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(71) Applicant *(for DE only)*: **ROCHE DIAGNOSTICS GMBH** [DE/DE]; Sandhofer Strasse 16, 68305 Mannheim (DE).

(71) Applicant *(for all designated States except DE, US)*: **F. HOFFMANN-LA ROCHE AG** [CH/CH]; Grenzacherstrasse 124, CH-4070 Bsel (CH).

(72) Inventors: **BENTLEY, Gordon**; Unit D, 1012 Walnut Street, Alameda, CA 94501 (US). **ERLICH, Henry, A.**; 3936 Rhoda Avenue, Oakland, CA 94602 (US). **HIGUCHI, Russell, Gene**; 3258 Liberty Avenue, Alameda, CA 94501 (US). **HOLCOMB, Cherie**; 3015 Partridge Avenue, Oakland, CA 94605 (US).

(74) Common Representative: **ROCHE DIAGNOSTICS GMBH**; Patent Department, 682985 Mannheim (DE).

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(54) Title: SYSTEM AND METHOD FOR DETECTION OF HLA VARIANTS

(57) Abstract: A method for detecting one or more HLA sequence types is described that comprises the steps of: amplifying a plurality of first amplicons from a double stranded nucleic acid sample, wherein the first amplicons are amplified with a plurality of pairs of nucleic acid primers that define exons 2 and 3 of both strands of HLA loci from the group consisting of HLA-A, HLA-B, and HLA-C; amplifying the first amplicons to produce a plurality of populations of second amplicons, wherein each population of second amplicons is clonally amplified from one of the first amplicons; sequencing the plurality of populations of second amplicons to generate a nucleic acid sequence composition for each of the plurality of second amplicons; and detecting variation in the sequence composition from one or more of the second amplicons for one or more of the HLA loci..

# SYSTEM AND METHOD FOR DETECTION OF HLA VARIANTS

## FIELD OF THE INVENTION

The invention provides methods, reagents and systems for detecting and analyzing sequence variants associated with HLA class I and class II loci. The variants may include single nucleotide polymorphisms (SNPs), polymorphic sequence motifs (i.e. complex polymorphisms involving adjacent nucleotides), insertion/deletion variation (referred to as "indels") and other types of polymorphism or variation known to those of ordinary skill in the related art that can occur in a population of target polynucleotides. The invention also relates to a method of investigating by massively parallel sequencing nucleic acids replicated by polymerase chain reaction (PCR), for the identification of mutations and polymorphisms of both known and unknown sequences. The invention involves using nucleic acid primers specifically designed to amplify a particular region and/or a series of overlapping regions of HLA DNA associated with a particular HLA characteristic or function. Also, the target sites for the primers were selected in part due to a low level of polymorphism enabling consistent amplification of the nucleic acids in a target HLA nucleic acid population which are suspected of containing variants to generate individual amplicons. Thousands of individual HLA amplicons are sequenced in a massively parallel, efficient, and cost effective manner to generate a distribution of the sequence variants found in the populations of amplicons that enables greater sensitivity of detection over previously employed methods.

## BACKGROUND OF THE INVENTION

The Human Leukocyte Antigen (generally referred to as HLA) class I and class II loci are the most polymorphic genes in the human genome, with a complex pattern of patchwork polymorphism (i.e. variants) localized primarily in exon 2 for the class II genes and exons 2 and 3 for the class I genes. For the current HLA typing methods, allele level resolution of HLA alleles, which is clinically important for hemapoetic stem cell transplantation, is technically challenging. Several large scale studies have demonstrated that precise, allele

level HLA matching between donor and patient significantly improves overall transplant survival by reducing the incidence and severity of both acute and chronic graft versus host disease and improving the rates of successful engraftment. When, for example, 8 of 8 of the most significant HLA loci are matched vs 6 of 8, survival after transplant was enhanced by 60% after 12 months.

It is current practice to maintain bone marrow donor registries in which millions of potential donors are HLA typed at low-medium resolution for the A, B, and, in many cases the DRB1 loci. Multiple potentially matched unrelated donors are selected, based on this initial typing, and then typed at the allele level resolution at these and additional loci to identify the donor best matched to the recipient.

Previously, the highest resolution HLA typing of variants has been obtained with fluorescent, Sanger-based DNA sequencing using capillary electrophoresis. However, ambiguities in the HLA typing data can persist due to multiple polymorphisms between alleles and the resultant phase ambiguities when both alleles are amplified and sequenced together. Resolving these ambiguities requires time-consuming approaches such as amplifying and then analyzing the two alleles separately.

Therefore, efficient detection of variation through improved sequencing methods enabled to generate sequence information in parallel from millions of DNA molecules is highly desirable. The clonal sequencing property of this system means that the allelic variants can be sequenced separately, thus allowing the setting of phase of linked polymoprhisms in the amplicon. Further, embodiments of improved sequencing methods include target specific high throughput sequencing techniques which have read lengths of about 250 nucleotides, about 400 nucleotides, or >400 nucleotides that enable complete sequence coverage of important HLA regions. For example, the target specific high throughput sequencing technologies employing HLA specific primers of the presently described invention are capable of setting the phase of the linked polymorphisms within an exon and make possible the unambiguous determination of the sequence of each HLA allele.

## SUMMARY OF THE INVENTION

Embodiments of the invention relate to the determination of the sequence of nucleic acids. More particularly, embodiments of the invention relate to methods and systems for correcting errors in data obtained during the sequencing of nucleic acids by SBS (sequencing by synthesis).

A method for detecting one or more HLA sequence types is described that comprises the steps of: amplifying a plurality of first amplicons from a double stranded nucleic acid sample, wherein the first amplicons are amplified with a plurality of pairs of nucleic acid primers that define exons 2 and 3 of both strands of HLA loci selected from the group consisting of HLA-A, HLA-B, and HLA-C; amplifying the first amplicons to produce a plurality of populations of second amplicons, wherein each population of second amplicons is clonally amplified from one of the first amplicons; sequencing the plurality of populations of second amplicons to generate a nucleic acid sequence composition for each of the plurality of second amplicons; and detecting variation in the sequence composition from one or more of the second amplicons for one or more of the HLA loci. In certain aspects the pairs of nucleic acid primers comprise sequence composition selected from a plurality of primers listed in Tables 4 and 5 below. In certain aspects the plurality of pairs of nucleic acid primers defines exons 1, 4, and 5 of the HLA loci. In certain aspects the first amplicons comprise an amplicon comprising sequence composition of exon 1, intron 1, and exon 2 of the HLA loci. In another aspect the first amplicons comprise an amplicon comprising sequence composition of exon 3 of the HLA loci. In another aspect the first amplicons comprise an amplicon comprising sequence composition of exon 4, intron 4, and exon 5 of the HLA loci. In certain aspects the plurality of pairs of nucleic acid primers defines exons 6, and 7 of the HLA-C locus. In particular aspects the first amplicons comprise an amplicon comprising sequence composition of exon 6, intron 6, and exon 7 of the HLA-C locus. In certain aspects plurality of pairs of nucleic acid primers for the HLA-A locus enable the sequencing of one or more exons in a forward and a reverse direction. In certain aspects the plurality of pairs of nucleic acid primers for the HLA-B locus enables the sequencing of one or more exons in a forward and a reverse direction. In certain aspects the plurality of pairs of nucleic acid primers for the HLA-C enables the sequencing of one or more exons in a forward and a reverse direction. In certain aspects the method further

comprises a plurality of adaptors each comprising an individual primer from the pairs of the nucleic acid primers. In certain aspects one or more of the plurality of adaptors comprise an MID identifier. In certain aspects the MID identifier enables pooling of the first amplicons derived from a plurality of the nucleic acid samples, wherein the populations of the second amplicons amplified from the pooled first amplicons are sequenced in parallel. In another aspect the plurality of adaptors comprises a general adaptor element and a key element. In certain aspects each population of second amplicons is immobilized on a bead substrate. In certain aspects the populations of second amplicons are clonally amplified using an emulsion PCR process. In certain aspects the plurality of populations of second amplicons are sequenced in parallel. In certain aspects the method further comprises the step of associating the variation with an HLA type. In particular aspects the association of variation and HLA type is known.

Additionally, a method for detecting one or more HLA sequence types is described that comprises the steps of: amplifying a plurality of first amplicons from a double stranded nucleic acid sample, wherein the first amplicons are amplified with a plurality of pairs of nucleic acid primers that define exon 2 of both strands of HLA loci selected from the group consisting of DRB1, DQA1, DQB1, DPA1, DPB1; amplifying the first amplicons to produce a plurality of populations of second amplicons, wherein each population of second amplicons is clonally amplified from one of the first amplicons; sequencing the plurality of populations of second amplicons to generate a nucleic acid sequence composition for each of the plurality of second amplicons; and detecting variation in the sequence composition from one or more of the second amplicons for one or more of the HLA loci. In certain aspects the pairs of nucleic acid primers comprise sequence composition selected from a plurality of primers listed in Tables 4 and 5. In certain aspects the plurality of pairs of nucleic acid primers for the DRB1 locus are generic and further enables amplification of loci selected from the group consisting of DRB3, DRB4, and DRB5 loci. In certain aspects the plurality of pairs of nucleic acid primers for the DRB1, 3, 4, and 5 loci enables the sequencing of exon 2 in a forward and a reverse direction. In certain aspects the plurality of pairs of nucleic acid primers for the DQA1 locus enables the sequencing of exon 2 in a forward and a reverse direction. In certain aspects the plurality of pairs of nucleic acid primers for the DQB1 locus enables the sequencing of exon 2 and exon 3 in a forward and

a reverse direction. In certain aspects the plurality of pairs of nucleic acid primers for the DPA1 locus enables the sequencing of exon 2 in a forward and a reverse direction. In certain aspects the plurality of pairs of nucleic acid primers for the DPB1 locus enables the sequencing of exon 2 in a forward and a reverse direction. In certain aspects the method further comprises a plurality of adaptors each comprising an individual primer from the pairs of the nucleic acid primers. In certain aspects one or more of the plurality of adaptors comprise an MID identifier. In certain aspects the MID identifier enables pooling of the first amplicons derived from a plurality of the nucleic acid samples, wherein the populations of the second amplicons amplified from the pooled first amplicons are sequenced in parallel. In particular aspects the plurality of adaptors comprises a general adaptor element and a key element. In certain aspects each population of second amplicons is immobilized on a bead substrate. In certain aspects the populations of second amplicons are clonally amplified using an emulsion PCR process. In certain aspects the plurality of populations of second amplicons are sequenced in parallel. In certain aspects the method further comprises the step of associating the variation with an HLA type. In particular aspects the association of variation and HLA type is known.

Also, an embodiment of a kit for detecting the one or more HLA types is described that comprises the pairs of nucleic acid primers employed to amplify the first amplicons of the embodiment of the methods described above.

The above embodiments and implementations are not necessarily inclusive or exclusive of each other and may be combined in any manner that is non-conflicting and otherwise possible, whether they are presented in association with a same, or a different, embodiment or implementation. The description of one embodiment or implementation is not intended to be limiting with respect to other embodiments and/or implementations. Also, any one or more function, step, operation, or technique described elsewhere in this specification may, in alternative implementations, be combined with any one or more function, step, operation, or technique described in the summary. Thus, the above embodiment and implementations are illustrative rather than limiting.

## BRIEF DESCRIPTION OF THE DRAWINGS

The above and further features will be more clearly appreciated from the following detailed description when taken in conjunction with the accompanying drawings. In the drawings, like reference numerals indicate like structures, elements, or method steps and the leftmost digit of a reference numeral indicates the number of the figure in which the references element first appears (for example, element 160 appears first in Figure 1). All of these conventions, however, are intended to be typical or illustrative, rather than limiting.

Figure 1 is a functional block diagram of one embodiment of a sequencing instrument under computer control and a reaction substrate;

Figure 2 is a simplified graphical representation of the relationship between the first amplicons to the HLA-A, B, and C genomic regions (exon and intron structure);

Figure 3 is a simplified graphical representation of the relationship between the first amplicons to the DPA1, DPB1, and DQA1 HLA regions; and

Figure 4 is a simplified graphical representation of the relationship between the first amplicons to the DQB1, and DRB1 HLA regions.

## DETAILED DESCRIPTION OF THE INVENTION

As will be described in greater detail below, embodiments of the presently described invention include systems and methods for designing primer species specific to HLA variants, and using those primers for highly sensitive detection of sequence variants.

*a. General*

The term "flowgram" generally refers to a graphical representation of sequence data generated by SBS methods, particularly pyrophosphate based sequencing methods (also referred to as "pyrosequencing") and may be referred to more specifically as a "pyrogram".

The term "read" or "sequence read" as used herein generally refers to the entire sequence data obtained from a single nucleic acid template molecule or a population of a plurality of substantially identical copies of the template nucleic acid molecule.

The terms "run" or "sequencing run" as used herein generally refer to a series of sequencing reactions performed in a sequencing operation of one or more template nucleic acid molecules.

The term "flow" as used herein generally refers to a serial or iterative cycle of addition of solution to an environment comprising a template nucleic acid molecule, where the solution may include a nucleotide species for addition to a nascent molecule or other reagent, such as buffers or enzymes that may be employed in a sequencing reaction or to reduce carryover or noise effects from previous flow cycles of nucleotide species.

The term "flow cycle" as used herein generally refers to a sequential series of flows where a nucleotide species is flowed once during the cycle (i.e. a flow cycle may include a sequential addition in the order of T, A, C, G nucleotide species, although other sequence combinations are also considered part of the definition). Typically, the flow cycle is a repeating cycle having the same sequence of flows from cycle to cycle.

The term "read length" as used herein generally refers to an upper limit of the length of a template molecule that may be reliably sequenced. There are numerous factors that contribute to the read length of a system and/or process including, but not limited to the degree of GC content in a template nucleic acid molecule.

The term "test fragment" or "TF" as used herein generally refers to a nucleic acid element of known sequence composition that may be employed for quality control, calibration, or other related purposes.

