654745	186655022	278	9	12	0.72	0.67	0.73	0.70
3	----	97907092	97907374	283	10	13	0.58	0.42	0.64	0.55
4	*SORCS2*	7447136	7447434	299	9	16	0.60	0.46	0.65	0.57
4	----	66995978	66996254	277	9	8	0.63	0.45	0.60	0.56
5	----	125498526	125498753	228	10	19	0.67	0.58	0.67	0.64
5	----	178259629	178259892	264	13	23	0.69	0.56	0.64	0.63
6	*HLA-DRB1*	32551483	32551765	283	9	306	0.98	0.95	0.96	0.96
6	*HLA-B*	31319390	31319668	279	10	27	0.93	0.91	0.88	0.91
7	----	64895079	64895355	277	10	12	0.59	0.61	0.66	0.62
7	----	9111002	9111297	296	9	11	0.60	0.64	0.59	0.61
8	----	6160141	6160442	302	10	13	0.56	0.67	0.63	0.62
8	*CSMD1*	3478267	3478568	302	10	13	0.63	0.51	0.66	0.60
9	----	132197723	132197947	225	11	11	0.66	0.45	0.53	0.55
9	----	95691312	95691534	223	10	14	0.39	0.35	0.46	0.40
10	----	133376146	133376435	290	9	14	0.74	0.74	0.78	0.75
10	----	123095093	123095377	285	10	18	0.74	0.78	0.69	0.74
11	*CNTN5*	99491238	99491538	301	9	7	0.62	0.50	0.60	0.57
11	----	5078880	5079122	243	10	13	0.65	0.40	0.61	0.55
12	----	82460264	82460531	268	9	7	0.65	0.66	0.66	0.66
12	----	17884472	17884719	248	9	10	0.67	0.61	0.66	0.65
13	*FARP1*	99084002	99084259	258	9	17	0.63	0.70	0.71	0.68

13	----	33553507	33553784	278	9	29	0.68	0.45	0.63	0.59
14	----	107094021	107094317	297	10	25	0.53	0.51	0.66	0.57
14	*TRA*	22736235	22736521	287	9	11	0.51	0.57	0.61	0.56
15	----	34750034	34750330	277	9	8	0.51	0.57	0.61	0.56
15	----	25047393	25047681	289	10	25	0.51	0.44	0.55	0.50
16	----	56376389	56376687	299	11	46	0.76	0.82	0.80	0.79
16	----	84340653	84340932	280	9	16	0.69	0.66	0.74	0.70
17	*TBCD*	80804085	80804372	288	9	33	0.70	0.70	0.74	0.71
17	*RBFOX3*	77148074	77148367	294	10	15	0.38	0.60	0.52	0.50
18	----	76597079	76597332	254	9	14	0.40	0.40	0.40	0.40
18	*CLUL1*	631210	631400	191	9	12	0.33	0.27	0.38	0.33
19	*CCDC61*	46500080	46500360	281	9	14	0.41	0.38	0.43	0.41
19	----	57525179	57525424	246	13	10	0.37	0.39	0.41	0.39
20	*PROER3*	5289890	5290161	272	9	7	0.64	0.51	0.59	0.58
20	*SIRPA*	1895467	1895674	208	11	24	0.49	0.58	0.56	0.54
21	----	20548907	20549196	290	9	6	0.44	0.35	0.42	0.40
21	*UMODL1*	43528819	43529079	261	9	10	0.33	0.38	0.39	0.36

* Chromosome 22 is absent due to the lack of good polymorphic loci. X and Y were excluded.

† Gene, if present.

‡ Hg19 coordinates.

[0040] HSCT has traditionally been used to treat malignant and non-malignant hematologic disorders. In addition, with the transplantation of solid organs, some amount of lymphoid tissue may be transferred by cell migration from the donor organs, thereby creating chimerism in the patient. In contrast, intentional induction of microchimerism, by injecting donor BM, is a strategy used to create donor-specific tolerance in extremity transplantation. The development and persistence of donor-recipient microchimerism may be associated with the acceptance of transplanted organs.

[0041] As such, in accordance with another embodiment, the inventive methods with NGS to detect low levels of donor cells, provides an opportunity to document such microchimerism (or lack thereof) and to manage immunosuppressive regimens to optimize engraftment.

[0042] NGS of highly polymorphic regions using the inventive methods has applications outside of transplantation medicine. Microchimerism resulting from bi-directional exchange

of cells between mother and fetus has been detected in women long after pregnancy using Y chromosome FISH and PCR for the SRY gene.

[0043] In accordance with some embodiments, the present invention can be applied for such studies with the additional benefit of also being able to detect exchanged cells between a mother and a daughter. NGS haplotyping using the inventive methods could be used to aid in the detection of rare tissue regenerative cells in the heart transplant setting. In sex-mismatched heart transplants and using Y chromosome FISH, studies have shown cardiac chimerism caused by migration of recipient cells to the grafted heart.

[0044] The field of forensics has relied upon STR loci for identification of suspects and human remains.

[0045] In accordance with another embodiment, testing haplotypes using the inventive methods can allow one to identify the presence of suspect in a large mixture of DNAs such as in "poly-suspect" cases. In addition to forensic applications, tools for quality control and sample tracking in large inventory of human DNA samples would be very valuable. Such tools have previously been reported using panels of SNPs, and the NGS-haplotyping assay of the present invention can be applied in similar situations. Similar markers could be developed to distinguish among species.

[0046] To ensure patient identity and exclude the possibility of a mislabeled specimen, in clinical laboratory settings, the present invention can be used to uniquely define the patient using one or more haplotypes, and then could easily be included in any NGS based genetic test. They can also be used for any absolutely critical test, such as ABO typing of blood products, and matched to the intended patient's genotype encoded in an implanted microchip immediately prior to transfusion. Although rare, wrong-patient adverse events occur in hospital settings, especially when patients share the same name or patients with similar appearances. To circumvent these preventable errors, a biological identifier, such as the haplotypes described in the present invention, could be implemented to unequivocally distinguish patients.

[0047] A kit is also provided comprising an array of oligonucleotides as described herein, or portions or fragments thereof, as well as a biochip as described herein, along with any or all of the following: assay reagents, buffers, probes and/or primers, and sterile saline or another pharmaceutically acceptable emulsion and suspension base. In addition, the kits may include instructional materials containing directions (e.g., protocols) for the practice of the methods described herein.

EXAMPLES

[0048] Sample collection and preparation. Cell lines HCT116 (A*01:01:01 and A*02:01:01) and DLD-1 (A*02:01:01 and A*24:02:01:01) were chosen due to their available HLA-A haplotypes, and they were confirmed in the Immunogenetics Laboratory of the Johns Hopkins University, as well as their DNA fingerprint profile using the AmpFlSTR® Profiler Plus® PCR Amplification Kit (Life Technologies, Carlsbad, CA). A large number of cells were expanded to permit making cell dilutions, since we have found that cell dilutions are generally more accurate then DNA dilutions (data not shown). Cell to cell dilutions were made, where appropriate number of HCT116 cells was added to 10 million DLD-1 cells for each dilution. DNA was extracted from each cell pellet using DNeasy Blood and Tissue kit (Qiagen, Valencia, CA). Extracted DNA 1 was quantified by Quantifiler (Applied Biosystems, Carlsbad, CA) and stored at -20 °C.

[0049] BME Samples. Samples from eighteen patients who underwent allogeneic HSCT were obtained from Molecular Diagnostic Laboratory at the Johns Hopkins Hospital, on an Institutional Review Board approved protocol. Samples were selected based on the disease type, the ability to distinguish patient from donor alleles, that they were all donor by STR analysis and that at least 600 ng (100,000 genomes) of DNA was available to test (Table 2). Each BM sample was prepared for sequencing as described below and sequenced on the Ion Torrent PGM to insure approximately 100,000 reads were obtained.

[0050] Table 2: BME patient characteristics and percent patient DNA detected.

