(12) STANDARD PATENT (11) Application No. **AU 2011274090 B2**
(19) AUSTRALIAN PATENT OFFICE

(54) Title
New PCR sequencing method and use thereof in HLA genotyping

(51) International Patent Classification(s)
C12Q 1/68 (2006.01) ***C12N 15/11*** (2006.01)

(21) Application No: **2011274090** (22) Date of Filing: **2011.06.30**

(87) WIPO No: **WO12/000445**

(30) Priority Data

(31) Number	(32) Date	(33) Country
201010213721.2	**2010.06.30**	**CN**
201010213717.6	**2010.06.30**	**CN**
PCT/CN2010/002150	**2010.12.24**	**CN**
PCT/CN2010/002149	**2010.12.24**	**CN**
201010213719.5	**2010.06.30**	**CN**

(43) Publication Date: **2012.01.05**
(44) Accepted Journal Date: **2015.04.09**

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(56) Related Art
WO2009/049889
BENTLEY, G. et al., Tissue Antigens, 2009 vol. 74, pages 393-403
US2009/0170713

(12) 按照专利合作条约所公布的国际申请

(19) 世界知识产权组织
国 际 局

(43) 国际公布日
2012 年 1 月 5 日 (05.01.2012)

PCT

(10) 国际公布号
WO 2012/000445 A1

(51) 国际专利分类号:
C12Q 1/68 (2006.01) *C12N 15/11* (2006.01)

(21) 国际申请号: PCT/CN2011/076688

(22) 国际申请日: 2011 年 6 月 30 日 (30.06.2011)

(25) 申请语言: 中文

(26) 公布语言: 中文

(30) 优先权:
201010213721.2 2010 年 6 月 30 日 (30.06.2010) CN
201010213719.5 2010 年 6 月 30 日 (30.06.2010) CN
201010213717.6 2010 年 6 月 30 日 (30.06.2010) CN
PCT/CN2010/002150 2010 年 12 月 24 日 (24.12.2010) CN
PCT/CN2010/002149 2010 年 12 月 24 日 (24.12.2010) CN

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(81) 指定国 (除另有指明，要求每一种可提供的国家保护): AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PE, PG, PH, PL, PT, RO, RS, RU, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW。

(84) 指定国 (除另有指明，要求每一种可提供的地区保护): ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), 欧亚 (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), 欧洲 (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG)。

本国际公布:
— 包括国际检索报告(条约第 21 条(3))。
— 包括说明书序列表部分(细则 5.2(a))。

(54) Title: NEW PCR SEQUENCING METHOD AND USE THEREOF IN HLA GENOTYPING

(54) 发明名称 : 新的的 PCR 测序方法及其在 HLA 基因分型中的应用

(57) Abstract: The present invention provides a PCR sequencing method, which comprises the steps of: 1) providing the sample; 2) amplifying; 3) mixing; 4) breaking; 5) sequencing and 6) splicing. The present invention also provides the primer tags used in the method, as well as the use of the method in genotyping, especially in HLA analysis. The present invention also provides PCR primers for HLA-A/B, HLA-C and HLA-DQB1.

(57) 摘要:

本发明提供了一种 PCR 测序方法，包括步骤：1）提供样品；2）扩增；3）混合；4）打断；5）测序以及 6）拼接。本发明还提供了用于该方法的引物标签，以及该方法用于基因分型，特别是 HLA 分析的用途。本发明还提供了针对 HLA-A/B，HLA-C 以及 HLA-DQB1 的 PCR 引物。

WO 2012/000445 A1

NEW PCR SEQUENCING METHOD AND USE THEREOF IN HLA GENOTYPING

Relevant applications

The present application claims the priority right of the Chinese Patent Application Nos. 201010213717.6, 201010213719.5, and 201010213721.2 as filed on Jun. 30, 2010 and the priority right of the International Application Nos. PCT/CN2010/002150 and PCT/CN2010/002149 as filed on Dec. 24, 2010, the contents of which are incorporated herein by reference in their entirety.

TECHNICAL FIELD

The present invention relates to the technical field of nucleic acid sequencing, in particular, the technical field of PCR sequencing. In addition, the present invention also relates to DNA barcoding technique and DNA incomplete shearing strategy. The method of the present invention is particularly applicable to the second generation sequencing technique, especially to the Paired-end sequencing technique of the second generation sequencing technique, and is also applicable to HLA genotyping. In particular, the present invention provides a method for HLA genotyping, in particular, a method for HLA-A, HLA-B, HLA-C and HLA-DQB1 genotyping, and also provides the primer pairs for PCR amplification used in the method.

BACKGROUND

A PCR sequencing method refers to a technique wherein DNA fragments of a gene of interest are obtained by a PCR method, and the obtained DNA fragments of the gene of interest are subjected to DNA sequencing to obtain the DNA sequence information of the gene of interest. PCR sequencing methods are widely applied to the fields such as detection of gene mutation and genotyping for a long time.

DNA sequencing technique is mainly classified into the first generation DNA sequencing technique represented by Sanger sequencing method and the second generation DNA sequencing technique represented by Illumina GA, Roche 454, ABI Solid, and the like. Sanger DNA sequencing technique is characterized by simple experimental operations, visual and accurate results, and short experimental period, and thus is wildly applied in fields such as clinical gene mutation detection and genotyping, wherein a fast turnaround time is highly required as to detection results. However, due to the characteristics such as low throughput and high cost, its application in fields where genotyping is performed in a large scale, is limited。

As compared with the first generation DNA sequencing technique, the second generation DNA sequencing technique has the characteristics such as high sequencing throughput, low cost, high level of automation, and single-molecule sequencing. Taken Illumina GA single-molecule sequencing as an example, a single sequencing run generates data of 50G (about 50 billion) bases, 5 billion bases data per day in average, and the average sequencing cost for a base is less than 1/1000 of the sequencing cost in Sanger method. Moreover, the analysis of results can be directly carried out by a computer. Thus, the second generation DNA sequencing technique is a technique quite suitable for large-scale sequencing projects. However, the contiguous sequencing length is generally short in the second generation DNA sequencing technique. Currently, the maximum bidirectional sequencing length is 200bp for Illumina GA; although the maximum sequencing length can be up to about 500 bp for Roche 454 GS-FLX, the sequencing cost is high and the throughput is low. When a PCR amplicon is of a length greater than the maximum sequencing length in a sequencer, the thorough sequencing of the whole amplicon cannot be accomplished by sequencing directly, and the whole DNA sequence information of the amplicon cannot be obtained. Due to short maximum sequencing length, the application of the second generation sequencing technique in PCR sequencing method is limited. In addition to gradual improvement of sequencing technique to obtain a longer maximum sequencing length, it is urgent need to develop a new technique to overcome the deficiency of the current maximum sequencing length of the second generation DNA sequencer in the PCR sequencing application field.

Human leukocyte antigen (HLA) is one of the gene systems found so far to be of the highest polymorphism. It is a primary gene system for modulating specific immune response in human bodies and determining individual difference in susceptibility to diseases, and is closely associated with allogeneic organ transplant rejection. It is found in studies that the higher the matching degree of genes, such as HLA-A, B, C, DRB1 and DQB1, as well as the resolution are in a donor and a receptor, the longer a transplant survives. It is already a regular testing item to subject a potential donor and a receptor to high-resolution HLA genotyping before hematopoietic stem cell transplantation.

The current international standard HLA high-resolution genotyping technique is a Sanger sequencing technique-based PCR sequencing method, which comprises PCR amplifying the corresponding HLA gene regions, sequencing the amplified product, subjecting the sequencing result to genotyping with a professional genotyping software, and finally obtaining the HLA genotype information of the sample. It is characterized by visual results, high resolution and capability of detecting new allele. However, due to the characteristics of Sanger sequencing, such as high cost and low throughput, its application in institutes like hematopoietic stem cell volunteer registration database (Bone Marrow Bank), in which large-scale HLA genotyping detection is required, is limited.

It was reported that a Roche 454 GS-FLX-based PCR sequencing method was used in HLA genotyping. However, since its cost for sequencing was relatively high, it was not significantly superior over the Sanger sequencing-based HLA genotyping technique in terms of sequencing throughput and sequencing cost. As compared with Roche 454 GS-FLX, Illumina GA has a shorter maximum sequencing length, but has obvious advantages in terms of sequencing throughput and sequencing cost. If the defect of the short maximum sequencing length of Illumina GA can be overcome, its application in HLA genotyping will make up for the shortage of the current HLA genotyping method.

CONTENTS OF THE INVENTION

When conducting sequencing analysis simultaneously to sequences associated

with a specific gene in a large number of samples by the second generation sequencing technique, PCR sequencing strategy is generally employed, wherein the combination of primer index and the second generation sequencing technique is employed directly. When the maximum sequencing length of the sequencer can cover the length of the whole PCR product, the above strategy meets the requirements. When the maximum sequencing length of the sequencer cannot cover the length of the whole PCR product, Illumina GA needs to be replaced with the second generation sequencer having a longer maximum sequencing length (such as Roche 454 GS-FLX). If the maximum sequencing length still cannot meet the requirements, a first generation sequencer has to be employed with the scarification of cost and throughput.

The actual situation is that Illumina GA has a super high sequencing throughput, but its maximum sequencing length is 200bp only; although the maximum sequencing length of Roche 454 GS-FLX can reach about 500 bp, the cost for sequencing is relative high and the throughput is relative low; although the maximum sequencing length of the first generation sequencer can reach above 1000bp, its throughput and cost are not comparable to those of the second generation sequencer.

Is there a technique capable of enhancing the length of PCR products that can be sequenced thoroughly by a sequencer without the scarification of cost and throughput? The combination of primer indexes, DNA incomplete shearing strategy, and the second generation sequencing technique in the present application can make the length of PCR products that can be sequenced by a sequencer longer than the maximum sequencing length of the sequencer whilst making full use of the characteristics of the second generation sequencing technique such as high throughput and low cost, thereby greatly broadening its applicable scope. The second generation sequencing technique employed in the present invention includes, among second generation sequencing techniques, a Paired-end sequencing technique, and a PCR sequencing technique which has a DNA reference sequence for the PCR template.

The present invention provides methods for PCR sequencing, by which the limitation resulted from short maximum sequencing length is alleviated and the application of the second generation DNA sequencing technique in the PCR

sequencing application field is broadened. For example, when performing sequencing with the second generation sequencing technique, index primers having a primer index added to the 5' end are used, the amplified PCR products are sheared, the sheared products are terminally repaired and have deoxyadenosine (A) ligated to their 3' ends, and then are ligated to different PCR-free adapters.

A PCR sequencing method, based on DNA barcoding technique and DNA incomplete shearing s trategy, can greatly increase the number of samples labeled specifically without increasing the number of primer indexes (Figure 5). In the present invention, the actually sequenced length of PCR products exceeds the maximum sequencing length of the sequencer by adding primer indexes to the forward and reverse PCR primers, in combination with using DNA incomplete shearing strategy, and applying the second generation sequencing technique.

The addition of an index sequence to the front end of an amplification primer is aimed to realize simultaneous sequencing of a plurality of samples. Concretely speaking, a unique primer index is added to each sample during PCR by using PCR-index/barcode technique in combination with synthesizing an index primer by adding a primer index to the 5' end of a PCR primer. As such, during the sequencing by the second generation sequencing technique, samples have to be processed one by one only in PCR step, and may be mixed together and processed simultaneously in the rest experimental steps, and the final result for each sample can be traced by virtue of its unique primer index.

"Adapter" or "library adapter" index technique refers to a library indexing technique comprising adding different library adapters to multiple sequencing libraries (different library adapters consist of different sequences, and the different portion among the sequences is called adapter index), constructing indexed sequencing libraries, then accomplishing sequencing of multiple different indexed sequencing libraries in a pool, wherein the final sequencing result for each indexed sequencing library is distinguishable. The term "PCR-Free library adapter" refers to a designed segment of bases, whose main role lies in auxiliary fixation of DNA molecule onto the sequencing chip and lies in providing the binding sites for universal

sequencing primers, wherein PCR-Free library adapter may be directly ligated to the two termini of the DNA fragments in the sequencing library. Since no PCR is involved in the introduction of the adapter, the adapter is called PCR-Free library adapter. For example, PCR-FREE library adapters used in the Examples of the present invention are from ILLUMIA.

A method of constructing PCR-FREE library, wherein a library adapter index technique is used, refers to direct ligation of library adapter to the two termini of the DNA fragment of the sequencing library. Since no PCR is involved in the introduction of the library adapter, it is called PCR-Free library construction. A DNA ligase may be used for ligation in the introduction process. Since no PCR is involved in the process of library construction, inaccuracy of the final results resulted from PCR bias is avoided during the construction of a library comprising PCR products of high sequence similarity.

DNA amplification methods, DNA extraction methods, DNA purification methods and DNA sequence alignment methods as involved in the present invention may be any methods available in the art. Said methods can be selected by a person skilled in the art according to practical situations. As to DNA sequencing methods, a person skilled in the art can carry out them according to conventional methods or following the instruction of the sequencer.

The design of primer indexes varies depending on the applied experimental platform. In view of the characteristics of Illumina GA sequencing platform, the following factors are primarily considered when designing the primer indexes in the present invention: 1: a mononucleotide repeat sequence comprising 3 or more base is avoided in primer index sequences, 2: the total amount of base A and base C at the same site of all the primer indexes accounts for 30%-70% of the amount of all the bases, 3: the GC content of the primer index sequence itself is between 40 and 60%, 4: primer indexes differ from one another by at least 4 bases, 5: sequences having a high sequence similarity to the Illumina GA sequencing primers are avoided in primer index sequences, and 6: the circumstance where the addition of primer index sequences to PCR primers results in serious hairpin and dimer, are reduced.

In the present invention, two primer indexes (which are either identical or

different) are added to two termini of a PCR product, respectively, so that the primer index at either terminal of the PCR product can specifically label the sample information of the PCR product. The resultant PCR product is subjected to incomplete shearing. The so-called "incomplete shearing" refers to the circumstance where the products comprise intact un-sheared PCR products and partially sheared PCR products. The shearing methods include, but are not limited to, chemical shearing methods (such as enzymatic digestion) and physical shearing methods. The physical shearing methods include ultrasonic shearing methods or mechanical shearing methods. The sheared DNA is subjected to 2% agarose electrophoresis, and all DNA bands between the maximum sequencing length and the maximum applicable DNA length of the sequencer are purified and recovered by slicing the gel (the longest DNA applicable to Illumina GA sequencer is 700 bp, and the length refers to the original DNA length, which does not comprise the length of the library adapter sequence). Methods for purification and recovery include, but are not limited to, recovery by electrophoresis and gel slicing, and recovery by magnetic beads. The recovered DNA fragments are subjected to the construction of sequencing libraries according to the procedures for constructing the sequencing libraries for the second generation sequencer, and then are subjected to sequencing. Preferably, the sequencing libraries are constructed according to the PCR-FREE procedures for constructing sequencing libraries, and Paired-End method is used as the sequencing method. PCR-Free construction of sequencing libraries is carried out according to methods known by a person skilled in the art. In the sequencing data obtained, the sequence information for all the test samples can be obtained by virtue of the primer index sequences. The sequence reads are aligned to the corresponding DNA reference sequences of the PCR products by BMA, and the complete sequence is assembled by the overlapping and linkage relationship between the sequence reads (Figure 1). The linkage here refers to the paired-end linkage relationship due to paired-End sequencing characteristics.

In Illumina GA sequencing (Genome Analyzer Sequencer from Illumina Inc., cited as Illumina GA for brief), DNA sequence analysis is carried out based on the principle of sequencing by synthesis. It may be applied to phase haplotype, and the finally

obtained data refers to a series of base sequences and may be directly applied to the alignment with the reference sequences in HLA database. Since it does not have the defect of misjudgment of peaks present in the traditional typing software, it is advantageous for automation of software typing. Illumina GA has a high sequencing throughput. Currently, one single sequencing run generates 50G (50 billion) base data, 5 billion base data per day in average. Due to the high data throughput, a high sequencing depth can be obtained for each sequence, thereby ensuring the reliability of the sequencing results.

There are no studies on applying Illumina GA to HLA typing field yet. The present invention applies Illumina GA sequencing to HLA typing field for the first time, and accomplishes HLA typing with low cost, high throughput, high accuracy and high resolution by using a PCR sequencing technique, based on DNA barcoding technique, DNA incomplete shearing and PCR-FREE library preparation.

In the present invention, by using a PCR sequencing technique which is based on DNA barcoding technique, DNA incomplete shearing and PCR-FREE library preparation, samples to be analyzed are grouped; the samples of each group are subjected to the amplification of a fragment of interest of HLA genes with primers labeled by bidirectional primer indexes (the maximum length of PCR products depends on the maximum length of the DNA that can be applied in a sequencer; the maximum applicable DNA length is 700bp in the current Illumina GA, and the length is the original DNA length, which does not comprise the length of the library adapter sequence); the PCR products are pooled together with the same amount, then subjected to incomplete shearing and indexed PCR-Free DNA sequencing library preparation. Different indexed sequencing libraries, as obtained from various groups of samples, are mixed in an equal mole, all the DNA fragments of a length longer than the maximum sequencing length of the sequencer are selectively recovered and are sequenced by Illumina GA sequencer. The DNA sequence reads for each sample can be obtained by screening the sequence information of adapter indexes, primer indexes and PCR primers in the total sequencing data. The resultant DNA sequences after assembly are aligned with the corresponding data in IMGT HLA professional database, thereby determining the HLA genotype of the sample finally.

In the methods described above, after shearing said DNA, DNA from samples of different groups is ligated to a different library adapter during indexed PCR-Free library preparation, and therefore in the following typing steps, the resultant sequencing data can be traced to the samples one by one based on the primer indexes and adapter indexes used in each sample. Sequences of each sample are aligned to the known DNA reference sequence corresponding to the PCR product by software. Based on the sequence overlapping and linkage relationship, an intact sequence for the PCR product is assembled from the sequences of the sheared DNA.

The present invention provides Illumina GA sequencing technique-based high-resolution HLA genotyping methods, thereby accomplishing haplotype sequencing and software typing automation, enhancing HLA genotyping throughput, and reducing cost.

Due to the requirement on the length of DNA template in the current sequencing techniques and the short read length in the current sequencing techniques, the original PCR primers for HLA-SBT methods are not applicable to new sequencing technique-based high-resolution HLA typing methods any more. The present invention designs new PCR primers with good specificity and conservation, which amplify Exons 2, 3, 4 of HLA-A, B gene independently, and whose PCR products have a length no more than 700 bp and are particularly applicable to Illumina GA (the maximum DNA length applicable to the current Illumina GA is 700bp). A set of PCR primers as provided in the present invention is applicable to HLA genotyping for subjects (in particular human) with a large scale, a high throughput and a low cost.

In the technical solutions employed in the present invention, all the latest HLA-A/B gene sequences are downloaded from IMGT/HLA internet website (http://www.ebi.ac.uk/imgt/hla/), and then are saved in the local disk as HLA-A data set; meanwhile, all the latest HLA-I gene sequences other than HLA-A sequences are downloaded as the comparison data set. Said two data sets are compared to look for conservative and specific sequences for each gene site at the two termini and internal portion of Exons 2, 3, 4, and the designed PCR primer sequence is compared with the whole human genome sequence for homology. Since HLA-A/B gene is highly similar

to other genes belonging to HLA-I molecules in terms of sequence, when designing PCR primers, the 3' terminal of the primer should be specific as far as possible so as to ensure the specificity of amplifying HLA-A/B gene with the primers. Meanwhile, the length of the PCR products is less than 700bp, and the annealing temperature of forward and reverse primers are substantially the same.

Multiple pairs of candidate HLA-A/B primers meeting the design requirements are used to amplify template DNAs of common HLA-A/B serotypes. Among them, two sets of PCR primers of HLA-A/B (6 pairs for each set) with the best conservatism and specificity, for amplification of Exons 2, 3 and 4, respectively, are screened out.

The two sets of PCR primers (6 pairs for each set) are used as the basic primers, on the basis of which, 95 sets of index primers which are used for amplification of 95 and 950 DNA templates of common serotypes of HLA-A/B (the serotypes of these templates include all the common serotypes of HLA-A/B), respectively, are designed. All the PCR products are sequenced with Illumina GA Pair-End 100 after mixing in an equal amount, and the sequencing results after assembly are compared with the original typing results to confirm the conservatism and specificity of the PCR primers.

HLA-A, B primers as designed in the present invention, i.e. two sets of PCR primers of HLA-A/B (6 pairs for each set) for amplification of Exons 2, 3 and 4, respectively, are shown in Table 1 and 2.

Table 1: PCR primers of HLA-A, B

SEQ ID NO:	primer No.	primer sequence	the use of primer	length of products
1	A-F2	CCTCTGYGGGGAGAAGCAA	Amplifying Exon 2 of HLA-A gene	480bp
2	A-R2	ATCTCGGACCCGGAGACTG		
3	A-F3	CGGGGCCAGGTTCTCACAC	Amplifying Exon 3 of HLA-A gene	410bp
4	A-R3	GGYGATATTCTAGTGTTGGTCCC AA		
5	A-F4	GTGTCCCATGACAGATGCAAAA	Amplifying Exon 4 of HLA-A gene	430bp
6	A-R4	GGCCCTGACCCTGCTAAAGG		

7	B-F2	AGGAGCGAGGGGACCGCA	Amplifying Exon 2 of HLA-B gene	400bp
8	B-R2	CGGGCCGGGGTCACTCAC		
9	B-F3	CGGGGCCAGGGTCTCACA	Amplifying Exon 3 of HLA-B gene	370bp
10	B-R3	GAGGCCATCCCCGGCGAC		
11	B-F4	GCTGGTCACATGGGTGGTCCTA	Amplifying Exon 4 of HLA-B gene	380bp
12	B-R4	CTCCTTACCCCATCTCAGGGTG		

Table 2: PCR primers of HLA-A, B

SEQ ID NO:	primer No.	primer sequence	the use of primer	length of products
13	A-F2s	CCTCTGYGGGGAGAAGCAA	Amplifying Exon 2 of HLA-A gene	481bp
14	A-R2s	GGATCTCGGACCCGGAGACTGT		
15	A-F3s	TGGGCTGACCGYGGGGTC	Amplifying Exon 3 of HLA-A gene	403bp
16	A-R3s	GGYGATATTCTAGTGTTGGTCCC AA		
17	A-F4s	GTGTCCCATKACAGATGCAAAA	Amplifying Exon 4 of HLA-A gene	405bp
18	A-R4s	GGCCCTGACCCTGCTAAAGG		
19	B-F2s	AGGAGCGAGGGGACCGCA	Amplifying Exon 2 of HLA-B gene	400bp
20	B-R2s	CGGGCCGGGGTCACTCAC		
21	B-F3s	CCAAAATCCCCGCGGGTT	Amplifying Exon 3 of HLA-B gene	405bp
22	B-R3s	GAGGCCATCCCCGGCGAC		
23	B-F4s	GCTGGTCACATGGGTGGTCCTA	Amplifying Exon 4 of	374bp
24	B-R4s	TGACCCCTCATCCCCCTCCT		

			HLA-B gene	

Degenerate primers refer to a mixture of all possible different sequences representing all different bases encoding a single amino acid. In order to increase specificity, degeneracy may be reduced according to bias of base usage in different organisms by referring to codon table, wherein R = A/G, Y = C/T, M = A/C, K = G/T, S = C/G, W = A/T, H = A/C/T, B = C/G/T, V = A/C/G, D = A/G /T, N=A/C/G/T.

The present invention designs 2 set of PCR primers (three pairs for each set) for amplification of Exons 2, 3 and 4 of HLA-C by using the method of designing PCR primers for amplification of Exons 2, 3 and 4 of HLA-A/B gene.

In the following Examples, 95 and 950 blood samples with known HLA genotypes are subjected to PCR amplification for H LA-C by using the selected 2 set of PCR primers (3 pairs for each set), respectively. The amplified products are sequenced by Sanger method and the second generation sequencing method. The sequencing results are applied to HLA-C typing, and are compared with the original typing results to confirm the conservatism and specificity of the PCR primers.

The present invention provides 2 set of PCR primers (three pairs for each set) for amplification of Exons 2, 3 and 4 of HLA-C gene, which are SEQ ID NOs: 25 and 26, 27 and 28, and 29 and 30 as shown in Table 3, and SEQ ID NOs: 31 and 32, 33 and 34, and 35 and 36 as shown in Table 4. Said 6 pairs of PCR primers have good conservatism and specificity, and can cover the full-length sequences of Exons 2, 3 and 4 of HLA-C, wherein the length of all the PCR products is less than 700bp, which meets the requirement of normal Illumina Solexa sequencing. In addition, the primers of the present invention are also applicable for Sanger sequencing.