The term "primer" as used herein generally refers to an oligonucleotide that acts as a point of initiation of DNA synthesis under conditions in which synthesis of a primer extension product complementary to a nucleic acid strand is induced in an appropriate buffer at a suitable temperature. A primer is preferably a single stranded oligodeoxyribonucleotide.

A "nascent molecule" generally refers to a DNA strand which is being extended by the template-dependent DNA polymerase by incorporation of nucleotide species which are complementary to the corresponding nucleotide species in the template molecule.

The terms "template nucleic acid", "template molecule", "target nucleic acid", or "target molecule" generally refer to a nucleic acid molecule that is the subject of a sequencing reaction from which sequence data or information is generated.

The term "nucleotide species" as used herein generally refers to the identity of a nucleic acid monomer including purines (Adenine, Guanine) and pyrimidines (Cytosine, Uracil, Thymine) typically incorporated into a nascent nucleic acid molecule.

The term "monomer repeat" or "homopolymers" as used herein generally refers to two or more sequence positions comprising the same nucleotide species (i.e. a repeated nucleotide species).

The term "homogeneous extension" as used herein, generally refers to the relationship or phase of an extension reaction where each member of a population of substantially identical template molecules is homogenously performing the same extension step in the reaction.

The term "completion efficiency" as used herein generally refers to the percentage of nascent molecules that are properly extended during a given flow.

The term "incomplete extension rate" as used herein generally refers to the ratio of the number of nascent molecules that fail to be properly extended over the number of all nascent molecules.

The term "genomic library" or "shotgun library" as used herein generally refers to a collection of molecules derived from and/or representing an entire genome (i.e. all regions of a genome) of an organism or individual.

The term "amplicon" as used herein generally refers to selected amplification products, such as those produced from Polymerase Chain Reaction or Ligase Chain Reaction techniques.

The term "variant" or "allele" as used herein generally refers to one of a plurality of species each encoding a similar sequence composition, but with a degree of distinction from each other. The distinction may include any type of genetic variation known to those of ordinary skill in the related art, that include, but are not limited to, polymorphisms such as single nucleotide polymorphisms (SNPs), insertions or deletions (the combination of

insertion/deletion events are also referred to as "indels"), differences in the number of repeated sequences (also referred to as tandem repeats), and structural variations.

The term "allele frequency" or "allelic frequency" as used herein generally refers to the proportion of all variants in a population that is comprised of a particular variant.

The term "key sequence" or "key element" as used herein generally refers to a nucleic acid sequence element (typically of about 4 sequence positions, i.e., TGAC or other combination of nucleotide species) associated with a template nucleic acid molecule in a known location (i.e., typically included in a ligated adaptor element) comprising known sequence composition that is employed as a quality control reference for sequence data generated from template molecules. The sequence data passes the quality control if it includes the known sequence composition associated with a Key element in the correct location.

The term "keypass" or "keypass well" as used herein generally refers to the sequencing of a full length nucleic acid test sequence of known sequence composition (i.e., a "test fragment" or "TF" as referred to above) in a reaction well, where the accuracy of the sequence derived from keypass test sequence is compared to the known sequence composition and used to measure of the accuracy of the sequencing and for quality control. In typical embodiments, a proportion of the total number of wells in a sequencing run will be keypass wells which may, in some embodiments, be regionally distributed.

The term "blunt end" as used herein is interpreted consistently with the understanding of one of ordinary skill in the related art, and generally refers to a linear double stranded nucleic acid molecule having an end that terminates with a pair of complementary nucleotide base species, where a pair of blunt ends is typically compatible for ligation to each other.

The term "sticky end" or "overhang" as used herein is interpreted consistently with the understanding of one of ordinary skill in the related art, and generally refers to a linear double stranded nucleic acid molecule having one or more unpaired nucleotide species at the end of one strand of the molecule, where the unpaired nucleotide species may exist on either strand and include a single base position or a plurality of base positions (also sometimes referred to as "cohesive end").

The term "bead" or "bead substrate" as used herein generally refers to any type of bead of any convenient size and fabricated from any number of known materials such as cellulose, cellulose derivatives, acrylic resins, glass, silica gels, polystyrene, gelatin, polyvinyl pyrrolidone, co-polymers of vinyl and acrylamide, polystyrene cross-linked with divinylbenzene or the like (as described, e.g., in Merrifield, Biochemistry 1964, 3, 1385-1390), polyacrylamides, latex gels, polystyrene, dextran, rubber, silicon, plastics, nitrocellulose, natural sponges, silica gels, control pore glass, metals, cross-linked dextrans (e.g., Sephadex™) agarose gel (Sepharose™), and other solid phase bead supports known to those of skill in the art.

Some exemplary embodiments of systems and methods associated with sample preparation and processing, generation of sequence data, and analysis of sequence data are generally described below, some or all of which are amenable for use with embodiments of the presently described invention. In particular, the exemplary embodiments of systems and methods for preparation of template nucleic acid molecules, amplification of template molecules, generating target specific amplicons and/or genomic libraries, sequencing methods and instrumentation, and computer systems are described.

In typical embodiments, the nucleic acid molecules derived from an experimental or diagnostic sample must be prepared and processed from its raw form into template molecules amenable for high throughput sequencing. The processing methods may vary from application to application, resulting in template molecules comprising various characteristics. For example, in some embodiments of high throughput sequencing, it is preferable to generate template molecules with a sequence or read length that is at least the length a particular sequencing method can accurately produce sequence data for. In the present example, the length may include a range of about 25-30 base pairs, about 50-100 base pairs, about 200-300 base pairs, about 350-500 base pairs, greater than 500 base pairs, or other length amenable for a particular sequencing application. In some embodiments, nucleic acids from a sample, such as a genomic sample, are fragmented using a number of methods known to those of ordinary skill in the art. In preferred embodiments, methods that randomly fragment (i.e. do not select for specific sequences or regions) nucleic acids and may include what is referred to as nebulization or sonication methods. It will, however, be appreciated that other methods of fragmentation, such as digestion using restriction

endonucleases, may be employed for fragmentation purposes. Also in the present example, some processing methods may employ size selection methods known in the art to selectively isolate nucleic acid fragments of the desired length.

Also, it is preferable in some embodiments to associate additional functional elements with each template nucleic acid molecule. The elements may be employed for a variety of functions including, but not limited to, primer sequences for amplification and/or sequencing methods, quality control elements (i.e. such as Key elements or other type of quality control element), unique identifiers (also referred to as a multiplex identifier or "MID") that encode various associations such as with a sample of origin or patient, or other functional element.

For example, some embodiments of the described invention comprise associating one or more embodiments of an MID element having a known and identifiable sequence composition with a sample, and coupling the embodiments of MID element with template nucleic acid molecules from the associated samples. The MID coupled template nucleic acid molecules from a number of different samples are pooled into a single "Multiplexed" sample or composition that can then be efficiently processed to produce sequence data for each MID coupled template nucleic acid molecule. The sequence data for each template nucleic acid is de-convoluted to identify the sequence composition of coupled MID elements and association with sample of origin identified. In the present example, a multiplexed composition may include representatives from about 384 samples, about 96 samples, about 50 samples, about 20 samples, about 16 samples, about 10 samples, or other number of samples. Each sample may be associated with a different experimental condition, treatment, species, or individual in a research context. Similarly, each sample may be associated with a different tissue, cell, individual, condition, or treatment in a diagnostic context. Those of ordinary skill in the related art will appreciate that the numbers of samples listed above are for the purposes of example and thus should not be considered limiting.

In preferred embodiments, the sequence composition of each MID element is easily identifiable and resistant to introduced error from sequencing processes. Some embodiments of MID element comprise a unique sequence composition of nucleic acid

species that has minimal sequence similarity to a naturally occurring sequence. Alternatively, embodiments of a MID element may include some degree of sequence similarity to naturally occurring sequence.

Also, in preferred embodiments the position of each MID element is known relative to some feature of the template nucleic acid molecule and/or adaptor elements coupled to the template molecule. Having a known position of each MID is useful for finding the MID element in sequence data and interpretation of the MID sequence composition for possible errors and subsequent association with the sample of origin.

For example, some features useful as anchors for positional relationship to MID elements may include, but are not limited to, the length of the template molecule (i.e. the MID element is known to be so many sequence positions from the 5' or 3' end), recognizable sequence markers such as a Key element and/or one or more primer elements positioned adjacent to a MID element. In the present example, the Key and primer elements generally comprise a known sequence composition that typically does not vary from sample to sample in the multiplex composition and may be employed as positional references for searching for the MID element. An analysis algorithm implemented by application 135 may be executed on computer 130 to analyze generated sequence data for each MID coupled template to identify the more easily recognizable Key and/or primer elements, and extrapolate from those positions to identify a sequence region presumed to include the sequence of the MID element. Application 135 may then process the sequence composition of the presumed region and possibly some distance away in the flanking regions to positively identify the MID element and its sequence composition.

Some or all of the described functional elements may be combined into adaptor elements that are coupled to nucleotide sequences in certain processing steps. For example, some embodiments may associate priming sequence elements or regions comprising complementary sequence composition to primer sequences employed for amplification and/or sequencing. Further, the same elements may be employed for what may be referred to as "strand selection" and immobilization of nucleic acid molecules to a solid phase substrate. In some embodiments, two sets of priming sequence regions (hereafter referred to as priming sequence A, and priming sequence B) may be employed for strand selection,

where only single strands having one copy of priming sequence A and one copy of priming sequence B is selected and included as the prepared sample. In alternative embodiments, design characteristics of the adaptor elements eliminate the need for strand selection. The same priming sequence regions may be employed in methods for amplification and immobilization where, for instance, priming sequence B may be immobilized upon a solid substrate and amplified products are extended there from.

Additional examples of sample processing for fragmentation, strand selection, and addition of functional elements and adaptors are described in U.S. Patent Application Serial No. 10/767,894, titled "Method for preparing single-stranded DNA libraries", filed January 28, 2004; U.S. Patent Application Serial No. 12/156,242, titled "System and Method for Identification of Individual Samples from a Multiplex Mixture", filed May 29, 2008; and U.S. Patent Application Serial No. 12/380,139, titled "System and Method for Improved Processing of Nucleic Acids for Production of Sequencable Libraries", filed February 23, 2009.

Various examples of systems and methods for performing amplification of template nucleic acid molecules to generate populations of substantially identical copies are described. It will be apparent to those of ordinary skill that it is desirable in some embodiments of SBS to generate many copies of each nucleic acid element to generate a stronger signal when one or more nucleotide species is incorporated into each nascent molecule associated with a copy of the template molecule. There are many techniques known in the art for generating copies of nucleic acid molecules such as, for instance, amplification using what are referred to as bacterial vectors, "Rolling Circle" amplification (described in U.S. Patent Nos. 6,274,320 and 7,211,390) and Polymerase Chain Reaction (PCR) methods, each of the techniques are applicable for use with the presently described invention. One PCR technique that is particularly amenable to high throughput applications include what are referred to as emulsion PCR methods (also referred to as emPCR™ methods).

Typical embodiments of emulsion PCR methods include creating a stable emulsion of two immiscible substances creating aqueous droplets within which reactions may occur. In particular, the aqueous droplets of an emulsion amenable for use in PCR methods may include a first fluid, such as a water based fluid suspended or dispersed as droplets (also

referred to as a discontinuous phase) within another fluid, such as a hydrophobic fluid (also referred to as a continuous phase) that typically includes some type of oil. Examples of oil that may be employed include, but are not limited to, mineral oils, silicone based oils, or fluorinated oils.

Further, some emulsion embodiments may employ surfactants that act to stabilize the emulsion, which may be particularly useful for specific processing methods such as PCR. Some embodiments of surfactant may include one or more of a silicone or fluorinated surfactant. For example, one or more non-ionic surfactants may be employed that include, but are not limited to, sorbitan monooleate (also referred to as Span™ 80), polyoxyethylenesorbitsan monooleate (also referred to as Tween™ 80), or in some preferred embodiments, dimethicone copolyol (also referred to as Abil® EM90), polysiloxane, polyalkyl polyether copolymer, polyglycerol esters, poloxamers, and PVP/hexadecane copolymers (also referred to as Unimer U-151), or in more preferred embodiments, a high molecular weight silicone polyether in cyclopentasiloxane (also referred to as DC 5225C available from Dow Corning).

The droplets of an emulsion may also be referred to as compartments, microcapsules, microreactors, microenvironments, or other name commonly used in the related art. The aqueous droplets may range in size depending on the composition of the emulsion components or composition, contents contained therein, and formation technique employed. The described emulsions create the microenvironments within which chemical reactions, such as PCR, may be performed. For example, template nucleic acids and all reagents necessary to perform a desired PCR reaction may be encapsulated and chemically isolated in the droplets of an emulsion. Additional surfactants or other stabilizing agent may be employed in some embodiments to promote additional stability of the droplets as described above. Thermocycling operations typical of PCR methods may be executed using the droplets to amplify an encapsulated nucleic acid template resulting in the generation of a population comprising many substantially identical copies of the template nucleic acid. In some embodiments, the population within the droplet may be referred to as a "clonally isolated", "compartmentalized", "sequestered", "encapsulated", or "localized" population. Also in the present example, some or all of the described droplets may further encapsulate a solid substrate such as a bead for attachment of template and amplified copies of the

template, amplified copies complementary to the template, or combination thereof. Further, the solid substrate may be enabled for attachment of other type of nucleic acids, reagents, labels, or other molecules of interest.

Embodiments of an emulsion useful with the presently described invention may include a very high density of droplets or microcapsules enabling the described chemical reactions to be performed in a massively parallel way. Additional examples of emulsions employed for amplification and their uses for sequencing applications are described in U.S. Patent Nos. 7,638,276; 7,622,280; and U.S. Patent Application Serial Nos. 10/767,899; and 11/045,678.