Pat#	Dis*	Patient HLA-A	Donor HLA-A	Diff†	%Pat
BME14	AML	A*02:05:01/A*01:01:01:01	A*02:05:01/A*01:01:01:01	11,6	0.000%
BME20	MDS	A*29:02:01:01/A*26:01:01	A*01:01:01:01/A*26:01:01	12,6	0.001%
BME18	AML	A*03:01:01:01/A*30:01:01	A*11:01:01/A*30:01:01	7,5	0.019%
BME10	AML	A*32:01:01/A*34:02:01	A*01:01:01:01/A*34:02:01	12,3	0.030%
BME19	AML	A*24:02:01:01/A*68:01:01:01	A*32:01:01/A*68:01:01:01	6,7	0.052%
BME25	ALL	A*01:01:01:01/A*03:01:01:01	A*33:01:01/A*03:01:01:01	12,11	0.060%
BME30	AML	A*03:01:01:01/A*24:02:01:01	A*03:01:01:01/A*01:01:01:01	7,6	0.136%
BME27	AML	A*02:01:01:01/A*24:02:01:01	A*02:01:01:01/A*02:01:01:01	7	0.140%
BME16	AML	A*02:XX/A*26:XX	A*02:xx/A*03:xx	6,9	0.189%
BME11	AML	A*30:02:01/A*74:01	A*68:02:01:01/A*74:01	8,2	0.190%
BME4	AML-M6	A*02:01:01:01/A*29:02:01:01	A*02:01:01:01/A*68:01:01:01	6,5	0.312%
BME26	ALL	A*11:01:01/A*30:01:01	A*23:01:01/A*30:01:01	6,9	0.328%
BME1	MM	A*02:01:01:01/A*23:01:01	A*30:xx/A*02:xx	4,7	0.416%
BME22	CLL	A*02:01:01:01/A*66:01	A*01:01:01:01/A*66:01	11,9	0.640%
BME21	MCL	A*68:02:01:01/A*29:02:01:01	A*30:01:01/A*29:02:01:01	8,6	0.750%
BME29	AML	A*11:01:01/A*26:01:01	A*11:01:01/A*11:01:01	6	0.863%
BME23	DLBCL	A*02:01:01:01/A*26:01:01	A*02:01:01:01/A*02:01:01:01	9	1.110%
BME24	HD	A*01:01:01:01/A*29:02:01:01	A*30:02:01/A*29:02:01:01	13,12	1.470%

*Disease: DLBCL, diffuse large B-cell lymphoma; HD, Hodgkin's disease; MM, multiple myeloma.

†. Number of SNP differences between unique alleles (bold) and between the unique patient and shared alleles.

[0051] HLA-A PCR amplification. Forward and reverse primers were ordered with Ion Torrent-specific adaptors (A16 and P1-adaptors) added at their 5' ends. Briefly, the first round PCR reaction included 600ng (100,000 genomes) of DNA, 200 nM forward and reverse primers, in Platinum® PCR SuperMix High Fidelity (Invitrogen, Carlsbad, CA) in a total of 100 µl reaction volume. The forward primer was (5'-CCATCTCATCCCTGCGTGTCTCCGAC-tcag-*AGGACCTGCGCTCTTGGAC*-3') (SEQ ID NO: 1), where A-adaptor (underlined), library key (lower case), and HLA-A primer (italics), and the reverse primer was (5'-CCTCTCTATGGGCAGTCGGTGAT-*CGTTCTCCAGGTATCTGCGGA*-3') (SEQ ID NO: 2), including the P1-adaptor (underlined) and HLA-A reverse (italics). The expected size of the full length PCR product is 298 base pairs, including adapters. Following amplification, adaptor containing PCR product was

visualized by gel electrophoresis (Novex® TBE Gels, Life Technologies, Carlsbad, CA) and quantified using High Sensitivity dsDNA reagents and Qubit 2.0 fluorometer (Invitrogen). With the desire to "paint" a single molecule on each bead for emulsion PCR, samples were diluted to a 0.03nM working concentration. During emulsion PCR each single molecule produces a "clone" of progeny molecules (estimated to be ~500,000) for sequencing.

[0052] Ion Torrent PGM library preparation and sequencing. Emulsion PCR using 20-25 µl working stock, next generation sequencing, and mapping to hg19 were all done per manufacturer's protocol (Life Technologies). Assessment of the percent amplicon containing beads was performed per manufacturer's protocol (Life Technologies) and measured with Qubit 2.0 fluorometer (Invitrogen, Carlsbad, CA). Amplicon coated beads were analyzed on 314 and 316 chips using the Ion PGM Sequencing 200 kit on the Life Technologies' Ion Torrent Personal Genome MachineTM (PGM), semiconductor sequencer, which detects dNTP incorporation using the hydrogen ion that is released (along with pyrophosphate) when a dNTP is incorporated into an elongating DNA strand (Nature 2011, 475:348-352 16; Electrophoresis 2012, 33:3397-3417).

[0053] Microsatellite analysis. PCR amplification of 9 microsatellites (AmpFlSTR® Profiler® kit; Applied Biosystems) or 15 microsatellites (Identifiler®, Applied Biosystems) was performed according to the manufacturer's instructions. Amplicons were resolved on a capillary electrophoresis ABI3130xl Genetic Analyzer (Applied Biosystems).

[0054] Bioinformatics/analysis. Initial processing of the data was done using the Ion Torrent platform-specific pipeline software Torrent Suite v3.2.1 to generate sequence reads, trim adapter sequences, and remove poor quality reads. Resulting sequence files were aligned to hg19 reference sequence. Further analysis of Fastq files was done using Geneious Pro 5.5.7 where perfectly matched reads were counted. Some samples were log10 transformed and graphed (GraphPad Prism v5.04).

[0055] Analysis of molecular specificity was done with homozygous HLA-A*01:01:01:01 and HLA-A*02:01:01:01 samples (hereafter simply A*01 and A*02, respectively). Each sample was analyzed for the other allele, followed by the introduction of the other allele's base at 11 SNP positions. For example, homozygous A*01 was analyzed for perfectly matched A*01 reads followed by perfect A*02 reads. Upon this, one A*02-specific base was introduced into A*01 sequence at each of the 11 SNP positions and analyzed. After introducing one A*02-specific base at all of the possible positions, 2 consecutive A*02-specific bases were introduced, repeating the process for 11 possible

combinations. The process was continued all the way to substituting all 11 consecutive A*02-specific bases. The number of reads found, for each combination, was averaged and percent of reads found was graphed.

[0056] Bone marrow transplant samples were analyzed for the amount of patient (patient unique allele) in the sample. After eliminating reads representing a haplotype shared by both individuals, we calculated a percent patient DNA (patient / patient + donor). Cases where three alleles were shared, the equation [(2 x unique patient) / (unique patient + shared patient-donor)] was used.

[0057] Bioinformatics analysis of 1000 Genomes database. Using 1000 Genomes release v3_20110521 from build hg18, we identified regions that were polymorphic and flanked by constant regions. We required a minimum of 9 variants with a MAF of $\geq$ 9% in three of the major populations: CEU, (CEPH, Utah Residents with Northern and Western European Ancestry); JPTCHB (a combined Asian population including JPT, Japanese in Tokyo, Japan, and CHB, Han Chinese in Beijing, Ching); and YRI (Yoruba in Ibadan, Nigeria) within 300 bp. A 300 bp size was chosen so that it could be amplified and sequenced in a single read, well within the limits of current NGS technology. We required potential regions to be flanked by constant regions of at least 20bp for primer placement.

[0058] Polymorphic regions were converted from hg18 to hg19 using UCSC's LiftOver tool (genome.ucsc.edu/cgi-bin/hgLiftOver, last accessed 3/28/2014). A custom script was written to convert phased variants from 1000 Genomes VCF files (both IMPUTE217 and SHAPEIT218 versions) into IMPUTE reference panel format haplotypes at every region. A custom program was then used to assess all possible haplotype combinations between two theoretical individuals for informativity and probability of occurrence. Particular haplotype combinations were defined as informative if at least one haplotype of individual B (patient) had at least 2 SNP differences from both haplotypes of individual A (donor). Once informative haplotype combinations were determined, the probabilities of these combinations occurring in two unrelated individuals was calculated (based on the 1000 Genomes frequencies) and summed. Results from the two methods of phasing the chromosomes were highly correlated (data not shown), so only the data from SHAPEIT2 phased haplotypes are presented. Thus, the calculated probability score reflects the informativity of the region for 2 unrelated individuals.