Table 3: PCR primers of Exons 2, 3 and 4 of HLA-C gene

SEQ ID NO:	No.	primer sequence	HLA-C Exons	length of products
25	C-F2	GACCCGGGGAGCCGCGCA	2	455bp
26	C-R2	TCGAGGGTCTGGGCGGGTT		
27	C-F3	CCTTTACCCGGTTTCATTTTCRGTTT	3	417bp
28	C-R3	CTACGGGAGATGGGGAAGGCT		

29	C-F4	GTGTCGCAAGAGAGATRCAAAGTGT	4	451bp
30	C-R4	GCTCTGGGAAAGGAGGRGAAGG		

Table 4: PCR primers of Exons 2, 3 and 4 of HLA-C gene

SEQ ID NO:	No.	primer sequence	HLA-C Exons	length of products
31	C-F2s	GACCCGGGGAGCCGCGCA	2	455bp
32	C-R2s	TCGAGGGTCTGGGCGGGTT		
33	C-F3s	GCCCAGACCCTCGRCCGGA	3	443bp
34	C-R3s	AGATRGGGAAGGCTCCCCACT		
35	C-F4s	TCTCAGGATRGTCACATGGGC	4	481bp
36	C-R4s	GCTCTGGGAAARGAGGRGAAGG		

According to the methods as described above, in order to apply the second generation sequencing technique to HLA-DQB1 genotyping, the present invention provides the PCR primers for amplification of Exons 2 and/or 3 of HLA-DQB1, which are SEQ ID NOs: 37-40 as shown in Table 5. The PCR primers have good conservatism and specificity, and can cover the full-length sequences of Exons 2, 3 of HLA-DQB1, wherein the length of all the PCR products is less than 700bp, which meets the requirement of normal Illumina Solexa sequencing. In addition, the primers of the present invention are also applicable to Sanger sequencing.

Table 5 PCR primers for amplification of the corresponding Exons of HLA-DQB1

SEQ ID NO:	Primer No.	primer sequence	amplification target	length of amplificated products
37	Q-F2	GATTCCYCGCAGAGGATTTCG	Exon 2 of HLA-DQB1	311bp
38	Q-R2	AGGGGCRACSACGCTCACCTC		
39	Q-F3	CCTGTCTGTTACTGCCCTCAGT	Exon 3 of HLA-DQB1	339bp
40	Q-R3	GGCCCATAGTAACAGAAACTCAATA		

Genotyping may be carried out on the basis of amplification of Exons 2 and/or 3 of HLA-DQB1 by using the primer pairs for amplification and the genotyping methods as provided in the present invention. In relative to the prior art, the genotyping methods use

Illumina Solexa sequencing technique, which is characterized by the capability of obtaining a high resolution HLA typing results with high throughput and low cost.

SPECIFIC MODE FOR CARRYING OUT THE INVENTION

A method for nucleic acid sequencing

In one aspect, the present invention provides a method for determining the nucleotide sequence of a nucleic acid of interest in a sample, comprising:

a) providing n samples, wherein n is an integer of ≥ 1; optionally, the n samples to be analyzed are divided into m groups, m is an integer and n≥m≥1 ;

b) amplifying: PCR amplification is performed under conditions suitable for amplifying the nucleic acid of interest in a sample using a pair or multiple pairs of index primers are used for each sample comprising a forward index primer and a reverse index primer; wherein each index primer comprises a primer index and a PCR primer optionally linked by a linker sequence at the 5' end of the PCR primer; wherein the primer indexes comprised in the forward index primer and reverse index primer may be identical or different; and wherein the primer indexes are selected from the primer indexes shown in Table 6 and the PCR primer are selected from SEQ ID NOS: 1-40 and 231-238;

c) pooling: PCR products from each of the samples are pooled together when n>1;

d) shearing: PCR products are subjected to incomplete shearing, purifying and recovering;

e) sequencing: the recovered sheared DNA mixture from step (d) are subjected to sequencing by second generation sequencing; and

f) assembling: matching the sequencing data from step (e) to samples based on the unique primer index for each sample, wherein each sequence read is aligned to the DNA reference sequence corresponding to the PCR products using an alignment program and assembling a complete sequence of the nucleic acid of interest from the sequences of the sheared DNA by virtue of sequence overlapping and linkage relationship.

In an embodiment of the present invention, said pair of index primers comprises at least one degenerate primer.

In one aspect of the present invention, each pair of primer indexes and a pair of PCR primers form a pair of index primers, forward and reverse PCR primers have a

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forward primer index and a reverse primer index at 5' end (or optionally linked by a linker sequence), respectively.

In one embodiment of the present invention, said PCR primers are PCR primers for amplification of HLA gene, particularly PCR primers for amplification of HLA-A/B gene, preferably PCR primers for amplification of Exons 2, 3 and 4 of HLA-A/B and Exon 2 of HLA-DRB1, preferably PCR primers for amplification of Exons 2, 3 and 4 of HLA-A/B as shown in Table 1 or Table 2, or preferably PCR primers for amplification of Exon 2 of HLA-DRB1 as shown in Table 7.

In one embodiment of the present invention, said PCR primers are PCR primers for amplification of HLA gene, particularly PCR primers for amplification of HLA-C gene, preferably PCR primers for amplification of Exons 2, 3 and/or 4 of HLA-C; preferably, said PCR primers are shown in Table 3 or Table 4.

In one embodiment of the present invention, said PCR primers are PCR primers for amplification of HLA gene, preferably PCR primers for amplification of Exon 2 and/3 of HLA-DQB1 gene; preferably, said PCR primers are shown in Table 5.

In one aspect of the present invention, said primer indexes are designed for PCR primers, preferably for PCR primers for amplification of specific gene of HLA, more preferably for PCR primers for amplification of Exons 2, 3 and 4 of HLA-A/B and Exon 2 for HLA-DRB1, particularly for PCR primers as shown in Table 1, Table 2 or Table 7; said primer indexes particularly comprise at least 10, or at least 20, or at least 30, or at least 40, or at least 50, or at least 60, or at least 70, or at least 80, or at least 90, or 95 pairs of 95 pairs of primer indexes as shown in Table 6 (or the set of primer indexes consisting of 10-95 pairs (for example, 10-95 pairs, 20-95 pairs, 30-95 pairs, 40-95 pairs, 50-95 pairs, 60-95 pairs, 70-95 pairs, 80-95 pairs, 90-95 pairs, or 95 pairs) of the 95 pairs of primer indexes as shown in Table 6); and

the set of index primers preferably comprises at least PI-1 to PI-10, or PI-11 to PI-20, or PI-21 to PI-30, or PI-31 to PI-40, or PI-41 to PI-50, or PI-51 to PI-60, or PI-61 to PI-70, or PI-71 to PI-80, or PI-81 to PI-90, or PI-91 to PI-95 of 95 pairs of primer indexes as shown in Table 6, or combinations of any two or more of them.

In one embodiment of the present invention, said DNA shearing includes chemical shearing methods and physical shearing methods, wherein the chemical shearing methods include enzymatic digestion, and the physical shearing methods include

ultrasonic shearing methods or mechanical shearing methods.

In one embodiment of the present invention, after said DNA shearing, all the DNA bands between the maximum read length of the sequencer and the applicable maximum DNA length of the sequencer are purified and recovered, wherein said purification and recovery methods include, but are not limited to, recovery by electrophoresis and gel slicing, and recovery by magnetic beads.

In another embodiment of the present invention, a method for sequencing the nucleotide sequence of a nucleic acid of interest in a test sample, comprising steps a) to d) of claim 1, and the following steps:

e) constructing a library: constructing a PCR-free sequencing library using the library of the sheared PCR products from step (d), wherein different library adapters may be added to distinguish different PCR-Free sequencing libraries, wherein all the DNA fragments between the maximum read length of the sequencer and the applicable maximum DNA length of the sequencer, preferably DNA fragments of 450 to 750 bp, are purified and recovered;

f) sequencing: the recovered PCR-Free sequencing library from step (e) is subjected to sequencing by second generation sequencing;

g) assembling: matching the sequencing data from step (f) to samples based on different library adapter sequences of the libraries and the unique primer index for each sample, wherein each sequence read is aligned to the DNA reference sequence corresponding to the PCR products using an alignment program and assembling a complete sequence of the nucleic acid of interest from the sequences of the PCR-Free sequencing library by virtue of sequence overlap and linkage relationship.

In one embodiment of the present invention, the samples are from a mammal. In another embodiment, the samples are from a human. In a further embodiment, the samples are human blood samples.

In one aspect, the present invention further provides the use of the above-mentioned method in HLA typing, characterized by comprising: sequencing a sample (particularly blood sample) from a patient by said method, and aligning the sequencing results with sequence data of Exons of HLA, preferably, Exons 2, 3, 4 of HLA-A/B, Exons 2, 3 and/or 4 of HLA-C, Exon 2 and/or 3 of HLA-DQB1 gene and/or Exon 2 of HLA-DRB1 in HLA database (such as IMGT HLA professional database); wherein if the result of

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sequence alignment shows 100% match, the HLA genotype of the corresponding sample is determined.

In one aspect of the present invention, said PCR primers are PCR primers for amplification of a specific gene of HLA, preferably PCR primers for amplification of Exons 2, 3, 4 of HLA-A/B gene and Exon 2 of HLA-DRB1, preferably PCR primers for amplification of Exons 2, 3 and 4 of HLA-A/B as shown in Table 1 or Table 2, or preferably PCR primers for amplification of Exon 2 of HLA-DRB1 as shown in Table 7; or preferably PCR primers for amplification of Exons 2, 3 and/or 4 of HLA-C, preferably said PCR primers are shown in Table 3 or Table 4; or preferably PCR primers for amplification of Exon 2 and/or 3 of HLA-DQB1, preferably said PCR primers are shown in Table 5.

In another aspect, the present invention provides a set of index primers comprising said set of primer indexes and a pair of PCR primers for amplification of a sequence of interest to be tested, wherein a pair of index primers comprises a pair of primer indexes and a pair of PCR primers, the forward and reverse PCR primer have a forward and a reverse primer index at 5' end (or optionally linked by a linker sequence), respectively.

In one embodiment of the present invention, said PCR primers are PCR primers for amplification of a specific gene of HLA, preferably PCR primers for amplification of Exons 2, 3, 4 of HLA-A/B gene and Exon 2 of HLA-DRB1, preferably PCR primers for amplification of Exons 2, 3 and 4 of HLA-A/B as shown in Table 1 or Table 2, or preferably PCR primers for amplification of Exon 2 of HLA-DRB1 as shown in Table 7; preferably PCR primers for amplification of Exons 2, 3 and/or 4 of HLA-C, preferably said PCR primers are shown in Table 3 or Table 4; or preferably PCR primers for amplification of Exon 2 and/or 3 of HLA-DQB1, preferably said PCR primers are shown in Table 5.

In another aspect, the present invention further provides the use of said index primers in PCR sequencing methods.

A HLA typing method

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In one aspect, the present invention provides a HLA typing method, comprising:

1) providing n samples, wherein n is an integer of ≥1, the sample is preferably from mammalian, more preferably human, particularly human blood sample;

2) dividing n samples to be analyzed into m groups, m is an integer and n≥m≥1 ;

3) amplifying: a pair of index primers is used for each sample, when there are templates from the sample, PCR amplification is performed under conditions suitable for amplifying the nucleic acid of interest, wherein each pair of index primers consists of a forward index primer and a reverse index primer (both of which may be degenerate primers) comprising primer indexes, wherein the primer indexes comprised in the forward index primer and reverse index primer may be identical or different: the primer indexes in the pairs of index primers used for different samples are different;

4) pooling: pooling PCR amplified products from each of the samples together to obtain PCR product libraries;

5) shearing: subjecting the resultant PCR product libraries to incomplete shearing;

6) constructing libraries: constructing PCR-free sequencing libraries from the library of the sheared PCR products with library adapter index technique, wherein different library adapters may be added to distinguish different PCR-Free sequencing libraries, all the DNA bands between the maximum read length of the sequencer and the applicable maximum DNA length of the sequencer, particularly DNA fragments of 450 to 750 bp, are recovered;

7) sequencing: subjecting the recovered DNA mixture to sequencing by using the second generation sequencing technique, preferably Paired-End technique (for example, Illumina GA, Illumina Hiseq 2000), obtaining the sequences of the sheared DNAs;

8) assembling: corresponding the obtained sequencing results to the samples one by one based on different library adapter sequences of the libraries and the unique primer index for each sample, aligning each sequence read to the DNA reference sequence corresponding to the PCR products by using alignment program (such as Blast, BWA program), assembling a complete sequence of the nucleic acid of interest from the sequences of the sheared DNA based on sequence overlapping and linkage

relationship; and

9) typing: aligning the sequencing results with sequence data of Exons of HLA, preferably, Exons 2, 3, 4 of HLA-A/B, Exons 2, 3 and/or 4 of HLA-C, Exon 2 and/or 3 of HLA-DQB1 gene and/or Exon 2 of HLA-DRB1 in HLA database (such as IMGT HLA professional database), wherein if the result of sequence alignment shows 100% match, the HLA genotype of the corresponding sample is determined.

In the HLA typing method of the present invention, a pair of index primers comprises a pair of primer indexes and a pair of PCR primers, the forward and reverse PCR primer have a forward and a reverse primer index at 5' end (or optionally linked by a linker sequence), respectively.

In one embodiment of the present invention, said PCR primers are PCR primers for amplification of a specific gene of HLA, preferably PCR primers for amplification of Exons 2, 3, 4 of HLA-A/B gene and Exon 2 of HLA-DRB1, preferably PCR primers for amplification of Exons 2, 3 and 4 of HLA-A/B as shown in Table 1 or Table 2, or preferably PCR primers for amplification of Exon 2 of HLA-DRB1 as shown in Table 7; preferably PCR primers for amplification of Exons 2, 3 and/or 4 of HLA-C, preferably said PCR primers are shown in Table 3 or Table 4; or preferably PCR primers for amplification of Exon 2 and/or 3 of HLA-DQB1, preferably said PCR primers are shown in Table 5.

In one embodiment of the present invention, said primer indexes are a set of primer indexes as described above.

In one embodiment of the HLA typing method of the present invention, said DNA shearing includes chemical shearing methods and physical shearing methods, wherein the chemical shearing methods include enzymatic digestion, and the physical shearing methods include ultrasonic shearing methods or mechanical shearing methods.

In one embodiment of the HLA typing method of the present invention, said purification and recovery methods include, but are not limited to, recovery by

electrophoresis and gel slicing, and recovery by magnetic beads.

In one embodiment of the HLA typing method of the present invention, the construction of PCR-free sequencing libraries from the libraries of the sheared PCR products with library adapter indexing technique comprises, adding m library adapters to the m PCR product libraries obtained in 2), wherein each PCR product library uses a different library adapter, thereby constructing m adapter indexed sequencing libraries; m adapter indexed sequencing libraries are pooled together at equal mole to construct a mixture of adapter indexed sequencing libraries, wherein the method for linking library adapters refers to direct linkage using DNA ligase without a PCR procedure.

PCR primers for HLA genotyping

In one aspect, the present invention provides PCR primers for HLA genotyping, characterized by that said PCR primers are PCR primers for amplification of Exons 2, 3, 4 of HLA-A/B gene and Exon 2 of HLA-DRB1, preferably PCR primers for amplification of Exons 2, 3 and 4 of HLA-A/B as shown in Table 1 or Table 2, or preferably PCR primers for amplification of Exon 2 of HLA-DRB1 as shown in Table 7; preferably PCR primers for amplification of Exons 2, 3 and/or 4 of HLA-C, preferably said PCR primers are shown in Table 3 or Table 4; or preferably PCR primers for amplification of Exons 2 and/or 3 of HLA-DQB1, preferably said PCR primers are shown in Table 5.

The present invention further provides a sequencing method using said PCR primers, comprising

providing a sample, particularly a blood sample, said blood sample is preferably from mammalian, particularly human;

amplifying: amplifying DNA from the blood sample with the PCR primers to obtain PCR products, and purifying the PCR products;

sequencing: subjecting the PCR products to sequencing, the sequencing method may be Sanger sequencing method, or the second generation sequencing method (such as Hiseq 2000, Illumina GA and Roche454).

In another aspect, the present invention further provides the use of said PCR primers in HLA genotyping, characterized by using said PCR primers, carrying out assembly and alignment analysis on the results obtained by the above sequencing method, and comparing the sequencing results with the standard sequences in the database to obtain the HLA genotyping results.

In another aspect, the present invention further provides a kit for HLA genotyping, comprising said PCR primers.

PCR primers for HLA-A, B genotyping

In one aspect, the present invention provides a set of PCR primers for HLA-A,B genotyping, characterized by that said PCR primers are as shown in Table 1 or Table 2.

In another aspect, the present invention provides a sequencing method using PCR primers for HLA-A,B genotyping, comprising:

providing a sample, particularly a blood sample, said blood sample is preferably from mammalian, particularly human;

amplifying: amplifying DNA from the blood sample with the PCR primers to obtain PCR products, and purifying the PCR products;

sequencing: subjecting the PCR products to sequencing, the sequencing method may be Sanger sequencing method, or the second generation sequencing method (such as Hiseq 2000, Illumina GA and Roche454).

In another aspect, the present invention further provides the use of said PCR primers in HLA genotyping, characterized by using said PCR primers, carrying out assembly and alignment analysis on the results obtained by the above sequencing method, and comparing the sequencing results with the standard sequences in the database to obtain the HLA genotyping results.

In another aspect, the present invention further provides a kit for HLA genotyping, comprising the PCR primers for HLA-A,B genotyping of the present invention.

PCR primers for HLA-C genotyping

The present invention further provides a new method for amplifying Exons 2, 3 and

4 of HLA-C gene, characterized by carrying out PCR amplification using the amplification primer pairs of the present invention, the sequences of the amplification primer pairs are as shown in Table 3 or Table 4.

Since Exons 2, 3 and 4 of HLA-C can be amplified by a PCR reaction, the method of the present invention is particularly suitable for HLA-C genotyping. As compared with the prior HLA-C genotyping methods, since the products obtained by using the method and the amplification primers of the present invention are controlled within 700 bp, Illumina Solexa sequencing technique-based HLA-SBT may be used during further genotyping.

The present invention further provides a method for sequencing Exons 2, 3 and 4 of HLA-C gene in samples, comprising the followings steps of:

1) providing a sample and extracting DNA of the sample;

2) amplifying the DNA with the PCR primer pair for HLA-C genotyping of the present invention to obtain PCR products, preferably purifying the PCR products, said PCR primer pair is preferably selected from the group consisting of the primer pair of SEQ ID NO: 25 and SEQ ID NO: 26, SEQ ID NO: 27 and SEQ ID NO: 28, SEQ ID NO: 29 and SEQ ID NO: 30, or SEQ ID NO: 31 and SEQ ID NO: 32, SEQ ID NO: 33 and SEQ ID NO: 34, SEQ ID NO: 35 and SEQ ID NO: 36;

3) subjecting the PCR products to sequencing, preferably by the second generation sequencing method, such as Illumina Solexa or Roche454.

The present invention further provides a HLA-C genotyping method, comprising:

1) PCR amplifying Exons 2, 3 and/or 4 of HLA-C gene of the sample to be tested with the PCR primer pair for HLA-C genotyping of the present invention, said PCR primer pair is preferably selected from the group consisting of the primer pair of SEQ ID NO: 25 and SEQ ID NO: 26, SEQ ID NO: 27 and SEQ ID NO: 28, SEQ ID NO: 29 and SEQ ID NO: 30, or SEQ ID NO: 31 and SEQ ID NO: 32, SEQ ID NO: 33 and SEQ ID NO: 34, SEQ ID NO: 35 and SEQ ID NO: 36;

2) subjecting the amplified exons to sequencing, comparing the sequencing results with the standard sequences in the database so as to determine the genotyping results, wherein the sequencing is carried out by Sanger sequencing method, or the second generation sequencing method, such as Illumina Solexa or Roche454.

In another aspect, the present invention further provides a kit for HLA-C genotyping, comprising the PCR primer pair for HLA-C genotyping of the present invention, preferably selected from the group consisting of the primer pair of SEQ ID NO: 25 and SEQ ID NO: 26, SEQ ID NO: 27 and SEQ ID NO: 28, SEQ ID NO: 29 and SEQ ID NO: 30, or SEQ ID NO: 31 and SEQ ID NO: 32, SEQ ID NO: 33 and SEQ ID NO: 34, SEQ ID NO: 35 and SEQ ID NO: 36. In one embodiment, said kit further comprises additional agents, for example, agents for DNA amplification, DNA purification, and/or DNA sequencing.

Genotyping may be performed on the basis of amplification of Exons 2, 3 and 4 of HLA-C, by using the amplification primer pair and the genotyping method as provided in the present invention. Hence, as compared with the prior art, the genotyping utilizes Illumina Solexa sequencing technique, enhances the throughput, simplifies the procedure, and meanwhile save time and cost.

PCR primers for HLA-DQB1 genotyping

The present invention further provides a new method for amplifying Exon 2 and/or 3 of HLA-DQB1, characterized by carrying out PCR amplification with the amplification primer pairs of the present invention, said amplification primer pairs are as shown in Table 5.

Since Exons 2 and/or 3 of HLA-DQB1 can be amplified by a PCR reaction, the method of the present invention is particularly suitable for HLA-DQB1 genotyping. As compared with the prior HLA-DQB1 genotyping methods, since the products obtained by using the method and the amplification primers of the present invention are controlled within 300-400 bp, Illumina Solexa sequencing technique-based HLA-SBT may be used during further typing.

The present invention further provides a method for sequencing Exon 2 and/or 3 of HLA-DQB1 in samples, comprising the following steps of:

1) providing a sample and extracting DNA of the sample;

2) amplifying the DNA with the PCR primer pair for HLA-DQB1 genotyping of the present invention, preferably PCR primer pairs shown in Table 5, to obtain PCR products, preferably purifying the PCR products;

3) subjecting the PCR products to sequencing, preferably by the second generation sequencing method, such as Illumina Solexa or Roche454.

In another aspect of the present invention, the present invention provides an improved method for HLA-DQB1 genotyping, comprising:

1) amplifying Exon 2 and/or 3 of HLA-DQB1 to be tested with the PCR primer pair for HLA-DQB1 genotyping of the present invention, preferably the PCR primer pairs as shown in Table 5;

2) subjecting the amplified exons to sequencing, comparing the sequencing results with the standard sequences in the database so as to determine the genotyping results, wherein the sequencing method may be Sanger sequencing method or the second generation sequencing method, such as Illumina Solexa or Roche454.

In another aspect, the present invention further provides a kit for HLA-DQB1 genotyping, comprising the PCR primer pair for HLA- DQB1 genotyping of the present invention, preferably, the PCR amplification primer pairs as shown in Table 5. In one embodiment, said kit further comprises additional agents, for example, agents for DNA amplification, DNA purification, and/or DNA sequencing.

DESCRIPTION OF DRAWINGS

Figure 1: A drawing illustrating the sequence assembling after labeling with primer indexes, DNA shearing and DNA sequencing. The forward and reverse primer index sequences Index-N-F/R (1) are introduced to the two termini of the PCR products of the sample No. N. The PCR products after shearing by a physical shearing method, comprises products carrying primer index sequences at one end, products carrying no primer index sequence at two termini, and completely unsheared products. All the DNA bands between the maximum read length of the sequencer and the applicable maximum DNA length of the sequencer are purified and recovered by gel slicing, and used for sequencing (2). The sequencing data of the PCR products belonging to the sample No. N are traced using Index-N-F/R. The known reference sequences of the PCR products are used to localize the relative positions of the sequence reads, and the sequencing results of the complete PCR products are assembled based on the overlapping and linkage relationship between

the sequence reads (3, 4).

Figure 2: A drawing illustrating the results of electrophoresis of PCR products of the corresponding Exons of HLA-A/B/DRB1 in Sample No.1 of Example 2. It can be seen from electrophoretogram that PCR products are a series of single bands of 300bp-500bp, wherein Lane M is a marker of molecular weight (DL 2000, Takara Co.), Lanes 1-7 are the PCR products of the exons (A2, A3, A4, B2, B3, B4, DRB1-2) of HLA-A/B/DRB1 of Sample No.1, and there is no amplification band in negative control (N). The results of other samples are similar.

Figure 3: A drawing illustrating results of DNA electrophoresis after shearing HLA-Mix in Example 4 (before and after gel slicing), wherein the gel-slicing area is the area of 450-750bp. Lane M is a marker of molecular weight (NEB-50bp DNA Ladder), and Lane 1 shows the electrophoretic result of HLA-Mix before gel slicing, and Lane 2 is a drawing showing the gel of HLA-Mix after slicing.

Figure 4: A screen-capture of the program for construction of consensus sequence of Sample No.1 in Example 6, illustrating assembling the complete sequence of the PCR products based on primer indexes and the overlapping relationship between DNA fragments. Please refer to http://www.ebi.ac.uk/imgt/hla/align.html for nomenclature of HLA genotypes. One could find the results of all the coding sequence of A*02:03:01 A*11:01:01 in the result output column on the left, wherein the sequence of Exon 2 is identical to the original known result of Template 1.