Also embodiments sometimes referred to as Ultra-Deep Sequencing, generate target specific amplicons for sequencing may be employed with the presently described invention that include using sets of specific nucleic acid primers to amplify a selected target region or regions from a sample comprising the target nucleic acid. Further, the sample may include a population of nucleic acid molecules that are known or suspected to contain sequence variants comprising sequence composition associated with a research or diagnostic utility where the primers may be employed to amplify and provide insight into the distribution of sequence variants in the sample. For example, a method for identifying a sequence variant by specific amplification and sequencing of multiple alleles in a nucleic acid sample may be performed. The nucleic acid is first subjected to amplification by a pair of PCR primers designed to amplify a region surrounding the region of interest or segment common to the nucleic acid population. Each of the products of the PCR reaction (first amplicons) is subsequently further amplified individually in separate reaction vessels such as an emulsion based vessel described above. The resulting amplicons (referred to herein as second amplicons), each derived from one member of the first population of amplicons, are sequenced and the collection of sequences are used to determine an allelic frequency of one or more variants present. Importantly, the method does not require previous knowledge of the variants present and can typically identify variants present at <1% frequency in the population of nucleic acid molecules.

Some advantages of the described target specific amplification and sequencing methods include a higher level of sensitivity than previously achieved. Further, embodiments that employ high throughput sequencing instrumentation, such as for instance embodiments that

employ what is referred to as a PicoTiterPlate® array (also sometimes referred to as a PTP™ plate or array) of wells provided by 454 Life Sciences Corporation, the described methods can be employed to generate sequence composition for over 100,000, over 300,000, over 500,000, or over 1,000,000 nucleic acid regions per run or experiment and may depend, at least in part, on user preferences such as lane configurations enabled by the use of gaskets, etc. Also, the described methods provide a sensitivity of detection of low abundance alleles which may represent 1% or less of the allelic variants. Another advantage of the methods includes generating data comprising the sequence of the analyzed region. Importantly, it is not necessary to have prior knowledge of the sequence of the locus being analyzed.

Additional examples of target specific amplicons for sequencing are described in U.S. Patent Application Serial No. 11/104,781, titled "Methods for determining sequence variants using ultra-deep sequencing", filed April 12, 2005; PCT Patent Application Serial No. US 2008/003424, titled "System and Method for Detection of HIV Drug Resistant Variants", filed March 14, 2008; and U.S. Patent Application Serial No. 12/456,528, titled "System and Method for Detection of HIV Tropism Variants", filed June 17, 2009.

Further, embodiments of sequencing may include Sanger type techniques, techniques generally referred to as Sequencing by Hybridization (SBH), Sequencing by Ligation (SBL), or Sequencing by Incorporation (SBI) techniques. Further, the sequencing techniques may include what is referred to as polony sequencing techniques; nanopore, waveguide and other single molecule detection techniques; or reversible terminator techniques. As described above, a preferred technique may include Sequencing by Synthesis methods. For example, some SBS embodiments sequence populations of substantially identical copies of a nucleic acid template and typically employ one or more oligonucleotide primers designed to anneal to a predetermined, complementary position of the sample template molecule or one or more adaptors attached to the template molecule. The primer/template complex is presented with a nucleotide species in the presence of a nucleic acid polymerase enzyme. If the nucleotide species is complementary to the nucleic acid species corresponding to a sequence position on the sample template molecule that is directly adjacent to the 3' end of the oligonucleotide primer, then the polymerase will extend the primer with the nucleotide species. Alternatively, in some embodiments the

primer/template complex is presented with a plurality of nucleotide species of interest (typically A, G, C, and T) at once, and the nucleotide species that is complementary at the corresponding sequence position on the sample template molecule directly adjacent to the 3' end of the oligonucleotide primer is incorporated. In either of the described embodiments, the nucleotide species may be chemically blocked (such as at the 3'-O position) to prevent further extension, and need to be deblocked prior to the next round of synthesis. It will also be appreciated that the process of adding a nucleotide species to the end of a nascent molecule is substantially the same as that described above for addition to the end of a primer.

As described above, incorporation of the nucleotide species can be detected by a variety of methods known in the art, e.g. by detecting the release of pyrophosphate (PPi) (examples described in U.S. Patent Nos. 6,210,891; 6,258,568; and 6,828,100), or via detectable labels bound to the nucleotides. Some examples of detectable labels include but are not limited to mass tags and fluorescent or chemiluminescent labels. In typical embodiments, unincorporated nucleotides are removed, for example by washing. Further, in some embodiments the unincorporated nucleotides may be subjected to enzymatic degradation such as, for instance, degradation using the apyrase or pyrophosphatase enzymes as described in U.S. Patent Application Serial Nos. 12/215,455, titled "System and Method for Adaptive Reagent Control in Nucleic Acid Sequencing", filed June 27, 2008; and 12/322,284, titled "System and Method for Improved Signal Detection in Nucleic Acid Sequencing", filed January 29, 2009.

In the embodiments where detectable labels are used, they will typically have to be inactivated (e.g. by chemical cleavage or photobleaching) prior to the following cycle of synthesis. The next sequence position in the template/polymerase complex can then be queried with another nucleotide species, or a plurality of nucleotide species of interest, as described above. Repeated cycles of nucleotide addition, extension, signal acquisition, and washing result in a determination of the nucleotide sequence of the template strand. Continuing with the present example, a large number or population of substantially identical template molecules (e.g. $10^3$, $10^4$, $10^5$, $10^6$ or $10^7$ molecules) are typically analyzed simultaneously in any one sequencing reaction, in order to achieve a signal which is strong enough for reliable detection.

In addition, it may be advantageous in some embodiments to improve the read length capabilities and qualities of a sequencing process by employing what may be referred to as a "paired-end" sequencing strategy. For example, some embodiments of sequencing method have limitations on the total length of molecule from which a high quality and reliable read may be generated. In other words, the total number of sequence positions for a reliable read length may not exceed 25, 50, 100, or 500 bases depending on the sequencing embodiment employed. A paired-end sequencing strategy extends reliable read length by separately sequencing each end of a molecule (sometimes referred to as a "tag" end) that comprise a fragment of an original template nucleic acid molecule at each end joined in the center by a linker sequence. The original positional relationship of the template fragments is known and thus the data from the sequence reads may be re-combined into a single read having a longer high quality read length. Further examples of paired-end sequencing embodiments are described in U.S. Patent No. 7,601,499, titled "Paired end sequencing"; and in U.S. Patent Application Serial No. 12/322,119, titled "Paired end sequencing", filed January 28, 2009.

Some examples of SBS apparatus may implement some or all of the methods described above and may include one or more of a detection device such as a charge coupled device (i.e., CCD camera) or a confocal type architecture, a microfluidics chamber or flow cell, a reaction substrate, and/or a pump and flow valves. Taking the example of pyrophosphate based sequencing, embodiments of an apparatus may employ a chemiluminescent detection strategy that produces an inherently low level of background noise.

In some embodiments, the reaction substrate for sequencing may include what is referred to as a PTP™ array available from 454 Life Sciences Corporation, as described above, formed from a fiber optics faceplate that is acid-etched to yield hundreds of thousands or more of very small wells each enabled to hold a population of substantially identical template molecules (i.e., some preferred embodiments comprise about 3.3 million wells on a 70 x 75mm PTP™ array at a 35 μm well to well pitch). In some embodiments, each population of substantially identical template molecule may be disposed upon a solid substrate, such as a bead, each of which may be disposed in one of said wells. For example, an apparatus may include a reagent delivery element for providing fluid reagents to the PTP plate holders, as well as a CCD type detection device enabled to collect photons of light emitted from each

well on the PTP plate. An example of reaction substrates comprising characteristics for improved signal recognition is described in U.S. Patent No. 7,682,816, titled "THIN-FILM COATED MICROWELL ARRAYS AND METHODS OF MAKING SAME", filed August 30, 2005. Further examples of apparatus and methods for performing SBS type sequencing and pyrophosphate sequencing are described in U.S. Patent Nos. 7,323,305 and 7,682,816.

In addition, systems and methods may be employed that automate one or more sample preparation processes, such as the emPCR™ process described above. For example, automated systems may be employed to provide an efficient solution for generating an emulsion for emPCR processing, performing PCR Thermocycling operations, and enriching for successfully prepared populations of nucleic acid molecules for sequencing. Examples of automated sample preparation systems are described in U.S. Patent Application Serial No. 11/045,678, titled "Nucleic acid amplification with continuous flow emulsion", filed January 28, 2005.

Also, the systems and methods of the presently described embodiments of the invention may include implementation of some design, analysis, or other operation using a computer readable medium stored for execution on a computer system. For example, several embodiments are described in detail below to process detected signals and/or analyze data generated using SBS systems and methods where the processing and analysis embodiments are implementable on computer systems.

An exemplary embodiment of a computer system for use with the presently described invention may include any type of computer platform such as a workstation, a personal computer, a server, or any other present or future computer. It will, however, be appreciated by one of ordinary skill in the art that the aforementioned computer platforms as described herein are specifically configured to perform the specialized operations of the described invention and are not considered general purpose computers. Computers typically include known components, such as a processor, an operating system, system memory, memory storage devices, input-output controllers, input-output devices, and display devices. It will also be understood by those of ordinary skill in the relevant art that there are many possible

configurations and components of a computer and may also include cache memory, a data backup unit, and many other devices.

Display devices may include display devices that provide visual information, this information typically may be logically and/or physically organized as an array of pixels. An interface controller may also be included that may comprise any of a variety of known or future software programs for providing input and output interfaces. For example, interfaces may include what are generally referred to as "Graphical User Interfaces" (often referred to as GUI's) that provides one or more graphical representations to a user. Interfaces are typically enabled to accept user inputs using means of selection or input known to those of ordinary skill in the related art.

In the same or alternative embodiments, applications on a computer may employ an interface that includes what are referred to as "command line interfaces" (often referred to as CLI's). CLI's typically provide a text based interaction between an application and a user. Typically, command line interfaces present output and receive input as lines of text through display devices. For example, some implementations may include what are referred to as a "shell" such as Unix Shells known to those of ordinary skill in the related art, or Microsoft Windows Powershell that employs object-oriented type programming architectures such as the Microsoft .NET framework.

Those of ordinary skill in the related art will appreciate that interfaces may include one or more GUI's, CLI's or a combination thereof.

A processor may include a commercially available processor such as a Celeron®, Core™, or Pentium® processor made by Intel Corporation, a SPARC® processor made by Sun Microsystems, an Athlon™, Sempron™, Phenom™,or Opteron™ processor made by AMD corporation, or it may be one of other processors that are or will become available. Some embodiments of a processor may include what is referred to as Multi-core processor and/or be enabled to employ parallel processing technology in a single or multi-core configuration. For example, a multi-core architecture typically comprises two or more processor "execution cores". In the present example, each execution core may perform as an independent processor that enables parallel execution of multiple threads. In addition, those of ordinary skill in the related will appreciate that a processor may be configured in

what is generally referred to as 32 or 64 bit architectures, or other architectural configurations now known or that may be developed in the future.

A processor typically executes an operating system, which may be, for example, a Windows®-type operating system (such as Windows® XP, Windows Vista®, or Windows®_7) from the Microsoft Corporation; the Mac OS X operating system from Apple Computer Corp. (such as Mac OS X v10.6 "Snow Leopard" operating systems); a Unix® or Linux-type operating system available from many vendors or what is referred to as an open source; another or a future operating system; or some combination thereof. An operating system interfaces with firmware and hardware in a well-known manner, and facilitates the processor in coordinating and executing the functions of various computer programs that may be written in a variety of programming languages. An operating system, typically in cooperation with a processor, coordinates and executes functions of the other components of a computer. An operating system also provides scheduling, input-output control, file and data management, memory management, and communication control and related services, all in accordance with known techniques.

System memory may include any of a variety of known or future memory storage devices. Examples include any commonly available random access memory (RAM), magnetic medium, such as a resident hard disk or tape, an optical medium such as a read and write compact disc, or other memory storage device. Memory storage devices may include any of a variety of known or future devices, including a compact disk drive, a tape drive, a removable hard disk drive, USB or flash drive, or a diskette drive. Such types of memory storage devices typically read from, and/or write to, a program storage medium (not shown) such as, respectively, a compact disk, magnetic tape, removable hard disk, USB or flash drive, or floppy diskette. Any of these program storage media, or others now in use or that may later be developed, may be considered a computer program product. As will be appreciated, these program storage media typically store a computer software program and/or data. Computer software programs, also called computer control logic, typically are stored in system memory and/or the program storage device used in conjunction with memory storage device.

In some embodiments, a computer program product is described comprising a computer usable medium having control logic (computer software program, including program code) stored therein. The control logic, when executed by a processor, causes the processor to perform functions described herein. In other embodiments, some functions are implemented primarily in hardware using, for example, a hardware state machine. Implementation of the hardware state machine so as to perform the functions described herein will be apparent to those skilled in the relevant arts.

Input-output controllers could include any of a variety of known devices for accepting and processing information from a user, whether a human or a machine, whether local or remote. Such devices include, for example, modem cards, wireless cards, network interface cards, sound cards, or other types of controllers for any of a variety of known input devices. Output controllers could include controllers for any of a variety of known display devices for presenting information to a user, whether a human or a machine, whether local or remote. In the presently described embodiment, the functional elements of a computer communicate with each other via a system bus. Some embodiments of a computer may communicate with some functional elements using network or other types of remote communications.

As will be evident to those skilled in the relevant art, an instrument control and/or a data processing application, if implemented in software, may be loaded into and executed from system memory and/or a memory storage device. All or portions of the instrument control and/or data processing applications may also reside in a read-only memory or similar device of the memory storage device, such devices not requiring that the instrument control and/or data processing applications first be loaded through input-output controllers. It will be understood by those skilled in the relevant art that the instrument control and/or data processing applications, or portions of it, may be loaded by a processor in a known manner into system memory, or cache memory, or both, as advantageous for execution.