EXAMPLE 1

[0059] Analysis of HLA-A alleles. We performed alignments of common HLA-A alleles in the European-origin population using dbMHC database, publically accessible platform for DNA and clinical data related to the human Major Histocompatibility Complex (ncbi.nlm.nih.gov/gv/mhc/main.cgi?cmd=init last accessed 3/28/2014). Regions with a high density of SNPs and flanked by non-polymorphic DNA were identified. One region, HLA-A exon 3 contained 18 possible SNPs and at least 15 major alleles in the European-origin population. We tested a series of primers surrounding this region and selected the best pair based on amplification efficiency and specificity (Figure 1D, see methods). The number of SNP differences in this region between the most common alleles of the European-origin population was tabulated (Figure 2). Some combinations of HLA-A alleles are easily differentiated whereas others are more difficult. For example, 11 SNPs differentiate allele A*01 from A*02 (double lined box), so that even with a relatively high base substitution error rate (e.g. 1% per base), a sample homozygous for A*01 should not contain any false positive A*02 allele reads. In contrast, A*02 and HLA-A*68:01:01:01 (hereafter A*68) have only 1 SNP difference (dashed lined box), so a pure sample homozygous for A*02 will likely contain reads matching A*68 due to the relative high error rate intrinsic to current NGS technologies.

EXAMPLE 2

[0060] Determination of "crosstalk" between molecules which vary by 11 SNPs. To test this experimentally, we sequenced two samples, one homozygous for A*01 and another homozygous for A*02, and analyzed each for the other allele (Figure 3). The A*01 and A*02 samples contained approximately 200,000, perfect matching reads. Neither pure sample contained any perfect reads of the other haplotype. We then examined the A*01 sample files for reads containing a single A*02 SNP at each of the 11 positions and found an average of 0.3-0.8% reads that contained a single error from the perfect haplotype (Figure 3A). When two SNPs of the opposite haplotype were searched for, no reads were obtained. Similar results were obtained with the pure A*02 sample (Figure 3B). A double waterfall plot shows that when enough discriminating SNPs between two individuals' alleles exist, the assay is highly specific. (Figure 3C).

EXAMPLE 3

[0061] HLA-A dose-response curve, accuracy, precision, and limit of detection (LD). To assess the accuracy and LD, we generated a dilution series from two cell lines with known HLA-A genotypes. These samples were chosen because the two alleles of interest (A*01 and A*02) vary from one another by 11 SNPs and both vary from the commonly shared allele (A*24) by 7 SNPs (Figure 2). Dilutions were made with cell mixes varying from 1 in 1 million (0.0001%) to 1 in 100 (1%) using a total of 10 million cells for each dilution. DNA was isolated and PCR performed using 600 ng of DNA. We chose this relatively large amount of DNA based on the desire to achieve a LD of at least 1:10,000 (0.01%) and to exceed that target LD by using 10x excess DNA. This relatively high DNA input reflects approximately 100,000 genomes (based on approximately 6 picograms/haploid genome) and was chosen to prevent "bottlenecking" and resultant allele dropout. For example, if DNA representing only 100 genomes were analyzed to 100,000X depth of coverage, the LD is input DNA limited, not depth of coverage limited, and is at best 1% (not accounting for Poisson sampling). A sample with a minor allele frequency of 0.1% would likely not be detected. Each sample was sequenced at least twice, and results graphed as the percent HLA-A*01 as a function of the percent HLA-A*01 bearing cells (Figure 4A). Least-squares analysis generated a straight line demonstrating excellent accuracy (R2=0.93). To determine precision we performed additional replicates at 0.1% and 0.01% dilutions (4 total). The assay was highly precise at 0.1% cell mix (mean= 0.065%, coefficient of variation = 0.12), but less so at the 0.01% cell mix as expected (mean= 0.012%, coefficient of variation=0.40). Because we detected the minor HLA-A allele in 4 out of 4 replicates at 0.01%, but only 1 out of 2 replicates at 0.003%, we concluded that 0.01% is the lower LD for this assay (data not shown). These results compare favorably with that of microsatellites, which are highly linear and generally accurate, but with a much poorer LD of only about 3% (Figure 4B).

EXAMPLE 4

[0062] SNP haplotype assay detects patient DNA in bone marrow samples that tested all donor by STR analysis.

[0063] We selected 18 patients whose donor-patient HLA genotypes varied by at least 4 SNPs (Table 2). The allele unique to donor varied from that unique to patient by 4-13 SNPs.

To further prevent false positive results, the unique patient allele varied from the shared allele by 2-12 SNPs. We also required that 600 ng be available for testing, so that the number of genomes (~100,000) exceeded by an order of magnitude the desired LD. Several samples were excluded that could not meet these criteria. All samples tested positive for some level of patient DNA, except one. The positives ranged from 0.001% to 1.47% patient DNA (Figure 5, mean 0.373), confirming that the haplotype based assay was more sensitive. We also noted that of the 4 samples with the highest levels of patient DNA, 3 of them were from patients with lymphoma.

EXAMPLE 5

[0064] The human genome contains clusters of SNPs that can be amplified to give information on haplotypes.

[0065] We used variant calls from the 1000 Genomes to identify regions containing at least 9 SNPs in all three populations (Europeans, Asians, and Africans, see Methods) within 300 bp. We further required these regions to be flanked by at least 20 bp of non-polymorphic DNA. The non-polymorphic regions provide potential primer sites, while the variable regions are suitable for haplotype counting. We identified 4,349 such genomic loci across the genome, requiring that the 9 or more variants have minor allele frequencies greater than 9% in all three populations (data not shown).

EXAMPLE 6

[0066] One concern is that a cluster of SNPs may not be that informative because it simply represents a high-frequency haplotype. For example, a haplotype that exists within a population at a 2% level and containing 20 SNPs, in conjunction with a second haplotype with the other base at each of the 20 positions present in 98% of the population, is a relatively uninformative marker. In contrast, a locus with 20 SNPs with 10 different haplotypes each of which is present at 10% in the population is highly informative.

[0067] Using the 4,349 regions identified as SNP dense, we analyzed the 1000 (1092) Genomes phased variant call files to calculate overall probability that two unrelated individuals would be different at each locus. Briefly, at each locus, a file was generated that contained all of the SNPs for each of the 2,184 (2 x 1092) alleles. We then determined the number of distinct alleles and the number of times each of them was represented. We then

calculated all possible combinations of alleles within donors and recipients, and finally determined the combined probability that two unrelated individuals would be informative. The most informative loci from each chromosome are shown in Table 1. These putative alternate loci will be experimentally validated in large cohorts of patients, and experiments are currently in progress to do this.

EXAMPLE 7

[0068] Use of haplotype counting to produce quality control DNA-mix standards to be used in evaluating ultrasensitive mutation detection methods.

[0069] Low-level point mutation detection is problematic using next generation sequencing (NGS) due to high error rates of the current platforms. Nevertheless, detecting point mutations with accuracy and high sensitivity is clinically important. Our initial work (Debeljak et al, JMD 2014, 16:495-503) showed that our haplotype counting (*HLA-A*) method with NGS was highly sensitive and accurate, thus providing a way to overcome intrinsically high error rates of NGS. Using this approach, we now demonstrate its utility to produce quality control samples that can be used to asses various ultrasensitive assays/technologies. To demonstrate this, we produced a dilution series, quality controlled it using the *HLA-A* haplotype counting method and then tested these controls using the AmpliSeq Cancer panel (Figure 1A) and digital droplet PCR (ddPCR, Figure 1B) technologies.

EXAMPLE 8

[0070] Ability to detect low levels of donor bone marrow in the setting of limb transplantation.

[0071] A patient underwent double arm transplantation, followed by infusion 2 weeks later of donor bone marrow (BM) cells. We evaluated various samples (PB, CD3, BM) at different time points post- infusion to determine whether donor-derived DNA can be detected. Donor-derived DNA was detected in PB samples at 0.003% and 0.001% in early days post-infusion (4 and 42 days, respectively, Table 3, Figure 7) using the haplotype counting method. These samples all tested as donor-negative by the conventional STR assay.