Figure 5: A drawing illustrating the PCR product after labeling with primer indexes and an adapter index. During experimentation, primer indexes are introduced to the two termini of the PCR product of each sample by PCR simultaneously; multiple PCR products carrying different primer indexes are pooled together to construct a sequencing library. During construction of sequencing libraries, when multiple sequencing libraries have to be constructed, the sequencing libraries may be labeled with the library adapters carrying different adapter indexes. After finishing the construction of libraries, multiple sequencing libraries labeled with different adapter indexes are pooled together and are sequenced by Illumina GA simultaneously (the primer indexes may be identical among sequencing libraries

labeled with different adapter indexes). After getting the sequencing results, DNA sequence information for each sample may be obtained by screening the sequence information of the adapter indexes and the primer indexes in the sequencing results.

Figure 6: A drawing illustrating the electrophoretic result of the PCR products of Exons 2, 3, 4 of HLA-C of some samples in Example 8. It can be seen from electrophoretogram that PCR products are a series of single bands of 400bp-500bp, wherein Lane M is reference for standard DNA molecular weights (DL 2000, Takara Co.).

Figure 7: A drawing illustrating results of DNA electrophoretic gel slicing after shearing HLA-Mix in Example 8, wherein the gel-slicing area is the area of 450-750bp. Lane M is a marker of molecular weight (NEB-50bp DNA Ladder), and Lane 1 is a drawing showing the gel of HLA-Mix before slicing, and Lane 2 is a drawing showing the gel of HLA-Mix after slicing.

Figure 8: A screen-capture of the program for construction of consensus sequence of Exon 2 of HLA-C site of Sample No. 2 in Example 8. Firstly, the sequence reads of C site of the sample are aligned with the reference sequence by BWA software, thereby constructing the consensus sequences of Exons 2, 3, 4 of C site of the sample; further, the haplotype sequence of each exon of C site is determined on the basis of the linkage relationship between SNPs; and finally the type of the sample is determined by the intersection of the haplotype sequences of the exons. As shown in the figure, two heterozygous SNP are comprised in 695-764 area of C gene sequence of sample No.2, and it can be determined from read1 and read2 that the linkage relationship of SNP is A-C, G-A ("..." in the figure represents the bases identical to those of the reference sequence). The sequences correspond to the shaded parts of the sequences of the C*010201 and C*07020101 types, respectively. The judgment of the linkage relationship of other areas is similar.

Figure 9: A drawing illustrating electrophoresis results of PCR products of Exons 2, 3 and 4 of HLA-C site of 26 samples in Example 9. As shown in the figure, all the PCR products are of a length less than 500 bp; the electrophoretic band is single; there is no obvious non-specific band; and the amplification efficiency of the same pair of primers is the same in various samples.

Figure 10: A drawing illustrating the analytic results of the sequencing data of PCR amplification products of Template 1 by using uType software in Example 9. The result output column on the left shows the result, C*08:01:01 C*15:05:01, which are identical to the original known type of Template 1.

Figure 11: A drawing illustrating the electrophoretic result of PCR products of Exon 2+3 of HLA-DQB1 in 94 samples of Example 10. It can be seen from electrophoretogram that PCR products are a series of single bands of 250bp-500bp, wherein Lane M is reference for standard DNA molecular weights (DL 2000, Takara Co.), Lanes PI-1 to PI-94 are the PCR amplification products of Exon 2+3 of HLA-DQB1 in 94 samples, and there is no amplification band in negative control (N).

Figure 12 shows the results of DNA electrophoretic gel slicing after shearing HLA-Q-Mix in Example 10, wherein the gel-slicing area is an area of 350-550bp. Lane M is a marker of standard DNA molecular weights (NEB-50bp DNA Ladder), and Lane 1 is a drawing showing the gel of HLA-Q-Mix before slicing, and Lane 2 is a drawing showing the gel of HLA-Q-Mix after slicing.

Figure 13 shows a screen-capture of the program for construction of consensus sequence of Sample No. 7 in Example 10, illustrating the main procedure of data analysis. Firstly, the sequence reads of the DQB1 site of the sample are aligned with the reference sequence by BWA software, thereby constructing the consensus sequences of Exons 2, 3 of DQB1 of the sample; and haplotype sequences of Exons 2, 3 of DQB1 are determined based on the linkage relationship between SNPs. As shown in the figure, six heterozygous SNPs are comprised in 2322-2412 area of DQB1 gene sequence of Sample No.7, and it can be determined from read1 that the linkage relationship of SNP1-SNP5 is T-G-T-C-C; it can be determined from read2 that the linkage relationship of another SNP1-SNP5 is C-C-A-G-T; it can be determined from read3 that the linkage relationship of SNP3-SNP6 is A-G-T-G; it can be determined from read4 that the linkage relationship of another SNP3-SNP6 is T-C-C-A; and it can be determined from the above linkage relationships of said SNPs that read1 is linked to read4, read2 is linked to read3, the complete SNP combination in this area is T-G-T-C-C-A and C-C-A-G-T-G, and the sequences correspond to the shaded parts of the sequences of DQB1*0303 and DQB1*0602 type. The judgment of the linkage

relationship of other areas is similar.

Figure 14 shows the electrophoretogram of the products in Example 11, resulted from amplification of each of Exons 2 and 3 of HLA-DQB1 site and amplification of Exons 2 and 3 with two pairs of PCR primers, respectively. The electrophoretogram shows three sets of PCR products from seven DNA templates, wherein all the PCR products have a length less than 500 bp; electrophoretic bands are single; and there is no obvious non-specific band. There is no amplification band in negative control (N), and Lane M is reference for standard DNA molecular weights (DL 2000, Takara Co.).

Figure 15 illustrates the analytic results of the sequencing data of PCR products resulted from amplification of Exons 2 and 3 of HLA-DQB1 of Template 7, by using uType software in Example 11. The result output column on the left shows the result, DQB1*03:03 DQB1*06:02, which is identical to the original known result of Template 7.

Figure 16 shows the electrophoretic results of PCR products from the corresponding Exons of HLA-A/B/C/DQB1 in Sample No. 1 in Example 12. It can be seen from electrophoretogram that PCR products are a series of single bands of 300bp-500bp, wherein Lane M is a marker of molecular weights (DL 2000, Takara Co.); Lanes 1-10 are the PCR amplified products of the Exons (A2, A3, A4, B2, B3, B4, C2, C3, C4, DQB1) of HLA-A/B/C/DQB1 of Sample No. 1; no amplification band is present in negative control (N). The results of other samples are similar.

Figure 17 illustrates the result of recovery from agarose gel after pooling HLA-1-Mix, HLA-2-Mix, HLA-3-Mix, HLA-4-Mix, HLA-5-Mix, HLA-6-Mix, HLA-7-Mix, HLA-8-Mix, HLA-9-Mix and HLA-10-Mix in equal mole in Example 12. Lane M is a marker of molecular weights, and Lane 1 is the electrophoretic result of the pool, and Lane 2 is the electrophoretogram after gel slicing containing the DNA fragments of a length ranging from 450 to 750 bp.

Figure 18 shows a screen-capture of the program for construction of consensus sequence of Exon 2 of HLA-C site of Sample No. 1 in Example 12. Firstly, the sequence reads of C site of the sample are aligned with the reference sequence by BWA software, thereby constructing the consensus sequences of Exons 2, 3, 4 of C site of the sample; further, the haplotype sequences of the exons of C site are

determined on the basis of the linkage relationship between SNPs; and finally the type of the sample is determined by the intersection of the haplotype sequences of the exons. As shown in the figure, two heterozygous SNPs are comprised in 695-764 area of C gene sequence of Sample No. 1, and it can be determined from read1 and read2 that the linkage relationship of SNPs is A-C, G-A ("..." in the figure represents the bases identical to those of the reference sequence). The sequences correspond to the shaded parts of the sequences of the C*010201 and C*07020101 type, respectively. The judgment of linkage relationship of other areas is similar.

EXAMPLES

The embodiments of the present invention are described in detail in the following examples. However, a person skilled in the art would understand that the following examples are used to illustrate the present invention rather than restricting the scope of the present invention.

In Examples 1-6 of the present invention, Exons 2, 3, 4 of HLA-A/B and Exon 2 of HLA-DRB1 in 95 samples were genotyped by using the combination of primer indexes + DNA incomplete shearing strategy + Illumia GA sequencer Paired-End 100 sequencing technique (PCR products have a length ranging from 290bp to 500bp), demonstrating that the method of the invention could accomplish the typing of gene fragments of a length exceeding the maximum read length of sequencer whilst sufficiently utilizing the characteristics of the second generation sequencer, such as high throughput and low cost.

Principle: for the sample to be analyzed, primer indexes were introduced to the two termini of the PCR products of Exons 2, 3, 4 of HLA-A/B and Exon 2 of HLA-DRB1 by PCR reaction so as to specifically label the sample information of the PCR products. The products of PCR amplification of three sites (HLA-A/B/DRB1) in each group of samples were pooled together to obtain a library of PCR products; after incomplete ultrasonic shearing of the library of PCR products, a PCR-free sequencing library was constructed. The sequencing library was subjected to 2% low melting point agarose gel electrophoresis, and all the DNA bands of a length ranging from 450bp to 750bp were purified and recovered by gel slicing (during the construction of the PCR-Free

sequencing library, since library adapters were added to the two termini of the DNA fragments, the length of the DNA band as shown in the electrophoretogram was about 250 bp longer than the actual length of the DNA fragments; therefore, the fragments of a length ranging from 450bp to 700bp as recovered here actually correspond to DNA fragments of an original length ranging from 200 bp to 500 bp). The recovered DNA was sequenced by Illumina GA PE-100. The sequence information of all the tested samples can be traced by primer index sequences, and the sequence of the whole PCR product can be assembled on the basis of the known reference sequences and the overlapping and linkage relationship between the sequences of DNA fragments, The complete sequence of the original PCR product can be assembled with the standard database of the corresponding exons of HLA-A/B/DRB1, thereby accomplishing HLA-A/B/DRB1 genotyping.

Example 1

Sample extraction

DNAs were extracted from 95 blood samples with known HLA-SBT typing results (China Marrow Donor Program cited hereafter as (CMDP)) by using KingFisher Automatic Extraction Instrument (US Thermo Co.). The main steps were as followed: as directed in the handbook, a certain amount of self-contained agents was added to six deep-well plates and one shallow-well plate equipped by the KingFisher Automatic Extraction Instrument, and all the plates, to which the agents were added, were placed in the corresponding positions as required. The program "Bioeasy_200ul Blood DNA_KF.msz" was selected, and was implemented to extract nucleic acids by pressing "star". Approximately 100 μl eluted products (i.e. the extracted DNA) were collected from plate Elution after the program was finished.

Example 2

PCR amplification

Different PCR index primers were made by synthesizing PCR primers having different primer indexes at 5' end, and such different PCR index primers may be applied to different samples, wherein the PCR primers were PCR primers for Exons 2,

3, 4 of HLA-A/B and Exon 2 of HLA-DRB1. Thereafter, primer indexes were introduced to the two termini of the PCR products by PCR reaction, thereby specifically labeling the PCR products from different samples.

95 sets of PCR index primers were used to amplify 95 DNA samples, respectively, wherein each set of PCR index primers consisted of a pair of bidirectional primer indexes (Table 6) and PCR primers for amplification of Exons 2, 3, 4 of HLA-A/B (Table 1) and of Exon 2 of HLA-DRB1 (Table 7), each forward PCR primer has the forward primer index in the pair of primer indexes linked at the 5' end, and the reverse PCR primer has the reverse primer index in the pair of primer indexes linked at the 5' end. During the synthesis of primers, the primer indexes were directly added to the 5' end of the PCR primers.

The 95 DNAs obtained from the sample extraction step of Example 1 were designated as No. 1-95. PCR reaction took place in 96-well plates, 7 plates in total, designated as HLA-P-A2, HLA-P-A3, HLA-P-A4, HLA-P-B2, HLA-P-B3, HLA-P-B4 and HLA-P-DRB1-2 (A2/A3/A4, B2/B3/B4, DRB1-2 represent the amplified sites), wherein a negative control without adding any template was set in each plate, and the primers used in the negative control were the same as those for Template 1. During experimentation, the numbering information of the samples corresponding to each pair of primer indexes was recorded.

Table 6: Relevant information of primer indexes

Primer index No.	SEQ ID NO:	Forward primer index	SEQ ID NO:	Reverse primer index	Corresponding position in 96-well plate	Corresponding template (Group 1)
PI-1	41	TCGCAGACATCA	42	TGACACGATGCT	A1	1
PI-2	43	TACATCGCACTA	44	TACAGATGCTGA	A2	2
PI-3	45	CTCGATGAGTAC	46	ACGTCTAGACAC	A3	3
PI-4	47	TCTGTATACTCA	48	TGCTGTAGTGAC	A4	4
PI-5	49	TATCTGCTCATA	50	AGATATCGAGCT	A5	5
PI-6	51	TACATGCTGAGC	52	ACGTGTCTATCA	A6	6
PI-7	53	TCATATCGCGAT	54	AGATCGTATAGC	A7	7
PI-8	55	ACAGATGCACGC	56	ATCTCGTGACAG	A8	8
PI-9	57	TAGATCGTACAT	58	ACTAGTACACGC	A9	9
PI-10	59	ACTACACGTCTC	60	ATAGTCACGCGT	A10	10
PI-11	61	AGACTCGCGTAT	62	TACTAGCTGACG	A11	11
PI-12	63	ATACTAGTGCTC	64	TGTATCGTGCTC	A12	12

PI-13	65	CACGATGACATC	66	TAGTGAGCGCAC	B1	13
PI-14	67	TGCTGTCTCGAG	68	CATAGCAGTGTC	B2	14
PI-15	69	TGTGCTCGAGTC	70	TCTGATCGAGCA	B3	15
PI-16	71	CACTCGTACATC	72	AGCGATGCTCAT	B4	16
PI-17	73	CGACGTGCTCGC	74	CGCGTACTGCAG	B5	17
PI-18	75	ACGCATCTATAC	76	CTAGTATCGCAG	B6	18
PI-19	77	CGAGATGACTCT	78	TGTATACACGAT	B7	19
PI-20	79	ACTGTCTCGAGC	80	ACGTAGCGCACA	B8	20
PI-21	81	CATCTGCTATAG	82	TCTAGCTCATGA	B9	21
PI-22	83	ACGCACTCTAGA	84	CTATGCACTGAT	B10	22
PI-23	85	TGAGATACAGTA	86	ATCTGCTATGAC	B11	23
PI-24	87	ACTCATCGTGCT	88	TAGAGCTGTCAC	B12	24
PI-25	89	TACACTGTCTAT	90	CAGCACATAGAT	C1	25
PI-26	91	CACAGTACTCGC	92	CTGCTAGTGTAT	C2	26
PI-27	93	TGTACTATCATA	94	TGTGATAGACAC	C3	27
PI-28	95	CTAGTACTGACG	96	AGCGAGTCTACT	C4	28
PI-29	97	TAGACTGAGCTA	98	ACATACTGAGAC	C5	29
PI-30	99	CAGACGCGTGAG	100	TACATCTCGTAT	C6	30
PI-31	101	CGCGACATCACG	102	TAGCGATGAGAC	C7	31
PI-32	103	ACACTCATAGAT	104	CTATCATGACAC	C8	32
PI-33	105	AGCGTATACTAG	106	CATACTCACGTA	C9	33
PI-34	107	TGTCGTGCTATC	108	ACATGACTCACG	C10	34
PI-35	109	CGCTAGACTGTA	110	TACTATAGTCGA	C11	35
PI-36	111	ACAGTGTAGCGC	112	TGATATGCTACA	C12	36
PI-37	113	CACTCTATCGAC	114	TCACGCGATGAG	D1	37
PI-38	115	ACACTCTAGTCA	116	ACGTAGATCTAT	D2	38
PI-39	117	CATATGAGATCG	118	AGCAGAGTGCTC	D3	39
PI-40	119	CAGCTATCATAC	120	CACTGCAGACGA	D4	40
PI-41	121	TATACTCTAGAT	122	TGCATAGAGCGC	D5	41
PI-42	123	TGTATGCTCGTC	124	TCGTGACAGATC	D6	42
PI-43	125	TAGTGATGCTCT	126	ACGAGCTGATAT	D7	43
PI-44	127	AGACTCTGAGTC	128	CTGATAGTATCA	D8	44
PI-45	129	CTCATAGACTAC	130	ATCGCGAGTGAC	D9	45
PI-46	131	TCGCTCACTACA	132	TGTCTCGACATC	D10	46
PI-47	133	ATAGAGTCTCAT	134	CGCATAGCGTAT	D11	47

PI-48	135	CGAGACACTCGC	136	TCGTAGTCTACA	D12	48
PI-49	137	CAGCATACTATC	138	TCGTGATACAGA	E1	49
PI-50	139	CAGCTATAGTCT	140	ATGCAGATATCT	E2	50
PI-51	141	TCTATCGATGCA	142	ACACGCAGATCG	E3	51
PI-52	143	CATGAGTATAGC	144	CTAGCTGACGTA	E4	52
PI-53	145	TAGCATATCGAG	146	TACACGTATGAG	E5	53
PI-54	147	ACGACTCGCTAC	148	TCATGACTAGTA	E6	54
PI-55	149	TAGCATACACGC	150	TGACGCGTATAC	E7	55
PI-56	151	CGTCATATGCAG	152	TATAGCGATGAC	E8	56
PI-57	153	TGCAGCGAGTAC	154	TCGACGCTAGCG	E9	57
PI-58	155	CGTGTCGACAGA	156	CAGTCGTGAGCA	E10	58
PI-59	157	ACTCGACGTGAG	158	ACGCGAGTGATA	E11	59
PI-60	159	ACTCGTCTGACG	160	TGCTATCACTGA	E12	60
PI-61	161	CATACTGTATCT	162	TACATAGATGTC	F1	61
PI-62	163	TCTACTCGTGAC	164	CACGTATAGTGA	F2	62
PI-63	165	CTGCACTAGACA	166	ACTCATATCGCA	F3	63
PI-64	167	ACACGAGCTCAT	168	CACTCATATCGA	F4	64
PI-65	169	TACAGATAGTCT	170	TCGTCTGTGATA	F5	65
PI-66	171	TACACTCGTGCT	172	TGACGCTCATCT	F6	66
PI-67	173	TACATGTGACGA	174	TCGTACATGCTC	F7	67
PI-68	175	TGTATGATCTCG	176	CACTGTGCTCAT	F8	68
PI-69	177	CAGTACACTCTA	178	ACTGCATGATCG	F9	69
PI-70	179	CATACTATCACG	180	TCGTGTCACTAC	F10	70
PI-71	181	CACTATACAGAT	182	CGACACGTACTA	F11	71
PI-72	183	ATATCGTAGCAT	184	TCGTGATCACTA	F12	72
PI-73	185	TAGTCTATACAT	186	AGACGCTGTCGA	G1	73
PI-74	187	TGTCACAGTGAC	188	TCATATGATCGA	G2	74
PI-75	189	ATCGACTATGCT	190	CGATCATATGAG	G3	75
PI-76	191	ATACTAGCATCA	192	TCATGCTGACGA	G4	76
PI-77	193	CACTGACGCTCA	194	CACTACATCGCT	G5	77
PI-78	195	TCGCTCATCTAT	196	TAGTACAGAGCT	G6	78
PI-79	197	TGTATCACGAGC	198	ATGATCGTATAC	G7	79
PI-80	199	TACTGCTATCTC	200	CGCTGCATAGCG	G8	80
PI-81	201	CGCGAGCTCGTC	202	ACTCGATGAGCT	G9	81
PI-82	203	TAGAGTCTGTAT	204	TGTCTATCACAT	G10	82

PI-83	205	TACTATCGCTCT	206	TATGTGACATAC	G11	83
PI-84	207	TAGATGACGCTC	208	TACTCGTAGCGC	G12	84
PI-85	209	TCGCGTGACATC	210	ATCTACTGACGT	H1	85
PI-86	211	ACACGCTCTACT	212	ACAGTAGCGCAC	H2	86
PI-87	213	TACATAGTCTCG	214	CTAGTATCATGA	H3	87
PI-88	215	TGAGTAGCACGC	216	TCGATCATGCAG	H4	88
PI-89	217	TAGATGCTATAC	218	TACATGCACTCA	H5	89
PI-90	219	ATCGATGTCACG	220	CAGCTCGACTAC	H6	90
PI-91	221	ATCATATGTAGC	222	CTCTACAGTCAC	H7	91
PI-92	223	TAGCATCGATAT	224	AGATAGCACATC	H8	92
PI-93	225	TGATCGACGCTC	226	CTAGATATCGTC	H9	93
PI-94	227	TGCAGCTCATAG	228	TACAGACTGCAC	H10	94
PI-95	229	CGACGTAGAGTC	230	CAGTAGCACTAC	H11	95

Table 7: PCR primers for amplification of the corresponding exons of DRB1 and without primer indexes

SEQ ID NO:	primer No.	primer sequence	use of primer	length of products
231	D2-F1	CACGTTTCTTGGAGTACTCTA	For amplification of Exon 2 of HLA-DRB1 gene	300bp
232	D2-F2	GTTTCTTGTGGCAgCTTAAgTT		
233	D2-F3	CCTGTGGCAGGGTAAGTATA		
234	D2-F4	GTTTCTTGAAGCAGGATAAGTT		
235	D2-F5	GCACGTTTCTTGGAGGAGG		
236	D2-F6	TTTCCTGTGGCAGCCTAAGA		
237	D2-F7	GTTTCTTGGAGCAGGTTAAAC		
238	D2-R	CCTCACCTCGCCGCTGCAC		

D2-F1, D2-F2, D2-F3, D2-F4, D2-F5, D2-F6, D2-F7 were forward primers for amplification of Exon 2 of HLA-DRB1, D2-R was a reverse primer for amplification of Exon 2 of HLA-DRB1.

PCR procedure for HLA-A/B/DRB1 was as followed:

96°C 2min

95°C 30s →60°C 30s →72°C 20s (32cycles)

15°C ∞

PCR reaction system for HLA-A/B was as followed, wherein all the agents were purchased from Promega (Beijing) Bio-Tech Co.

Promega 5×buffer I (Mg2+ plus)	5.0ul
dNTP Mixture (2.5mM/ μl each)	2.0ul
PI_{nf}-A/B-$F_{2/3/4}$ (2pmol/ ul)	1.0ul
PI_{nr}-A/B-$R_{2/3/4}$ (2pmol/ ul)	1.0ul
Promega Taq (5U/ul)	0.2ul
DNA (about 20 ng/ul)	5.0ul

ddH_2O	10.8ul
Total	25.0ul

The PCR reaction system for HLA-DRB1 was as followed :

Promega 5×buffer I (Mg2+ plus)	5.0ul
dNTP Mixture (2.5mM/ μl each)	2.0ul
PI_{nf}-D2-F1 (2pmol/ ul)	1.0ul
PI_{nf}-D2-F2 (2pmol/ ul)	1.0ul
PI_{nf}-D2-F3 (2pmol/ ul)	1.0ul
PI_{nf}-D2-F4 (2pmol/ ul)	1.0ul
PI_{nf}-D2-F5 (2pmol/ ul)	1.0ul
PI_{nf}-D2-F6 (2pmol/ ul)	1.0ul
PI_{nf}-D2-F7 (2pmol/ ul)	1.0ul
PI_{nr}-D2-R (2pmol/ ul)	1.0ul
Promega Taq (5U/ul)	0.2ul
DNA (about 20 ng/ul)	5.0ul
ddH_2O	4.8ul
Total	25.0ul

Wherein PI_{nf}-A/B/D2-$F_{1/2/3/4/5/6/7}$ represents the F primer of HLA-A/B/DRB1 having the forward primer index sequence No. n (Table 6) at 5' end, PI_{nr}-A/B/D2-$R_{2/3/4}$ represents the R primer of HLA-A/B/DRB1 having the reverse primer index sequence No. n at 5' end (here $n \leq 95$), and the rest may be deduced similarly. Moreover, each sample corresponds to a specific set of PCR primers (PI_{nf}-A/B/D2-$F_{1/2/3/4/5/6/7}$, PI_{nr}-A/B/D2-$R_{2/3/4}$).

PCR reaction was carried out in PTC-200 PCR apparatus from Bio-Rad Co.. After PCR reaction, 2ul PCR products were subjected to 1% agarose gel electrophoresis. Figure 2 showed the electrophoretic result of the PCR products of the corresponding exons of HLA-A/B/DRB1 of Sample No. 1, and the marker for DNA molecular weights was DL 2000 (Takara Co.). There were a series of single bands of a length ranging from 300 bp to 500 bp in the electrophorogram, indicating successful PCR amplification of the exons (A2, A3, A4, B2, B3, B4, DRB1-2) of HLA-A/B/DRB1 of Sample No.1. There

was no amplification band in the negative control (N). The results of other samples were similar.