Also, a computer may include one or more library files, experiment data files, and an internet client stored in system memory. For example, experiment data could include data related to one or more experiments or assays such as detected signal values, or other values associated with one or more SBS experiments or processes. Additionally, an internet client

may include an application enabled to accesses a remote service on another computer using a network and may for instance comprise what are generally referred to as "Web Browsers". In the present example, some commonly employed web browsers include Microsoft® Internet Explorer 8 available from Microsoft Corporation, Mozilla Firefox® 3.6 from the Mozilla Corporation, Safari 4 from Apple Computer Corp., Google Chrome from the Google™ Corporation, or other type of web browser currently known in the art or to be developed in the future. Also, in the same or other embodiments an internet client may include, or could be an element of, specialized software applications enabled to access remote information via a network such as a data processing application for biological applications.

A network may include one or more of the many various types of networks well known to those of ordinary skill in the art. For example, a network may include a local or wide area network that employs what is commonly referred to as a TCP/IP protocol suite to communicate. A network may include a network comprising a worldwide system of interconnected computer networks that is commonly referred to as the internet, or could also include various intranet architectures. Those of ordinary skill in the related arts will also appreciate that some users in networked environments may prefer to employ what are generally referred to as "firewalls" (also sometimes referred to as Packet Filters, or Border Protection Devices) to control information traffic to and from hardware and/or software systems. For example, firewalls may comprise hardware or software elements or some combination thereof and are typically designed to enforce security policies put in place by users, such as for instance network administrators, etc.

*b. Embodiments of the presently described invention*

As described above, embodiments of the invention relate to methods of identifying or diagnosing a number of sequence variants associated with HLA (e.g., allelic variants, single nucleotide polymorphism variants, indel variants) by the identification of specific DNA. Examples of HLA alleles are described in Mason and Parham (1998) Tissue Antigens 51: 417-66, which lists HLA-A, HLA-B, and HLA-C alleles and Marsh et al. (1992) Hum.

Immunol. 35:1, which list HLA class II alleles for DRA, DRB, DQA1, DQB1, DPA1, and DPB1.

Typically, one or more instrument elements may be employed that automate one or more process steps. For example, embodiments of a sequencing method may be executed using instrumentation to automate and carry out some or all process steps. Figure 1 provides an illustrative example of sequencing instrument 100 that for sequencing processes requiring capture of optical signals typically comprise an optic subsystem and a fluidic subsystem for execution of sequencing reactions and data capture that occur on reaction substrate 105. It will, however, be appreciated that for sequencing processes requiring other modes of data capture (i.e. PH, temperature, electrochemical, etc.) a subsystem for the mode of data capture may be employed which are known to those of ordinary skill in the related art.

Embodiments of sequencing instrument 100 employed to execute sequencing processes may include various fluidic components in the fluidic subsystem, various optical components in the optic subsystem, as well as additional components not illustrated in Figure 1 that may include microprocessor and/or microcontroller components for local control of some functions. In some embodiments samples may be optionally prepared for sequencing in an automated or partially automated fashion using sample preparation instrument 180 configured to perform some or all of the necessary preparation for sequencing using instrument 100. Further, as illustrated in Figure 1 sequencing instrument 100 may be operatively linked to one or more external computer components such as computer 130 that may for instance execute system software or firmware such as application 135 that may provide instructional control of one or more of the instruments such as sequencing instrument 100 or sample preparation instrument 180, and/or data analysis functions. Computer 130 may be additionally operatively connected to other computers or servers via network 150 that may enable remote operation of instrument systems and the export of large amounts of data to systems capable of storage and processing. In the present example, sequencing instrument 100 and/or computer 130 may include some or all of the components and characteristics of the embodiments generally described above.

In general, embodiments of the invention include a two stage PCR technique (i.e. producing first and second amplicons as described above) targeted to specific regions of HLA, coupled with a sequencing technique that produces sequence information from thousands of nucleic acid molecules in parallel which enables identification of the frequency of occurrence of HLA types present, even those types occurring at a very low frequency in a sample. It will be appreciated that in typical HLA typing embodiments the HLA type for an individual would be completely homozygous where the type would be detected at about a 100% frequency or completely heterozygous where each type would be detected at about 50% frequency. However, embodiments of the invention can detect HLA types present in a sample containing HLA in non-stoichiometric allele amounts, such as, for example, HLA types present at greater than 50%, less than 50%, less than 25%, less than 10%, less than 5% or less than 1%. For example, for a sample derived from a single individual using specific amplification one would expect to detect 100% or 50% (in a heterozygote) of an HLA allele. However one might detect, for example, 5% or 10%, in a complex mixture derived from more than one individual, such as a forensics specimen with multiple contributors (blood from suspect and victim) or in a blood sample monitoring engraftment following a bone marrow transplant (mixture of donor and recipient) or in the SCIDS example with 1-2% maternal cells. The described embodiments enable such identification in a rapid, reliable, and cost effective manner.

In the described embodiments the second round of amplification typically occurs using the emulsion based PCR amplification strategy described above that results in the immobilized clonal population of "second" amplicons on a bead substrate that effectively sequesters the second amplicons preventing diffusion when the emulsion is broken. Typically, thousands of the second amplicons are then sequenced in parallel as described elsewhere in this specification. For example, beads with immobilized populations of second amplicons may be loaded onto reaction substrate 105 and processed using sequencing instrument 100 which generates >1000 clonal reads from each sample and outputs the sequence data to computer 130 for processing. Computer 130 executes specialized software (such as for instance application 135) to identify the HLA type(s) for the loci of interest present in the sample.

As described above, sequencing many nucleic acid templates in parallel provides the sensitivity for the presently described invention as described above. For example, based on binomial statistics the lower limit of detection (i.e., one event) for a fully loaded 60 mm x 60 mm PicoTiterPlate (2 X $10^6$ high quality bases, comprised of 200,000 x 100 base reads) with 95% confidence, is for a population with allelic frequency of at least 0.002%, and with 99% confidence for a population with allelic frequency of at least 0.003% (it will also be appreciated that a 70 x 75 mm PicoTiterPlate could be employed as described above, which allows for an even greater number of reads and thus increased sensitivity). For comparison, SNP detection via pyrophosphate based sequencing has reported detection of separate allelic states on a tetraploid genome, so long as the least frequent allele is present in 10% or more of the population (Rickert et al., 2002 BioTechniques. 32:592-603). Conventional fluorescent DNA sequencing is even less sensitive, experiencing trouble resolving 50/50 (i.e., 50 %) heterozygote alleles (Ahmadian et al., 2000 Anal. BioChem. 280:103-110).

For the purposes of example, Table 1 shows the probability of detecting zero, or one or more, events, based on the incidence of SNP's in the total population, for a given number N (=100) of sequenced amplicons. "*" indicates a probability of 3.7% of failing to detect at least one event when the incidence is 5.0%; similarly, "**" reveals a probability of 0.6% of failing to detect one or more events when the incidence is 7%.

The table thus indicates that the confidence level to detect a SNP present at the 5% level is 95% or better and, similarly, the confidence of detecting a SNP present at the 7% level is 99% or better.

Table 1

| Incidence (%) | Prob. of at least 1 event (N = 100) | Prob. of no event (N = 100) |
|---|---|---|
| 1 | 0.264 | 0.736 |
| 2 | 0.597 | 0.403 |
| 3 | 0.805 | 0.195 |
| 4 | 0.913 | 0.087 |
| 5 | 0.963 | 0.037 * |
| 6 | 0.985 | 0.015 |
| 7 | 0.994 | 0.006 ** |

| | | |
|---|---|---|
| 8 | 0.998 | 0.002 |
| 9 | 0.999 | 0.001 |
| 10 | 1.000 | 0.000 |

Naturally, multiplex analysis is of greater applicability than depth of detection and Table 2 displays the number of SNPs that can be screened simultaneously on a single PicoTiterPlate array, with the minimum allelic frequencies detectable at 95% and 99% confidence.

Table 2

| SNP Classes | Number of Reads | Minimum frequency of SNP in population detectable with 95% confidence | Minimum frequency of SNP in population detectable with 99% confidence |
|---|---|---|---|
| 1 | 200000 | 0.002% | 0.003% |
| 2 | 100000 | 0.005% | 0.007% |
| 5 | 40000 | 0.014% | 0.018% |
| 10 | 20000 | 0.028% | 0.037% |
| 50 | 4000 | 0.14% | 0.18% |
| 100 | 2000 | 0.28% | 0.37% |
| 200 | 1000 | 0.55% | 0.74% |
| 500 | 400 | 1.39% | 1.85% |
| 1000 | 200 | 2.76% | 3.64% |

Embodiments of the described invention provide methods of HLA genotyping based the discovery that a multiplex, parallel clonal sequencing analysis can be used to genotype at least 3, typically at least 6, and preferably at least 8 HLA loci in multiple individuals at the same time. The sequencing platforms described herein clonally propagate in parallel millions of single DNA molecules which are then also sequenced in parallel. It will be appreciated that the read lengths obtainable by the described sequencing platforms (i.e. GS FLX or GS Junior sequencing platforms available from 454 Life Sciences Corporation) are typically > 500 nucleotides. These clonal read lengths make possible setting the phase of

the linked polymorphisms within an exon and thus the unambiguous determination of the sequence of each HLA allele. It is important to note that the described sequencing technologies with read lengths of 500 bases or more enable the acquisition of the complete sequence composition for the loci of interest as a single read in both directions. For example, each strand of the double stranded DNA for the region comprising one or more loci may be simultaneously sequenced in the 5'-3' direction producing a complete read across said loci enabling unambiguous HLA typing. Thus a higher level of confidence is achieved due to the fact that each nucleotide position in the loci of interest has been interrogated and reviewed in both the forward and reverse directions.

In the described invention, the system is sufficiently high throughput to enable a complete, 8-locus HLA typing for multiple individuals, e.g., 24, 48, or more subjects, in a single sequencing run using a next-generation sequencing platform as described herein.

The highly multiplexed amplicon sequencing of the described embodiments employ sample-specific internal sequence tags (i.e. MIDs as described above) in the primers that allow pooling of samples yet maintain the ability to assign sequences to a specific individual. In the described embodiments, the HLA genotypes for at least eight loci (HLA-A, B, C, DRB1, DQA1, DQB1, DPA1, DPB1), as well as for DRB 3, 4, and 5 can be obtained from the data generated by sequencing. This HLA sequencing system can also detect chimeric mixtures, e.g., the detection of the rare non-transmitted maternal allele present in the blood of SCID patients as referenced above. For example, those of ordinary skill in the related art appreciate that SCIDs (also sometimes referred to as "Bubble Boy Disease") can include the presence of a third allele in cells of maternal origin in circulation within an individual. The individuals containing cells with the non-transmitted maternal alleles (i.e. maternal cells) are sometimes referred to as "Micro Chimeras" and the maternal cells typically occur at a very low frequency (i.e. ~1-2%) yet have a profound effect upon the individual who often lacks a functional immune system.

Those of ordinary skill in the related art appreciate that the human leukocyte antigen system (HLA) complex spans approximately 3.5 million base pairs on the short arm of chromosome 6. The major regions are the class I and class II regions. The major Class I antigens are HLA-A, HLA-B, and HLA-C and the major Class II antigens are HLA-DP,

HLA-DQ and HLA-DR. The HLA-DP, HLA-DQ and HLA-DR loci encode the α and β chains of the HLA-DR, DP and DQ antigens. The HLA genes are among the most polymorphic genes in the genome. Polymorphisms that are expressed in the HLA antigen (and therefore of great interest for typing for transplantation) are localized primarily in exon 2 for the class II genes and exons 2 and 3 for the class I genes. In the presently described embodiments, the read lengths attainable employing the HLA primers and sequencing system described herein enable complete sequencing through the HLA regions important for accurate typing including exon 2 and exon 3. For example, those of ordinary skill in the related art will appreciate that in most individuals HLA-A*01010101 typically comprises about 73 sequence positions in exon 1, about 130 sequence positions in intron 1, about 270 sequence positions in exon 2, about 241 sequence positions in intron 2, about 276 sequence positions in exon 3, about 578 sequence positions in intron 3, about 276 sequence positions in exon 4, about 102 sequence positions in intron 4, about 117 sequence positions in exon 5, about 442 sequence positions in intron 5, about 33 sequence positions in exon 6, about 142 sequence positions in intron 6, about 48 sequence positions in exon 7, about 169 sequence positions in intron 7, and about 5 sequence positions in exon 8.

In embodiments of the described invention, the genotype of an HLA gene as described herein refers to determining the variations in HLA type (which include various polymorphisms) present in the HLA loci. For HLA-A, the variants present in exon 2 and exon 3 are determined by sequencing the products of first amplicons generated by PCR from an individual. In typical embodiments, the sequence of exon 4 is also determined. Exon 2, exon 3, and exon 4, or regions thereof that comprise the allelic determinants, are each amplified in individual PCR reactions to obtain first amplicons. Similarly, first amplicons are obtained for exon 2 and exon 3, and in some embodiments, exon 4, for the HLA-B and HLA-C alleles for an individual. For genotyping HLA class II alleles, first amplicons are obtained for exon 2 of DRB1, DPB1, DPA1, DQA1 and exons 2 and 3 of DQB1. Each exon can be sequenced completely by sequencing the products of first amplicons generated from both strands with sufficient overlap between the reads from either end that specific HLA alleles can be unambiguously assigned.

Figures 2-4 provide a simplified graphical example of the relationship between the first amplicons generated in embodiments of the invention to the respective HLA region. For

instance, Figure 2 illustrates first amplicon 203 that spans a region comprising exon 1, intron 1, and exon 2; first amplicon 205 that spans a region comprising exon 3; and first amplicon 207 that spans a region comprising exon 4, intron 4, and exon 5 of the HLA-A allele using HLA specific forward primer 250 and reverse primer 260. Figure 2, also illustrates similar relationships for first amplicons 213, 215, and 217 of the HLA-B allele; and first amplicons 223, 225, and 227 of the HLA-C allele with an additional first amplicon 229 that spans a region comprising exon 6, intron 6, and exon 7. Similarly, Figure 3 illustrates first amplicons 303, 313, and 323 that span a region comprising exon 2 of the DPA1, DPB1, and DQA1 alleles respectively; and Figure 4 illustrates first amplicons 403, and 413 that span a region comprising exon 2 of the DQB1, and DRB1 alleles with the addition of first amplicon 405 that spans a region comprising exon 3 of the DRB1 allele. It will be appreciated that the graphical representations provided in Figures 2-4 are for the purposes of illustration and should not be considered limiting.