[0072] Table 3: Samples tested and percent donor DNA detected.

				NGS Results			
Sample Alias	Type	Days Post BM Infusion	STR Results	Patient Unique (A*01)	Donor Unique (A*24)	Shared (A*02:05/A*2)	% Donor
ArmTransplant Case 1-S1	PB	1	0% Donor	5,352	0	2,117	0%
ArmTransplant Case 1-S2	PB	4	0% Donor	804,061	22	328,699	**0.003%**
ArmTransplant Case 1-S7	PB	42	0% Donor	1,331,462	15	1,218,256	**0.001%**
ArmTransplant Case 1-S9	PB	70	0% Donor	18,311	0	48,929	0%
ArmTransplant Case 1-S10	PB	165	0% Donor	13,900	0	37,420	0%
ArmTransplant Case 1-S11	PB	252	0% Donor	1,523,636	0	1,519,307	0%
ArmTransplant Case 1-S16	PB	564	0% Donor	1,544,569	0	1,216,153	0%

EXAMPLE 9

[0073] Ability to predict early relapse of AML patients after bone marrow transplantation.

[0074] Currently, relapse of AML post BM transplantation is tested by STR analysis, but this technology is relatively insensitive (limit of detection ~3%). We have applied our haplotype counting assay to determine if we can detect relapse earlier in patients who underwent haploidentical transplants. A total of 15 patient samples (7 control/ non-relapse and 8 relapse) were evaluated and results compared for significance. Samples evaluated in control group were on average 291 days post transplantation. Relapse samples evaluated were taken 53-145 days prior to STR-relapsed sample (see Table 4 below).

[0075] In controls (non-relapse), average patient-derived DNA was present at 0.05%. In contrast, average patient-derived DNA in relapse group was present at 0.32%. There is statistical significance between the two groups (Figure 8, p= 0.0011).

[0076] Table 4: Evaluation of AML Relapse post-BM transplantation

Patient ID	Disease	Number Differences[a]	Days post-transplant	Days pre-STR relapse	Days clean after our sample	Percent (%) Pre	
Non-Relapse Patients							
BME 10	AML	12,3	150	---	1254	0.03%	
BME 11	AML	8,2	180	---	558	0.19%	
BME 14	AML	11,6	360	---	778	0.00%	
BME 18	MDS/AML	7,5	365	---	.....[b]	0.019%	Average % Pre: 0.05%
BME 19	AML	6,7	423	---	.....[b]	0.052%	Avg Days Post Transplant: 391.1
BME 20	MDS	12,6	378	---	721	0.001%	
BME 25	ALL	12,11	182	---	545	0.06%	
Relapse Patients							
BME 1	IgG multiple myeloma	4,7	56	180	---	0.416%	
BME 4	Acute erythroid leukemia	6,5	86	90	---	0.312%	
BME 16	AML	6,8	86	111	---	0.389%	
BME 26	Refractory acute lymphoblastic leukemia	6, 9	179	148	---	0.328%	Average % Pre: 0.322%
BME 27	Secondary AML	7	56	131	---	0.14%	Avg Days Post Transplant: 88.8
BME 29	High risk AML	6	94	130	---	0.863%	
BME 30	AML-Trisomy 8 with neg FLT-3	7,6	83	53	---	0.136%	
BME 31	Refractory multiple myeloma	2,5	141	129	---	0.191%	

a. Number of SNP differences between unique alleles.

b. Two patients transferred and sample used in our assay was the last sample collected at our institution.

EXAMPLE 10

[0077] Detecting maternal microchimerism in fetal tissues

[0078] We evaluated the presence of maternal microchimerism in various fetal tissues for 2 different cases. Each sample was also evaluated by STR (Identifiler) assay, which was unable to detect maternal DNA. Using our NGS-based haplotyping counting approach, we were able to detect maternal DNA in various fetal tissues (see Table 5). Quantity of the DNA used for testing varied from 140ng (~23,000 genomes) to 600ng (100,000 genomes). Samples in each case were barcoded, pooled, enriched, and sequenced on a 316v2 chip using 400bp chemistry kit.

[0079] Table 5: Detection of Maternal and Fetal DNA

	Sample	STR	NGS
Case 1	Case 1- Placenta	No maternal DNA detected	**99.4% Fetus; 0.60% Mother**
	Case 1-Spleen	No maternal DNA detected	100% Fetus
	Case 1-Lung	No maternal DNA	**99.99% Fetus; 0.01%**

		detected	**Mother**
	Case 1-Thymus	No maternal DNA detected	100% Fetus
	Case 1-Liver	No maternal DNA detected	100% Fetus
Case 2	Case 2 -Spleen	No maternal DNA detected	100% Fetus
	Case 2 -Lung	No maternal DNA detected	100% Fetus
	Case 2 -Thymus	No maternal DNA detected	**99.99% Fetus; 0.01% Mother**
	Case 2 -Liver	No maternal DNA detected	100% Fetus
	Case 2 -Lymph Node	No maternal DNA detected	100% Fetus

EXAMPLE 11

[0080] Validation of additional loci

[0081] Previously (Debeljak et al, JMD 2014, 16:495-503), the present inventors bioinformatically discovered over 4,000 additional loci in the human genome that can be used for our haplotype counting assay. This is of value since a given transplanted patient may be identical to their donor at the *HLA-A* locus, or possess *HLA-A* alleles with too few SNPs that are different between the donor and recipient. The present inventors are working on experimentally validating some of the 4,000 additional loci. Table 6 shows some of the loci we are validating. Using an independent set of 8 normal samples, and using whole genome sequencing, we provide an alignment of *FARP1* as an example (Figure 9), and the haplotypes of FARP1 locus that were compared (Figure 10).

[0082] Table 6: Loci used for Validation of Methods

Chr	Gene Name	Start	Stop	# Sanger Sequenced	# Sanger Hets	# SNPs	# Alleles Experimental (imputed)
1	CDK11B	1650786	1651004	4	0	21	1
1	TFB2M	246714691	246714887	4	3	9	4
4	SORCS2	7447136	7447434	10	8	18	7
6	DQB1	32629192	32629348	4	2	22	3
6	DRB1	32551493	32551765	2	0	44	2
8	CSMD1	3,478,267	3,478,568	4	1	22	3
10	(TCERG1L)	133376146	133376435	5	4	17	6
10	(FGFR2)	123095093	123095377	5	2	18	5
13	FARP1	99084002	99084259	10	7	15	7
16	(MT4)	56576389	56576687	4	3	14	4
16	(MT4)	56576537	56576811	4	3	15	5
16	(MT4)	56576537	56576764	4	3	15	5
17	RBFOX3	77,148,074	77,148,367	4	2	12	2
18	CLUL1	631210	631400	8	0	14	2
20	SIRPA	1,895,490	1,895,707	4	4	12	2
21	(TMPRSS15)	20237591	20237839	5	3	12	4
21	(TMPRSS15)	20237647	20237905	5	0	11	3
21	(TMPRSS15)	20237737	20238011	5	3	13	5
21	(TMPRSS15)	20548907	20549196	5	5	15	5
21	(TMPRSS15)	20549081	20549365	5	5	13	5

*Boxed loci indicates they overlap

[0083] All references, including publications, patent applications, and patents, cited herein are hereby incorporated by reference to the same extent as if each reference were individually and specifically indicated to be incorporated by reference and were set forth in its entirety herein.

[0084] The use of the terms "a" and "an" and "the" and similar referents in the context of describing the invention (especially in the context of the following claims) are to be construed to cover both the singular and the plural, unless otherwise indicated herein or clearly contradicted by context. The terms "comprising," "having," "including," and "containing" are to be construed as open-ended terms (i.e., meaning "including, but not limited to,") unless otherwise noted. Recitation of ranges of values herein are merely intended to serve as a shorthand method of referring individually to each separate value falling within the range, unless otherwise indicated herein, and each separate value is incorporated into the specification as if it were individually recited herein. All methods described herein can be performed in any suitable order unless otherwise indicated herein or

otherwise clearly contradicted by context. The use of any and all examples, or exemplary language (e.g., "such as") provided herein, is intended merely to better illuminate the invention and does not pose a limitation on the scope of the invention unless otherwise claimed. No language in the specification should be construed as indicating any non-claimed element as essential to the practice of the invention.