Example 3

Pooling and purification of PCR products

20 μl of the rest PCR products was taken from each well of the 96-well plate HLA-P-A2 (except for the negative control), and was mixed homogeneously under shaking in a 3 ml EP tube (designated as HLA-A2-Mix). The same operation was applied to the other 6 96-well plates, designated as HLA-A3-Mix, HLA-A4-Mix, HLA-B2-Mix, HLA-B3-Mix, HLA-B4-Mix and HLA-D2-Mix. 200ul was taken from each of HLA-A2-Mix, HLA-A3-Mix, HLA-A4-Mix, HLA-B2-Mix, HLA-B3-Mix, HLA-B4-Mix and HLA-D2-Mix, and was mixed in a 3 ml EP tube, designated as HLA-Mix. 500ul DNA mixture from HLA-Mix was subjected to column purification with Qiagen DNA Purification kit (QIAGEN Co.) (For the specific purification steps, please refer to the manufacturer's instruction). It was determined by Nanodrop 8000 (Thermo Fisher Scientific Co.) that the 200ul DNA obtained by purification has a HLA-Mix DNA concentration of 48 ng/ul.

Example 4

Shearing of PCR products, and construction of Illumina GA PCR-Free sequencing libraries

1. DNA shearing

A total amount of 5ug DNA, taken from the purified HLA-Mix, was contained in a Covaris microtube with an AFA fiber and Snap-Cap and was subjected to the shearing in Covaris S2DNA Shearer (Covaris Co.). The shearing conditions were as followed:

Frequency sweeping

Duty Cycle	10%
Intensity	5
Cycles/Burst	200
Time (second)	300

2. Purification after shearing

All the sheared products of HLA-Mix were recovered and purified by QIAquick PCR Purification Kit, and were dissolved in 37.5ul EB (QIAGEN Elution Buffer), respectively.

3. Terminal repairing reaction

The purified HLA-Mix after the shearing was subject to DNA terminal repairing reaction, and the reaction system was as followed (all the agents were purchased from Enzymatics Co.):

DNA	37.5μL
H_2O	37.5μL
10x Polynucleotide Kinase Buffer (B904)	10 μL
dNTP mixture (Solution Set (10mM each))	4 μL
T4 DNA Polymerase	5μL
Klenow Fragment	1μL
T4 Polynucleotide Kinase	5μL
Total volume	100 μL

Reaction conditions: incubating at 20°C for 30 min in a Thermomixer (Thermomixer, Eppendorf Co.).

The reaction products were recovered and purified by the QIAquick PCR Purification Kit, and were dissolved in 34 μl EB (QIAGEN Elution Buffer).

4. Addition of A at 3' end

A was added to 3' end of the DNA recovered in the last step, and the reaction system was as followed (all the agents were purchased from Enzymatics Co.):

DNA obtained in the last step	32 μL
10x blue buffer	5 μL
dATP (1mM, GE Co.)	10 μL
Klenow (3'-5' exo-)	3 μL
Total volume	50 μL

Reaction conditions: incubating at 37°C for 30 min in a Thermomixer (Thermomixer, Eppendorf Co.).

The reaction products were recovered and purified by MiniElute PCR Purification Kit (QIAGEN Co.), and were dissolved in 13 μl EB (QIAGEN Elution Buffer).

5. Ligation of Illumina GA PCR-Free library adapter

The term "PCR-Free library adapter" refers to a segment of designed bases, whose main role lies in auxiliary fixation of DNA molecule onto the sequencing chip and lies in providing the binding sites for universal sequencing primers, wherein PCR-Free library adapter may be directly ligated to the two termini of the DNA fragments in the sequencing library; since no PCR was involved in the introduction of the library adapter, the library adapter was called PCR-Free library adapter.

The products having A added were ligated to the Illumina GA PCR-Free library adapters, and the reaction system was as followed (all the agents were purchased from Illumina Co.):

DNA obtained in the last step	11μL
2x Rapid ligation buffer	15μL
PCR-free adapter oligo mix (30mM)	1μL
T4 DNA Ligase (Rapid, L603-HC-L)	3μL
Total volume	30 μL

Reaction conditions: incubating at 20°C for 15 min in a Thermomixer (Thermomixer, Eppendorf Co.).

The reaction products were purified by Ampure Beads (Beckman Coulter Genomics), and were dissolved in 50ul deionized water, and the DNA concentration determined by Fluorescence quantitative PCR (QPCR) was as followed:

	result determined by qPCR (nM)
HLA-Mix	78.90

6. Recovery by gel slicing

30μL HLA-Mix was subjected to 2% low melting point agarose gel electrophoresis. The electrophoretic condition was 100V, 100min. DNA marker was the 50bp DNA marker from NEB Co.. The gel containing the DNA fragments ranging from 450 to 750bp was sliced (Figure 3). The products in the sliced gel were recovered and purified by QIAquick PCR Purification Kit (QIAGEN Co.), the volume after purification was 32ul, and the DNA concentration measured by Fluorescence quantitative PCR (QPCR) was 10.16nM.

Example 5

Illumina GA sequencing

According to the results of QPCR, 10pmol DNA was taken and subjected to the sequencing by Illumina GA PE-100 program. For the specific operation procedure, please refer to the Illumina GA operation instruction (Illumina GA IIx).

Example 6

Analysis of the results

The sequencing results from Illumina GA were a series of DNA sequences, and by searching the forward and reverse primer index sequences and primer sequences in the sequencing results, databases comprising the sequencing results of the PCR products of various exons of HLA-A/B/DRB1 for each sample corresponding to respective primer index were constructed. The sequencing results of each exon were aligned to the reference sequence (reference sequences were from http://www.ebi.ac.uk/imgt/hla/) of the corresponding exon by BWA (Burrows-Wheeler Aligner), and meanwhile, the consensus sequences of each database were constructed, and the DNA sequences in the database were selected and corrected. The corrected DNA sequences were assembled into

the corresponding sequences of exons of HLA-A/B/DRB1 on the basis of sequence overlapping and linkage (Paired-End linkage) relationship. The resultant DNA sequence was aligned with the sequence database of the corresponding exon of HLA-A/B/DRB1 in IMGT HLA professional database. If the result of sequence alignment shows 100% match, the HLA-A/B/DRB1 genotype of the corresponding sample was determined. Please refer to the screen-capture of the program for construction of consensus sequence of Exon 2 of HLA-A site in Sample No.1 as illustrated in Figure 4.

For all 95 samples, the typing results obtained by the above method were completely consistent with the known typing results, wherein the results of Samples No. 1-32 were as followed:

Sample No.	Original known HLA-A/B/DRB1 type					
1	A*02:03	A*11:01	B*38:02	B*48:01	DRB1*14:54	DRB1*15:01
2	A*01:01	A*30:01	B*08:01	B*13:02	DRB1*03:01	DRB1*07:01
3	A*01:01	A*02:01	B*15:11	B*47:01	DRB1*13:02	DRB1*15:01
4	A*24:08	A*26:01	B*40:01	B*51:01	DRB1*04:04	DRB1*09:01
5	A*01:01	A*24:02	B*54:01	B*55:02	DRB1*04:05	DRB1*09:01
6	A*01:01	A*03:02	B*15:11	B*37:01	DRB1*10:01	DRB1*14:54
7	A*11:01	A*30:01	B*13:02	B*15:18	DRB1*04:04	DRB1*07:01
8	A*01:01	A*02:01	B*35:03	B*81:01	DRB1*11:01	DRB1*15:01
9	A*02:06	A*31:01	B*27:07	B*40:02	DRB1*03:01	DRB1*13:02
10	A*01:01	A*66:01	B*37:01	B*49:01	DRB1*10:01	DRB1*13:02
11	A*01:01	A*03:01	B*35:01	B*52:01	DRB1*01:01	DRB1*15:02
12	A*11:01	A*11:01	B*15:01	B*15:05	DRB1*04:06	DRB1*15:01
13	A*01:01	A*11:02	B*07:02	B*15:02	DRB1*09:01	DRB1*15:01
14	A*01:01	A*02:01	B*52:01	B*67:01	DRB1*15:02	DRB1*16:02
15	A*01:01	A*02:05	B*15:17	B*50:01	DRB1*07:01	DRB1*15:01
16	A*01:01	A*11:01	B*37:01	B*40:02	DRB1*10:01	DRB1*12:02
17	A*24:07	A*32:01	B*35:05	B*40:01	DRB1*03:01	DRB1*04:05
18	A*11:01	A*24:02	B*13:01	B*35:01	DRB1*16:02	DRB1*16:02

19	A*11:01	A*11:01	B*40:02	B*55:12	DRB1*04:05	DRB1*15:01
20	A*02:11	A*24:02	B*40:01	B*40:06	DRB1*11:01	DRB1*15:01
21	A*01:01	A*02:06	B*51:01	B*57:01	DRB1*07:01	DRB1*12:01
22	A*01:01	A*29:01	B*07:05	B*15:01	DRB1*04:05	DRB1*07:01
23	A*01:01	A*02:07	B*37:01	B*46:01	DRB1*04:03	DRB1*10:01
24	A*24:85	A*30:01	B*13:02	B*55:02	DRB1*07:01	DRB1*15:01
25	A*11:01	A*31:01	B*07:06	B*51:01	DRB1*12:02	DRB1*14:05
26	A*01:01	A*11:01	B*46:01	B*57:01	DRB1*07:01	DRB1*08:03
27	A*01:01	A*02:01	B*15:18	B*37:01	DRB1*04:01	DRB1*15:01
28	A*01:01	A*24:02	B*37:01	B*46:01	DRB1*09:01	DRB1*10:01
29	A*26:01	A*66:01	B*40:40	B*41:02	DRB1*12:01	DRB1*15:01
30	A*02:01	A*29:02	B*13:02	B*45:01	DRB1*03:01	DRB1*12:02
31	A*01:01	A*11:03	B*15:01	B*57:01	DRB1*07:01	DRB1*15:01
32	A*11:01	A*26:01	B*35:03	B*38:01	DRB1*11:03	DRB1*14:04

Sample No.	The determined HLA-A/B/DRB1 type					
1	A*02:03	A*11:01	B*38:02	B*48:01	DRB1*14:54	DRB1*15:01
2	A*01:01	A*30:01	B*08:01	B*13:02	DRB1*03:01	DRB1*07:01
3	A*01:01	A*02:01	B*15:11	B*47:01	DRB1*13:02	DRB1*15:01
4	A*24:08	A*26:01	B*40:01	B*51:01	DRB1*04:04	DRB1*09:01
5	A*01:01	A*24:02	B*54:01	B*55:02	DRB1*04:05	DRB1*09:01

6	A*01:01	A*03:02	B*15:11	B*37:01	DRB1*10:01	DRB1*14:54
7	A*11:01	A*30:01	B*13:02	B*15:18	DRB1*04:04	DRB1*07:01
8	A*01:01	A*02:01	B*35:03	B*81:01	DRB1*11:01	DRB1*15:01
9	A*02:06	A*31:01	B*27:07	B*40:02	DRB1*03:01	DRB1*13:02
10	A*01:01	A*66:01	B*37:01	B*49:01	DRB1*10:01	DRB1*13:02
11	A*01:01	A*03:01	B*35:01	B*52:01	DRB1*01:01	DRB1*15:02
12	A*11:01	A*11:01	B*15:01	B*15:05	DRB1*04:06	DRB1*15:01
13	A*01:01	A*11:02	B*07:02	B*15:02	DRB1*09:01	DRB1*15:01
14	A*01:01	A*02:01	B*52:01	B*67:01	DRB1*15:02	DRB1*16:02
15	A*01:01	A*02:05	B*15:17	B*50:01	DRB1*07:01	DRB1*15:01
16	A*01:01	A*11:01	B*37:01	B*40:02	DRB1*10:01	DRB1*12:02
17	A*24:07	A*32:01	B*35:05	B*40:01	DRB1*03:01	DRB1*04:05
18	A*11:01	A*24:02	B*13:01	B*35:01	DRB1*16:02	DRB1*16:02
19	A*11:01	A*11:01	B*40:02	B*55:12	DRB1*04:05	DRB1*15:01
20	A*02:11	A*24:02	B*40:01	B*40:06	DRB1*11:01	DRB1*15:01
21	A*01:01	A*02:06	B*51:01	B*57:01	DRB1*07:01	DRB1*12:01
22	A*01:01	A*29:01	B*07:05	B*15:01	DRB1*04:05	DRB1*07:01
23	A*01:01	A*02:07	B*37:01	B*46:01	DRB1*04:03	DRB1*10:01
24	A*24:85	A*30:01	B*13:02	B*55:02	DRB1*07:01	DRB1*15:01
25	A*11:01	A*31:01	B*07:06	B*51:01	DRB1*12:02	DRB1*14:05
26	A*01:01	A*11:01	B*46:01	B*57:01	DRB1*07:01	DRB1*08:03

27	A*01:01	A*02:01	B*15:18	B*37:01	DRB1*04:01	DRB1*15:01
28	A*01:01	A*24:02	B*37:01	B*46:01	DRB1*09:01	DRB1*10:01
29	A*26:01	A*66:01	B*40:40	B*41:02	DRB1*12:01	DRB1*15:01
30	A*02:01	A*29:02	B*13:02	B*45:01	DRB1*03:01	DRB1*12:02
31	A*01:01	A*11:03	B*15:01	B*57:01	DRB1*07:01	DRB1*15:01
32	A*11:01	A*26:01	B*35:03	B*38:01	DRB1*11:03	DRB1*14:04

Note: among HLA-DRB1 types, DRB1*1201 does not exclude the possibility of DRB1*1206/1210/1217, and DRB1*1454 does not exclude the possibility of DRB1*1401, because said alleles were completely identical in the sequence of Exon 2 of HLA-DRB1.

Example 7

HLA-A,B and DRB1 genotyping by using the second generation sequencing technique (Illumina GA)

Sample extraction

DNAs were extracted from 950 blood samples with known HLA-SBT typing results (China Marrow Donor Program cited hereafter as (CMDP)) by using KingFisher Automatic Extraction Instrument (US Thermo Co.). The method was as described in Example 1.

PCR amplification

The 950 DNAs obtained from the sample extraction step were designated as No. 1-950, and were divided into 10 groups (95 DNAs for each), which were designated as HLA-1, HLA-2, HLA-3, HLA-4, HLA-5, HLA-6, HLA-7, HLA-8, HLA-9, HLA-10. For each group of samples, 95 DNA samples were amplified by 95 sets of PCR primers (Table 1) carrying bidirectional primer indexes (Table 6) for amplification of Exons 2, 3, 4 of HLA-A/B and PCR primers (Table 7) carrying bidirectional primer indexes (Table 6) for amplification of Exon 2 of HLA-DRB1.

PCR reaction took place in 96-well plates, using 70 plates in total, designated as HLA-X-P-A2, HLA-X-P-A3, HLA-X-P-A4, HLA-X-P-B2, HLA-X-P-B3, HLA-X-P-B4 and HLA-X-P-DRB1-2 ("X" represents the information of the group number 1/2/3/4/5/6/7/8/9/10, "A2/3/4", "B2/3/4", "DRB1-2" represent the amplification sites), wherein a negative control without adding any template was set in each plate, and the primers used for the negative control were primers labeled by PI-1 (Table 6). During experimentation, the information of each sample on the group number and primer indexes was recorded. The method was as described in Example 2.

Pooling and purification of PCR products

For samples of Group X ("X" is 1/2/3/4/5/6/7/8/9/10), 20 μl of rest PCR products was taken from each well of the 96-well plate HLA-X-P-A2 (except for the negative control), and was mixed homogeneously under shaking in a 3 ml EP tube (designated as HLA-X-A2-Mix). The same operation was applied to the other 6 96-well plates of the samples of Group X, designated as HLA-X-A3-Mix, HLA-X-A4-Mix, HLA-X-B2-Mix, HLA-X-B3-Mix, HLA-X-B4-Mix and HLA-X-D2-Mix. 200ul was taken from each of HLA-X-A2-Mix, HLA-X-A3-Mix, HLA-X-A4-Mix, HLA-X-B2-Mix, HLA-X-B3-Mix, HLA-X-B4-Mix and HLA-X-D2-Mix, and was mixed in a 3ml EP tube, designated as HLA-X-Mix. 500ul DNA mixture from HLA-X-Mix was subjected to column purification with Qiagen DNA Purification kit (QIAGEN Co.) (For the specific purification steps, please refer to the manufacturer's instruction) to obtain 200ul DNA, and its DNA concentration was determined by Nanodrop 8000 (Thermo Fisher Scientific Co.). The same operation was also applied to other groups. The finally determined DNA concentrations were as followed.

	HLA-1-Mix	HLA-2-Mix	HLA-3-Mix	HLA-4-Mix	HLA-5-Mix	HLA-6-Mix	HLA-7-Mix	HLA-8-Mix	HLA-9-Mix	HLA-10-Mix
concentration (ng/ul)	48.0	52.1	49.3	50.2	47.6	48.5	49.1	48.6	51.3	50.8

The method was as described in Example 3.

The construction of Illumina GA Sequencing libraries was performed by the

method of Example 4. The corresponding relationships between the sample groups and the library adapters were as followed.

Sample group No.	HLA-1	HLA-2	HLA-3	HLA-4	HLA-5	HLA-6	HLA-7	HLA-8	HLA-9	HLA-10
Library adapter No.	1	2	3	4	5	6	7	8	9	10

The reaction products were purified by Ampure Beads (Beckman Coulter Genomics), and were dissolved in 50ul deionized water, and the DNA molar concentrations determined by Fluorescence quantitative PCR (QPCR) were as followed:

	HLA-1-Mix	HLA-2-Mix	HLA-3-Mix	HLA-4-Mix	HLA-5-Mix	HLA-6-Mix	HLA-7-Mix	HLA-8-Mix	HLA-9-Mix	HLA-10-Mix
Conc. (nM)	78.90	72.13	79.33	80.21	77.68	78.50	89.12	78.60	81.32	80.82

Recovery by gel slicing

HLA-1-Mix, HLA-2-Mix, HLA-3-Mix, HLA-4-Mix, HLA-5-Mix, HLA-6-Mix, HLA-7-Mix, HLA-8-Mix, HLA-9-Mix and HLA-10-Mix were mixed at an equal mole (final concentration was 72.13 nM/ul), designated as HLA-Mix-10. 30μL HLA-Mix-10 was subjected to 2% low melting point agarose gel electrophoresis. The electrophoretic condition was 100V, 100min. DNA marker was the 50bp DNA marker from NEB Co.. The gel containing the DNA fragments ranging from 450 to 750bp was sliced. The products in the sliced gel were recovered and purified by QIAquick PCR Purification Kit (QIAGEN Co.), the volume after purification was 32ul, and the DNA concentration measured by Fluorescence quantitative PCR (QPCR) was 9.96nM.

The sequencing and result analysis were performed as described in Examples 5 and 6. For all 950 samples, the typing results obtained by the above method were completely consistent with the known typing results.

Example 8

HLA-C genotyping by using the second generation sequencing technique (Illumina GA)

1 . DNA sample extraction

The steps were as described in Example 1.

2 . PCR amplification

The steps were as described in Example 2, except that the PCR primers used were PCR primers for Exons 2, 3 and 4 of HLA-C, as shown in Table 3.

95 sets of PCR index primers were used to amplify 95 DNA samples, respectively, wherein each set of PCR index primers consisted of PCR primers for amplification of Exons 2, 3, 4 of HLA-C (Table 3) and a pair of bidirectional primer indexes (as described below), each forward PCR primer has the forward primer index of a pair of primer indexes linked at the 5' end, and the reverse PCR primer has the reverse primer index of a pair of primer indexes linked at the 5' end. During the synthesis of primers, the primer indexes were directly added to the 5' end of the PCR primers.

The 95 DNAs obtained from the sample extraction step were designated as No. 1-95. PCR reaction took place in 96-well plates, 3 plates in total, designated as HLA-P-C2, HLA-P-C3, HLA-P-A4 (C2/3/4 represent the amplification sites), wherein a negative control without adding any template was set in each plate, and the primers used in the negative control were the same as the primer PI-96. During experimentation, the numbering information of the sample corresponding to each pair of primer indexes was recorded.

The primer indexes used were the primer indexes PI-1 to PI-95 as listed in Table 6, and the following negative control pirmer index PI-96 (Table 8)

Table 8: Relevant information of the primer index used for the negative control

PI-96	CACTGTATAGCT	CGACTAGTACTA	H12	Negative control

The DNAs, extracted by using KingFisher Automatic Extraction Instrument in step 1, were used as the templates, and PCR amplification was carried out in single

tubes by using primers for exons of HLA-C, wherein the primers have indexes at 5' end. PCR procedure was as followed:

C2: 96°C 2 min

95°C 30s →62°C 30s →72°C 20s (35 cycles)

15°C ∞

C3: 96°C 2 min

95°C 30s →56°C 30s →72°C 20s (35 cycles)

15°C ∞

C4: 96°C 2 min

95°C 30s →60°C 30s →72°C 20s (35 cycles)

15°C ∞

PCR reaction system of HLA-C was as followed:

Promega 5×buffer I (Mg2+ plus)	5.0 μL
dNTP mixture (2.5 mM each)	2.0 μL
PInr-C-F2/3/4 (50 ng/ul)	1.5 μL
PInf-C-R2/3/4 (50 ng/ul)	1.5 μL
Promega Taq (5 U/ul)	0.2 μL
DNA (about 20 ng/ul)	2.0 μL
ddH_2O	12.8 μL
Total	25.0 μL

Wherein, PI_{nf}-C-$F_{2/3/4}$ represents the F primer of HLA-C having the forward primer index sequence No. n (Table 2) at 5' end, PI_{nr}-C-$R_{2/3/4}$ represents the R primer of HLA-C having the reverse primer index sequence No. n at 5' end (here $n \leq 96$), and the rest may be deduced similarly. Moreover, each sample corresponds to a specific set of PCR primers.

PCR reaction was carried out in PTC-200 PCR apparatus from Bio-Rad Co.. After PCR reaction, 2ul PCR products were subjected to 1.5% agarose gel electrophoresis. Figure 6 showed the electrophoretic result of the PCR products of the corresponding exons of HLA-C of the first 20 samples, and the DNA molecular marker was DL 2000 (Takara Co.). There were a series of single bands of a length ranging from 400 bp to 500

bp in the electrophorogram, indicating successful PCR amplification of exons (C2, C3, C4) of HLA-C of the samples. The results of other samples were similar.

Pooling and purification of PCR products

20 μl of the rest PCR products was taken from each well of the 96-well plate HLA-P-C2 (except for the negative control), and was pooled homogeneously under shaking in a 3 ml EP tube (designated as HLA-C2-Mix). The same operation was applied to the other 2 96-well plates, designated as HLA-C3-Mix and HLA-C4-Mix. 200ul was taken from each of HLA-C2-Mix, HLA-C3-Mix and HLA-C4-Mix, and was mixed in a 1.5 ml EP tube, designated as HLA-Mix. 500ul DNA mixture from HLA-Mix was subjected to column purification with Qiagen DNA Purification kit (QIAGEN Co.) (For the specific purification steps, please refer to the manufacturer's instruction). It was determined by Nanodrop 8000 (Thermo Fisher Scientific Co.) that the 200ul DNA obtained by purification has a HLA-Mix DNA concentration of 50ng/ul.

4 . Construction of Illumina GA PCR-Free sequencing libraries

4.1 Shearing of PCR products

A total amount of 5ug DNA, taken from the purified HLA-Mix, was contained in a Covaris microtube with AFA fiber and Snap-Cap and was subjected to the shearing in Covaris S2 (Covaris Co.). The shearing conditions were as followed:

Frequency sweeping

Duty Cycle	10%
Intensity	3
Cycles/Burst	200
Time (s)	180

4.2 Purification of the sheared PCR products

All the sheared products of HLA-Mix were recovered and purified by QIAquick PCR Purification Kit, and were dissolved in 37.5ul EB (QIAGEN Elution Buffer), respectively.

4.3 Terminal repairing reaction

The purified products were subject to DNA terminal repairing reaction, the reaction system was as followed (all the agents were purchased from Enzymatics Co.):

Products purified in the last step	37.5 μL
10x Polynucleotide Kinase Buffer (B904)	5 μL
dNTP mixture (Solution Set (10mM each))	2 μL
T4 DNA Polymerase	2.5 μL
Klenow Fragment	0.5 μL
T4 Polynucleotide Kinase	2.5 μL
Total volume	50 μL

Reaction conditions: incubating at 20°C for 30 min in a Thermomixer (Thermomixer, Eppendorf Co.).

The reaction products were recovered and purified by the QIAquick PCR Purification Kit, and were dissolved in 32 μl EB (QIAGEN Elution Buffer).

4.4 Addition of A at 3' end

A was added to 3' end of the DNA recovered in the last step, and the reaction system was as followed (all the agents were purchased from Enzymatics Co.):

DNA obtained in the last step	32 μL
10x blue buffer	5 μL
dATP (1mM, GE Co.)	10 μL
Klenow (3'-5' exo-)	3 μL
Total volume	50 μL

Reaction conditions: incubating at 37°C for 30 min in a Thermomixer (Thermomixer, Eppendorf Co.).

The reaction products were recovered and purified by MiniElute PCR Purification Kit (QIAGEN Co.), and were dissolved in 38 μl EB (QIAGEN Elution Buffer).