Each sample from an individual is amplified at one or more loci individually using primers that target the loci of interest that typically include a polymorphic region of one or more exons of interest. The primers employed in the amplification reaction may include additional sequence element such as adapter sequences for emulsion PCR and an identifying MID sequence element that serves as a marker for the DNA from a single individual.

The invention employs amplification primers that amplify the loci of interest of the HLA genes. Typically, the primers are designed to ensure that the entire polymorphic portion of an exon is obtained.

In the described embodiments, primer sequences for the multiplex amplification of the invention are incorporated into adaptors that include sequence elements that can be used to facilitate the clonal sequencing and the analysis. The adaptors of some or all of the described embodiments therefore include the following components: a general adaptor element, a unique identification (i.e. MID) tag and a primer sequence that hybridizes to an HLA gene of interest to use in an amplification reaction to obtain a first HLA amplicon. For example, a schematic representation of an adaptor may include:

General Adapter Sequence +MID Sequence + Target-specific Sequence

The general adaptor elements of the described embodiments may comprise various sequence elements and are typically present at the 5' end of the adaptors. For example, the general adapter regions may comprise sequences that serve as the site of annealing of primers for the sequencing reaction and also correspond to sequences present on beads, or a solid surface, so that the first amplicon can be annealed to the surface for emulsion PCR. The forward primer for amplifying an HLA exon includes an adapter sequence at the 5' end, referred to here as the adapter region A. The reverse primer comprises a region that contains an adapter sequence at the 5' end, referred to here as adapter region B. As noted, the sequences present in the adaptor region and their complements allow for annealing of the first amplicons to beads for emulsion PCR as well as the populations of second amplicons which result from the emPCR process. Optionally, the adaptor may further include a unique discriminating key sequence comprised of a non-repeating nucleotide sequence (i.e., ACGT, CAGT, etc.). This key sequence is typically incorporated to bioinformaticly distinguish the sequenced populations of second amplicons for HLA genotyping from control sequences that are included in the reaction.

In the described embodiments the general adaptor sequence may include the following sequences:

Forward A: GCCTCCCTCGCGCCATCCGACTCAG (SEQ ID NO: 1);

Reverse B: GCCTTGCCAGCCCGCGCAGTCTCAG (SEQ ID NO: 2)

OR

Forward A: CGTATCGCCTCCCTCGCGCCATCAG (SEQ ID NO: 3);

Reverse B: CTATGCGCCTTGCCAGCCCGCTCAG (SEQ ID NO: 4)

It will be appreciated that the described invention is not limited to the exact composition of the general adaptor sequences described above and that different sequence compositions may be used.

PCR primers for use in the described embodiments of HLA genotyping method further comprise MID sequence elements as described above. These MID sequence elements are used to bioinformaticly distinguish the sequenced HLA second amplicons from each

individual tested. In the described embodiments, HLA regions of interest are amplified from a nucleic acid sample from a subject to be genotyped. For example, the HLA exons, or regions of the exons, comprising the variants that act as allelic determinants are individually amplified. The first amplicons obtained from the subject are marked with the same MID sequence element associating the first amplicons with the subject. In the present example, the MID sequence element is included in the adaptors that are used to amplify each first amplicon for that subject as well as subsequent amplification producing the populations of second amplicons. Accordingly, the MID sequence elements are also sequenced in the sequencing reaction and the sequence composition of each first amplicon (i.e. via sequencing of the respective population of second amplicons) are bioinformaticly deconvoluted to associate the sequence composition, and variants contained therein, with the subject.

Table 3 provides examples of MID sequence element useable with embodiments of the described invention.

| 4 BP MID's | 5 BP MID's | | | | 10 BP MID's | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | SEQ ID | | SEQ ID | | SEQ ID | | SEQ ID |
| | 5'T | 5'C | 5'A | 5'G | 5'T | NO: | 5'C | NO: | 5'A | NO: | 5'G | NO: |
| TCAG | TCAGC | TCAGA | TCAGC | TCAGA | ACGCTCGACA | 5 | ACGAGTGCGT | 17 | ACGAGTGCGT | 17 | ACGAGTGCGT | 17 |
| TCAT | TCATC | TCATG | TCATG | TCATC | AGACGCACTC | 6 | ACGCTCGACA | 5 | AGACGCACTC | 6 | ACGCTCGACA | 5 |
| TCTC | TCTCA | TCTCT | TCTCT | TCTCA | AGCACTGTAG | 7 | AGCACTGTAG | 7 | AGCACTGTAG | 7 | AGACGCACTC | 6 |
| TCTG | TCTGC | TCTGA | TCTGC | TCTGA | ATCAGACACG | 8 | ATCAGACACG | 8 | ATCAGACACG | 8 | CGTGTCTCTA | 10 |
| TGAT | TGATC | TGATG | TGATG | TGATC | ATATCGCGAG | 9 | ATATCGCGAG | 9 | ATATCGCGAG | 9 | CTCGCGTGTC | 11 |
| TGAG | TGAGC | TGAGA | TGAGC | TGAGA | CGTGTCTCTA | 10 | CGTGTCTCTA | 10 | CTCGCGTGTC | 11 | TAGTATCAGC | 12 |
| TGCT | TGCTC | TGCTG | TGCTG | TGCTC | CTCGCGTGTC | 11 | TCTCTATGCG | 13 | TAGTATCAGC | 12 | TGATACGTCT | 18 |
| TGCA | TGCAG | TGCAT | TGCAG | TGCAT | TAGTATCAGC | 12 | TGATACGTCT | 18 | TCTCTATGCG | 13 | TACTGAGCTA | 14 |
| CAGA | CAGAG | CAGAT | CAGAG | CAGAT | TCTCTATGCG | 13 | TACTGAGCTA | 14 | TGATACGTCT | 18 | CGAGAGATAC | 16 |
| CAGC | CAGCA | CAGCT | CAGCT | CAGCA | TACTGAGCTA | 14 | CATAGTAGTG | 15 | CATAGTAGTG | 15 | CATAGTAGTA | 20 |
| CATC | CATCA | CATCT | CATCT | CATCA | CATAGTAGTG | 15 | CGTGTCTCTG | 19 | CGAGAGATAC | 16 | ACGAGTGCGA | 21 |
| CATG | CATGC | CATGA | CATGC | CATGA | CGAGAGATAC | 16 | CATAGTAGTA | 20 | CGTGTCTCTG | 19 | CATAGTAGTC | 22 |
| CTCT | CTCTC | CTCTG | CTCTG | CTCTC | | | | | | | | |
| CTCA | CTCAG | CTCAT | CTCAG | CTCAT | | | | | | | | |
| CTGA | CTGAG | CTGAT | CTGAG | CTGAT | | | | | | | | |
| CTGC | CTGCA | CTGCT | CTGCT | CTGCA | | | | | | | | |
| ATCA | ATCAG | ATCAT | ATCAG | ATCAT | | | | | | | | |
| ATCT | ATCTC | ATCTG | ATCTG | ATCTC | | | | | | | | |
| ATGA | ATGAG | ATGAT | ATGAG | ATGAT | | | | | | | | |
| ATGC | ATGCA | ATGCT | ATGCT | ATGCA | | | | | | | | |
| AGCA | AGCAG | AGCAT | AGCAG | AGCAT | | | | | | | | |
| AGCT | AGCTC | AGCTG | AGCTG | AGCTC | | | | | | | | |
| AGAT | AGATC | AGATG | AGATG | AGATC | | | | | | | | |
| AGAG | AGAGC | AGAGA | AGAGC | AGAGA | | | | | | | | |

Table 3- MID Sequences

In embodiments of the described invention the MID sequences can be designed taking into account certain parameters which may include some or all of the parameters described above. For example, in designing a 4-residue MID tag, it is desirable to choose 4 bases that take into account the flow cycle of the nucleotides in the sequencing reaction. In the present example, if the nucleotides are added in the order T, A, C, and G, it is typically desirable to design the MID sequence such that a nucleotide that is positive (i.e. nucleotide in the flow is complementary to the next nucleotide in MID sequence) is followed by a residue that would be negative (i.e. nucleotide in the flow is non-complementary to the next nucleotide in MID sequence). Accordingly, in this example, if an MID sequence begins with an "A" nucleotide such that the nucleotide incorporated in the sequencing reaction is T, the second nucleotide in the tag sequence would be a nucleotide such that A would not be incorporated. In addition, it is desirable to avoid forming homopolymers, either within the MID sequence or through creating them based on the last nucleotide of the adapter region or the first nucleotide of the HLA-specific primer region of the adaptor.

The target-specific sequence (also referred to herein as HLA priming region, HLA binding region, or HLA hybridizing region) of the described adaptors is the region of the primer that hybridizes to the HLA sequence of interest to amplify the desired locus that may include an exon, combination of two exons and intervening intron sequence, or in some embodiments, a limited region of the exon. Typically, the HLA priming region of the adaptor hybridizes to intronic sequence adjacent to the exon to be amplified in order to obtain the entire exon sequence. The HLA primer sequences are preferably selected to selectively amplify the HLA exon of interest, although in some embodiments, a primer pair may also amplify a highly similar region of a related region of HLA gene. For example, the primers for exon 2 of DRB1 described in the example section below also amplify the DRB3, DRB4, and DRB5 loci (i.e. they are "generic" to those loci). The primer sequences are selected such that the exon is amplified with sufficient specificity to allow unambiguous determination of the HLA genotype from the sequence.

Consensus sequences of HLA genes and alleles are known and available through various databases, including GenBank and other gene databases and have been published (see e.g., Mason and Parham (1998) Tissue Antigens 51: 417-66, listing

HLA-A, HLA-B, and HLA-C alleles; Marsh et al. (1992) Hum. Immunol. 35:1, listing HLA Class II alleles--DRA, DRB, DQA1, DQB1, DPA1, and DPB1).

The PCR primers were designed based on principles known in the art. Strategies for primer design may be found throughout the scientific literature, for example, in Rubin, E. and A. A. Levy, Nucleic Acids Res, 1996.24 (18): p. 3538-45; and Buck et al., Biotechniques, 1999.27 (3): p. 528-36. For example, the HLA-specific primer is typically about 20 nucleotides or greater, e.g., 20 to 35 nucleotides in length. Other parameters that are considered are G/C content, design considerations to avoid internal secondary structure, and prevent the formation of primer dimers, as well as melting temperatures (Tm).

Examples of HLA target specific primers for use in embodiments of the invention are provided in Table 4.

| **HLA-A** | **SEQ ID No:** | |
|---|---|---|
| HLA-A Exon 2 5' | 23 | GAAACGGCCTCTGTGGGGAGAAGCAA |
| HLA-A Exon 1-2 3' | 24 | GGTGGATCTCGGACCCGGAGACTGT |
| HLA-A Exon 3 5' | 25 | GACTGGGCTGACCGTGGGGT |
| HLA-A Exon 3 3' | 26 | CCCCTGGTACCVGTGCGCTGCA |
| HLA-A Exon 3 5' | 27 | GACTGGGCTGACCKYGGGGT |
| HLA-A Exon 3 3' | 28 | GAGGGTGATATTCTAGTGTTGGTCCCAA |
| HLA-A Exon 4 5' | 29 | TGCCTGAATGWTCTGACTCTTCCCGTMAGA |
| HLA-A Exon 4 3' | 30 | TGACCCTGCTAAAGGTCTCCAGAG |
| HLA-A Exon 4 3' | 30 | TGACCCTGCTAAAGGTCTCCAGAG |
| HLA-A Exon 4 5' | 31 | CTGGGTTCTGTGCTCYCTTCCCCAT |
| HLA-A Exon 4 3' | 32 | CTCCAGAGAGGCTCCTGCTTTCCSTA |
| **HLA-B** | | |
| HLA-B Exon 2 5' | 33 | AGAGCTCGGGAGGAGCGAGGGGACCSCAG |
| HLA-B Exon 2 3' | 34 | ACTCGAGGCCTCGCTCTGGTTGTAGTA |
| HLA-B Exon 2 3' | 35 | CGGTCGAGGGTYTGGGC |
| HLA-B Exon 3 5' | 36 | AGAGCTCGGGCCAGGGTCTCACA |
| HLA-B Exon 3 3' | 37 | ACTCGAGGGAGGCCATCCCCGGCGACCTAT |
| HLA-B Exon 3 5' | 38 | CCCGGTTTCATTTTCAGTTGAGG |
| HLA-B Exon 4 5' | 39 | GCGCCTGAATTTTCTGACTCTTCCCA |
| HLA-B Exon 4 3' | 40 | GGCTCCTGCTTTCCCTGAGAA |
| HLA-B Exon 4 5' | 41 | CTGGTCACATGGGTGGTCC |
| HLA-B Exon 4 3' | 42 | AGATATGACCCCTCATCCC |