[0085] Preferred embodiments of this invention are described herein, including the best mode known to the inventors for carrying out the invention. Variations of those preferred embodiments may become apparent to those of ordinary skill in the art upon reading the foregoing description. The inventors expect skilled artisans to employ such variations as appropriate, and the inventors intend for the invention to be practiced otherwise than as specifically described herein. Accordingly, this invention includes all modifications and equivalents of the subject matter recited in the claims appended hereto as permitted by applicable law. Moreover, any combination of the above-described elements in all possible variations thereof is encompassed by the invention unless otherwise indicated herein or otherwise clearly contradicted by context.

Claims:

1. A method for identifying informative haplotypes useful for identity testing comprising:

a) obtaining the DNA sequences of a plurality of individual genomes of a mammal;

b) identifying within the genomes of a) haplotypes comprising both allelic DNA sequences of about 100 to 400 or more base pairs in length, having at least one or more polymorphic regions which are flanked at both the 5' and 3' ends with constant regions of at least about 20 base pairs in length;

c) identifying within the haplotypes of b) those haplotypes which have at least about 2 or more single nucleotide polymorphism variants of the polymorphic regions; and

d) identifying those haplotypes of c) as informative if at least 1 haplotype from a first individual genome has at least 2 or more single nucleotide polymorphism differences from both alleles of a second individual genome.

2. The method of claim 1, wherein the DNA sequences of a plurality of individual genomes is from humans.

3. The method of claim 1, wherein the haplotypes of b) are 300 base pairs in length.

4. The method of claim 1, wherein in c), identifying within the haplotypes of b) those haplotypes which have at least about 2 or more single nucleotide polymorphism variants of the polymorphic regions

5. The method of claims 1 to 4, wherein the haplotypes of b) are located within a gene, or an intron region within a gene, or intragenic regions of the genomic DNA.

6. A method for determining the DNA sequence of one or more informative haplotypes in a DNA sample of a mammal comprising:

a) obtaining a sample containing a sufficient amount of DNA which comprises at least about 100,000 genomes of the mammal;

b) purifying the DNA from a);

c) amplifying the DNA from b) using PCR and primers and probes specific for one or more informative haplotypes and for the sequencing method (need list of possible types) being used to analyze the DNA sequences of the informative haplotypes;

d) analyzing the plurality of DNA sequences of the amplified informative haplotypes for single nucleotide polymorphisms in c);

e) comparing the DNA sequence single nucleotide polymorphisms found in d) to the DNA sequence single nucleotide polymorphisms for one or more reference informative haplotypes, wherein when a DNA sequence of the one or more informative haplotypes from d) does not contain all of the single nucleotide polymorphisms of the one or more reference informative haplotypes, the DNA sequence is discarded as erroneous; and

g) establishing that when a DNA sequence of the one or more informative haplotypes from d) contains all of the single nucleotide polymorphisms of the one or more reference informative haplotypes, the DNA sequence is a match and the haplotype identity is confirmed.

FIGURES 1A-1D

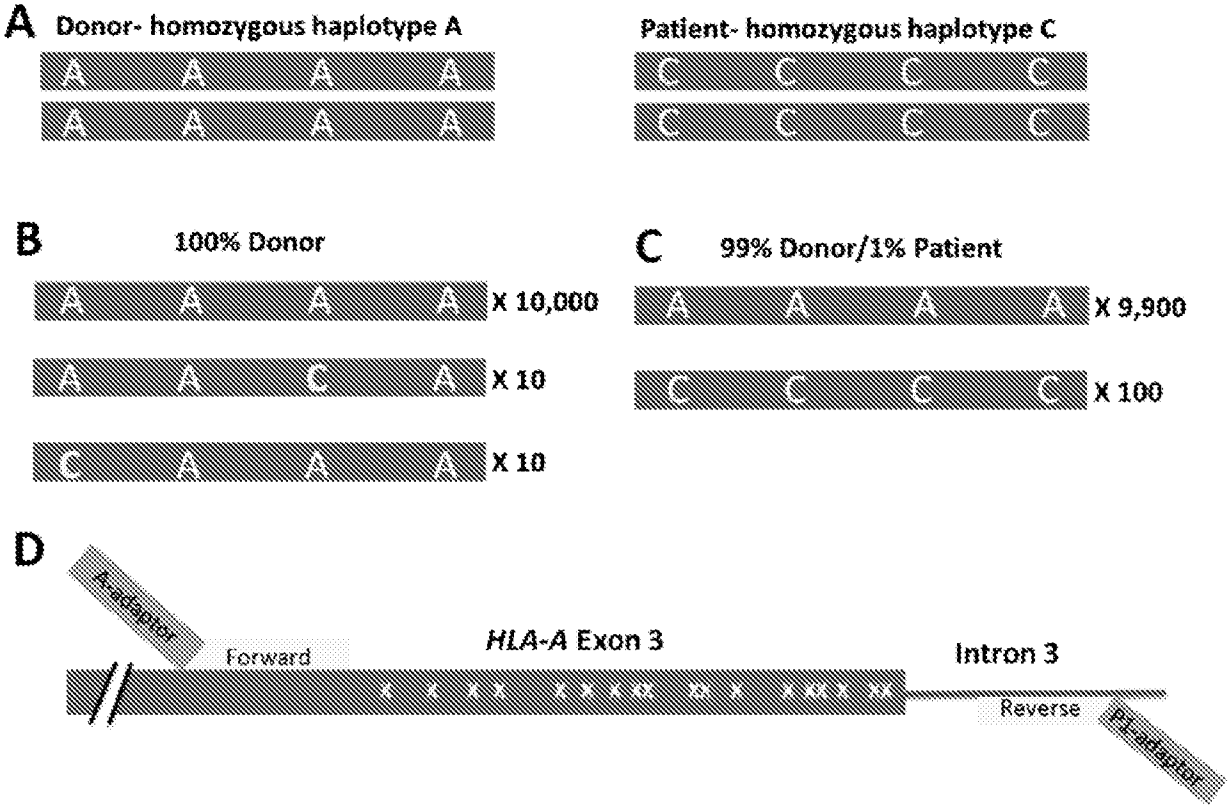


FIGURE 2

Frequency
Allele
16.6
A*01:01:01
29.1
A*02:01:01
14.7
A*03:01:01:01
5.9
A*11:01:01
1.6
A*23:01:01
8.4
A*24:02:01:01
1.5
A*25:01:01
3.0
A*26:01:01
3.3
A*29:02:01:01
1.7
A*30:01:01
1.0
A*30:02:01
2.2
A*31:01:02
3.6
A*32:01:01
1.0
A*33:01:01
2.4
A*68:01:01:01
99.9%
A*01:01:01
A*02:01:01
A*03:01:01:01
A*11:01:01
A*23:01:01
A*24:02:01:01
A*25:01:01
A*26:01:01
A*29:02:01:01
A*30:01:01
A*30:02:01
A*31:01:02
A*32:01:01
A*33:01:01
A*68:01:01:01