4.5 Ligation of Illumina GA PCR-Free library adapter

The products having A added were ligated to the Illumina GA PCR-Free library adapters, and the reaction system was as followed (all the agents were purchased from Illumina Co.):

DNA obtained in the last step	38 μL
10x Ligation buffer	5 μL
PCR-free adapter oligo mix (30mM)	2 μL
T4 DNA Ligase (Rapid, L603-HC-L)	5 μL
Total volume	50 μL

Reaction conditions: incubating at 16°C overnight in a Thermomixer (Thermomixer, Eppendorf Co.).

The reaction products were purified by Ampure Beads (Beckman Coulter Genomics), and were dissolved in 50ul deionized water, and the DNA concentration determined by Fluorescence quantitative PCR (QPCR) was as followed:

	result determined by qPCR (nM)
HLA-Mix	122.71

4.6 Recovery by gel slicing

30μL HLA-Mix was subjected to 2% low melting point agarose gel electrophoresis. The electrophoretic condition was 100V, 100min. DNA marker was the 50bp DNA Ladder from NEB Co.. The gel containing the DNA fragments ranging from 400 to 750bp was sliced (Figure 7). The products in the sliced gel were recovered and purified by QIAquick PCR Purification Kit (QIAGEN Co.), the volume after purification was 32ul, and the DNA concentration measured by Fluorescence quantitative PCR (QPCR) was 17.16nM.

5. Illumina GA sequencing

According to the detection results of QPCR, 10pmol DNA was taken and subjected to the sequencing by Illumina GA PE-100 program. For the specific operation procedure, please refer to the Illumina GA operation instruction (Illumina GA IIx).

6. Analysis of the results

The sequencing results from Illumina GA were a series of DNA sequences, and by searching the forward and reverse primer index sequences and primer sequences in the

sequencing results, databases comprising the sequencing results of the PCR products of various exons of HLA-C for each sample corresponding to respective primer index were constructed were constructed. The sequencing results of each exon was aligned to the reference sequence (reference sequences were from http://www.ebi.ac.uk/imgt/hla/) of the corresponding exon by BWA (Burrows-Wheeler Aligner), and consensus sequences of each database were constructed; and the sequence reads were selected and corrected based on the quality value of base sequencing, and difference between the sequence reads and consensus sequences. The corrected DNA sequences were assembled into the corresponding sequences of exons of HLA-C on the basis of sequence overlapping and linkage (Paired-End linkage) relationship. The screen-capture of Figure 8 illustrates the procedure for construction of consensus sequence of Exon 2 of HLA-C site in Sample No.2.

The resultant DNA sequence was aligned with the sequence database of the corresponding exon of HLA-C in IMGT HLA professional database. If the result of sequence alignment showed 100% match, the HLA-C genotype of the corresponding sample was determined. For all 95 samples, the typing results obtained by the above method were completely consistent with the known typing results, wherein the typing results of Samples No. 1-32 were as followed: (as shown in Table 9, all the obtained results were identical to the original known results).

Table 9: Comparison of the typing results obtained by the above method with the original known typing results of the samples

Sample No.	Original known HLA-C genotype		Results for HLA-C obtained at this time		Identical or not
1	C*08:01	C*15:05	C*08:01	C*15:05	yes
2	C*01:02	C*07:02	C*01:02	C*07:02	yes
3	C*08:01	C*16:02	C*08:01	C*16:02	yes
4	C*01:02	C*03:02	C*01:02	C*03:02	yes
5	C*01:02	C*02:02	C*01:02	C*02:02	yes
6	C*01:02	C*15:02	C*01:02	C*15:02	yes
7	C*01:02	C*03:04	C*01:02	C*03:04	yes
8	C*03:02	C*07:02	C*03:02	C*07:02	yes
9	C*06:02	C*16:02	C*06:02	C*16:02	yes
10	C*01:02	C*03:04	C*01:02	C*03:04	yes
11	C*03:04	C*07:02	C*03:04	C*07:02	yes
12	C*07:02	C*08:01	C*07:02	C*08:01	yes
13	C*01:02	C*15:02	C*01:02	C*15:02	yes
14	C*01:02	C*03:04	C*01:02	C*03:04	yes
15	C*01:02	C*03:04	C*01:02	C*03:04	yes
16	C*07:02	C*12:02	C*07:02	C*12:02	yes
17	C*04:01	C*08:01	C*04:01	C*08:01	yes
18	C*08:01	C*16:02	C*08:01	C*16:02	yes
19	C*14:02	C*15:02	C*14:02	C*15:02	yes
20	C*01:02	C*03:03	C*01:02	C*03:03	yes
21	C*03:03	C*08:01	C*03:03	C*08:01	yes
22	C*03:04	C*07:02	C*03:04	C*07:02	yes
23	C*07:02	C*08:01	C*07:02	C*08:01	yes
24	C*07:02	C*12:02	C*07:02	C*12:02	yes
25	C*07:02	C*12:03	C*07:02	C*12:03	yes
26	C*03:04	C*08:01	C*03:04	C*08:01	yes
27	C*01:02	C*03:04	C*01:02	C*03:04	yes

28	C*07:02	C*12:02	C*07:02	C*12:02	yes
29	C*03:02	C*07:02	C*03:02	C*07:02	yes
30	C*01:02	C*03:03	C*01:02	C*03:03	yes
31	C*01:02	C*07:02	C*01:02	C*07:02	yes
32	C*01:02	C*07:02	C*01:02	C*07:02	yes

Note: among HLA-C types, C*0303 does not exclude the possibility of C*0320N, C*0401 does not exclude the possibility of C*0409N/0430, C*0702 does not exclude the possibility of C*0750, C*0801 does not exclude the possibility of C*0822, C*1505 does not exclude the possibility of C*1529, because said alleles were completely identical in the sequences of Exons 2, 3, 4 of HLA-C.

Example 9: HLA-C genotyping by using Sanger sequencing method

1. Sample DNA extraction

As described in Example 1, DNAs were extracted by using KingFisher Automatic Extraction Instrument from 26 out of 95 samples with known HLA genotypes.

2. PCR amplification

The above DNAs, extracted by using KingFisher Automatic Extraction Instrument, were used as templates, and PCR amplification was carried out in single tubes by using three pairs of PCR primers C-F2/C-R2, C-F3/C-R3, C-F4/C-R4 (Table 3), respectively. The PCR procedure for each pair of primers was as followed:

C2: 96°C 2 min

95°C 30s →62°C 30s →72°C 20s (35 cycles)

15°C ∞

C3: 96°C 2min

95°C 30s →56°C 30s →72°C 20s (35 cycles)

15°C ∞

C4: 96°C 2min

95°C 30s →60°C 30s →72°C 20s (35 cycles)

15°C ∞

PCR reaction system of HLA-C was as followed:

Promega 5×buffer I (Mg2+ plus)	5.0 μL
dNTP Mixture (2.5 mM each)	2.0 μL
Primer mix (50 ng/μL)	3.0 μL
Promega Taq (5 U/μL)	0.2 μL
DNA (about 20 ng/μL)	2.0 μL
ddH_2O	12.8 μL
total	25.0 μL

PCR products were subjected to agarose gel electrophoresis (Figure 9) before purification.

3 . Purification of PCR products

PCR products were purified by using Millipore purification plates. The main steps were as followed. The wells to be used were marked with a marker pen in the 96-well purification plate for PCR products, and 50 μl ultrapure water was added to each of the wells to be used. The rest wells were sealed by sealing film. The plate was stood for 15 min or was connected to a drawing and filtering system (-10 pa) for 5 min. When the purification plate was taken from the drawing and filtering system, liquid in the discharge port at the bottom of the purification plate was sipped up with absorbent paper.

PCR products to be purified were centrifugated at 4000 rpm for 1 min; the cover or silica gel pad for the PCR products to be purified was removed, and 100 μl ultrapure water was added to each PCR reaction system. Then, the purification plate, to which the PCR products to be purified were added, was connected to the drawing and filtering system, and the vacuum degree was adjusted to -10 pa as shown in barometer. The drawing and filtering were continued until no liquid was left on the microporous regeneratable cellulose film at the bottom of the purification plate, and no reflection gloss of intact liquid surface was found when observing under light.

In the wells containing PCR products to be purified, 50 μl ultrapure water or TE was added to the microporous regeneratable cellulose film; the purification plate was vibrated at room temperature in a trace vibrator for 5 min; and the whole liquids

contained in the corresponding wells were transferred to the corresponding wells of a new 96-well PCR plate.

4. Performance of sequencing reaction and purification of products of the sequencing reaction

The above purified PCR products were used as templates for sequencing reaction.

Conditions for sequencing reaction

96°C 2 min

96°C 10s → 55°C 5s → 60°C 2min (25 cycles)

15°C ∞

The system for sequencing reaction was

Purified PCR products	1 μL
primers (3.2 pmol/l)	1 μL
2.5 *Bigdye	0.3 μL
5*Buffer	0.85 μL
water	1.85 μL
Total volume	5 μL

The products of the sequencing reaction were purified by the following steps: the sequencing reaction plate was balanced, and centrifugated at 3000 g for 1 min. In the 96-well plate, to each 5 μL reaction system, 2μL 0.125 mol/L EDTA-Na2 solution, 33 μL 85% ethanol were added, and the plate was covered by a silica gel pad and was sufficiently vibrated for 3 min. The plate was then centrifugated at 4□, 3000 g for 30 min. The sequencing plate was taken out after centrifugation, the silica gel pad was removed, and the sequencing plate was placed downwardly onto absorbent paper, and was then subjected to inverted centrifugation until the centrifugal force reached 185g. To each well of the 96-well plate, 50 μl 70% ethanol was added. The plate was covered with a silica gel pad, and vibrated for 1.5 min, and centrifugated at 4□, 3000 g for 15 min. The sequencing reaction plate was then placed in a dark and ventilative place for 30 min so as to be air-dried until no ethanol odor was felt. To each well of the 96-well plate, 10 μL HI-DI formamide was added (alternatively,

to each well of a 384-well plate, 8 μL was added), and then the plate was covered by sealing film, and was centrifugated to 1000 rpm after vibrating for 5s.

5. Sequencing and result analysis

Purified products of the sequencing reaction were subjected to capillary electrophoresis sequencing in ABI 3730XL. The sequencing peaks were analyzed by uType software (Invitrogen) to obtain HLA typing results (Figure 10). All the results obtained by the above method were identical to the original known results, as shown in Table 10.

Table 10: Comparison of the typing results obtained by the above method with the original known typing results

Sample No.	Original known HLA-C genotype		Results for HLA-C obtained at this time		Identical or not
1	C*08:01	C*15:05	C*08:01	C*15:05	yes
2	C*01:02	C*07:02	C*01:02	C*07:02	yes
3	C*08:01	C*16:02	C*08:01	C*16:02	yes
4	C*01:02	C*03:02	C*01:02	C*03:02	yes
5	C*01:02	C*02:02	C*01:02	C*02:02	yes
6	C*01:02	C*15:02	C*01:02	C*15:02	yes
7	C*01:02	C*03:04	C*01:02	C*03:04	yes
8	C*03:02	C*07:02	C*03:02	C*07:02	yes
9	C*06:02	C*16:02	C*06:02	C*16:02	yes
10	C*01:02	C*03:04	C*01:02	C*03:04	yes
11	C*03:04	C*07:02	C*03:04	C*07:02	yes
12	C*07:02	C*08:01	C*07:02	C*08:01	yes
13	C*01:02	C*15:02	C*01:02	C*15:02	yes
14	C*01:02	C*03:04	C*01:02	C*03:04	yes
15	C*01:02	C*03:04	C*01:02	C*03:04	yes
16	C*07:02	C*12:02	C*07:02	C*12:02	yes
17	C*04:01	C*08:01	C*04:01	C*08:01	yes
18	C*08:01	C*16:02	C*08:01	C*16:02	yes
19	C*14:02	C*15:02	C*14:02	C*15:02	yes

20	C*01:02	C*03:03	C*01:02	C*03:03	yes
21	C*03:03	C*08:01	C*03:03	C*08:01	yes
22	C*03:04	C*07:02	C*03:04	C*07:02	yes
23	C*07:02	C*08:01	C*07:02	C*08:01	yes
24	C*07:02	C*12:02	C*07:02	C*12:02	yes
25	C*07:02	C*12:03	C*07:02	C*12:03	yes
26	C*01:02	C*07:02	C*01:02	C*07:02	yes

Example 10: HLA-DQB1 genotyping by using the second generation sequencing technique (Illumina Solexa)

94 blood samples with known HLA-SBT typing results were subjected to HLA-DQB1 genotyping, according to the methods as described in Example 8, except for the following items.

94 sets of PCR index primers were used to amplify 94 DNA samples, respectively, wherein each set of PCR index primers consisted of PCR primers for amplification of Exon 2 or 3 of HLA-DQB1 (Table 5) and a pair of bidirectional primer indexes (as described above), each forward PCR primer has the forward primer index of a pair of primer indexes linked at the 5' end, and the reverse PCR primer has the reverse primer index of a pair of primer indexes linked at the 5' end. During the synthesis of primers, the primer indexes were directly added to the 5' end of the PCR primers, wherein the primers were synthesized by Shanghai Invitrogen Co.

The 94 DNAs obtained in the sample extraction step, were designated as No. 1-94. PCR reaction was carried out in 96-well plates, Exons 2, 3 of DQB1 in each sample was amplified in the same well. Two negative controls without adding any template were set in each plate, and the primer indexes used in negative controls are PI-95 and PI-96. During experimentation, the numbering information of the sample corresponding to each pair of primer indexes was recorded.

The primer indexes used were the primer indexes PI-1 to PI-94 as listed in Table 6, and the following primer indexes PI-95 and PI-96 (Table 11) for negative controls.

Table 11: Relevant information on the primer indexes used for negative controls

PI-95	CGACGTAGAGTC	CAGTAGCACTAC	H11	Negative

				control
PI-96	CACTGTATAGCT	CGACTAGTACTA	H12	Negative control

PCR procedure for HLA-DQB1 was as followed:

96°C 2min

95°C 30s→60°C 30s→72°C 20s (32 cycles)

15°C ∞

PCR reaction system for HLA-DQB1 was as followed:

Promega 5×buffer I (Mg2+ plus)	5.0 ul
dNTP mixture (2.5 mM each)	2.0 ul
PInf-Q-F2 (2 pmol/ul)	1.0 ul
PInf-Q-R2 (2 pmol/ul)	1.0 ul
PInf-Q-F3 (2 pmol/ul)	1.0 ul
PInf-Q-R3 (2 pmol/ul)	1.0 ul
Promega Taq (5U/ul)	0.2 ul
DNA (about 20 ng/ul)	5.0 ul
ddH_2O	8.8 ul
Total	25.0 ul

Wherein, PInf-Q-F2/3 represents the F primer of HLA-DQB1 having the forward primer index sequence No. n (Table 1) at 5' end; PInf-Q-R 2/3 represents the R primer of HLA-DQB1 having the reverse primer index sequence No. n at 5' end (here n≤96); and the rest may be deduced similarly. Moreover, each sample corresponds to a specific set of PCR primers.

PCR reaction was carried out in PTC-200 PCR apparatus from Bio-Rad Co.. After PCR reaction, 2ul PCR products were subjected to 1.5% agarose gel electrophoresis. Figure 11 showed the electrophoretic result of the PCR products of Exons 2+3 of HLA-DQB1 of 94 samples, and the DNA molecular marker was DL 2000 (Takara Co.).

Pooling and purification of PCR products

20 μl of the rest PCR products was taken from each well of the 96-well plate HLA-P-DQB1 (except for the negative control), and was mixed homogeneously in a 3 ml EP tube (designated as HLA-Q-Mix). 500ul DNA mixture from HLA-Q-Mix was subjected to column purification with Qiagen DNA Purification kit (QIAGEN Co.) (For the specific purification steps, please refer to the manufacturer's instruction). It was determined by Nanodrop 8000 (Thermo Fisher Scientific Co.) that the 200ul DNA obtained by purification has a HLA-Q-Mix DNA concentration of 48ng/ul.

Conditions for shearing were as followed:

Frequency sweeping

Duty Cycle	10%
Intensity	5
Cycles/Burst	200
Time (s)	300

The reaction products were subjected to terminal repairing reaction, and then were recovered and purified by QIAquick PCR Purification Kit, and were dissolved in 34ul EB (QIAGEN Elution Buffer).

The reaction products were further subjected to the addition of A at 3' end, and then were recovered and purified by MiniElute PCR Purification Kit (QIAGEN Co.), and were dissolved in 13 μl EB solution (QIAGEN Elution Buffer).

After ligation of library adapters, the reaction products were purified by Ampure Beads (Beckman Coulter Genomics), and were dissolved in 50 μl deionized water, and the DNA concentration determined by Fluorescence quantitative PCR (QPCR) was as followed:

	result determined by qPCR (nM)
HLA-Q-Mix	115.3

The gel containing the DNA fragments ranging from 350 to 550bp was sliced (Figure 12). After purification and recovery of the products from the gel, the DNA concentration, as determined by Fluorescence quantitative PCR (QPCR), was 18.83 nM.

Analysis of the results

The sequencing results from Illumina GA were a series of DNA sequences, and by searching the forward and reverse primer index sequences and primer sequences in the sequencing results, databases, comprising the sequencing results of the PCR products of various exons of HLA-DQB1 for each sample corresponding to respective primer index were constructed. The sequencing results of each exon was aligned to the reference sequence (reference sequences were from http://www.ebi.ac.uk/imgt/hla/) of the corresponding exon by BWA (Burrows-Wheeler Aligner), and consensus sequences of each database were constructed, and the sequence reads were selected and corrected based on the quality value of base sequencing, and difference between the sequence reads and consensus sequences. The corrected DNA sequences were assembled into the corresponding sequences of Exons 2, 3 of HLA-DQB1 on the basis of sequence overlapping and linkage (Paired-End linkage) relationship. The screen-capture of Figure 13 illustrates the procedure for construction of consensus sequence of Exon 2 of HLA-DQB1 site in Sample No.7.

The resultant DNA sequence for Exons 2, 3 of HLA-DQB1 was aligned with the sequence database of the corresponding exon of HLA-DQB1 in IMGT HLA professional database. If the result of sequence alignment showed 100% match, the HLA-DQB1 genotype of the corresponding sample was determined.

For all 94 samples, the typing results obtained by the above method were completely consistent with the original known typing results, wherein the results of Samples No. 1-32 were shown in Table 12.

Table 12: The typing results of Samples No. 1-32

Sample No.	Original known DQB1 genotype		Results for DQB1 obtained at this time		Identical or not
1	DQB1*02:02	DQB1*03:01	DQB1*02:02	DQB1*03:01	yes
2	DQB1*02:02	DQB1*04:01	DQB1*02:02	DQB1*04:01	yes
3	DQB1*05:02	DQB1*02:02	DQB1*05:02	DQB1*02:02	yes
4	DQB1*02:02	DQB1*06:03	DQB1*02:02	DQB1*06:03	yes
5	DQB1*03:03	DQB1*04:02	DQB1*03:03	DQB1*04:02	yes
6	DQB1*05:02	DQB1*03:17	DQB1*05:02	DQB1*03:17	yes
7	DQB1*03:03	DQB1*06:02	DQB1*03:03	DQB1*06:02	yes
8	DQB1*05:03	DQB1*04:02	DQB1*05:03	DQB1*04:02	yes
9	DQB1*04:02	DQB1*06:01	DQB1*04:02	DQB1*06:01	yes
10	DQB1*05:01	DQB1*06:10	DQB1*05:01	DQB1*06:10	yes
11	DQB1*03:01	DQB1*03:03	DQB1*03:01	DQB1*03:03	yes
12	DQB1*05:01	DQB1*05:01	DQB1*05:01	DQB1*05:01	yes
13	DQB1*02:02	DQB1*04:02	DQB1*02:02	DQB1*04:02	yes
14	DQB1*05:02	DQB1*02:01	DQB1*05:02	DQB1*02:01	yes
15	DQB1*02:01	DQB1*06:02	DQB1*02:01	DQB1*06:02	yes
16	DQB1*03:03	DQB1*04:01	DQB1*03:03	DQB1*04:01	yes
17	DQB1*05:01	DQB1*03:02	DQB1*05:01	DQB1*03:02	yes
18	DQB1*03:03	DQB1*06:01	DQB1*03:03	DQB1*06:01	yes
19	DQB1*03:03	DQB1*06:10	DQB1*03:03	DQB1*06:10	yes
20	DQB1*05:03	DQB1*04:01	DQB1*05:03	DQB1*04:01	yes
21	DQB1*05:02	DQB1*04:01	DQB1*05:02	DQB1*04:01	yes
22	DQB1*03:01	DQB1*03:03	DQB1*03:01	DQB1*03:03	yes
23	DQB1*05:02	DQB1*05:03	DQB1*05:02	DQB1*05:03	yes
24	DQB1*05:02	DQB1*03:02	DQB1*05:02	DQB1*03:02	yes
25	DQB1*03:03	DQB1*06:01	DQB1*03:03	DQB1*06:01	yes
26	DQB1*05:02	DQB1*06:09	DQB1*05:02	DQB1*06:09	yes

27	DQB1*02:02	DQB1*06:02	DQB1*02:02	DQB1*06:02	yes
28	DQB1*05:02	DQB1*03:01	DQB1*05:02	DQB1*03:01	yes
29	DQB1*02:01	DQB1*03:01	DQB1*02:01	DQB1*03:01	yes
30	DQB1*06:03	DQB1*06:09	DQB1*06:03	DQB1*06:09	yes
31	DQB1*05:02	DQB1*02:02	DQB1*05:02	DQB1*02:02	yes
32	DQB1*05:01	DQB1*06:01	DQB1*05:01	DQB1*06:01	yes

Example 11: HLA-DQB1 genotyping by using Sanger sequencing method

1. Sample DNA extraction

As described in Example 1, DNAs were extracted by using KingFisher Automatic Extraction Instrument from 20 out of 94 samples with known HLA genotypes.

2. PCR amplification

The above DNAs, extracted by using KingFisher Automatic Extraction Instrument, were used as templates, and PCR amplification was carried out in single tubes by using two pairs of PCR primers (Q-F2 and Q-R2, Q-F3 and Q-R3) as listed in Table 5, respectively. The PCR procedure for each pair of primers was as followed:

96°C 2min

95°C 30s→56°C 30s→72°C 20s (35 cycles)

15°C ∞

PCR reaction system for HLA-Q was as followed:

Promega 5×buffer I (Mg2+ plus)	5.0 μL
dNTP Mixture (2.5 mM each)	2.0 μL
Primer mixture (25 ng/μL)	3.0 μL
Promega Taq (5 U/μL)	0.2 μL
DNA (about 20 ng/μL)	2.0 μL
ddH2O	12.8 μL
total	25.0 μL

PCR products were subjected to agarose gel electrophoresis before purification.

3. Purification of PCR products

The method and steps were the same as those described in Example 9.

4 . Performance of sequencing reaction and purification of products of the sequencing reaction

The method and steps were the same as those described in Example 9.

5. Sequencing and result analysis

Purified products of the sequencing reaction were subjected to capillary electrophoresis sequencing in ABI 3730XL. The sequencing peaks were analyzed by uType software (Invitrogen) to obtain HLA typing results (Figure 15). All the results obtained by the above method were identical to the original known results, as shown in Table 13.

Table 13: Comparison of the typing results obtained by the above method with the original known typing results

Sample No.	Original known DQB1 genotype		Results for DQB1 obtained at this time		Identical or not
1	DQB1*02:02	DQB1*03:01	DQB1*02:02	DQB1*03:01	yes
2	DQB1*02:02	DQB1*04:01	DQB1*02:02	DQB1*04:01	yes
3	DQB1*05:02	DQB1*02:02	DQB1*05:02	DQB1*02:02	yes
4	DQB1*02:02	DQB1*06:03	DQB1*02:02	DQB1*06:03	yes
5	DQB1*03:03	DQB1*04:02	DQB1*03:03	DQB1*04:02	yes
6	DQB1*05:02	DQB1*03:17	DQB1*05:02	DQB1*03:17	yes
7	DQB1*03:03	DQB1*06:02	DQB1*03:03	DQB1*06:02	yes
8	DQB1*05:03	DQB1*04:02	DQB1*05:03	DQB1*04:02	yes
9	DQB1*04:02	DQB1*06:01	DQB1*04:02	DQB1*06:01	yes
10	DQB1*05:01	DQB1*06:10	DQB1*05:01	DQB1*06:10	yes
11	DQB1*03:01	DQB1*03:03	DQB1*03:01	DQB1*03:03	yes
12	DQB1*05:01	DQB1*05:01	DQB1*05:01	DQB1*05:01	yes
13	DQB1*02:02	DQB1*04:02	DQB1*02:02	DQB1*04:02	yes
14	DQB1*05:02	DQB1*02:01	DQB1*05:02	DQB1*02:01	yes
15	DQB1*02:01	DQB1*06:02	DQB1*02:01	DQB1*06:02	yes

16	DQB1*03:03	DQB1*04:01	DQB1*03;03	DQB1*04:01	yes
17	DQB1*05:01	DQB1*03:02	DQB1*05:01	DQB1*03:02	yes
18	DQB1*03:03	DQB1*06:01	DQB1*03:03	DQB1*06:01	yes
19	DQB1*03:03	DQB1*06:10	DQB1*03:03	DQB1*06:10	yes
20	DQB1*05:03	DQB1*04:01	DQB1*05:03	DQB1*04:01	yes

Example 12: Genotyping of Exons 2, 3, 4 of HLA-A/B/C and Exons 2, 3 of HLA-DQB1 in 950 samples

In the present example, Exons 2, 3, 4 of HLA-A/B/C and Exons 2, 3 of HLA-DQB1 in 950 samples were genotyped by using the combination of primer indexes, DNA incomplete shearing strategy, library indexes, PCR-Free libraries preparation, and Illumia GA Paired-End 100 sequencing technique (PCR products having a length ranging from 300bp to 500bp), demonstrating that the method of the present invention could accomplish the genotyping of gene fragments of a length exceeding the maximum read length of sequencer, and also demonstrating that the present invention could accomplish HLA genotyping with low cost, high throughput, high accuracy and high resolution.