| | | |
|---|---|---|
| **HLA-C** | | |
| HLA-C Exon 2 5' | 43 | AGTCGACGAADCGGCCTCTGSGGA |
| HLA-C Exon 2 3' | 44 | ACTCGAGGGGCYGGGGTCACTCAC |
| HLA-C Exon 3 5' | 45 | ACGTCGACGGGCCAGGKTCTCACA |
| HLA-C Exon 3 3' | 46 | ACCTCGAGGTCAGCAGCCTGACCACA |
| HLA-C Exon 3 3' nested | 47 | CTCCCCACTGCCCCTGGTAC |
| HLA-C Exon 4 5' | 48 | CAAAGTGTCTGAATTTTCTGACTCTTCCC |
| HLA-C Exon 4 3' | 49 | TGAAGGGCTCCAGAAGGACTT |
| HLA-C Exon 4 3' | 50 | TGAAGGGCTCCAGGACTT |
| HLA-C Exon 4 5' | 51 | GTGTCGCAAGAGAGATRCAAAGTGT |
| HLA-C Exon 4 3' | 52 | GAGGRGAAGGTGAGGGGCC |
| **DPB1** | | |
| DPB1 Exon 2 5' | 53 | GCTGCAGGAGAGTGGCGCCTCCGCTCAT |
| DPB1 Exon 2 3' | 54 | CGGATCCGGCCCAAAGCCCTCACTC |
| **DQ** | | |
| DQA1 Exon 2 5' | 55 | GTTTCTTYCATCATTTTGTGTATTAAGGT |
| DQA1 Exon 2 3' | 56 | CGGTAGAGTTGTAGCGTTTA |
| DQA1 Exon 2 5' | 57 | GTCAGTTTCTTYCATCATTTTGTGTATTAAGGT |
| DQA1 Exon 2 5' | 58 | GAAAGTCAGTTTCTTYCATCATTTTGTGTATTAA |
| DQA1 Exon 2 3' | 59 | CCATGASAAGATCTGGGGACCTCT |
| DQA1 Exon 2 3' | 56 | CGGTAGAGTTGTAGCGTTTA |
| DQB1 Exon 2 5' | 60 | AGGATCCCCGCAGAGGATTTCGTGTACCA |
| DQB1 Exon 2 3' | 61 | TCCTGCAGGACGCTCACCTCTCCGCTGCA |
| DQB1 Exon 3 5' | 62 | TGGAGCCCACAGTGACCATCTCC |
| DQB1 Exon 3 3' | 63 | GCTGGGGTGCTCCACGTGGCA |
| DQB1 Exon 3 5' | 62 | TGGAGCCCACAGTGACCATCTCC |
| DQB1 Exon 3 3' | 64 | AGTGACATCAGGGATAAGAGATGGGAA |
| **DRB1** | | |
| DRB1 generic 5' | 65 | CCGGATCCTTCGTGTCCCCACAGCACG |
| DRB1 generic 3' | 66 | CCGAATTCCGCTGCACTGTGAAGCTCTC |
| DRB1 generic 5' | 67 | CCGGATCCTTCGTGTCCCCACAG |
| DRB1 generic 3' | 68 | GATTCTRAATGCTCACAGATGGCG |

Table 4- HLA Target Specific Primer Sequences

Further Table 5 provides additional examples of HLA target specific primers useable for embodiments of the described invention. Those of ordinary skill in the related art will appreciate that the target specific primer sequences in Tables 4 and 5 may be used interchangeably with one another for the same target loci. It will also be noted that some

of the HLA specific primer sequences in the adaptor sequences of Table 5 may be the same as those in Table 4 however for some HLA loci some differences exist.

| Locus | | Name | Sequence | SEQ Id No: |
|---|---|---|---|---|
| A1-2 | 5' | PM1283 | GTTTCCAGAGAAGCCAATCAGTGTCGT | 69 |
| A1-2 | 5' | PM1277 | TAAAGTCCGCACGCACCCACCG | 70 |
| A4-5 | 3' | PM1280 | CTTGGAACCCTCAGTGAGACAAGAAAT | 71 |
| A4-5 | 3' | PM1281 | TTGGAACCCTCAGTGAGACAAGAAAT | 72 |
| A4-5 | 3' | PM1282 | CTGGGGCTTGGAACCCTCAGTGA | 73 |
| A4-5 | 5' | PM1288 | GGTTCTGTGCTCYCTTCCCCAT | 74 |
| A4-5 | 3' | PM1289 | GGAACCCTCAGTGAGACAAGAAAT | 75 |
| A4-5 | 3' | PM1290 | GGGCTTGGAACCCTCAGTGA | 76 |
| B1-2 | 5' | FHLAB1-2TV1 | GCACCCACCCGGACTCAGARTCTCCT | 77 |
| B1-2 | 5' | FHLAB1-2TV2 | CCACCCGGACTCAGARTCTCCT | 78 |
| B1-2 | 3' | RHLAB1-2TV1 | CCGGGCCGGGGTCACTCAC | 79 |
| B1-2 | 3' | RHLAB1-2TV2 | GGGCCGGGGTCACTCAC | 80 |
| B1-2 | 3' | RHLAB1-2TV3 | CCCGCGGGGATTTTGGCCTC | 81 |
| B1-2 | 3' | RHLAB1-2TV4 | CGCGGGGATTTTGGCCTC | 82 |
| B3 | 5' | FHLAB3TV1 | CGCGTTTACCCGGTTTCATTTTCAGTTG | 83 |
| B3 | 5' | FHLAB3TV2 | CGTTTACCCGGTTTCATTTTCAGTTG | 84 |
| B3 | 5' | FHLAB3TV3 | CCCGGTTTCATTTTCAGTTGAGGYCAA | 85 |
| B3 | 5' | FHLAB3TV4 | GGTTTCATTTTCAGTTGAGGYCAA | 86 |
| B3 | 3' | RHLABC3TV1 | GGAGATGGGGAAGGCTCCCCACT | 87 |
| B3 | 3' | RHLABC3TV2 | ATGGGGAAGGCTCCCCACT | 88 |
| B3 | 3' | RHLABC3TV3 | AGGGGGCCCTCAGAGGAAACT | 89 |
| ABC3 | 5' | FCLASS13TV1 | GTTTAGGCCAAAATCCCCGCGG | 90 |
| B4-5 | 5' | FHLAB4-5TV1 | AAAGCGCCTGAATTTTCTGACTCTTCCCA | 91 |
| B4-5 | 5' | FHLAB4-5TV2 | CGCCTGAATTTTCTGACTCTTCCCA | 92 |
| B4-5 | 3' | RHLAB4-5TV1 | GCTGCTTCCCAGTAATGAGGCAGGGA | 93 |
| B4-5 | 3' | RHLAB4-5TV2 | GCTTCCCAGTAATGAGGCAGGGA | 94 |
| B4-5 | 3' | RHLAB4-5TV3 | TGCGTTAGCCCCTGTGTGSATGC | 95 |
| B4-5 | 3' | RHLAB4-5TV4 | CGTTAGCCCCTGTGTGSATGC | 96 |
| C1-2 | 5' | FHLAC1-2TV1 | CGGGTTCTAGAGAAGCCAATCAGCGTCT | 97 |
| C1-2 | 5' | FHLAC1-2TV2 | GGTTCTAGAGAAGCCAATCAGCGTCT | 98 |
| C1-2 | 5' | FHLAC1-2TV3_1 | TTCTAGAGAAGCCAATCAGCGTCT | 99 |
| C1-2 | 3' | RHLAC1-2TV1 | GGTCGAGGGTCTGGGCGGGTT | 100 |

| | | | | |
|---|---|---|---|---|
| C1-2 | 3' | RHLAC1-2TV2 | CGAGGGTCTGGGCGGGTT | 101 |
| C1-2 | 3' | RHLAC1-2TV3_1 | CCGGGCYGGGGTCACTCAC | 102 |
| C3 | 5' | FHLAC3TV1 | CGCCCAGACCCTCGACCGGA | 103 |
| C3 | 5' | FHLAC3TV2 | CCCAGACCCTCGACCGGA | 104 |
| C3 | 3' | RHLAC3TV4_1 | GAGAGAAAGGTCAGCAGCCTGACCACA | 105 |
| C3 | 3' | RHLAC3TV5_1 | AAAGGTCAGCAGCCTGACCACA | 106 |
| C3 | 5' | FHLAC3TV3_1 | CCTCGACCGGAGAGAGCCCYAGT | 107 |
| C3 | 5' | FHLAC3TV4_1 | CGACCGGAGAGAGCCCYAGT | 108 |
| C4-5 | 5' | FHLAC4-5TV1 | TCCATTCTCAGGATGGTCACATGGGC | 109 |
| C4-5 | 5' | FHLAC4-5TV2 | ATTCTCAGGATGGTCACATGGGC | 110 |
| C4-5 | 3' | RHLAC4-5TV1 | GGGCACACTTCTACCTGGGGCTTGAAACT | 111 |
| C4-5 | 3' | RHLAC4-5TV2 | CACACTTCTACCTGGGGCTTGAAACT | 112 |
| C4-5 | 3' | RHLAC4-5TV3 | CACACAGGGTCCCAGGCTGGGA | 113 |
| C4-5 | 3' | RHLAC4-5TV4 | ACAGGGTCCCAGGCTGGGA | 114 |
| C6-7 | 5' | FHLAC6-7TV1 | ACTTCTCTTGGGTCCAAGACTAGGAGGTTCCC | 115 |
| C6-7 | 5' | FHLAC6-7TV2 | TGGGTCCAAGACTAGGAGGTTCCC | 116 |
| C6-7 | 3' | RHLAC6-7TV1 | CCCACCCCCGACCACTTCAGCT | 117 |
| C6-7 | 3' | RHLAC6-7TV2 | CACCCCCGACCACTTCAGCT | 118 |
| C6-7 | 3' | RHLAC6-7TV3 | GAAACGTCCCAATCAAAGRATCCCCATTA | 119 |
| C6-7 | 3' | RHLAC6-7TV4 | CGTCCCAATCAAAGRATCCCCATTA | 120 |
| DPA1 | 5' | PM1272 | GACCACTTGCATATTCAAACTGA | 121 |
| DPA1 | 3' | PM1274 | GGCTACAGAGGAAGAGGCAAAGATA | 122 |
| DPA1 | 5' | PM1273 | GACCACTTGCATATTCAAACTGACA | 123 |
| DPA1 | 3' | PM059 | GGCTACAGAGGAAGAGGCAAAGATAGG | 124 |
| DRB1 | 5' | PM1283 | GTTTCCAGAGAAGCCAATCAGTGTCGT | 69 |
| DRB1 | 5' | PM1284 | CGGATGCTTTGTGGACCCGCA | 125 |
| DRB1 | 5' | PM1285 | GGATGCTTTGTGGACCCGCA | 126 |
| DRB1 | 3' | PM1286 | GGATAGAGAGGATTCTGAATGCTCACAGAT | 127 |
| DRB1 | 3' | PM1287 | GGATAGAGAGGATTCTGAATGCTCACAGA | 128 |

Table 5- HLA Adaptors

Those of ordinary skill in the art will appreciate that some variability of sequence composition for primer sets exist and that 90% or greater homology to the disclosed

primer sequences are considered within the scope of the presently described invention. For example, the target regions for the sets of primers may be slightly shifted and thus some difference in primer sequence composition is expected. Also, refinements to the consensus sequence may be made or new sequence degeneracy at certain positions may be discovered resulting in a slight difference of sequence composition in the target region, and similarly some variation in primer sequence composition is expected.

The template nucleic acid used to amplify the HLA first amplicon of interest is typically from genomic DNA isolated from a subject to be genotyped. In the current method, more than one subject is HLA genotyped in parallel reactions. In the current invention, at least 12 subjects, and typically at least 16, 20, 24, 30, 36, or 48 subjects are HLA genotyped. The HLA amplicons may be obtained using any type of amplification reaction. In the described embodiments, first amplicons are typically made by PCR using HLA primer pairs as described herein, where it is typically desirable to use a polymerase with a low error rate, e.g., such as a high-fidelity Taq polymerase (Roche Diagnostics). The PCR conditions can be optimized to determine suitable conditions for obtaining first HLA amplicons from a subject. Each first HLA amplicon may be individually amplified in separate PCR reactions. In some embodiments, the first HLA amplicons for a subject may be obtained in one or more multiplex reactions that comprise primer pairs to amplify individual amplicons.

In the described embodiments, populations of HLA second amplicons are amplified and immobilized on beads via an emulsion PCR process as described above. For example, the first HLA amplicons are, preferably, individually compartmentalized within an aqueous droplet of a water in oil emulsion and attached to a single bead compartmentalized within the droplet by annealing a bead bound primer to the first amplicon, via an complementary primer element in the adaptor region. The bead comprises a large number of the primer species complementary to the primer element in the adapter portion. In the present example, the discrete aqueous phase microdroplets, are approximately 60 to 200 μm in diameter, enclosed by a thermostable oil phase where the emulsion droplets are formed such that on average, the emulsion comprises only one target nucleic acid and one bead. Each microdroplet contains, preferably, amplification reaction solution (i.e., the reagents necessary for nucleic acid amplification, such as polymerase, salts, and appropriate primers, e.g., corresponding to the adaptor region).

In the described embodiments, emulsion PCR is typically performed with two populations of beads, as the first HLA amplicons are sequenced in both directions. In one population of beads, a first primer complementary to the "reverse" primer element in the adapter sequence (i.e. the "B" adaptor) is attached to a bead. In the second population of beads, a second primer complementary to the "forward" primer element in the adapter sequence (i.e. the "A" adaptor) is attached to a bead. Thus, a primer for use in the emulsion amplification reaction typically has the sequence of the adapter region, without additional sequences such as "key" sequences. In some embodiments, the emulsion amplification reaction may be performed with asymmetric primer concentrations in the aqueous solution (i.e. typically the primer species immobilized on the bead will have the lower concentration in solution). For example, the PCR primers may be present in an 8:1 or 16:1 ratio (i.e., 8 or 16 of one primer to 1 of the second primer) to perform asymmetric PCR. However it will be appreciated that the asymmetric primer concentrations may not be necessary and equal primer concentrations may instead be employed in the aqueous solution, or in some preferred embodiments the primer species immobilized on the bead will not be present in the the aqueous solution (i.e. the B primer species is immobilized and the A primer species is in solution).