FIGURES 3A-3C

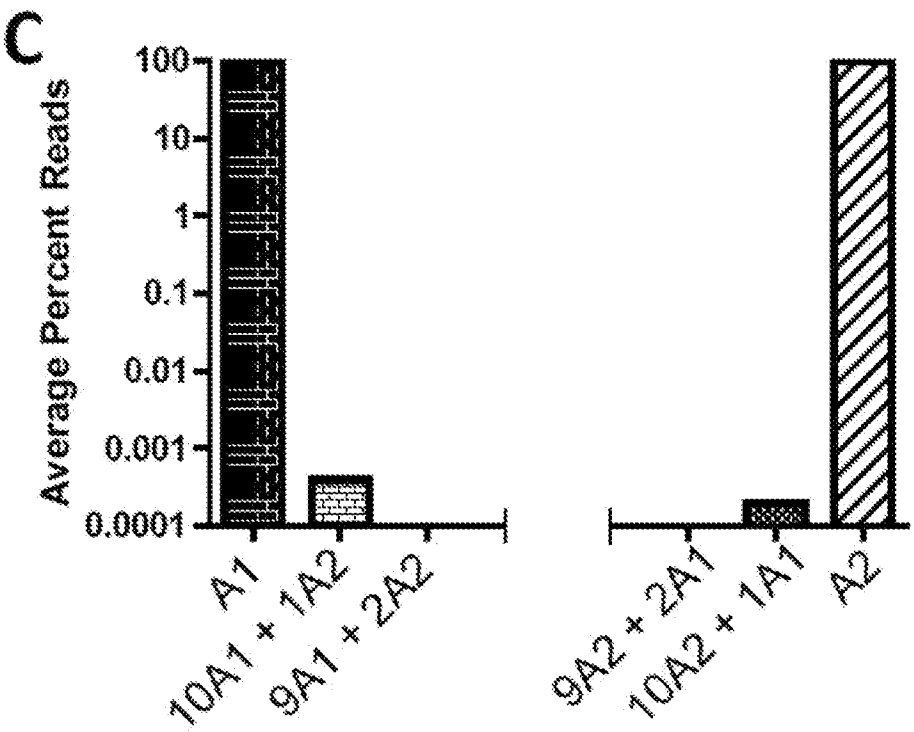
A

Exon 3 position	154 (C/T)	163 (A/G)	178 (C/T)	184 (A/C/G/T)	195 (C/T)	196 (A/G/T)	202 (C/T)	216 (A/G)	217 (C/G)	227 (C/G)	228 (G/T)
cDNA position	497	506	521	527	538	539	545	559	560	570	571
Sample Probe											Number of Reads
A1 A1	T	G	T	C	C	G	T	C	G	C	G 201,605
A2	C	A	C	T	T	T	C	A	C	G	T 0
	-	A	-	-	-	-	-	-	-	-	- 1
	-	-	-	-	T	-	-	-	-	-	- 1
	-	-	-	-	-	-	C	-	-	-	- 6
	-	-	-	-	-	-	-	-	C	-	- 1
Total 10A1 + 1A2											9

B

Sample Probe												
A2 A2	C	A	C	T	T	T	C	A	C	G	T	187,630
A1	T	G	T	C	C	G	T	C	G	C	G	0
	-	-	T	-	-	-	-	-	-	-	-	2
	-	-	-	-	-	-	T	-	-	-	-	1
Total 10A2 + 1A1												3

C



FIGURES 4A-4B

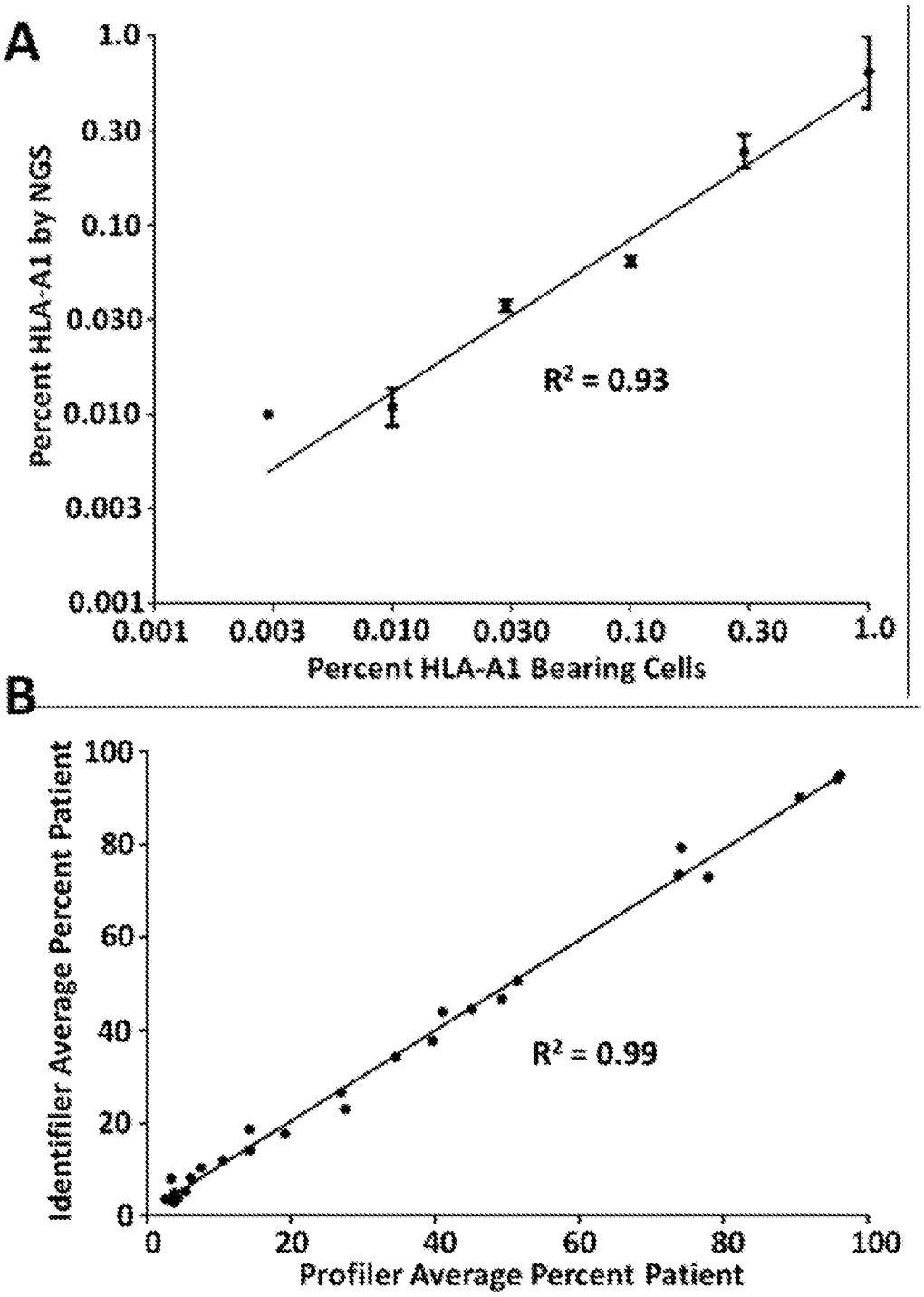
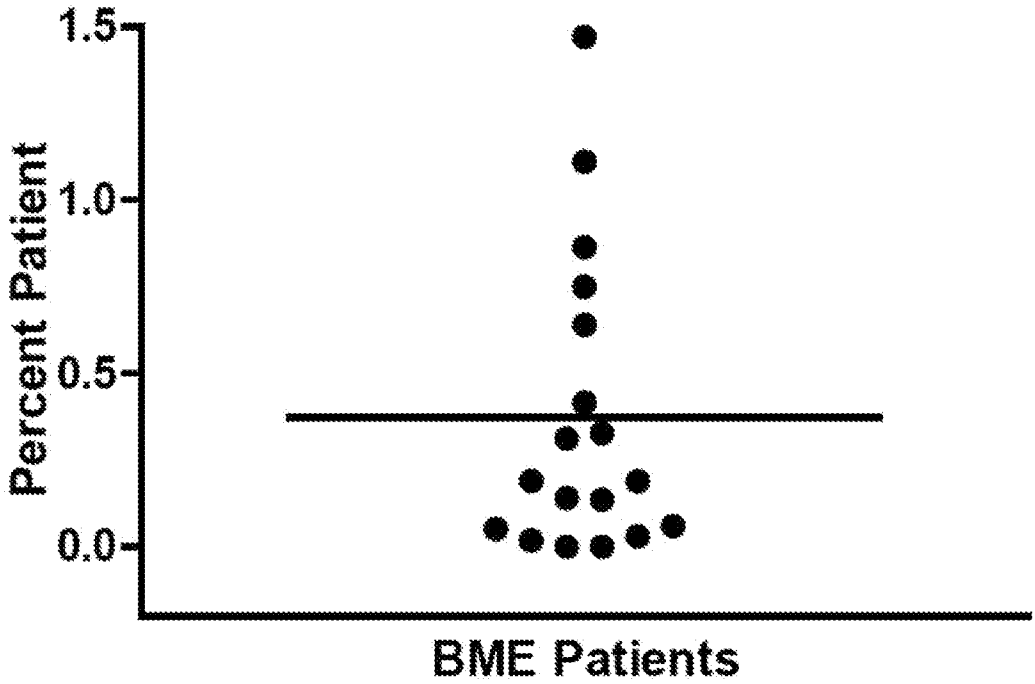