Principle: the samples to be analyzed were divided into 10 groups; for samples of each group, primer indexes were introduced to the two termini of the PCR products of Exons 2, 3, 4 of HLA-A/B/C and Exons 2, 3 of HLA-DQB1 by PCR reaction so as to specifically label the sample information of the PCR products. The products of PCR amplification of four sites (HLA-A/B/C/DQB1) in each group of samples were mixed together to obtain a library of PCR products; after incomplete ultrasonic shearing of the libraries of PCR products, indexed PCR-free sequencing libraries were constructed (wherein for the PCR product library of each sample group, a different adapter was used, thereby constructing 10 indexed sequencing libraries). The 10 indexed sequencing libraries were pooled together at an equal mole to construct a mixed index sequencing library. The mixed index sequencing library was subjected to 2% low melting point agarose gel electrophoresis, and all the DNA bands of a length ranging from 450bp to 750bp were recovered and purified by gel slicing. The recovered DNA was sequenced by the Illumina GA PE-100 method. The sequence

information of all the tested samples can be traced by primer index sequences and library index sequences, and the sequence of the whole PCR product can be assembled on the basis of the known reference sequences and the overlapping and linkage relationship between the sequences of DNA fragments, The complete sequence of the original PCR product can be aligned with the standard database of the corresponding exons of HLA-A/B/C/DQB1, thereby accomplishing HLA-A/B/C/DQB1 genotyping.

1. Sample extraction

DNAs were extracted from 950 blood samples with known HLA-SBT typing results (China Marrow Donor Program cited hereafter as (CMDP)) by using KingFisher Automatic Extraction Instrument (US Thermo Co.). The process was the same as that described in Example 1.

2. PCR amplification

The 950 DNAs obtained from the sample extraction step were designated as No. 1-950, and were divided into 10 groups (95 DNAs for each group), which were designated as HLA-1, HLA-2, HLA-3, HLA-4, HLA-5, HLA-6, HLA-7, HLA-8, HLA-9, HLA-10. For each group of samples, 95 DNA samples were amplified by 95 sets of PCR primers carrying bidirectional primer indexes (Table 6) for amplification of Exons 2, 3, 4 of HLA-A/B (Table 2), for amplification of Exons 2, 3, 4 of HLA-C (Table 4), and for amplification of Exons 2, 3 of HLA-DQB1 (Table 5), respectively. PCR reaction took place in 96-well plates, using 100 plates in total, designated as HLA-X-P-A2, HLA-X-P-A3, HLA-X-P-A4, HLA-X-P-B2, HLA-X-P-B3, HLA-X-P-B4, HLA-X-P-C2, HLA-X-P-C3, HLA-X-P-C4 and HLA-X-P-DQB1 ("X" represents the information of the group number 1/2/3/4/5/6/7/8/9/10, "A2/3/4", "B2/3/4", "C2/3/4", "DQB1" represent the amplification sites), wherein a negative control without adding any template was set in each plate, and the primers used for the negative control were primers labeled by PI-1 (Table 6). During experimentation, the information of each sample on the group number and primer indexes was recorded. For example, the relevant information on primer indexes PI-1 and PI-2 was as followed, and the rest may be deduced similarly.

primer index No.	forward primer index	reverse primer index	Corresponding position in 96-well plate	Corresponding template (Group 1)	Corresponding template (Group n+1, wherein 1≤ n<10, n was an integer)
PI-1	TCGCAGACATCA	TGACACGATGCT	A1	1	1+95*n
PI-2	TACATCGCACTA	TACAGATGCTGA	A2	2	2+95*n

The PCR procedure and PCR reaction system for HLA-A/B/C were the same as those described in Example 2. The PCR primers for amplification of the corresponding exons of HLA-A/B were shown in Table 2, and the PCR primers for amplification of the corresponding exons of HLA-C were shown in Table 4.

PCR procedure for HLA-DQB1 was as followed.

96°C 2min

95°C 30s→55°C 30s→72°C 20s (32cycles)

15°C ∞

Multiple PCR reaction system for HLA-DQB1 (amplification of Exons 2, 3 simultaneously) is the same as the one described in Example 10, and the PCR primers for amplification of the corresponding exons of HLA-DQB1 were as shown in Table 5.

Wherein, PI_{nf}-A/B/C-$F_{2/3/4}$ and PI_{nf}-Q-F2/F3 represent the F primers of HLA-A/B/C/DQB1 having the forward primer index sequence No. n (Table 6) at 5' end, PI_{nr}-A/B/C-$R_{2/3/4}$ and PI_{nr}-Q-R2/R3 represent the R primers of HLA-A/B/C/DQB1 having the reverse primer index sequence No. n at 5' end (here n≤95), and the rest may be deduced similarly. Moreover, each sample corresponds to a specific set of PCR primers (PI_{nf}-A/B/C-$F_{2/3/4}$, PI_{nr}-A/B/C-$R_{2/3/4}$, PI_{nf}-Q-F2/F3, PI_{nr}-Q-R2/R3).

PCR reaction was carried out in PTC-200 PCR apparatus from Bio-Rad Co.. After

PCR reaction, 3ul PCR products were subjected to 2% agarose gel electrophoresis. Figure 16 showed the electrophoretic result of the PCR products of the corresponding exons of HLA-A/B/C/DQB1 of Sample No. 1, and the DNA molecular marker was DL 2000 (Takara Co.). There were a series of single bands of a length ranging from 300 bp to 500 bp in the electrophorogram, indicating successful PCR amplification of exons (A2, A3, A4, B2, B3, B4, C2, C3, C4, DRB1) of HLA-A/B/C/DQB1 of Sample No.1. There was no amplification band in negative control (N). The results of other samples were similar.

3. Pooling and purification of PCR products

For samples of Group X ("X" is 1/2/3/4/5/6/7/8/9/10), 20 μl of the rest PCR products was taken from each well of the 96-well plate HLA-X-P-A2 (except for the negative control), and was mixed homogeneously under shaking in a 3 ml EP tube (designated as HLA-X-A2-Mix). The same operation was applied to the other 9 96-well plates of the samples of Group X, designated as HLA-X-A3-Mix, HLA-X-A4-Mix, HLA-X-B2-Mix, HLA-X-B3-Mix, HLA-X-B4-Mix, HLA-X-C2-Mix, HLA-X-C3-Mix, HLA-X-C4-Mix, and HLA-X-DQB1-Mix. 200ul was taken from each of HLA-X-A2-Mix, HLA-X-A3-Mix, HLA-X-A4-Mix, HLA-X-B2-Mix, HLA-X-B3-Mix, HLA-X-B4-Mix, HLA-X-C2-Mix, HLA-X-C3-Mix, HLA-X-C4-Mix, and HLA-X-DQB1-Mix, and was mixed in a 3ml EP tube, designated as HLA-X-Mix. 500ul DNA mixture from HLA-X-Mix was subjected to column purification with Qiagen DNA Purification kit (QIAGEN Co.) (For the specific purification steps, please refer to the manufacturer's instruction) to obtain 200ul purified DNA, of which the DNA concentration were determined by Nanodrop 8000 (Thermo Fisher Scientific Co.). The same operation was also applied to the other 9 groups of samples. The finally determined DNA concentrations of the 10 groups of samples were as followed.

	HLA-1-Mix	HLA-2-Mix	HLA-3-Mix	HLA-4-Mix	HLA-5-Mix	HLA-6-Mix	HLA-7-Mix	HLA-8-Mix	HLA-9-Mix	HLA-10-Mix
Conc. (ng/ul)	53.1	52.3	56.1	57.2	50.5	55.7	54.2	58.6	53.9	54.8

4. Construction of Illumina GA sequencing libraries

As described in Example 4, a total amount of 5ug DNA, taken from the purified HLA-X-Mix, was subjected to DNA shearing, purification after shearing, terminal repairing reaction, addition of A at 3' end, and ligation of Illumina GA PCR-Free library adapter.

The corresponding relationship between the sample groups and library adapters was as followed.

Sample group No.	HLA-1	HLA-2	HLA-3	HLA-4	HLA-5	HLA-6	HLA-7	HLA-8	HLA-9	HLA-10
Library adapter No.	1	2	3	4	5	6	7	8	9	10

The obtained reaction products were purified by Ampure Beads (Beckman Coulter Genomics), and were dissolved in 50ul deionized water, and the DNA concentrations determined by Fluorescence quantitative PCR (QPCR) were as followed:

	HLA-1-Mix	HLA-2-Mix	HLA-3-Mix	HLA-4-Mix	HLA-5-Mix	HLA-6-Mix	HLA-7-Mix	HLA-8-Mix	HLA-9-Mix	HLA-10-Mix
Conc./nM	86.60	78.21	54.56	87.35	84.37	85.09	96.21	85.81	88.14	88.26

6. Recovery by gel slicing

HLA-1-Mix, HLA-2-Mix, HLA-3-Mix, HLA-4-Mix, HLA-5-Mix, HLA-6-Mix, HLA-7-Mix, HLA-8-Mix, HLA-9-Mix and HLA-10-Mix were mixed at an equal mole (final concentration was 70.86nM/ul), designated as HLA-Mix-10. 30μL HLA-Mix-10 was subjected to 2% low melting point agarose gel electrophoresis. The electrophoretic condition was 100V, 100min. DNA marker was the 50bp DNA marker from NEB Co.. The gel containing the DNA fragments ranging from 450 to 750bp was sliced (Figure 17). The products in the sliced gel were recovered and purified by QIAquick PCR Purification Kit (QIAGEN Co.), the volume after purification was 32ul, the DNA concentration measured by Fluorescence quantitative PCR (QPCR)

was 10.25nM.

5 . Illumina GA sequencing and result analysis

Sequencing and result analysis were carried out according to the methods as described in Examples 5 and 6.

Databases, comprising the sequencing results of the PCR products of various exons of HLA-A/B/C/DQB1 for each sample corresponding to respective primer index were constructed. The resultant DNA sequence was aligned with the sequence database of the corresponding exon of HLA-A/B/C/DQB1 in IMGT HLA professional database. If the result of sequence alignment showed 100% match, the HLA-A/B/C/DQB1 genotype of the corresponding sample was determined. Please refer to the screen-capture of the program for construction of consensus sequence of Exon 2 of HLA-C site in Sample No.1, as illustrated in Figure 18. For all 950 samples, the typing results obtained by the above method were completely consistent with the original known typing results, wherein the results of Samples No. 1-32 were as followed:

No.	Original known HLA-A/B/C/DQB1 type							
1	A*02: 07	A*03: 01	B*07: 02	B*46: 01	C*01: 02	C*07: 02	DQB1*03 :03	DQB1*06 :02
2	A*11: 01	A*31: 01	B*15: 11	B*38: 02	C*03: 03	C*07: 02	DQB1*03 :03	DQB1*04 :01
3	A*02: 07	A*24: 02	B*13: 01	B*46: 01	C*01: 02	C*03: 04	DQB1*03 :02	DQB1*06 :01
4	A*24: 02	A*33: 03	B*40: 01	B*51: 01	C*01: 02	C*14: 02	DQB1*03 :03	DQB1*03 :03
5	A*31:	A*31:	B*15:	B*35:	C*04:	C*04:	DQB1*03	DQB1*06

	01	01	01	01	01	01	:02	:02
6	A*02:	A*03:	B*44:	B*46:	C*01:	C*05:	DQB1*03	DQB1*06
	07	01	02	01	02	01	:01	:02
7	A*02:	A*30:	B*07:	B*13:	C*06:	C*07:	DQB1*02	DQB1*06
	01	01	02	02	02	02	:02	:01
8	A*02:	A*02:	B*46:	B*46:	C*01:	C*01:	DQB1*05	DQB1*03
	07	07	01	01	02	02	:02	:03
9	A*01:	A*33:	B*49:	B*58:	C*03:	C*07:	DQB1*06	DQB1*06
	01	03	01	01	02	01	:04	:09
10	A*02:	A*11:	B*46:	B*48:	C*01:	C*08:	DQB1*05	DQB1*03
	07	01	01	01	03	01	:03	:02
11	A*02:	A*30:	B*13:	B*15:	C*06:	C*08:	DQB1*03	DQB1*03
	06	01	02	02	02	01	:01	:01
12	A*24:	A*31:	B*35:	B*51:	C*03:	C*14:	DQB1*03	DQB1*06
	02	01	01	01	03	02	:03	:01
13	A*11:	A*33:	B*46:	B*46:	C*01:	C*01:	DQB1*03	DQB1*03
	01	03	01	01	02	02	:02	:03
14	A*01:	A*02:	B*38:	B*57:	C*06:	C*07:	DQB1*05	DQB1*03
	01	03	02	01	02	02	:02	:03
15	A*02:	A*24:	B*13:	B*15:	C*03:	C*07:	DQB1*03	DQB1*06
	06	02	01	25	04	02	:01	:01

16	A*11: 01	A*24: 02	B*15: 02	B*15: 27	C*04: 01	C*08: 01	DQB1*03 :01	DQB1*03 :03
17	A*24: 02	A*24: 02	B*40: 01	B*46: 01	C*01: 02	C*03: 04	DQB1*03 :03	DQB1*06 :02
18	A*24: 02	A*24: 02	B*40: 01	B*46: 01	C*01: 02	C*03: 04	DQB1*03 :03	DQB1*06 :02
19	A*11: 01	A*33: 03	B*40: 02	B*58: 01	C*03: 02	C*03: 04	DQB1*02 :01	DQB1*03 :02
20	A*24: 02	A*30: 01	B*13: 02	B*40: 01	C*03: 04	C*06: 02	DQB1*06 :02	DQB1*06 :03
21	A*02: 01	A*24: 02	B*40: 01	B*40: 01	C*07: 02	C*14: 02	DQB1*04 :02	DQB1*06 :02
22	A*02: 01	A*33: 03	B*15: 01	B*44: 03	C*01: 02	C*14: 03	DQB1*03 :01	DQB1*06 :04
23	A*26: 01	A*33: 03	B*15: 01	B*58: 01	C*03: 02	C*08: 01	DQB1*02 :01	DQB1*03 :01
24	A*02: 01	A*11: 01	B*13: 01	B*55: 02	C*01: 06	C*03: 04	DQB1*03 :01	DQB1*03 :03
25	A*02: 01	A*32: 01	B*40: 01	B*52: 01	C*03: 04	C*12: 02	DQB1*05 :02	DQB1*06 :01
26	A*02: 03	A*02: 07	B*40: 01	B*46: 01	C*01: 02	C*07: 02	DQB1*03 :02	DQB1*06 :01

	03	07	01	01	02	02	:02	:01
27	A*02:	A*02:	B*46:	B*46:	C*01:	C*01:	DQB1*03	DQB1*06
	07	07	01	01	02	02	:03	:01
28	A*24:	A*30:	B*13:	B*39:	C*06:	C*07:	DQB1*02	DQB1*06
	02	01	02	05	02	02	:02	:01
29	A*31:	A*33:	B*15:	B*58:	C*03:	C*07:	DQB1*04	DQB1*06
	01	03	18	01	02	04	:01	:09
30	A*02:	A*03:	B*27:	B*40:	C*02:	C*03:	DQB1*03	DQB1*03
	06	01	05	02	02	04	:01	:01
31	A*02:	A*33:	B*15:	B*58:	C*03:	C*08:	DQB1*05	DQB1*06
	06	03	02	01	02	01	:01	:01
32	A*03:	A*30:	B*13:	B*51:	C*06:	C*15:	DQB1*02	DQB1*03
	01	01	02	01	02	02	:02	:01

No.	HLA-A/B/C/DQB1 type determined by the method of the invention							
1	A*02:	A*03:	B*07:	B*46:	C*01:	C*07:	DQB1*03	DQB1*06
	07	01	02	01	02	02	:03	:02
2	A*11:	A*31:	B*15:	B*38:	C*03:	C*07:	DQB1*03	DQB1*04
	01	01	11	02	03	02	:03	:01
3	A*02:	A*24:	B*13:	B*46:	C*01:	C*03:	DQB1*03	DQB1*06
	07	02	01	01	02	04	:02	:01

4	A*24: 02	A*33: 03	B*40: 01	B*51: 01	C*01: 02	C*14: 02	DQB1*03 :03	DQB1*03 :03
5	A*31: 01	A*31: 01	B*15: 01	B*35: 01	C*04: 01	C*04: 01	DQB1*03 :02	DQB1*06 :02
6	A*02: 07	A*03: 01	B*44: 02	B*46: 01	C*01: 02	C*05: 01	DQB1*03 :01	DQB1*06 :02
7	A*02: 01	A*30: 01	B*07: 02	B*13: 02	C*06: 02	C*07: 02	DQB1*02 :02	DQB1*06 :01
8	A*02: 07	A*02: 07	B*46: 01	B*46: 01	C*01: 02	C*01: 02	DQB1*05 :02	DQB1*03 :03
9	A*01: 01	A*33: 03	B*49: 01	B*58: 01	C*03: 02	C*07: 01	DQB1*06 :04	DQB1*06 :09
10	A*02: 07	A*11: 01	B*46: 01	B*48: 01	C*01: 03	C*08: 01	DQB1*05 :03	DQB1*03 :02
11	A*02: 06	A*30: 01	B*13: 02	B*15: 02	C*06: 02	C*08: 01	DQB1*03 :01	DQB1*03 :01
12	A*24: 02	A*31: 01	B*35: 01	B*51: 01	C*03: 03	C*14: 02	DQB1*03 :03	DQB1*06 :01
13	A*11: 01	A*33: 03	B*46: 01	B*46: 01	C*01: 02	C*01: 02	DQB1*03 :02	DQB1*03 :03
14	A*01:	A*02:	B*38:	B*57:	C*06:	C*07:	DQB1*05	DQB1*03

	01	03	02	01	02	02	:02	:03
15	A*02:	A*24:	B*13:	B*15:	C*03:	C*07:	DQB1*03	DQB1*06
	06	02	01	25	04	02	:01	:01
16	A*11:	A*24:	B*15:	B*15:	C*04:	C*08:	DQB1*03	DQB1*03
	01	02	02	27	01	01	:01	:03
17	A*24:	A*24:	B*40:	B*46:	C*01:	C*03:	DQB1*03	DQB1*06
	02	02	01	01	02	04	:03	:02
18	A*24:	A*24:	B*40:	B*46:	C*01:	C*03:	DQB1*03	DQB1*06
	02	02	01	01	02	04	:03	:02
19	A*11:	A*33:	B*40:	B*58:	C*03:	C*03:	DQB1*02	DQB1*03
	01	03	02	01	02	04	:01	:02
20	A*24:	A*30:	B*13:	B*40:	C*03:	C*06:	DQB1*06	DQB1*06
	02	01	02	01	04	02	:02	:03
21	A*02:	A*24:	B*40:	B*40:	C*07:	C*14:	DQB1*04	DQB1*06
	01	02	01	01	02	02	:02	:02
22	A*02:	A*33:	B*15:	B*44:	C*01:	C*14:	DQB1*03	DQB1*06
	01	03	01	03	02	03	:01	:04
23	A*26:	A*33:	B*15:	B*58:	C*03:	C*08:	DQB1*02	DQB1*03
	01	03	01	01	02	01	:01	:01
24	A*02:	A*11:	B*13:	B*55:	C*01:	C*03:	DQB1*03	DQB1*03
	01	01	01	02	06	04	:01	:03

25	A*02: 01	A*32: 01	B*40: 01	B*52: 01	C*03: 04	C*12: 02	DQB1*05 :02	DQB1*06 :01
26	A*02: 03	A*02: 07	B*40: 01	B*46: 01	C*01: 02	C*07: 02	DQB1*03 :02	DQB1*06 :01
27	A*02: 07	A*02: 07	B*46: 01	B*46: 01	C*01: 02	C*01: 02	DQB1*03 :03	DQB1*06 :01
28	A*24: 02	A*30: 01	B*13: 02	B*39: 05	C*06: 02	C*07: 02	DQB1*02 :02	DQB1*06 :01
29	A*31: 01	A*33: 03	B*15: 18	B*58: 01	C*03: 02	C*07: 04	DQB1*04 :01	DQB1*06 :09
30	A*02: 06	A*03: 01	B*27: 05	B*40: 02	C*02: 02	C*03: 04	DQB1*03 :01	DQB1*03 :01
31	A*02: 06	A*33: 03	B*15: 02	B*58: 01	C*03: 02	C*08: 01	DQB1*05 :01	DQB1*06 :01
32	A*03: 01	A*30: 01	B*13: 02	B*51: 01	C*06: 02	C*15: 02	DQB1*02 :02	DQB1*03 :01

Notice: In case that the sequences of Exons 2, 3, 4 of HLA-A/B/C were completely identical, a common type was selected.

950 samples with known HLA-SBT typing results were subjected to genotyping of HLA-A/B/C/DQB1 sites by the technical strategy of the present invention, and the results showed that the typing results obtained by the technical strategy of the present invention were completely consistent with the original known results.

Although the embodiments of the present invention have been already described in detail, a person skilled in the art would understand that based on all the teaching as disclosed, various modification and substitution may be made to the embodiments without departing from the spirit and scope of the present invention. The scope of the present invention is defined by the claims appended and any equivalent thereof.

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Throughout this specification and the claims which follow, unless the context requires otherwise, the word "comprise", and variations such as "comprises" and "comprising", will be understood to imply the inclusion of a stated integer or step or group of integers or steps but not the exclusion of any other integer or step or group of integers or steps.

The reference in this specification to any prior publication (or information derived from it), or to any matter which is known, is not, and should not be taken as an acknowledgment or admission or any form of suggestion that that prior publication (or information derived from it) or known matter forms part of the common general knowledge in the field of endeavour to which this specification relates.

Claims

1. A method for determining the nucleotide sequence of a nucleic acid of interest in a sample, comprising:

a) providing n samples, wherein n is an integer of ≥ 1; optionally, the n samples to be analyzed are divided into m groups, m is an integer and n≥m≥1 ;

b) amplifying: PCR amplification is performed under conditions suitable for amplifying the nucleic acid of interest in a sample using a pair or multiple pairs of index primers for each sample comprising a forward index primer and a reverse index primer; wherein each index primer comprises a primer index and a PCR primer optionally linked by a linker sequence at the 5' end of the PCR primer; wherein the primer indexes comprised in the forward index primer and reverse index primer may be identical or different; wherein the primer indexes in the pairs of index primers used for different samples are different; and wherein the primer indexes are selected from the primer indexes shown in Table 6 and the PCR primers are selected from SEQ ID NOS: 1-40 and 231-238;

c) pooling: PCR products from each of the samples are pooled together where n > 1;

d) shearing: PCR products are subjected to incomplete shearing, purifying and recovering;

e) sequencing: the recovered sheared DNA mixture from step (d) are subjected to sequencing by second generation sequencing ; and

f) assembling: matching the sequencing data from step (e) to samples based on the unique primer index for each sample, wherein each sequence read is aligned to the DNA reference sequence corresponding to the PCR products using an alignment program and assembling a complete sequence of the nucleic acid of interest from the sequences of the sheared DNA by virtue of sequence overlapping and linkage relationship.

2. The method of claim 1, wherein said PCR primers for amplifying samples according to step (b) are selected from Tables 1, 2, 3, 4, 5 or 7.

3. The method of claim 1 or 2, wherein said primer indexes according to step (b) comprise at least 10, 20, 30, 40, 50, 60, 70, 80, 90 or 95 pairs of the primer indexes as shown in Table 6; and/or

wherein said primer indexes consist of 10-95 pairs 20-95 pairs, 30-95 pairs, 40-95 pairs, 50-95 pairs, 60-95 pairs, 70-95 pairs, 80-95 pairs, 90-95 pairs, or 95 pairs of the primer indexes as shown in Table 6; and/or

wherein said primer indexes comprise at least PI-1 to PI-10, or PI-11 to PI-20, or PI-21 to PI-30, or PI-31 to PI-40, or PI-41 to PI-50, or PI-51 to PI-60, or PI-61 to PI-70, or PI-71 to PI-80, or PI-81 to PI-90, or PI-91 to PI-95 of the primer indexes as shown in Table 6, or combinations of any two or more of them.