Following emulsion PCR amplification, the beads that have the singled-stranded second HLA amplicon template are isolated, e.g., via a moiety such as a biotin that is present on an amplification primer during the emulsion PCR, and the template is sequenced using DNA sequencing technology described elsewhere in this specification. For example, clonal second amplicons are sequenced using a sequencing primer (e.g., primer A or primer B) and adding four different dNTPs or ddNTPs subjected to a polymerase reaction. As each dNTP or ddNTP is added to the primer extension product, a pyrophosphate molecule is released. Pyrophosphate release can be detected enzymatically, such as, by the generation of light in a luciferase-luciferin reaction. Additionally, a nucleotide degrading enzyme, such as apyrase, can be present during the reaction in order to degrade unincorporated nucleotides. In other embodiments, the reaction can be carried out in the presence of a sequencing primer, polymerase, a nucleotide degrading enzyme, deoxynucleotide triphosphates, and a pyrophosphate detection system comprising ATP sulfurylase and luciferase.

Once the sequencing data is obtained for the sequence of the individual DNA molecules, the unambiguous HLA sequence can be determined by comparing these sequence files to an HLA sequence database for the known HLA alleles. The read lengths achieved by the 454 Sequencing system (454 Life Sciences Corporation) (typically at least 500bp) are sufficient to enable unambiguous determination of the sequence composition of each exon. The assignment of genotypes at each locus based on the exon sequence data files can be performed by application 135. For example, application 135 may include a software application developed by Conexio Genomics. An important aspect of the software is the ability to filter out related sequence reads (pseudogenes and other unwanted HLA genes) that were co-amplified by the primers along with the target sequence. In the same or alternative examples, application 135 may include the Amplicon Variant Analyzer software application (generally referred to as the AVA software) (454 Life Sciences Corporation) that compares the sequence composition generated from each first amplicon against a consensus sequence and identifies all variation that deviates from the consensus. In some embodiments, the AVA software may be additionally enabled to associate variants (or combinations of variation) with variation known type (i.e. HLA type) or variation known to confer a phenotype associated with a disease, condition, resistance, etc. Alternatively, the AVA software may be employed for pre-processing the sequence data where the pre-processed data may subsequently be uploaded into the Conexio software for further processing.

Further, embodiments of the described invention include packaging some or all of the compositions and reagents described herein into kits. A kit of the described embodiments typically comprises multiple adaptor pairs as described herein that are suitable for amplifying the regions of interest in an HLA allele. The adaptor pairs comprise a forward primer comprising a general adapter region, an MID tag and an HLA primer region; and a reverse primer that comprises a general adapter region, an MID tag, and an HLA primer region. It will, however, be appreciated that only one MID tag may be necessary depending on the number of sample associations necessary. The kits of the described embodiments often comprise primer pairs to amplify first amplicons for determining the genotype of multiple subjects for at least HLA-A, HLA-B, and DRB1. Often, a kit of the described embodiments comprise sufficient HLA

primer pairs to determine the genotype of HLA-A, HLA-B, HLA-C, DRB1, DQA1, DQB1, DPA1, and DPB1 genes for multiple individuals, e.g., 12 or more individuals.

In some embodiments, a kit can additionally comprise one or more populations of beads that have a primer attached that corresponds to an adapter region that can be used in emulsion PCR. In some embodiments, a kit can comprise one or more reaction compartments comprising reagents suitable for performing a reaction selected at the discretion of a practitioner. For example, in some embodiments, a kit can comprise one or more reaction compartments comprising one more sequencing reagents.

The various components included in the kit are typically contained in separate containers, however, in some embodiments one or more of the components can be present in the same container. Additionally, kits can comprise any combination of the compositions and reagents described herein. In some embodiments, kits can comprise additional reagents that may be necessary or optional for performing the disclosed methods. Such reagents include, but are not limited to, buffers, control polynucleotides, and the like.

Having described various embodiments and implementations, it should be apparent to those skilled in the relevant art that the foregoing is illustrative only and not limiting, having been presented by way of example only. Many other schemes for distributing functions among the various functional elements of the illustrated embodiment are possible. The functions of any element may be carried out in various ways in alternative embodiments.

CLAIMS

1. A method for detecting one or more HLA sequence variants, comprising the steps of:

(a) amplifying a plurality of first amplicons from a double stranded nucleic acid sample, wherein the first amplicons are amplified with a plurality of pairs of nucleic acid primers that define exons 2 and 3 of both strands of HLA loci selected from the group consisting of HLA-A, HLA-B, and HLA-C;

(b) amplifying the first amplicons to produce a plurality of populations of second amplicons, wherein each population of second amplicons is clonally amplified from one of the first amplicons;

(c) sequencing the plurality of populations of second amplicons to generate a nucleic acid sequence composition for each of the plurality of second amplicons; and

(d) detecting variation in the sequence composition from one or more of the second amplicons for one or more of the HLA loci.

2. A method for detecting one or more HLA sequence variants, comprising the steps of:

(a) amplifying a plurality of first amplicons from a double stranded nucleic acid sample, wherein the first amplicons are amplified with a plurality of pairs of nucleic acid primers that define exon 2 of both strands of HLA loci selected from the group consisting of DRB1, DQA1, DQB1, DPA1, DPB1;

(b) amplifying the first amplicons to produce a plurality of populations of second amplicons, wherein each population of second amplicons is clonally amplified from one of the first amplicons;

(c) sequencing the plurality of populations of second amplicons to generate a nucleic acid sequence composition for each of the plurality of second amplicons; and

(d) detecting variation in the sequence composition from one or more of the second amplicons for one or more of the HLA loci.

3. The method of claim 1 or 2, wherein the pairs of nucleic acid primers comprise sequence composition selected from a plurality of primers listed in Tables 4 and 5.

4. The method of claim 1, wherein the plurality of pairs of nucleic acid primers define exons 1, 4, and 5 of the HLA loci.

5. The method of claim 1, wherein the plurality of pairs of nucleic acid primers defines exons 6, and 7 of the HLA-C locus.

6. The method of claim 2, wherein the plurality of pairs of nucleic acid primers for the DRB1 locus are generic and further enable amplification of loci selected from the group consisting of DRB3, DRB4, and DRB5 loci.

7. The method of any one of claims 1 to 6, further comprising a plurality of adaptors each comprising an individual primer from the pairs of the nucleic acid primers.

8. The method of claim 7, wherein one or more of the plurality of adaptors comprise an MID identifier and wherein the MID identifier enables pooling of the first amplicons derived from a plurality of the nucleic acid samples, wherein the populations of the second amplicons amplified from the pooled first amplicons are sequenced in parallel.

9. The method of any one of claims 7 or 8 , wherein the plurality of adaptors comprise a general adaptor element and a key element.

10. The method of any one of claims 1 to 9, wherein each population of second amplicons is immobilized on a bead substrate.

11. The method of any one of claims 1 to 10, wherein the populations of second amplicons are clonally amplified using an emulsion PCR process.

12. The method of any one of claims 1 to 11, wherein the plurality of populations of second amplicons is sequenced in parallel.

13. The method of any one of claims 1 to 12 further comprising the step of:

(e) associating the variation with an HLA type.

14. A kit for detecting one or more HLA sequence variants, comprising a plurality of the pairs of nucleic acid primers employed to amplify the first amplicons of claim 1.

15. A kit for detecting one or more HLA sequence variants, comprising a plurality of the pairs of nucleic acid primers employed to amplify the first amplicons of claim 2.

**FIGURE 1**

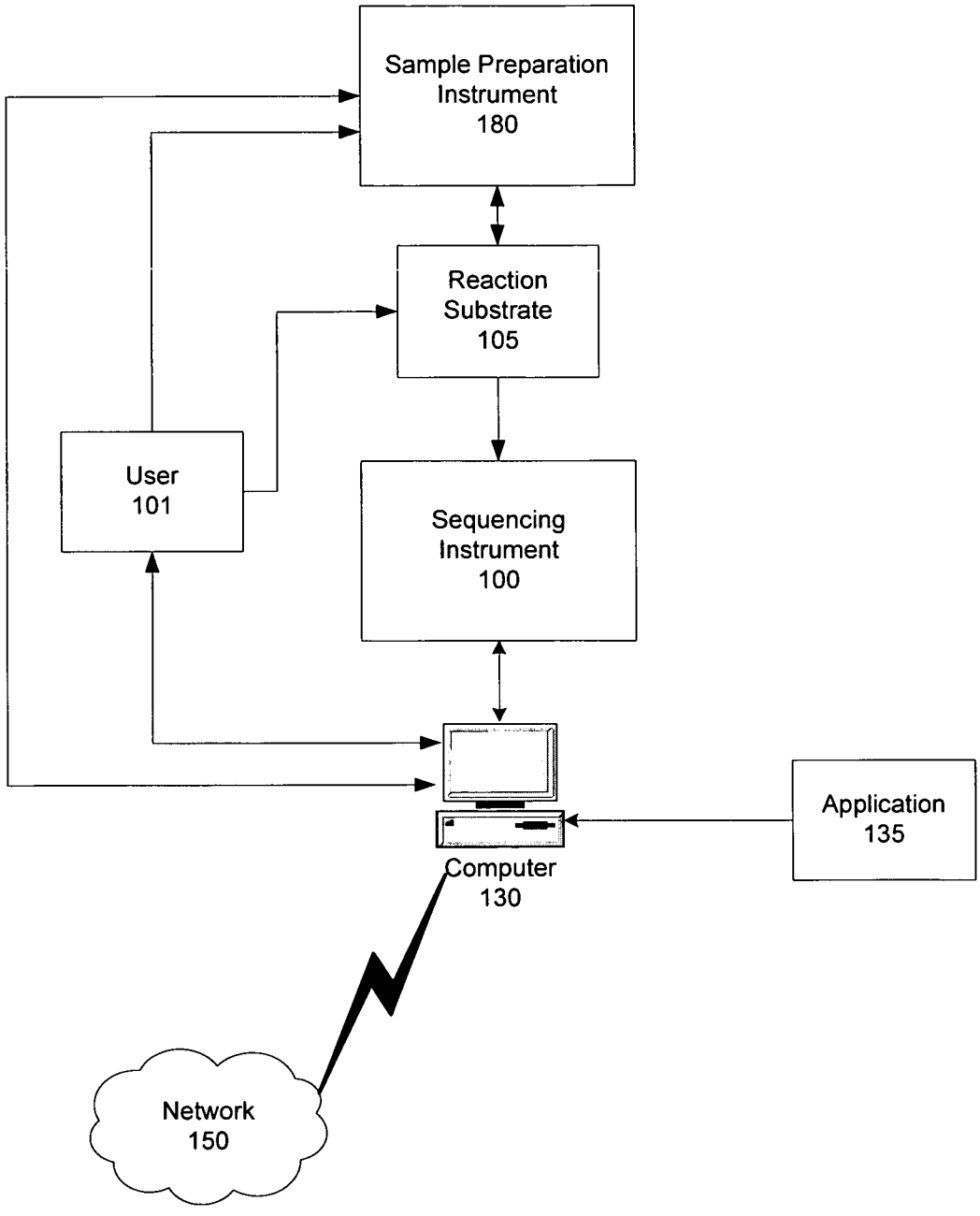

Sample Preparation Instrument 180
Reaction Substrate 105
User 101
Sequencing Instrument 100
Application 135
Computer 130
Network 150

FIGURE 2

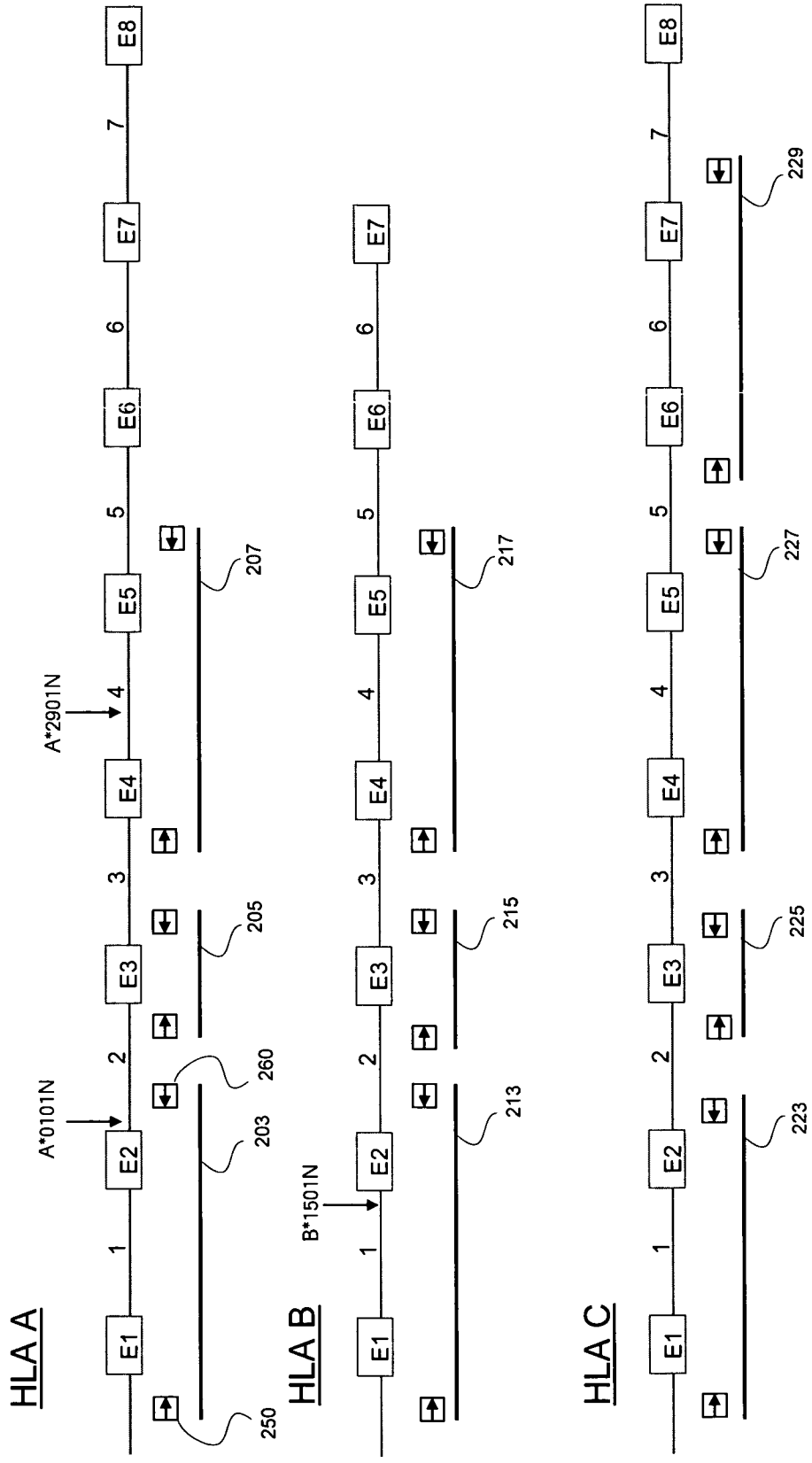

HLA A
A*0101N
A*2901N
E1
E2
E3
E4
E5
E6
E7
E8
1
2
3
4
5
6
7
250
203
260
205
207
HLA B
B*1501N
213
215
217
HLA C
223
225
227
229