FIGURE 5



FIGURES 6A-6B

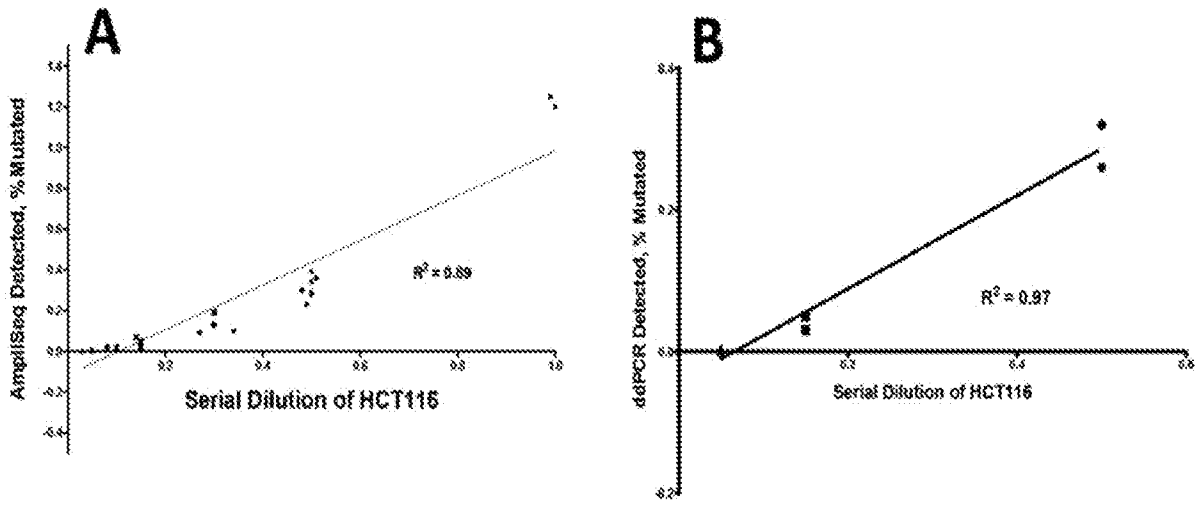


FIGURE 7

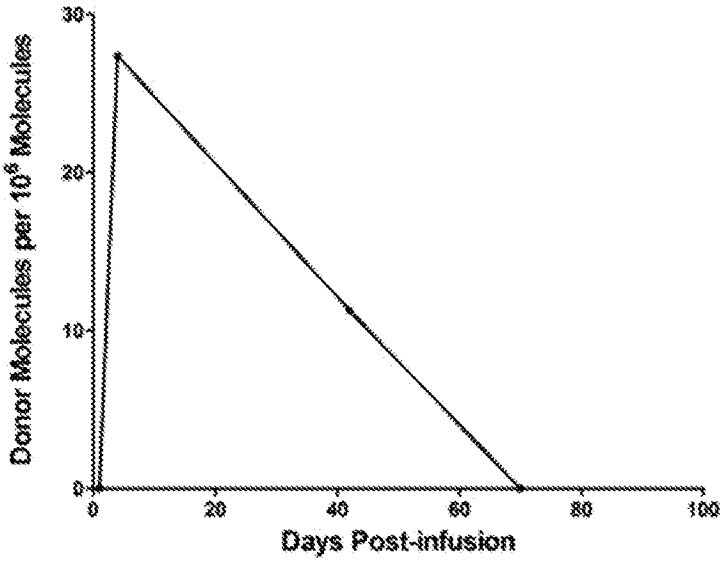

Donor Molecules per 10^6 Molecules
30
20
10
0
0
20
40
60
80
100
Days Post-infusion

FIGURE 8

	Mean	SEM	Mann-Whitney (one-tailed) p Value
Non-Relapse	0.050	0.025	0.0011
Relapse	0.322	0.085	