4. The method of any one of claims 1to 3, wherein said DNA shearing includes chemical and physical shearing methods.

5. A method for determining the nucleotide sequence of a nucleic acid of interest in a sample comprising:

a) providing n samples, wherein n is an integer of ≥ 1; optionally, the n samples to be analyzed are divided into m groups, m is an integer and $n \geq m \geq 1$;

b) amplifying: PCR amplification is performed under conditions suitable for amplifying the nucleic acid of interest in a sample using a pair or multiple pairs of index primers for each sample comprising a forward index primer and a reverse index primer; wherein each index primer comprises a primer index and a PCR primer optionally linked by a linker sequence at the 5' end of the PCR primer; wherein the primer indexes comprised in the forward index primer and reverse index primer may be identical or different; wherein the primer indexes in the pairs of index primers used for different samples are different; and wherein the primer indexes are selected from the primer indexes shown in Table 6 and the PCR primers are selected from SEQ ID NOS: 1-40 and 231-238;

c) pooling: PCR products from each of the samples are pooled together where $n > 1$;

d) shearing: PCR products are subjected to incomplete shearing, purifying and recovering;

e) constructing a library: constructing a PCR-free sequencing library using the library of the sheared PCR products from step (d), wherein different library adapters may be added to distinguish different PCR-Free sequencing libraries, wherein all the DNA fragments between the maximum read length of the sequencer and the applicable maximum DNA length of the sequencer are purified and recovered;

f) sequencing: the recovered PCR-free sequencing library from step (e) is subjected to sequencing by second generation sequencing;

g) assembling: matching the sequencing data from step (f) to samples based on the different library adapter sequences of the libraries and the unique primer index for each sample, wherein each sequence read is aligned to the DNA reference sequence corresponding to the PCR products using an alignment program and assembling a complete sequence of the nucleic acid of interest from the sequences of the PCR-free sequencing library by virtue of sequence overlap and linkage relationship.

6. The method of claim 5, wherein constructing a library according to step (e) comprises the recovery and purification of 450 to 750 bp DNA fragments.

7. The method of claim 1 or 5, wherein the pair of index primers comprises at least one degenerate primer.

8. The method of any one of claims 1 to 7, wherein the samples are from a mammal.

9. The method of claim 8 wherein the samples are from a human.

10. The method of claim 9 wherein the samples are human blood samples.

11. Use of the method of any one of claims 1 to 10 in HLA genotyping comprising: sequencing a sample according to the method of any one of claims 1 to 10, and aligning the sequencing results with sequence data in a HLA database; wherein the HLA genotype of a sample is determined if the results of a sequence alignment of Exons 2, 3, 4 of HLA-A/B, Exons 2, 3 and/or 4 of HLA-C, Exon 2 and/or 3 of HLA-DQB1 gene and/or Exon 2 of HLA-DRB1 shows 100% match.

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12. The method of any one of claims 1 to 10, wherein the method is used for HLA genotyping wherein sequencing data is aligned to sequence data of exons 2, 3, 4 of HLA-A/B; exons 2, 3 and/or 4 of HLA-C; exon 2 and/or 3 of HLA-DQB1 gene and/or exon 2 of HLA-DRB1 in a HLA database to determine the HLA genotype of the corresponding sample.

13. The method of any one of claims 1 to 10 and 12; and the use of claim 11 substantially described herein with reference to the Examples and/or Figures.

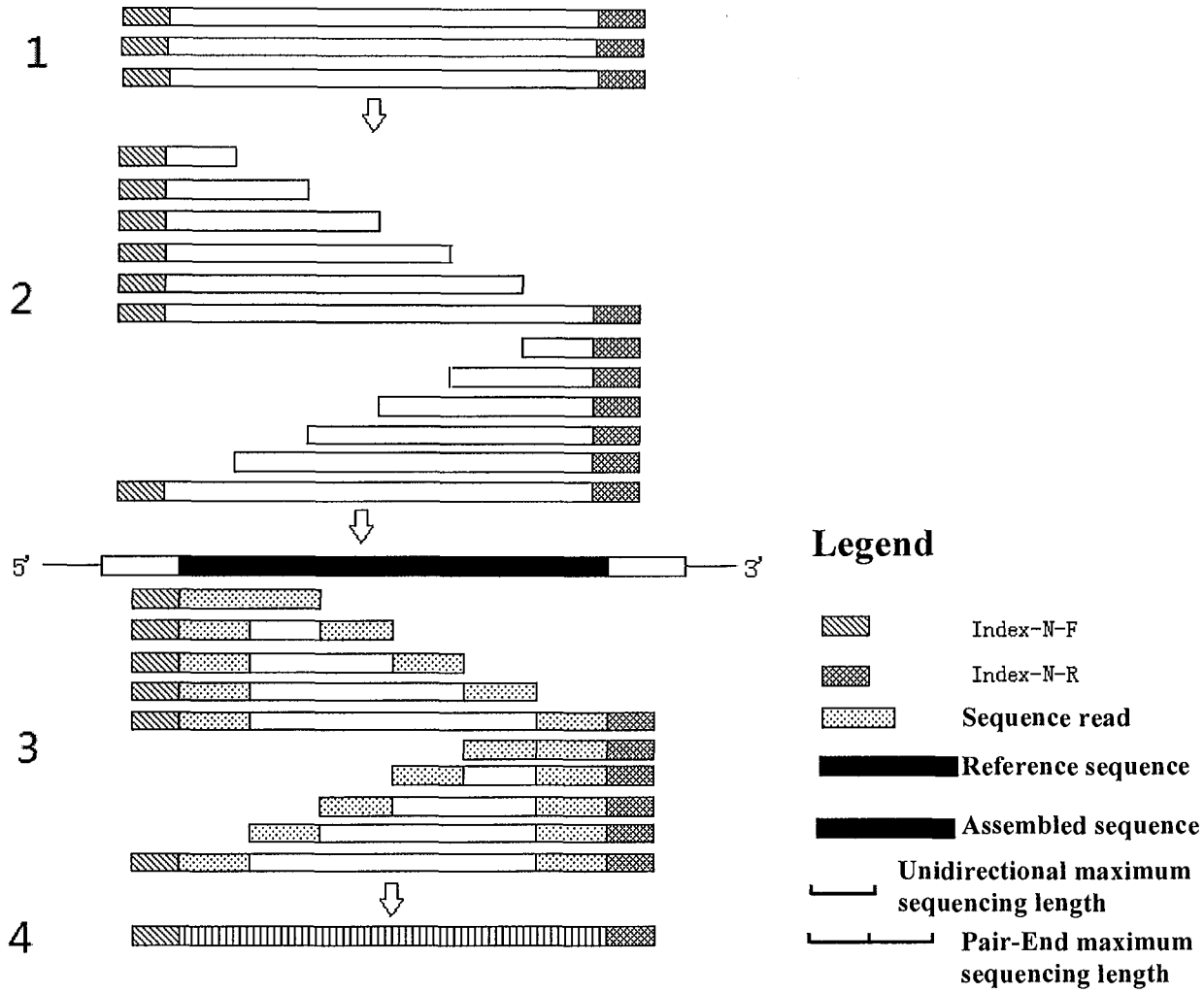
1
2
5'
3'
3
4
Legend
Index-N-F
Index-N-R
Sequence read
Reference sequence
Assembled sequence
Unidirectional maximum sequencing length
Pair-End maximum sequencing length

Fig. 1

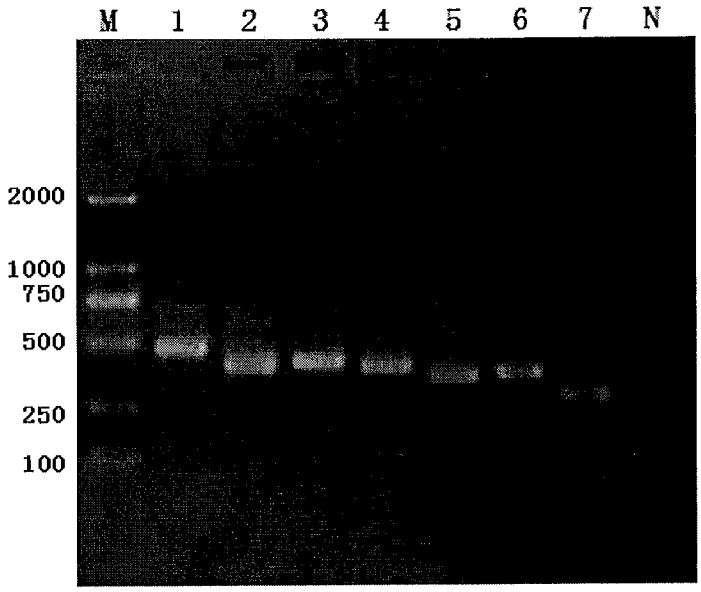
M
1
2
3
4
5
6
7
N
2000
1000
750
500
250
100

Fig. 2

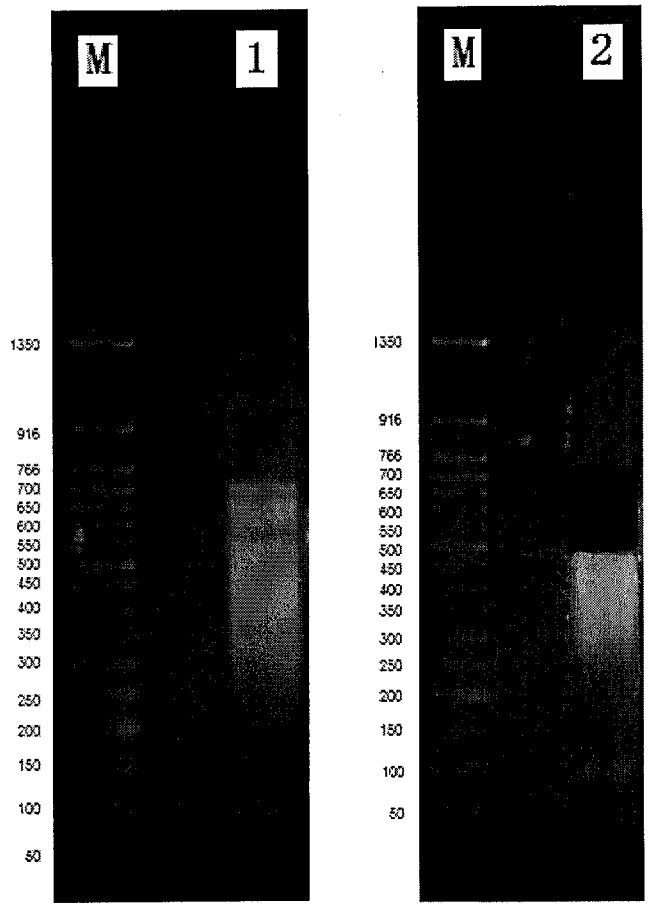
M
1
M
2
1350
916
766
700
650
600
550
500
450
400
350
300
250
200
150
100
50

Fig. 3


Reference sequence
A*01:01:01:01
Constructed consensus sequence
Pair-End Sequencing Results
Assembled sequence
Assembled sequence
>A*02:03:01
GCTCTCACTCCATGAGGTATTTCTTCACATCCGTGTCCCGGCCCGGCCGCGGGGAGCCCC
GCTTCATCGCAGTGGGCTACGTGGACGACACGCAGTTCGTGCGGTTCGACAGCGACGCCG
CGAGCCAGAGGATGGAGCCGCGGGGCGCCGTGGATAGAGCAGGAGGGTCCGGAGTATTGGG
ACGGGGAGACACGGAAAGTGAAGGCCCACTCACAGACTCACCGAGTGGACCTGGGGACCC
TGCGCGGCTACTACAACCAGAGCGAGGCCG
>A*11:01:01
GCTCCCACTCCATGAGGTATTTCTACACCTCCGTGTCCCGGCCCGGCCGCGGGGAGCCCC
GCTTCATCGCCGTGGGCTACGTGGACGACACGCAGTTCGTGCGGTTCGACAGCGACGCCG
CGAGCCAGAGGATGGAGCCGCGGGGCGCCGTGGATAGAGCAGGAGGGGCCGGAGTATTGGG
ACCAGGAGACACGGAATGTGAAGGCCCAGTCACAGACTGACCGAGTGGACCTGGGGACCC
TGCGCGGCTACTACAACCAGAGCGAGGACG

Fig. 4

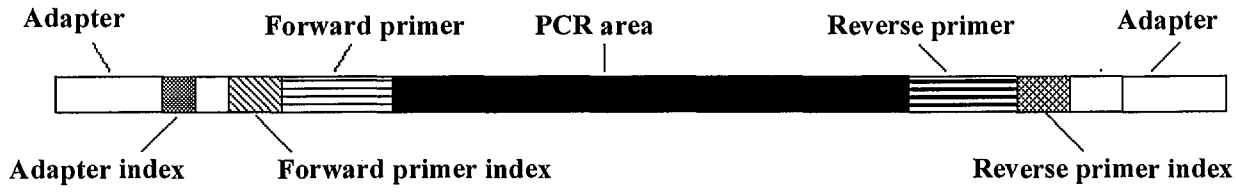
Adapter
Forward primer
PCR area
Reverse primer
Adapter
Adapter index
Forward primer index
Reverse primer index

Fig. 5

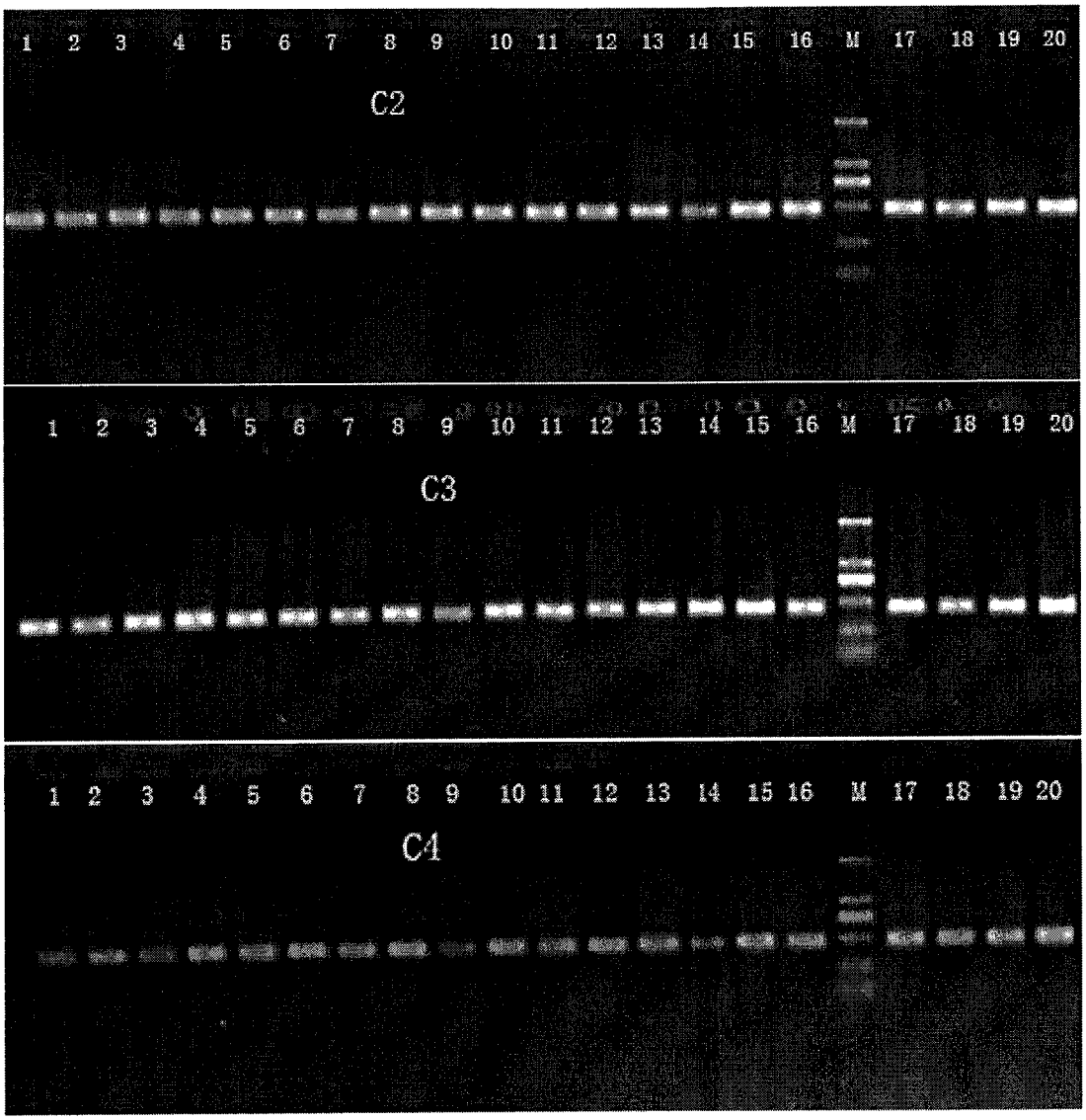
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 M 17 18 19 20
C2
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 M 17 18 19 20
C3
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 M 17 18 19 20
C4

Fig. 6

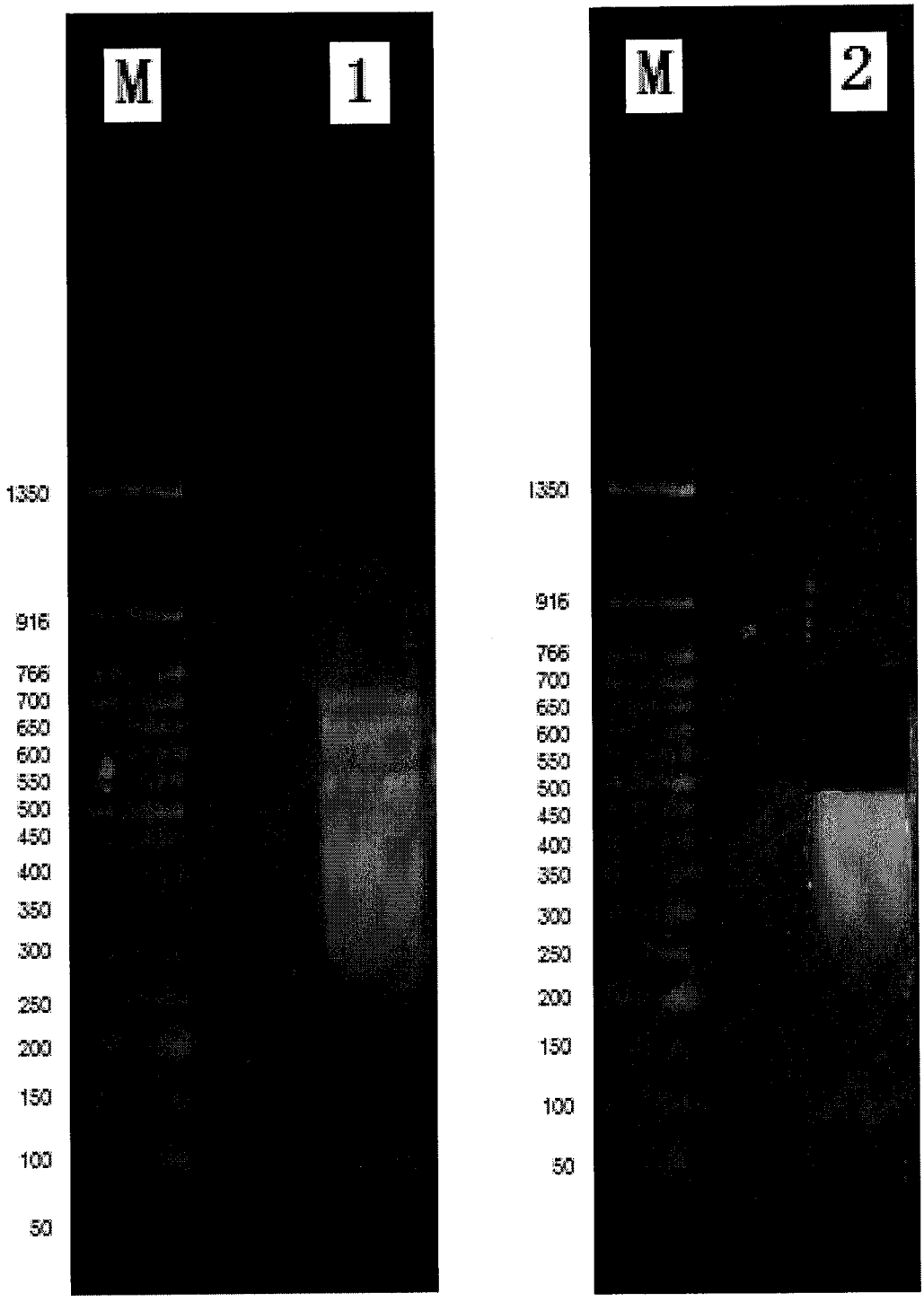
M
1
M
2
1350
916
766
700
650
600
550
500
450
400
350
300
250
200
150
100
50
1350
916
766
700
650
600
550
500
450
400
350
300
250
200
150
100
50

Fig. 7

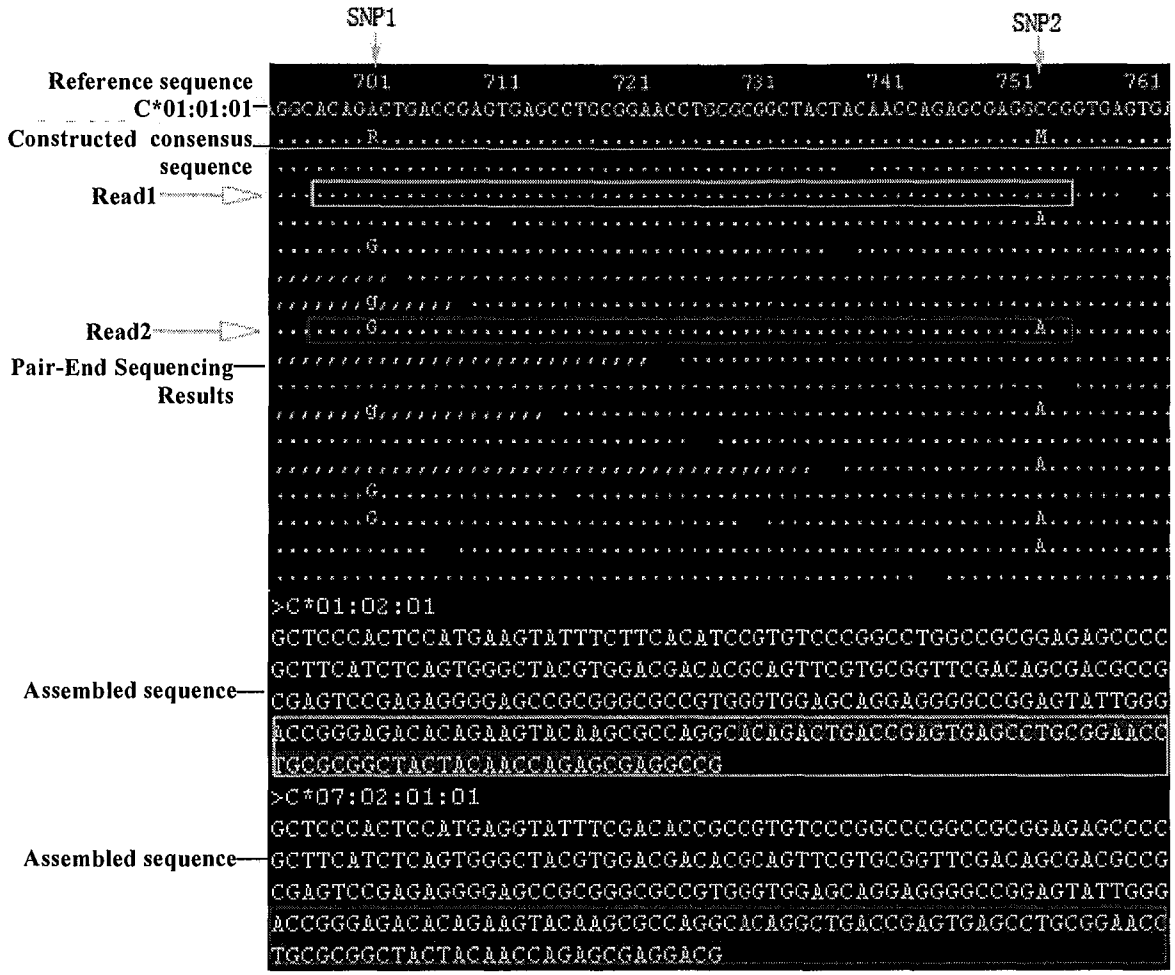
SNP1
SNP2
Reference sequence
C*01:01:01
Constructed consensus sequence
Read1
Read2
Pair-End Sequencing Results
Assembled sequence
Assembled sequence
701
711
721
731
741
751
761
>C*01:02:01
GCTCCCACTCCATGAAGTATTTCTTCACATCCGTGTCCCGGCCTGGCCGCGGAGAGCCCC
GCTTCATCTCAGTGGGCTACGTGGACGACACGCAGTTCGTGCGGTTCGACAGCGACGCCG
CGAGTCCGAGAGGGGAGCCGCGGGCGCCGTGGGTGGAGCAGGAGGGGCCGGAGTATTGGG
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Fig. 8