**FIGURE 3**

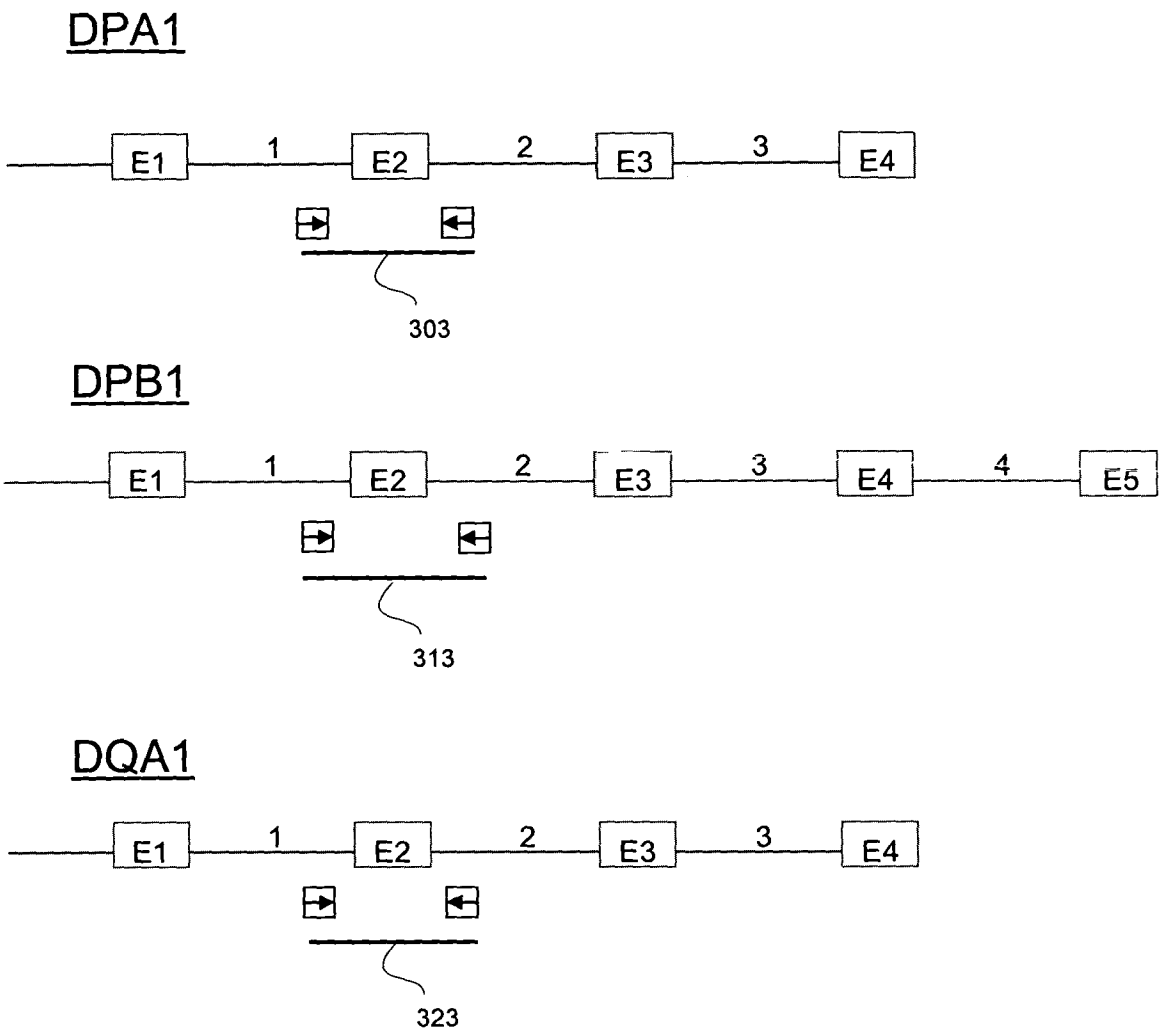
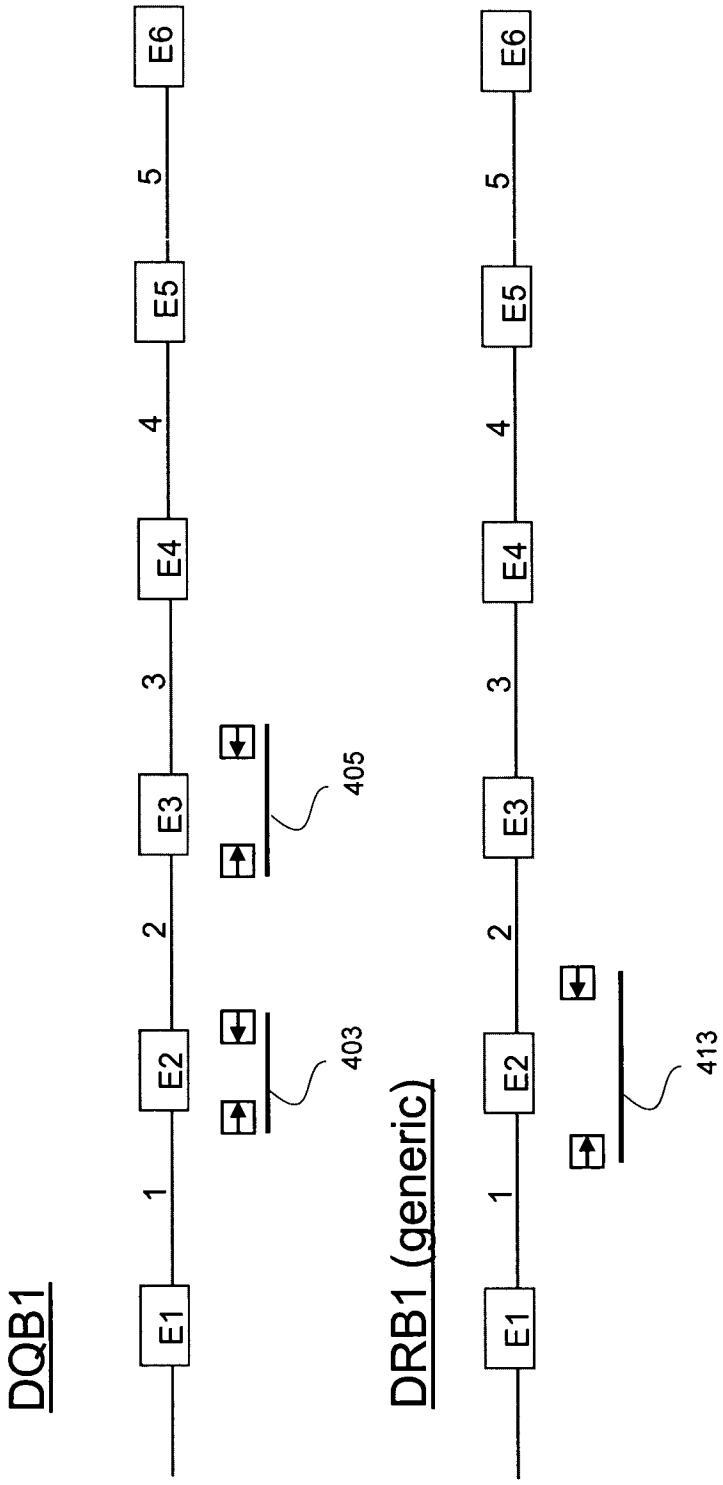

DPA1
E1
1
E2
2
E3
3
E4
303
DPB1
E1
1
E2
2
E3
3
E4
4
E5
313
DQA1
E1
1
E2
2
E3
3
E4
323

FIGURE 4


DQB1
E1
1
E2
2
E3
3
E4
4
E5
5
E6
403
405
DRB1 (generic)
E1
1
E2
2
E3
3
E4
4
E5
5
E6
413

# INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2010/002307

**A. CLASSIFICATION OF SUBJECT MATTER**
INV. C12Q1/68
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

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 practical, search terms used)
EPO-Internal, BIOSIS, Sequence Search, EMBASE, WPI Data

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| X | US 5 550 039 A (TRACHTENBERG ELIZABETH A [US]) 27 August 1996 (1996-08-27)<br>column 2, line 18 - line 30<br>column 4, line 45 - line 49<br>column 7, line 32 - column 8, line 2<br>column 3, line 1 - line 8 | 1,3-5,<br>7-14 |
| A | US 5 310 893 A (ERLICH HENRY A [US] ET AL)<br>10 May 1994 (1994-05-10)<br>column 6, line 4 - line 9<br>column 9, line 3 - line 33<br>example 1<br>the whole document | 2,3,<br>6-13,15 |

-/--

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

[X] See patent family annex.

* Special categories of cited documents :

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

"E" earlier document 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 |
|---|---|
| 11 August 2010 | 17/08/2010 |

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

Form PCT/ISA/210 (second sheet) (April 2005)

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| A | US 5 541 065 A (ERLICH HENRY A [US] ET AL)<br>30 July 1996 (1996-07-30)<br>column 5, line 57 - line 62<br>column 8, line 63 - column 9, line 6<br>example 1<br>the whole document | 2,3,<br>6-13,15 |
| X<br>Y | US 5 604 099 A (ERLICH HENRY A [US] ET AL)<br>18 February 1997 (1997-02-18)<br>column 1, line 22 - line 26<br>column 31, line 65 - column 33, line 3<br>the whole document | 2,3,13,<br>15<br>6-12 |
| Y | BUNCE M ET AL: "Phototyping:<br>comprehensive DNA typing for HLA-A, B, C,<br>DRB1, DRB3, DRB4, DRB5 & DQB1 by PCR with<br>144 primer mixes utilizing<br>sequence-specific primers (PCR-SSP)"<br>TISSUE ANTIGENS, MUNKSGAARD, COPENHAGEN,<br>DK LNKD-<br>DOI:10.1111/J.1399-0039.1995.TB03127.X,<br>vol. 46, 1 January 1995 (1995-01-01),<br>pages 355-367, XP008096257<br>ISSN: 0001-2815<br>* abstract<br>the whole document | 6 |
| Y | WO 2005/073410 A2 (454 CORP [US]; NOBILE<br>JOHN R [US]; LEE WILLIAM [US]; LEAMON JOHN<br>H [US]) 11 August 2005 (2005-08-11)<br>the whole document | 7-12 |
| Y | WO 2008/076842 A2 (APPLERA CORP [US]; LAO<br>KAI QIN [US]; STRAUS NEIL A [US])<br>26 June 2008 (2008-06-26)<br>the whole document | 7-12 |
| X,P | WO 2009/049889 A1 (ROCHE DIAGNOSTICS GMBH<br>[DE]; HOFFMANN LA ROCHE [CH])<br>23 April 2009 (2009-04-23)<br>claim 1<br>page 9, line 14 - line 16<br>the whole document | 1,3-5,<br>7-14 |

# INTERNATIONAL SEARCH REPORT

International application No.
PCT/EP2010/002307

**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.:
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:

see additional sheet

1. ☐ As all required additional 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 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.:

1-15(partially)

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.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. claims: 1, 3-5, 7-14(all partially)

   - A method for detecting one or more HLA-A sequence variants.
   - A kit.

---

2. claims: 1, 3-5, 7-14(all partially)

   - A method for detecting one or more HLA-B sequence variants.
   - A kit.

---

3. claims: 1, 3-5, 7-14(all partially)

   - A method for detecting one or more HLA-C sequence variants.
   - A kit.

---

4. claims: 2, 3, 6-13, 15(all partially)

   - A method for detecting one or more HLA-DRB1 sequence variants.
   - A kit.

---

5. claims: 2, 3, 6-13, 15(all partially)

   - A method for detecting one or more HLA-DQA1 sequence variants.
   - A kit.

---

6. claims: 2, 3, 6-13, 15(all partially)

   - A method for detecting one or more HLA-DQB1 sequence variants.
   - A kit.

---

7. claims: 2, 3, 6-13, 15(all partially)

   - A method for detecting one or more HLA-DPA1 sequence variants.
   - A kit.

---

8. claims: 2, 3, 6-13, 15(all partially)

   - A method for detecting one or more HLA-DPB1 sequence

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

variants.
- A kit.

---

# INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/EP2010/002307

| Patent document cited in search report | Publication date | Patent family member(s) | Publication date |
|---|---|---|---|
| US 5550039 A | 27-08-1996 | NONE | |
| US 5310893 A | 10-05-1994 | NONE | |
| US 5541065 A | 30-07-1996 | NONE | |
| US 5604099 A | 18-02-1997 | NONE | |
| WO 2005073410 A2 | 11-08-2005 | CA 2553833 A1<br>EP 1735458 A2 | 11-08-2005<br>27-12-2006 |
| WO 2008076842 A2 | 26-06-2008 | US 2009325183 A1<br>US 2008145898 A1 | 31-12-2009<br>19-06-2008 |
| WO 2009049889 A1 | 23-04-2009 | CA 2701411 A1<br>EP 2203567 A1 | 23-04-2009<br>07-07-2010 |