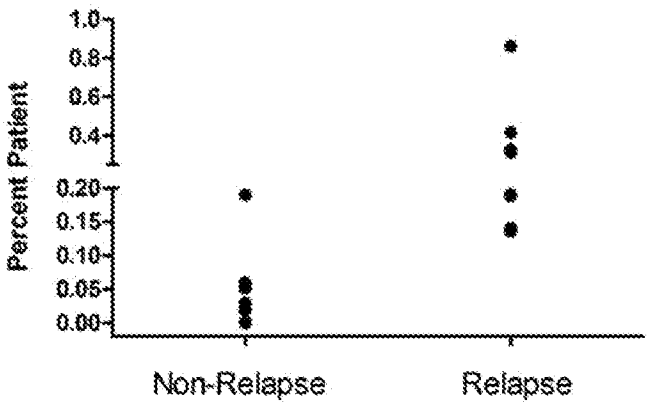


FIGURE 9

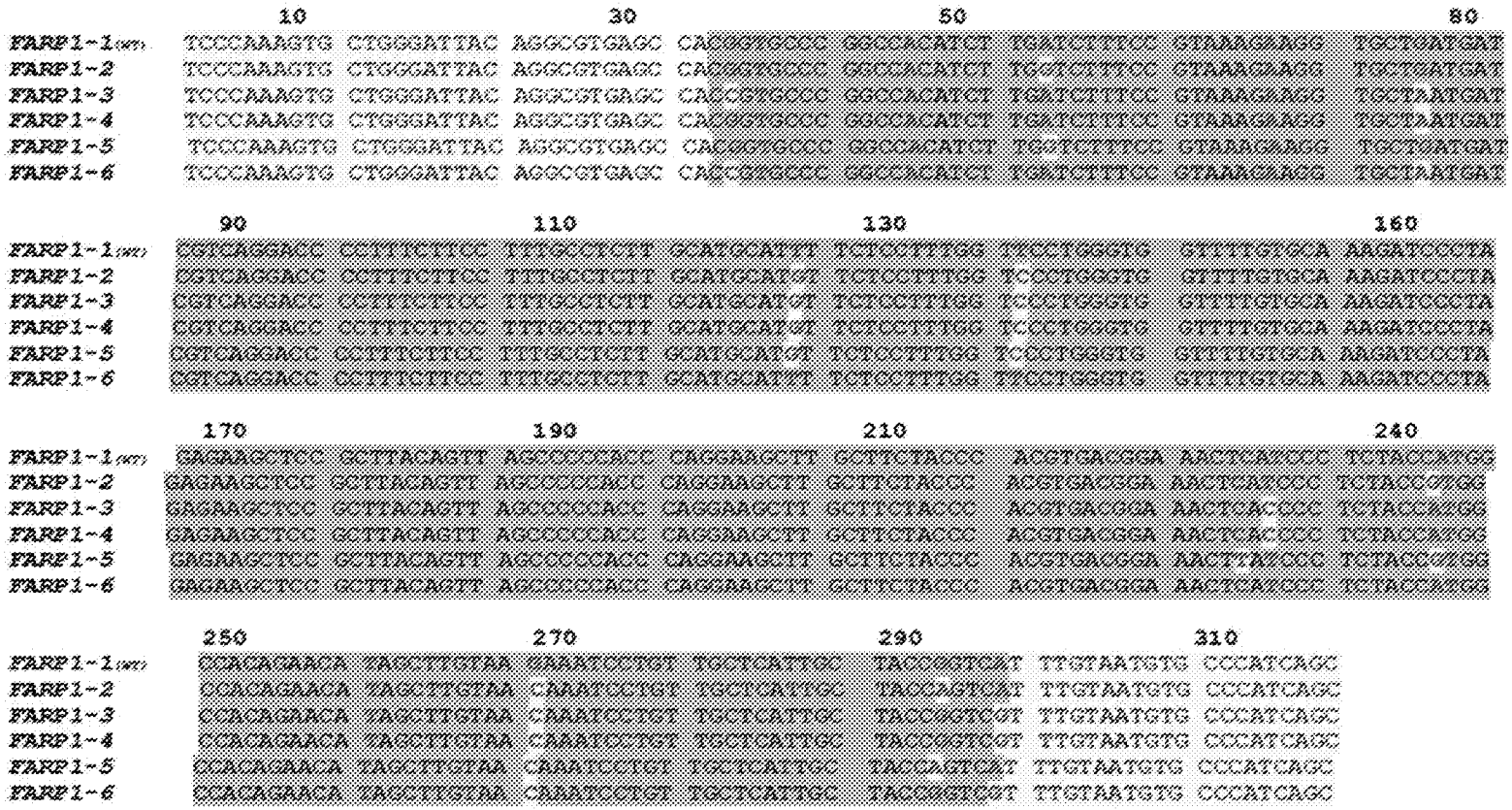

10 30 50 80
FARP1-1(wt) TCCCAAAGTG CTGGGATTAC AGGCGTGAGC CACCGTGCCC GGCCAGATCT TGATCTTTCC GTAAAGAAGG TGCTGATGAT
FARP1-2 TCCCAAAGTG CTGGGATTAC AGGCGTGAGC CACCGTGCCC GGCCAGATCT TGGTCTTTCC GTAAAGAAGG TGCTGATGAT
FARP1-3 TCCCAAAGTG CTGGGATTAC AGGCGTGAGC CACCGTGCCC GGCCAGATCT TGATCTTTCC GTAAAGAAGG TGCTAATGAT
FARP1-4 TCCCAAAGTG CTGGGATTAC AGGCGTGAGC CACCGTGCCC GGCCAGATCT TGATCTTTCC GTAAAGAAGG TGCTAATGAT
FARP1-5 TCCCAAAGTG CTGGGATTAC AGGCGTGAGC CACCGTGCCC GGCCAGATCT TGGTCTTTCC GTAAAGAAGG TGCTGATGAT
FARP1-6 TCCCAAAGTG CTGGGATTAC AGGCGTGAGC CACCGTGCCC GGCCAGATCT TGATCTTTCC GTAAAGAAGG TGCTAATGAT
90 110 130 160
FARP1-1(wt) CGTCAGGACC CCTTTCTTCC TTTGCCTCTT GCATGCATTT TCTCCTTTGG TGCCTGGGTG GTTTTGTGCA AAGATCCCTA
FARP1-2 CGTCAGGACC CCTTTCTTCC TTTGCCTCTT GCATGCATGT TCTCCTTTGG TCCCTGGGTG GTTTTGTGCA AAGATCCCTA
FARP1-3 CGTCAGGACC CCTTTCTTCC TTTGCCTCTT GCATGCATGT TCTCCTTTGG TCCCTGGGTG GTTTTGTGCA AAGATCCCTA
FARP1-4 CGTCAGGACC CCTTTCTTCC TTTGCCTCTT GCATGCATGT TCTCCTTTGG TCCCTGGGTG GTTTTGTGCA AAGATCCCTA
FARP1-5 CGTCAGGACC CCTTTCTTCC TTTGCCTCTT GCATGCATGT TCTCCTTTGG TCCCTGGGTG GTTTTGTGCA AAGATCCCTA
FARP1-6 CGTCAGGACC CCTTTCTTCC TTTGCCTCTT GCATGCATTT TCTCCTTTGG TGCCTGGGTG GTTTTGTGCA AAGATCCCTA
170 190 210 240
FARP1-1(wt) GAGAAGCTCC GCTTACAGTT AGCCCCCACC CAGGAAGCTT GCTTCTACCC ACGTGACGGA AACTCATCCC TCTACCATGG
FARP1-2 GAGAAGCTCC GCTTACAGTT AGCCCCCACC CAGGAAGCTT GCTTCTACCC ACGTGACGGA AACTCATCCC TCTACCATGG
FARP1-3 GAGAAGCTCC GCTTACAGTT AGCCCCCACC CAGGAAGCTT GCTTCTACCC ACGTGACGGA AACTCACCCC TCTACCATGG
FARP1-4 GAGAAGCTCC GCTTACAGTT AGCCCCCACC CAGGAAGCTT GCTTCTACCC ACGTGACGGA AACTCACCCC TCTACCATGG
FARP1-5 GAGAAGCTCC GCTTACAGTT AGCCCCCACC CAGGAAGCTT GCTTCTACCC ACGTGACGGA AACTTATCCC TCTACCGTGG
FARP1-6 GAGAAGCTCC GCTTACAGTT AGCCCCCACC CAGGAAGCTT GCTTCTACCC ACGTGACGGA AACTCATCCC TCTACCATGG
250 270 290 310
FARP1-1(wt) CCACAGAACA TAGCTTGTAA CAAATCCTGT TGCTCATTGC TACCAGTCAT TTGTAATGTG CCCATCAGC
FARP1-2 CCACAGAACA TAGCTTGTAA CAAATCCTGT TGCTCATTGC TACCAGTCAT TTGTAATGTG CCCATCAGC
FARP1-3 CCACAGAACA TAGCTTGTAA CAAATCCTGT TGCTCATTGC TACCAGTCGT TTGTAATGTG CCCATCAGC
FARP1-4 CCACAGAACA TAGCTTGTAA CAAATCCTGT TGCTCATTGC TACCAGTCGT TTGTAATGTG CCCATCAGC
FARP1-5 CCACAGAACA TAGCTTGTAA CAAATCCTGT TGCTCATTGC TACCAGTCAT TTGTAATGTG CCCATCAGC
FARP1-6 CCACAGAACA TAGCTTGTAA CAAATCCTGT TGCTCATTGC TACCAGTCGT TTGTAATGTG CCCATCAGC

FIGURE 10


Allele
FARP1-1 ---
FARP1-2 6 ---
FARP1-3 7 7 ---
FARP1-4 6 6 1 ---
FARP1-5 7 1 8 7 ---
FARP1-6 4 8 2 2 9 ---
FARP1-1
FARP1-2
FARP1-3
FARP1-4
FARP1-5
FARP1-6

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2015/043748

A. CLASSIFICATION OF SUBJECT MATTER

C12Q 1/68(2006.01)i

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 1/68; G01N 33/50

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
Korean utility models and applications for utility models
Japanese utility models and applications for utility models

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
eKOMPASS(KIPO internal) & Keywords: SNP haplotype assay, hematopoietic stem cell transplantation, polymorphic region, identity test

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2013-128199 A1 (NHS BLOOD & TRANSPLANT) 06 September 2013 See abstract; pages 9-10; claims 1-8, 11; figure 1.	1-4,6
X	US 2005-0266410 A1 (WALSH et al.) 01 December 2005 See abstract; claims 1-10.	1-4,6
A	PETERSDORF et al., 'MHC haplotype matching for unrelated hematopoietic cell transplantation' PLoS Medicine, Vol.4, Issue no.1, Article no.e8, pp.0059-0068 (2007) See the whole document.	1-4,6
A	PETERSDORF, 'The major histocompatibility complex: a model for understanding graft-versus-host disease' Blood, 2013, Vol.122, No.11, pp.1863-1872 (2013) See the whole document.	1-4,6
A	DE BAKKER et al., 'A high-resolution HLA and SNP haplotype map for disease association studies in the extended human MHC' Nature Genetics, Vol.38, No.10, pp.1166-1172 (2006) See the whole document.	1-4,6

☒ Further documents are listed in the continuation of Box C. ☒ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents,such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
10 November 2015 (10.11.2015)	**10 November 2015 (10.11.2015)**

Name and mailing address of the ISA/KR
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Korean Intellectual Property Office
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Authorized officer
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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2015/043748

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
PX	DEBELJAK et al., 'Haplotype counting by next-generation sequencing for ultrasensitive human DNA detection' The Journal of Molecular Diagnostics, Vol.16, No.5, pp.495-503 (September 2014) See the whole document.	1-4,6

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2015/043748

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☒ Claims Nos.: 5
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required addtional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fees, this Authority did not invite payment of any additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.
PCT/US2015/043748

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2013-128199 A1	06/09/2013	EP 2820152 A1	07/01/2015
		US 2015-0017643 A1	15/01/2015
US 2005-0266410 A1	01/12/2005	WO 2005-123951 A2	29/12/2005
		WO 2005-123951 A3	19/10/2006