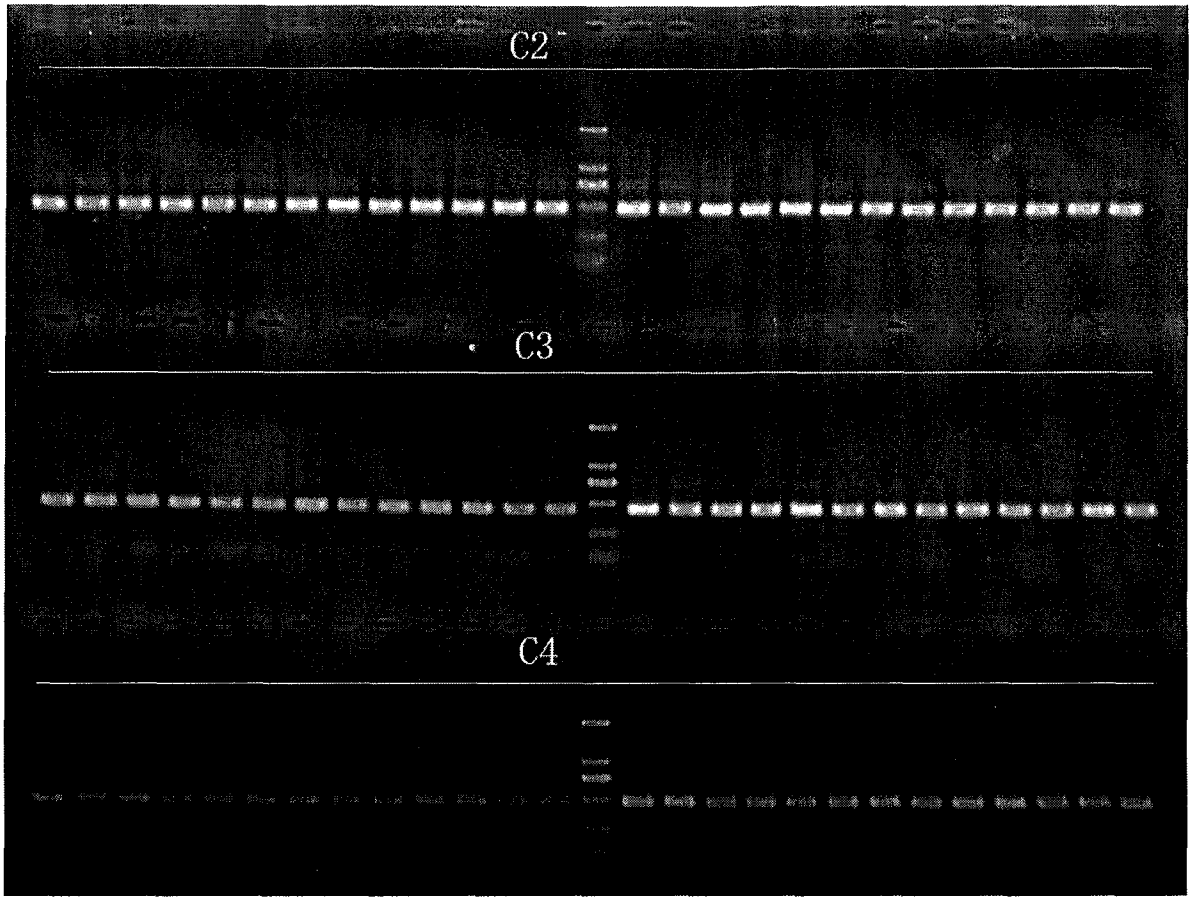
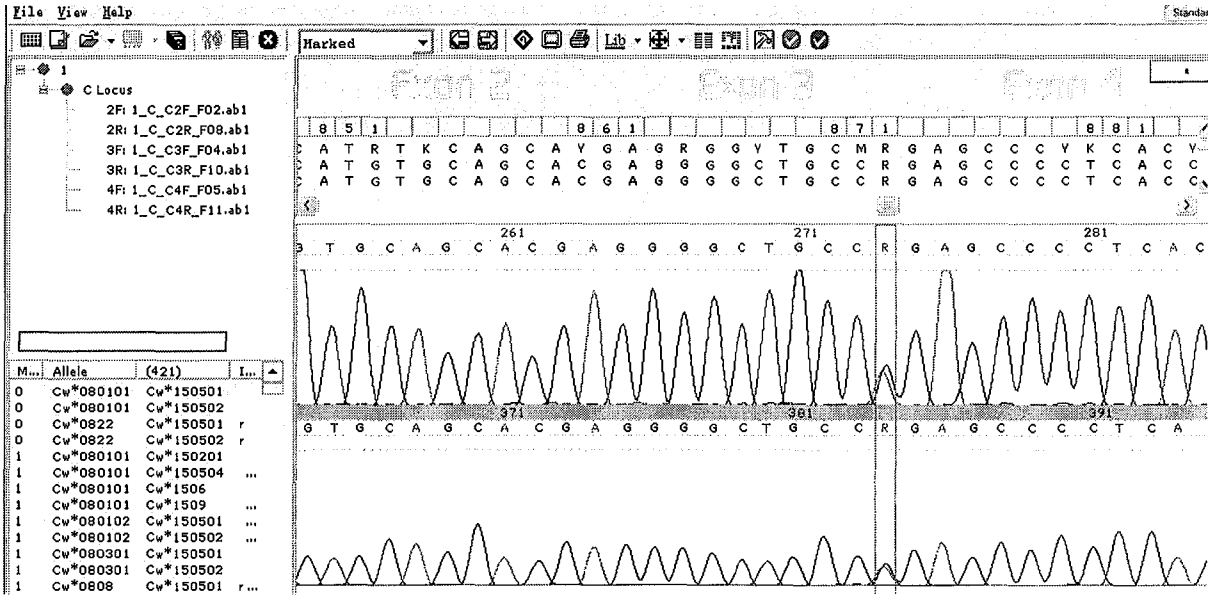

C2
C3
C4

Fig. 9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Marked
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3F: 1_C_C3F_F04.ab1
3R: 1_C_C3R_F10.ab1
4F: 1_C_C4F_F05.ab1
4R: 1_C_C4R_F11.ab1
Allele
(421)
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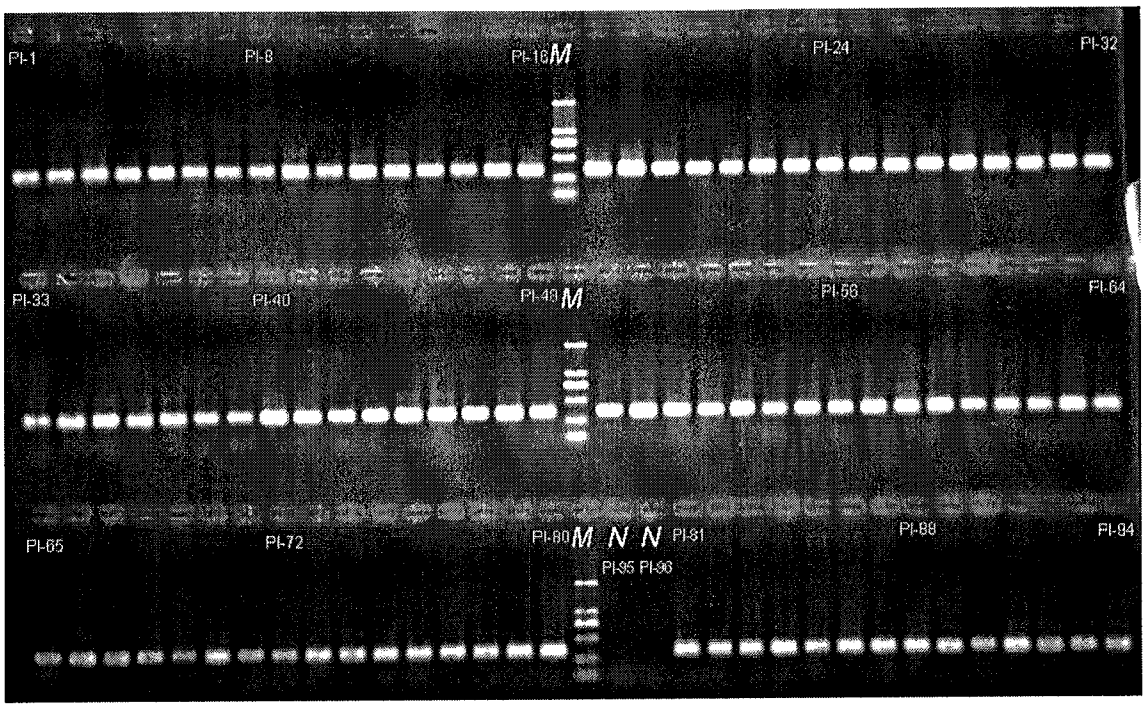
Fig. 10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PI-16
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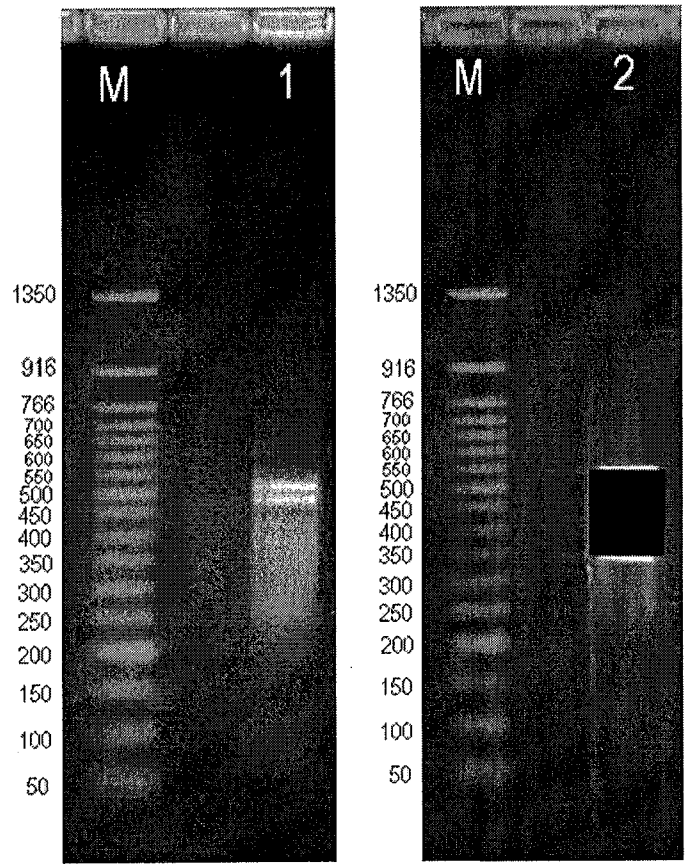
PI-95 PI-96

Fig. 11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100
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Fig. 12


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SNP3
SNP4
SNP5
SNP6
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2341
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2401
2411
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read3
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Fig. 13

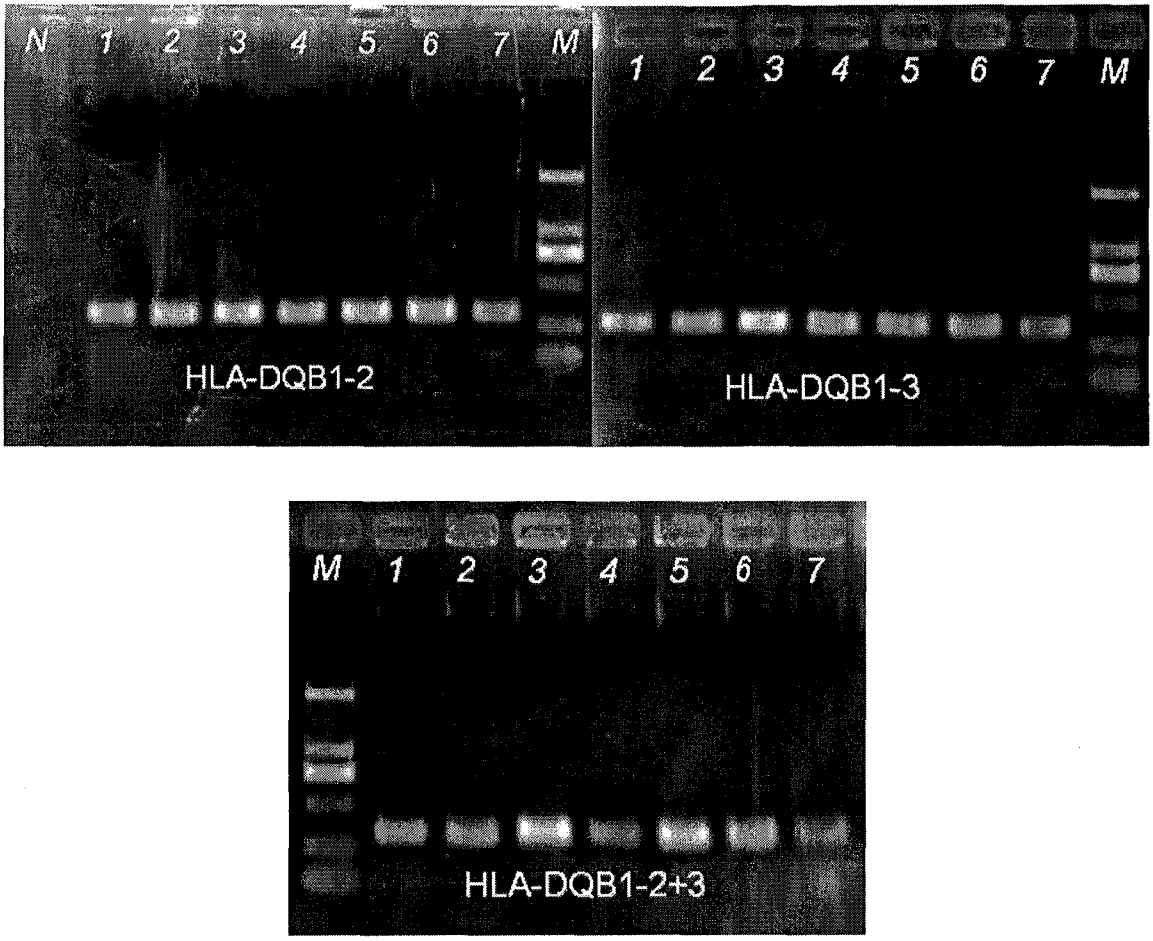
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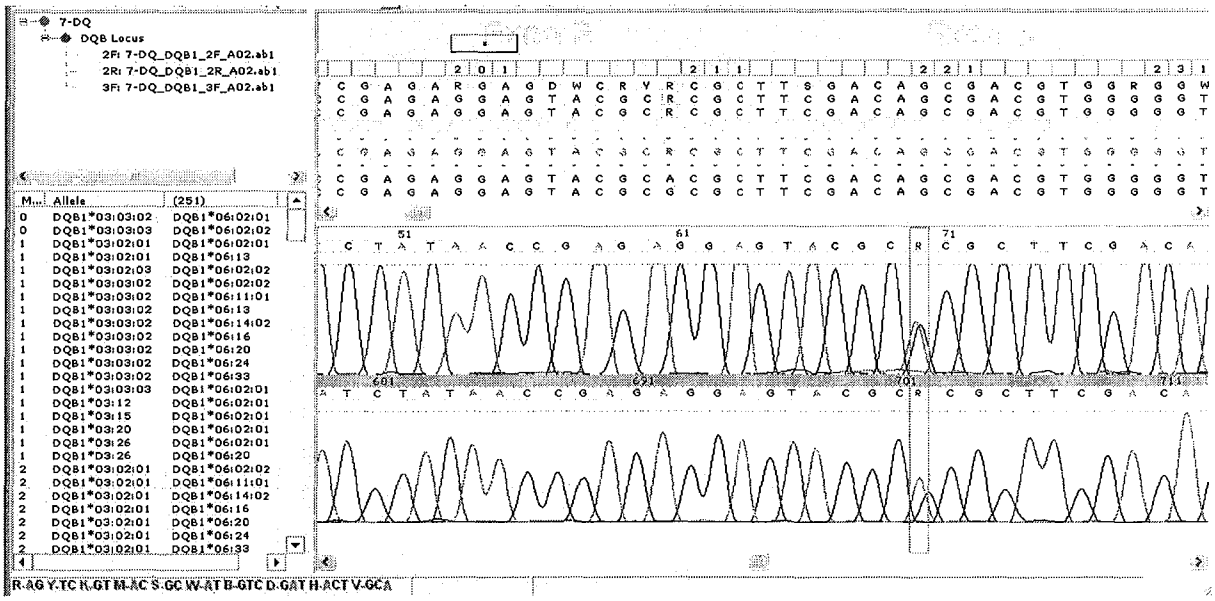
Fig. 14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1 DQB1*03:02:01 DQB1*06:13
1 DQB1*03:02:03 DQB1*06:02:02
1 DQB1*03:03:02 DQB1*06:02:02
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2 DQB1*03:02:01 DQB1*06:14:02
2 DQB1*03:02:01 DQB1*06:16
2 DQB1*03:02:01 DQB1*06:20
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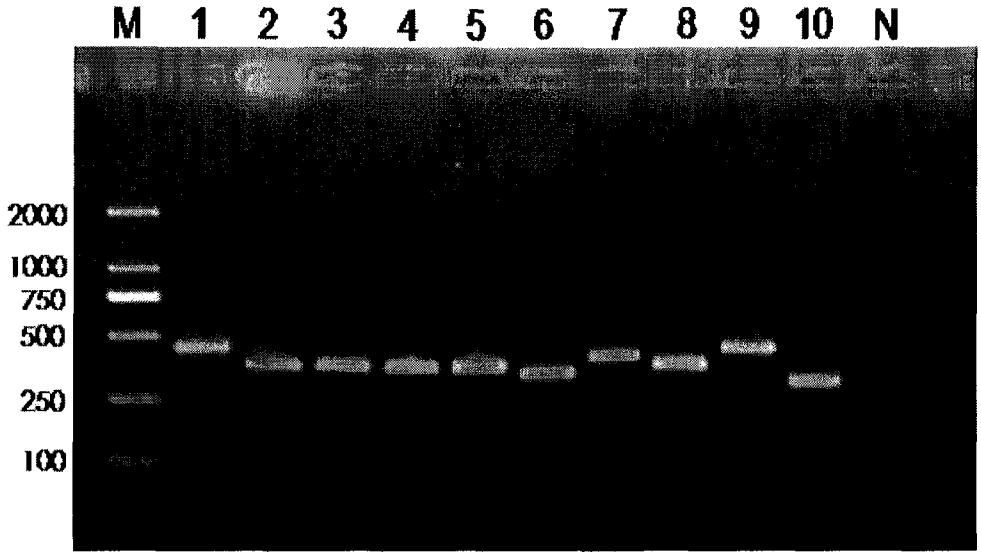
Fig. 15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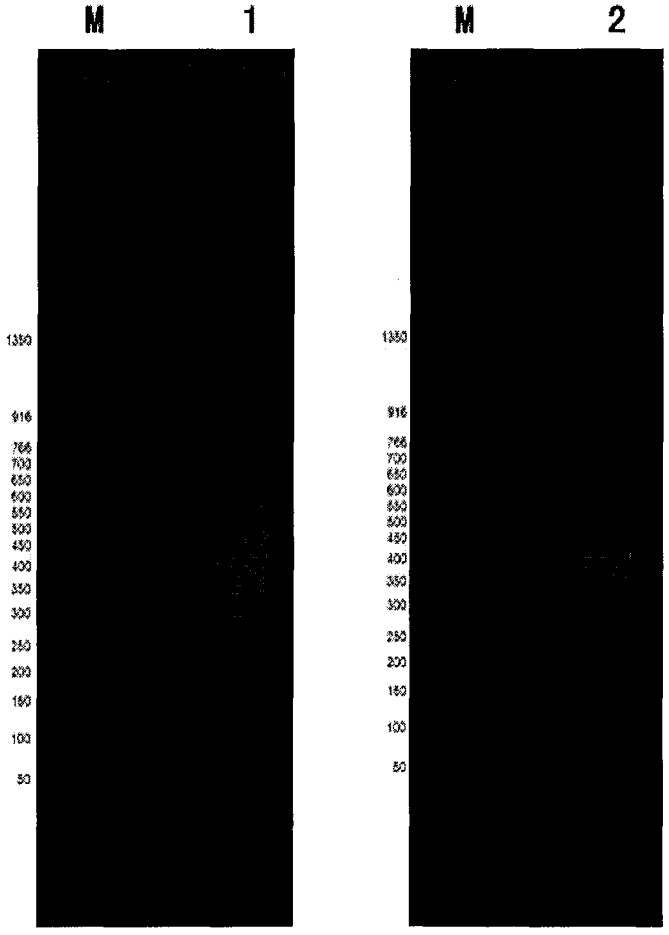
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Fig. 17

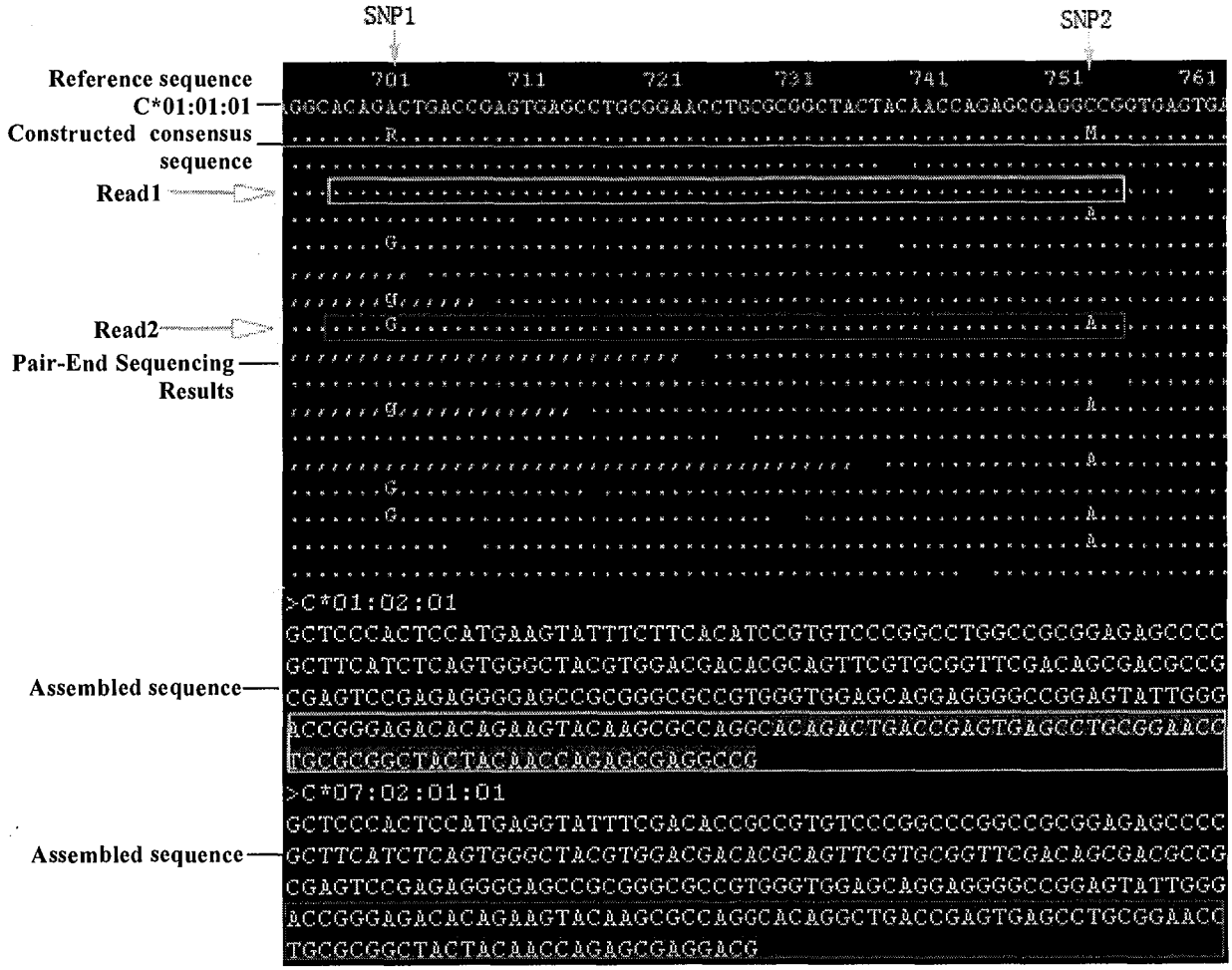
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Fig. 18

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